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Identification of the Phr-dependent heat shock regulon in the hyperthermophilic archaeon, *Thermococcus kodakaraensis*

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The hyperthermophilic archaeon *Thermococcus kodakaraensis* harbors a putative transcriptional regulator (*Tk*-Phr) that is orthologous to the *Pyrococcus furiosus* Phr (*Pf*-Phr). *Pf*-Phr, a transcriptional regulator, represses genes encoding the small heat shock protein (sHSP), AAA$^+$ ATPase and *Pf*-Phr itself under normal growth temperatures. Here we constructed a gene disruption strain of *Tk*-Phr (strain KHR1). KHR1 cells showed similar specific growth rates with those of the wild-type strain under various temperatures. A whole genome microarray analysis was performed between KHR1 and wild-type cells grown at 80°C. Transcript levels of more than 20 genes were significantly higher in KHR1 cells. Most genes contained a sequence motif virtually identical to that of *Pf*-Phr in their 5′-flanking regions. The *Tk*-Phr regulon included genes encoding sHSP, AAA$^+$ ATPase, prefoldin, RecA superfamily ATPase and Tip49. On the other hand, more than half of the members in the regulon encoded conserved/hypothetical proteins, raising the possibility that these proteins participate in unidentified processes of the heat shock response. In contrast, *Tk*-Phr deletion did not lead to dramatic increase in transcript and protein levels of a chaperonin (CpkB) previously shown to respond to heat shock, suggesting the presence of a second, Phr-independent heat shock response mechanism in *T. kodakaraensis*.

Keywords: Archaea/heat shock response/microarray/Phr/transcriptional regulation.

Abbreviations: BRE, transcription factor B-responsive element; HSPs, heat shock proteins; *Pf*-Phr, *Pyrococcus furiosus* Phr; sHSP, small heat shock protein; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TBP, TATA-binding protein; TFB, transcription factor B; *Tk*-Phr, *Thermococcus kodakaraensis* Phr.

Heat shock response is a homeostatic mechanism that occurs in the entire range of living organisms (*1*, *2*). Heat stress, in many cases, causes an induction of a group of proteins called heat shock proteins (HSPs). Key members of HSPs are molecular chaperones that repress protein aggregation and assist refolding of partially denatured proteins, as well as proteases that degrade proteins suffering unrepairable damage.

Although the heat shock response is observed in all three domains of life (*Eucarya*, *Bacteria* and *Archaea*), the transcriptional regulation mechanisms underlying the heat shock response are quite different in each domain. In many eukaryotes, transcriptional activation of HSP genes is mediated by an oligomeric heat shock activation factor(s), called HSF, that binds to a highly conserved *cis*-acting element, termed the heat shock element (*3*, *4*). In the gram-negative *Escherichia coli*, an alternative sigma factor, σ^{32}, is responsible for transcription of the HSP genes. A temperature upshift increases both the activity and the stability of σ^{32}, thereby facilitating transcription of the HSP genes (*5*, *6*). Similarly, in the gram-positive *Bacillus subtilis*, an alternative sigma factor σ^B specifically promotes the transcription of HSP genes (*7*). In addition to the σ^B-dependent control, *B. subtilis* has two other regulation mechanisms mediated by individual transcriptional repressors (HrcA and CtsR) (*8*–*11*).

In *Archaea*, however, much less is known about the mechanisms that contribute to the heat shock response. An analysis on the heat-responsive *hsp5* promoter from the halophilic archaeon, *Halobacterium salinarum*, revealed that the transcription factor B-responsive element (BRE) and TATA box sequences themselves were the direct determinants of the heat-inducible activity (*12*). Another report on the *cct1* promoter from another halophilic archaeon, *Haloferax volcanii*, also indicated that sequence elements located adjacent to the TATA-box were necessary for heat-induced transcription (*13*). As halophilic archaea generally contain multiple copies of genes encoding TATA-binding protein (TBP) and transcription factor B (TFB) (*14*), it was suggested that the heat-responsive properties of these genes are ascribed to the core promoter elements (i.e. TATA-box and/or BRE) that are recognized by specific TBPs and/or TFBs (*12*).

On the other hand, heat shock response mechanisms modulated by specific transcriptional regulators have been reported in thermophilic archaea. One example is Phr, a regulator identified in the hyperthermophilic archaeon, *Pyrococcus furiosus* (encoded by PF1790) (*15*). *In vitro* studies indicated that Phr acts as a transcriptional repressor of its own and of two heat-inducible genes (PF1883 and PF1882) coding for the small heat shock protein (sHSP) and an AAA$^+$

ATPase, respectively. A chromatin immunoprecipitation experiment revealed that Phr actually binds to these promoters in cells under normal growth temperatures and is released from them upon heat shock (*16*). The 3D structure of Phr demonstrated that this protein consists of two domains, an amino-terminal winged helix DNA-binding domain and a carboxy-terminal antiparallel coiled coil helical domain (*16*).

A genetic analysis is a straightforward approach to examine the function of transcriptional regulators *in vivo*. In the hyperthermophilic archaea, however, genetic tools are still limited to a small number of organisms. *Thermococcus kodakaraensis* is a hyperthermophilic archaeon that grows within a temperature range between 60 and 100°C with an optimum at 85°C (*17*, *18*). The development of a gene disruption system (*19*–*21*), along with the complete genome sequence (*22*), makes this archaeon an attractive model organism to study genes of unknown function in hyperthermophilic archaea (*23*–*28*).

In this report, we have characterized a gene (TK2291) disruption mutant of a Phr ortholog in *T. kodakaraensis*. Whole genome microarray analysis suggests that TK2291 encodes a transcriptional repressor regulating over 20 genes, including those encoding sHSP, AAA^+ ATPase and prefoldin, as well as a number of conserved/hypothetical proteins.

Materials and methods

Microorganisms, plasmids and media

E. coli DH5α was used for general DNA manipulation. *E. coli* cells were cultivated in LB medium (10 g l^{-1} of tryptone, 5 g l^{-1} of yeast extract and 10 g l^{-1} of NaCl) at 37°C with 100 μg ml^{-1} ampicillin. Plasmid DNA was purified using QIAGEN Plasmid Kits (Qiagen, Hilden, Germany). Restriction enzymes and other modification enzymes were purchased from Takara Bio (Kyoto, Japan) or Toyobo (Osaka, Japan). KOD Plus (Toyobo) was used as a DNA polymerase for PCR, and the QIAEX Gel Extraction Kit (Qiagen) was used to recover DNA fragments from agarose gels after electrophoresis. *T. kodakaraensis* strains were routinely cultivated under anaerobic conditions in a rich growth medium (MA-YT) containing 30.2 g l^{-1} artificial sea salts (Marine Art SF; Tomita Pharmaceutical, Tokushima, Japan), 5 g l^{-1} yeast extract and 5 g l^{-1} tryptone (*29*). After autoclaving, elemental sulfur powder (Wako Pure Chemical Industries, Osaka, Japan) was added at a concentration of 5 g l^{-1}.

Construction of the *T. kodakaraensis* Δ*phr* strain

Disruption of the *phr* gene (TK2291) of *T. kodakaraensis* by double-crossover homologous recombination was performed using previously described methods (*19*, *20*). The plasmid DNA used for disruption of *phr* was constructed as follows. A DNA fragment containing the *phr*-coding region together with its flanking regions (~1000 bp) was amplified from genomic DNA with the primer set PKHR-L1 (5′-TGTCGTTCCAAAGCCAAAGG-3′) and PKHR-R2 (5′-TGTCTCTCCCTCTTCCCTGG-3′), and inserted into the HincII site of pUC118. Using the plasmid as a template, the flanking regions of *phr* along with the plasmid backbone were amplified using the primer set PKHR-L2 (5′-CCCTTTCC CTAACCCAAAGT-3′) and PKHR-R1 (5′-GAAGTCGTTAAAG GAGAAAG-3′), and the amplified fragment was designated as L-Phr. A PvuII–PvuII restriction fragment (763 bp) containing the *pyrF* marker gene was excised from pUD2 (*20*), and ligated with L-Phr to obtain the plasmid for *phr* disruption (pUPhr). A *T. kodakaraensis* uracil-auxotroph strain, KU216 (Δ*pyrF*) (*20*), was used as the host strain, and transformants exhibiting uracil prototrophy were selected. Genotypes were confirmed by PCR using the primer set phr-US1 (5′-GGCTGGGGAGCGCTAAGG-3′) and phr-DS2 (5′-CCATGGATACATCATGGTC-3′). Genotypes were also confirmed by Southern blot analyses. Two micrograms of genomic DNA from KU216 and a transformant were digested with XbaI, separated by 1% agarose gel electrophoresis and transferred to a nylon membrane Hybond^{TM}-N^+ (GE healthcare, Buckinghamshire, UK). The preparation of specific probes, hybridization and signal detection were performed with the DIG-DNA labeling and detection kit (Roche Diagnostics, Basel, Switzerland) according to the instructions from the manufacturer. The constructed Δ*phr* strain was designated KHR1.

Growth measurements

Growth characteristics of wild-type cells (strain KOD1) and Δ*phr* cells (strain KHR1) were measured as follows. Each strain was precultured in MA-YT medium supplemented with 0.5 g l^{-1} elemental sulfur powder (S^0) (MA-YT-S^0) at 85°C for 10 h. After preculture, cells were inoculated into 10 ml of MA-YT-S^0, and cultured at 75, 80, 85 and 90°C. Cell densities were measured at appropriate intervals at A_{660} with a UV spectrometer Ultraspec 3300 *pro* (GE Healthcare).

Microarray analysis

T. kodakaraensis KOD1 and KHR1 were cultivated at 80°C in MA-YT-S^0. Cells were harvested in the early log phase ($A_{660} \approx 0.2$), and total RNA was extracted using the RNeasy Midi kit (Qiagen). The microarray plate used in this study (Array Tko1) was manufactured at Takara Bio (Otsu, Japan) and covers 2226 genes among the total predicted 2306 genes of *T. kodakaraensis* KOD1 (96.5% coverage). Two identical sets (left and right) were loaded on each plate. Therefore, two sets of data are obtained from each microarray plate. In the data files, individual signal intensity ratios obtained from each set as well as the average ratio value and the SD are shown. The experimental procedure for microarray analysis was performed as described previously (*23*).

Protein methods

Protein concentration was measured using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA), with bovine serum albumin as the standard. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed with 12.5% gels. Western blot analysis was performed using rabbit antibodies raised against recombinant CpkA and CpkB proteins.

Results

Phr ortholog of *T. kodakaraensis*

The *T. kodakaraensis* genome harbors a single Phr ortholog encoded by TK2291. *T. kodakaraensis* Phr (*Tk*-Phr) shares 63.3% overall identity with *P. furiosus* Phr (*Pf*-Phr). The degree of similarity was higher in the amino-terminal DNA-binding domains (residues 1–99 in *Tk*-Phr, 80.4% identical), whereas similarity in the carboxy-terminal domains was relatively lower (residues 100–198 in *Tk*-Phr, 46.5% identical).

Construction of a *phr*-disrupted mutant strain

To clarify the physiological role of *Tk*-Phr and identify genes that are regulated by the protein, a gene disruption strain of *Tk-phr* was constructed. A disruption vector that harbors the *pyrF* gene (pUPHR) was constructed and used to transform the host strain KU216 (Δ*pyrF*) via double-crossover homologous recombination (Fig. 1A). A transformant exhibiting uracil prototrophy was selected and confirmed to have the expected genotype. PCR amplification resulted in a DNA fragment with a length corresponding to that of the Δ*phr* locus (Fig. 1B). The genetic recombination at the Δ*phr* locus and the absence of the *phr* gene on the chromosome was further confirmed by Southern blot analysis (Fig. 1C), and the mutant strain was designated KHR1.

Growth properties of KHR1

Growth characteristics of the KHR1 strain at various temperatures were examined and compared with those of the wild-type strain, KOD1 (Fig. 2). At the optimal growth temperature of 85°C, the two strains exhibited similar specific growth rates. We noticed that a decrease in cell density observed in the stationary phase was less evident in the KHR1 cells than in the KOD1 cells. The difference was more apparent when cultivation temperatures were lower. In contrast, the growth characteristics of these strains were almost indistinguishable at 90°C.

SDS–PAGE analysis of KHR1 cell-free extract

Pf-Phr is presumed to be a transcriptional regulator that represses HSP genes in cells under non-heat stress conditions (*15*, *16*). If *Tk*-Phr has a similar function, derepression of HSP genes should occur in the knockout strain, resulting in overproduction of HSPs under normal growth temperatures. To examine whether this was the case, cell-free extracts from KHR1 cells grown at 80°C, a suboptimal growth temperature for *T. kodakaraensis*, were analyzed by SDS–PAGE and compared with those of the wild-type strain. As a result, we observed a dramatic increase in the levels of at least nine proteins in the KHR1 strain (Fig. 3, arrows). We identified the nine proteins by determining their N-terminal amino acid sequences. The proteins were products of TK1157, TK0131, TK1492, TK2294, TK1591, TK1155, TK0750, TK1121 and TK1746. Among these, TK1155 (encoding sHSP) and TK1157 (encoding AAA^+ ATPase) are the orthologs of *P. furiosus* PF1883 and PF1882, respectively, both of which have been shown to be regulated by *Pf*-Phr in *P. furiosus* (*15*). TK1121 encodes a heat-inducible prefoldin α-subunit, whose

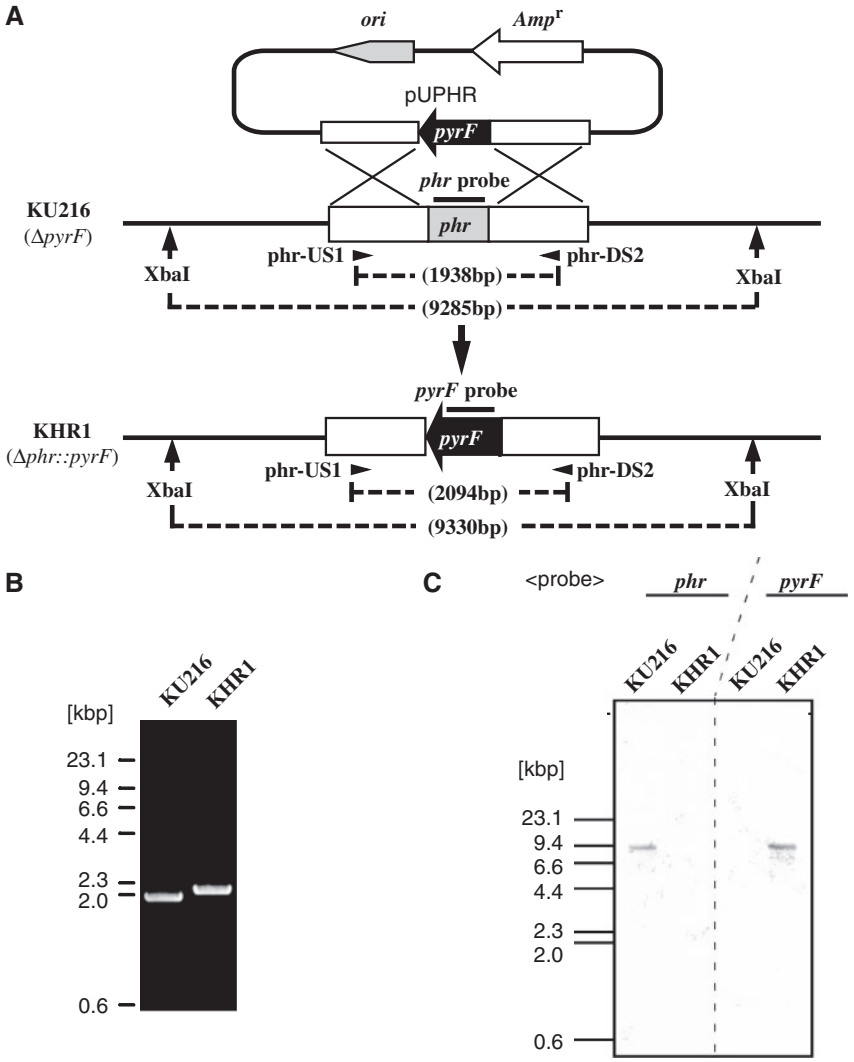

Fig. 1 (A) A schematic diagram illustrating the expected homologous recombination that should occur using the disruption vector pUPHR. The homologous 5′- and 3′-flanking regions of *phr* (1000 bp each) are shown in white boxes. The positions and orientations of primers used for PCR analysis are indicated by arrowheads. The expected lengths of linear DNA fragments amplified by PCR or obtained by XbaI digestion are indicated. (B) Confirmation of gene disruption by PCR. PCR analyses of the *phr* locus with the primer set phr-US1/phr-DS2 using the genomic DNA of the KU216 strain (left lanes) and the KHR1 strain (right lanes) as a template. (C) Confirmation of gene disruption by Southern blot analyses. Southern blot analyses of XbaI digested genomic DNA of KU216 and the KHR1 using probes corresponding to regions within the coding regions of *phr* (left lanes) and *pyrF* (right lanes).

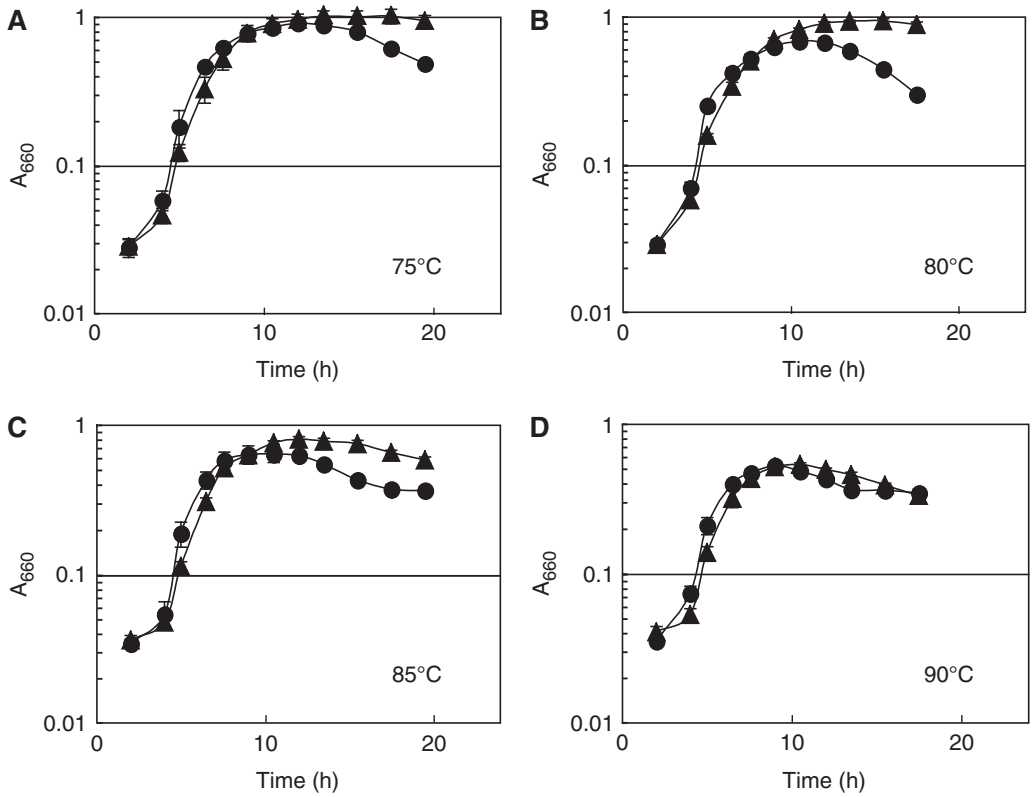

Fig. 2 Growth of *T. kodakaraensis* KOD1 (circles) and KHR1 (triangles) in MA-YT medium containing 0.5 % (w/v) S^0. Batch cultivations of each strain were carried out at 75°C (A), 80°C (B), 85°C (C) or 90°C (D). Error bars represent standard deviations for three independent experiments. A_{660}, Absorbance at 660 nm.

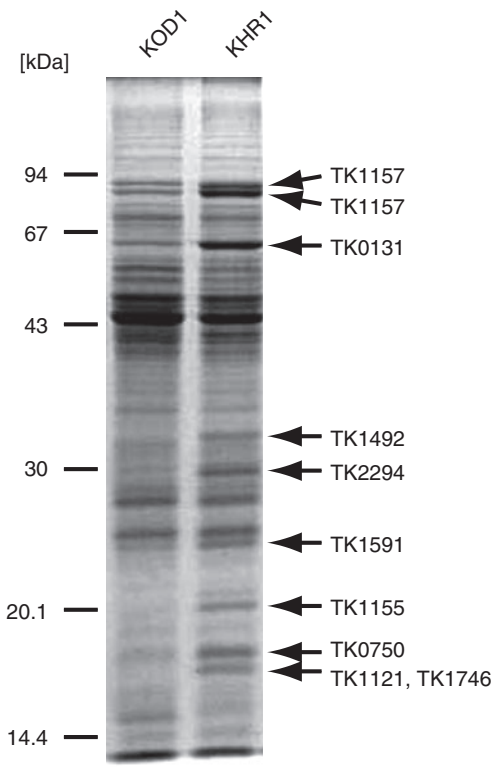

Fig. 3 SDS–PAGE analysis (12.5% acrylamide) of cell-free extracts obtained from KOD1 (wild-type) and KHR1 (Δ*phr*) strains cultivated at 80°C. Ten micrograms of protein were applied in each lane. The protein bands identified by N-terminal amino acid analysis are indicated by arrows together with their ORF number.

transcription was recently found to be under the control of *Tk*-Phr (*30*). The remaining six ORFs encode either conserved or hypothetical proteins whose functions are currently unknown.

Transcriptome analysis of the KOD1 and KHR1 strains

The results of the protein analysis suggest that *Tk*-Phr functions in *T. kodakaraensis* as a negative regulator for a group of genes which include chaperone genes. In order to determine the entire set of genes that are regulated by *Tk*-Phr (the Phr regulon), a whole genome microarray analysis was performed using the wild-type strain KOD1 and KHR1, both grown at 80°C (see Supplementary Table S1). A scatter plot analysis (Fig. 4) showed that transcript levels of dozens of genes increased significantly in KHR1 compared to KOD1. The number of ORFs that displayed an increase of over 2-fold in signal intensity was 39. Among these, 25 ORFs with signal intensity ratios (KHR1/KOD1) >4-fold are shown in Table 1. Several chaperone-like genes were found among the genes with the highest ratios, such as TK1121 (prefoldin, α subunit), TK1155 (sHSP), TK1157 (AAA^+ ATPase, CDC48/VCP type) and TK1590 (Predicted ATPase, RecA superfamily), which may reflect a strong repression of these genes by Phr under non-heat stress conditions. A sharp increase in signal intensity ratio was also observed for TK1122 (prefoldin, β subunit), which together with the adjacent TK1121 constitute a heat-inducible prefoldin operon (*30*). TK1199

encodes a TBP-interacting protein, Tip49, a member of the AAA^+ family of helicases. It is worthy to note that an orthologous gene of TK1199 in *A. fulgidus* (AF1813) is heat-inducible (*31*). TK2279 encodes a bifunctional enzyme (CTP:inositol-1-phosphate cytidylyltransferase and CDP-inositol:inositol-1-phosphate transferase) (*32*, *33*), which is the key enzyme for the synthesis of di-*myo*-inositol-phosphate. This compound is a thermophile-specific compatible solute, the intracellular levels of which in *Thermococcus* spp. increase in response to growth at temperatures above their optimal growth temperatures (*34*). A majority of the remaining ORFs in Table 1 encode either conserved or hypothetical proteins. TK2294, which encodes a conserved protein of the UPF0128 family, is an ortholog of MJ1463 of *Methanocaldococcus jannaschii*. This gene has also been reported to be upregulated upon heat shock (*35*).

In order to confirm the effects brought about by *phr* gene disruption, a second microarray experiment was performed using cells from a separate culture (see Supplementary Table S2). The results obtained were in good agreement with the data described above. For example, among the top 25 ORFs shown in Table 1, 22 ORFs were again found in the top 25 ORFs in the second experiment. Moreover, the top nine ORFs in the two experiments are occupied by the same ORFs. The results strongly suggest that the results of our microarray experiments are biologically relevant.

Our transcriptome analyses imply that *Tk*-Phr functions as a transcriptional repressor for a group of genes that are involved in heat shock response. The consensus DNA binding sequence of *Pf*-Phr has been proposed to be 5′-$TTTN_2TN_2CN_5GN_2AN_2AAA$-3′ (*16*). As the DNA-binding domain of *Tk*-Phr shows a strikingly high similarity to that of *Pf*-Phr, this consensus sequence was employed to search for putative *Tk*-Phr-binding sequences. Of the 25 ORFs shown in Table 1, six of them are clustered with other ORFs found in this table and can be considered to comprise operons with these ORFs. Among the other 19 ORFs with an upstream promoter region, 17 ORFs contained sequences highly similar to the *Pf*-Phr consensus sequence ($\leqslant$4 mismatches) (Table 1). The presence of these sequences suggests that the two Phr proteins recognize similar, if not identical, DNA sequences. Two putative binding sites could be found in each of the promoter regions of TK1155, TK1157, TK1576 and TK1896. In most cases, the putative binding sequences either overlapped or were located downstream of the TATA box sequence, near the transcriptional initiation site. Intriguingly, although transcript levels of TK1493 and TK0803 displayed a significant increase in strain KHR1, potential binding sequences could not be observed in their upstream regions. This may be due to the presence of Phr-binding sequence(s) showing low levels of similarities with the proposed binding motif, or their transcription may be under direct control of other factor(s), which are themselves regulated by Phr.

The 21 putative binding motifs identified in the 17 ORFs shown in Table 1 were aligned, and the consensus motif was represented graphically as a sequence logo (http://weblogo.berkeley.edu/logo.cgi) (Fig. 5). The logo illustrated that, besides nucleotides suggested by Liu *et al.* (*16*), nucleotide A at position 8 and nucleotide T at position 16 were also highly conserved, and may be recognized by *Tk*-Phr. Therefore, a modified motif, 5′-$TTTN_2TNACN_5GTNAN_2AAA$-3′, is proposed here as the consensus DNA binding sequence for *Tk*-Phr. As these two nucleotides are also conserved in all of the *Pf*-Phr-binding sites identified to date, the motif may also apply for *Pf*-Phr.

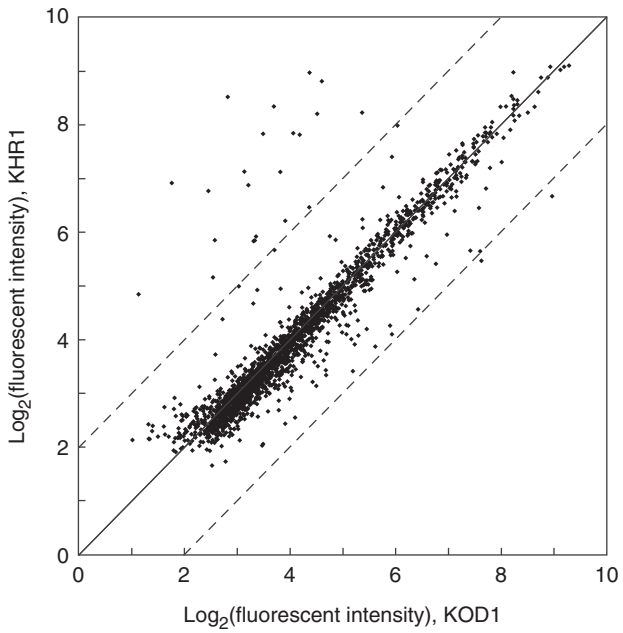

Fig. 4 Scatter plot of signal intensities for each ORF derived from a total RNA sample of KHR1 mutant (Δ*phr*) compared with that of KOD1 (wild-type, control) in a DNA microarray hybridization experiment. The upper and lower dotted lines indicate a 4-fold increase and decrease, respectively, in the signal intensities in the KHR1 cells.

Table 1. Top 25 ORFs whose signal intensities increased in KHR1 at 80°C.

Gene ID	Annotation	Candidate Phr-binding site(s)[a]	Start position[b]	Mismatch bases[c]	Protein analysis[d]	Mean intensity ratio[e] [$\log_2$(KHR1/KOD1) ± SD]
TK1121	Prefoldin, α subunit	TaTttTaACaatggGTaAgaAAA	−43	1	Identified	5.75 ± 0.04
TK1155	Small heat shock protein	TTTggTaACtttcgGagAgaAAA	−73	1	Identified	5.20 ± 0.25
		TTTaaTaACaattgGTgAgaAAA	−32	0		
TK1590	Predicted ATPase, RecA superfamily	aaTggTaACtaaaaGTaAatttA	−37	4	–	4.70 ± 0.02
TK1157	CDC48/VCP homolog, AAA superfamily	TTTggTaACctcactTaAgaAAA	−91	1	Identified	4.65 ± 0.20
		TTTagTaACttacgGTaAccAAA	−41	0		
TK1097	Conserved protein	TTTaaatACaaaatGTtAttAAA	−53	1	–	4.39 ± 0.01
TK0750	Conserved protein	TTTatatACtcaagGTtActAAA	−56	1	Identified	4.36 ± 0.29
TK0131	Conserved protein, DUF505 family	TTTaaaaACctgttGTtAatAAA	−54	1	Identified	4.26 ± 0.10
TK1591	Predicted transcription regulator, encoded next to RecA superfamily ATPase	OP (with TK1590)	–	–	Identified	4.04 ± 0.14
TK1492	Conserved protein	TTTatTttCttcagGTaAgaAAA	−38	1	Identified	3.83 ± 0.02
TK1746	Hypothetical protein	aaaatTaACgaaagGTtAatAAA	−57	3	Identified	3.76 ± 0.42
TK1897	Conserved protein, DUF473 family	OP (with TK1896)	–	–	–	3.74 ± 0.00
TK1576	Conserved membrane protein	TTTgaTaACacaaatacAgaAAA	−75	2	–	3.72 ± 0.01
		TaaatTaACctttgGTgAgaAtA	−39	3		
TK1896	Conserved protein, DUF75 family	TTgccTttCcagtgGaaAccAAA	−74	3	–	3.69 ± 0.15
		TTTaaTaACctttgGaaActAAA	−42	1		
TK1096	Conserved membrane protein, DUF340 family	OP (with TK1095)	–	–	–	3.35 ± 0.14
TK1122	Prefoldin, β subunit	OP (with TK1121)	–	–	–	3.33 ± 0.05
TK1199	TBP-interacting protein Tip49 homolog	TTTagTaACcttcgGTattctAA	−42	2	–	2.91 ± 0.07
TK1575	Conserved membrane protein, containing TrkA-C domain	OP (with TK1576)	–	–	–	2.67 ± 0.10
TK2279	Bifunctional sugar nucleotidyltransferase/ phosphatidylglycerophosphate synthase	TTTaaatACtaaggGTtAatttA	−84	3	–	2.62 ± 0.05
TK2294	Conserved protein, UPF0128 family	TTTacTaACctttgGTgAtgAAA	−23	0	Identified	2.57 ± 0.17
TK2295	Hypothetical membrane protein	OP (with TK2294)	–	–	–	2.56 ± 0.16
TK1493	Conserved protein, FUN14 family	–	–	–	–	2.35 ± 0.02
TK0141	Hydrolase, metallo-β-lactamase superfamily	TTaagTaAgcttcgGTtAttttA	−41	4	–	2.15 ± 0.03
TK2165	Conserved protein	TTTatTaACtttggaTtAcaAAA	−42	1	–	2.01 ± 0.04
TK1095	Conserved membrane protein	aTTaaTggCagtcaGTttttcAA	−27	4	–	2.01 ± 0.04
TK0803	ABC-type manganese/zinc transport system, ATPase component	–	–	–	–	2.00 ± 0.01

[a]Capital letters indicate nucleotides that match the modified Phr-binding motif (5′-TTTN$_2$TNACN$_5$GTNAN$_2$AAA-3′); –, no Phr-binding motif; OP, ORFs constituting potential operons with other ORFs in this Table. [b]Positions relative to the translation initiation codon. [c]Numbers of mismatch nucleotides compared with the modified Phr-binding motif. [d]The ORFs whose products were detected by amino-terminus amino acid sequence analysis in Figure 3 are indicated as 'identified'. [e]The mean intensity ratio (Δ*phr*/wild-type) is expressed as a $\log_2$ value with standard deviation (SD).

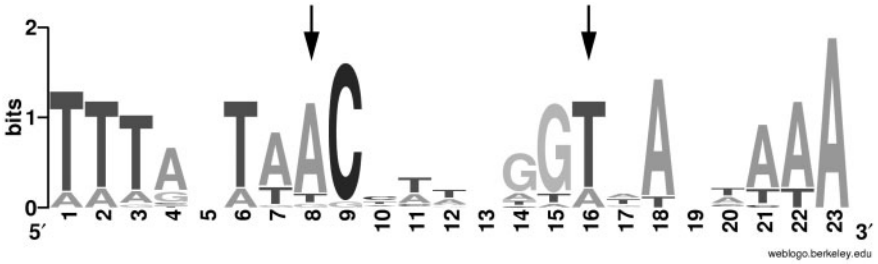

Fig. 5 Sequence logo of putative *Tk*-Phr-binding sequence based on 21 candidate sequences shown in Table 1. The sequence logos were generated using WebLogo (*47*). Positions of a nucleotide pair newly identified as a part of Phr-binding sequences in this study are indicated with arrows.

The *P. furiosus phr* gene itself contains a Phr-binding sequence, and, accordingly, its transcription is activated by heat shock (*15*, *16*). Although our *T. kodakaraensis* microarray analysis cannot examine the change in transcript levels of the *Tk-phr* gene (TK2291) because of the absence of this ORF in KHR1 cells, a putative Phr-binding sequence is present in the TK2291 promoter region ($(^{-37})$TTTN$_2$TN ACN$_5$GTNAN$_2$GAA) (bases matching the above consensus sequence are underlined; numbers in parentheses indicate the positions relative to the initiation codon). This implies that a similar auto-regulation mechanism for Phr is also present in *T. kodakaraensis*.

The microarray analysis also indicated the presence of a small number of genes whose transcript levels decreased in the KHR1 strain. For example, signal levels of an operon composed of TK1024, encoding a hypothetical protein, and TK1025, encoding a putative ATP/GTP-binding protein, both decreased over 4-fold. Signal levels of another operon (TK0166–TK0161) encoding a putative membrane-associated protein complex, and TK2114 encoding a conserved protein also showed a decrease of over 2-fold. When we examined their promoter regions, none of these promoters contained sequences similar to the Phr-binding motif, suggesting that Phr does not directly control these ORFs/operons.

Transcriptional regulation of chaperonin-encoding genes

Archaea harbor group II chaperonin (HSP60) genes, and their up-regulation in response to heat shock have been observed in various species (*31*, *35–39*), including *Pyrococcus* spp. (*40*) and *Thermococcus* spp. (*41*, *42*). *T. kodakaraensis* contains two chaperonin subunits, CpkA (encoded by TK0678) and CpkB (encoded by TK2303) (*43*, *44*). CpkA is abundant at temperatures lower than the optimal growth temperature, while CpkB is mainly expressed at higher temperatures (*41*, *45*). The results of this study reveal that in the case of *cpkB*, slightly higher signal intensities were observed in KHR1 cells [$\log_2$(KHR1/KOD1) = 0.92 ± 0.04], whereas in the case of *cpkA*, transcript levels were nearly equivalent in KHR1 and KOD1 cells [$\log_2$(KHR1/KOD1) = 0.16 ± 0.07]. These data agree with the presence of a weakly related Phr-binding motif in the *cpkB* upstream region [$(^{-67})$GGAN$_2$AN ACN$_5$GTNAN$_2$AAA] and the absence of such a sequence in the *cpkA* upstream region. In order to examine the effects of Phr disruption on the protein levels of these chaperonins, cell-free extracts of KHR1 and KOD1 cells grown at 80°C were subjected to SDS–PAGE, and western blot analysis was carried out with antibodies specific to CpkA and CpkB. Consistent with the results of our microarray experiments, protein levels of CpkB were slightly higher in KHR1 cells compared to those observed in KOD1 cells, while levels of CpkA protein were similar in the two strains (Fig. 6).

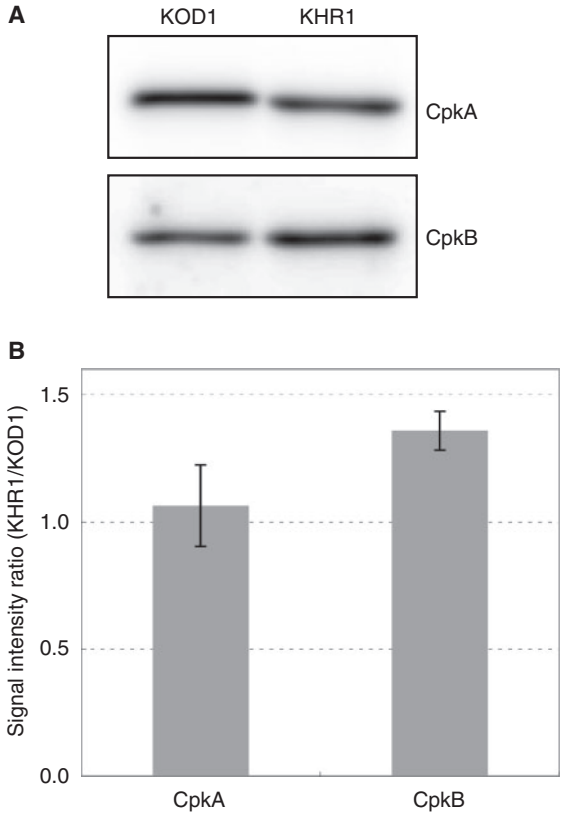

Fig. 6 (A) Western blot analysis of cell-free extracts prepared from *T. kodakaraensis* KOD1 and KHR1 cells using anti-CpkA and anti-CpkB antibodies. Cells were cultivated at 80°C and harvested in the mid-exponential phase. Five micrograms of protein were applied for each lane. (B) The signal intensity ratios of each protein in KHR1 (Δ*phr*) and KOD1 (wild-type) cells. Two biologically independent experiments were performed and error bars represent the difference from the average values.

Discussion

In this study, transcriptomes of a *T. kodakaraensis* Δ*phr* mutant were analyzed and compared with those of the wild-type strain. Disruption of *Tk-phr* resulted in increases in transcript levels for a group of more than 20 genes, including those encoding sHSP (TK1155) and the AAA^+ ATPase (TK1157). The promoter analysis revealed the presence of putative Phr-binding sequences, almost identical to that of *Pf*-Phr, on most of their upstream regions. The present results obtained by *in vivo* analysis are clearly consistent with the model presented for *Pf*-Phr mainly based on *in vitro* research. Furthermore, our data strongly suggest that the function of *Tk*-Phr as a transcriptional factor is global, and is most likely involved in the regulation of various processes of the heat shock response.

Genome sequences imply that the Phr-dependent transcriptional regulation is a ubiquitous event in the order Thermococcales. All sequenced genomes of this order (i.e. *P. furiosus*, *T. kodakaraensis*, *Pyrococcus abyssi*, *Pyrococcus horikoshii*, *Thermococcus onnurineus*, *Thermococcus barophilus, Thermococcus gammatolerans, Thermococcus sibiricus* and *Thermococcus* sp. AM4) contain single Phr orthologs showing high sequence similarity to one another (over 60% identical). In addition, when the genomes of *P. furiosus* and *T. onnurineus* were examined, we found that among the upstream regions of ORFs whose orthologs in *T. kodakaraensis* contain the Phr-binding motif, 9 out of 12 ORFs in *P. furiosus* and 10 out of 11 ORFs in *T. onnurineus* also contained putative Phr-binding motifs, suggesting that analogous Phr regulons are present in these organisms.

In the hyperthermophilic, sulfate-reducing euryarchaeon, *Archaeoglobus fulgidus*, the protein HSR1 (encoded by AF1298) was proposed as a transcriptional factor responsible for heat shock regulation in this organism (*31*). HSR1 is a protein ortholog distantly related to Phr, and both are classified in the same protein family (COG1777). Similar to Phr, HSR1 binds to the upstream region of an operon constituted by three genes (AF1298–AF1296) encoding HSR1 itself, AAA^+ ATPase and sHSP, and also to the upstream region of AF1971, encoding a second sHSP. Similarities are also found in their binding sequences; a palindromic *cis*-acting motif (CTAAC N_5GTTAG) found for HSR1 highly resembles the central part of the modified Phr-binding motif (NTNACN_5GTNAN). As proteins of this COG family are widely distributed in the Euryarchaeota (*12*), this raises the possibilities that the Phr/HSR1-mediated heat shock regulation is an evolutionary-linked mechanism conserved among the members of the Euryarchaeota.

The *Tk*-Phr regulon contains genes that can be presumed to be involved in the response towards high temperature conditions, such as those encoding sHSP, heat-inducible prefoldin, and the key enzyme for compatible solute biosynthesis. On the other hand, it was surprising that a majority of the members in the *Tk*-Phr regulon encode either conserved or hypothetical proteins. Although hyperthermophilic archaea are supposed to have reduced sets of chaperone genes (*46*), the present microarray analysis raises the possibility that *T. kodakaraensis* (and other hyperthermophiles in this order) harbors many, as of yet unidentified, molecular chaperones, some of which are encoded by these ORFs. There is also the possibility that Phr is involved in the response towards other various stress conditions in addition to high temperature, and that the conserved/hypothetical proteins controlled by Phr function in their response.

Although *Tk*-Phr has been shown to regulate an abundant number of genes, its gene disruption did not lead to a dramatic difference in phenotype, at least under the conditions examined in this study. We did notice that the decrease in cell density in the stationary phase observed in wild-type cells was less evident in the KHR1 cells at temperatures of 85°C and lower. Further studies will be necessary to determine what this observation reflects and how it is brought about. At present we can only speculate that the increase in heat-shock proteins or stress-response proteins brought about by Phr inactivation may have a protective effect against cell lysis in the stationary phase.

Although a notable increase (~2-fold) in the transcript levels of *cpkB* was observed in the Δ*phr* strain, these induction levels were far below the levels of other chaperone-encoding heat shock genes. On the other hand, strong transcriptional activations of chaperonin genes were observed by heat shock in *P. furiosus* (*40*) and in *A. fulgidus* (*31*), reaching induction levels comparable to the sHSP- or AAA^+ ATPase-encoding genes. In *T. kodakaraensis* also, a strong induction of the *cpkB* transcripts upon heat shock was observed (*41*, *45*). Therefore, the low induction levels of *cpkB* transcripts in the Δ*phr* strain suggest the presence of another, Phr-independent regulation pathway(s) during heat shock activation of chaperonin genes. Recently, the presence of a Phr-independent pathway was also suggested for the heat-inducible prefoldin gene operon (*pfdCD*, TK1121–TK1122) (*30*). Although Phr seems to play a key role in the heat shock response, the cellular process of heat shock response in *T. kodakaraensis* is more complex, and needs further investigations to elucidate the entire mechanism.

Supplementary data

Supplementary data are available at *JB* online.

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Conflict of interest

None declared.

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