A Mutant Chaperonin That Is Functional at Lower Temperatures Enables Hyperthermophilic Archaea To Grow under Cold-Stress Conditions

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ABSTRACT

Thermococcus kodakarensis **grows optimally at 85°C and possesses two chaperonins, cold-inducible CpkA and heat-inducible CpkB, which are involved in adaptation to low and high temperatures, respectively. The two chaperonins share a high sequence identity (77%), except in their C-terminal regions. CpkA, which contains tandem repeats of a GGM motif, shows its highest ATPase activity at 60°C to 70°C, whereas CpkB shows its highest activity at temperatures higher than 90°C. To clarify the effects of changes in ATPase activity on chaperonin function at lower temperatures, various CpkA variants were constructed by introducing single point mutations into the C-terminal region. A CpkA variant in which Glu530 was replaced with Gly (CpkA-E530G) showed increased ATPase activity, with its highest activity at 50°C. The efficacy of the CpkA variants against denatured indole-3-glycerol-phosphate synthase of *T. kodakarensis* (TrpC$_{Tk}$), which is a CpkA target, was then examined *in vitro*. CpkA-E530G was more effective than wild-type CpkA at facilitating the refolding of chemically unfolded TrpC$_{Tk}$ at 50°C. The effect of *cpkA-E530G* on cell growth was then examined by introducing *cpkA-E530G* into the genome of *T. kodakarensis* KU216 (*pyrF*). The mutant strain, DA4 (*pyrF cpkA-E530G*), grew as well as the parental KU216 strain at 60°C. In contrast, DA4 grew more vigorously than KU216 at 50°C. These results suggested that the CpkA-E530G mutation prevented cold denaturation of proteins under cold-stress conditions, thereby enabling cells to grow in cooler environments. Thus, a single base pair substitution in a chaperonin gene allows cells to grow vigorously in a new environment.**

IMPORTANCE

Thermococcus kodakarensis **possesses two group II chaperonins, cold-inducible CpkA and heat-inducible CpkB, which are involved in adaptation to low and high temperatures, respectively. CpkA might act as an "adaptive allele" to adapt to cooler environments. In this study, we compared the last 20 amino acids within the C termini of the chaperonins and found a clear correlation between the CpkA-type chaperonin gene copy number and growth temperature. Furthermore, we introduced single mutations into the CpkA C-terminal region to clarify its role in cold adaptation, and we showed that a single base substitution allowed the organism to adapt to a lower temperature. The present data suggest that hyperthermophiles have evolved by obtaining mutations in chaperonins that allow them to adapt to a colder environment.**

Chaperonin, also known as heat shock protein 60 (HSP60), belongs to an evolutionarily conserved protein family that enables host cells to survive under stressful conditions, including restricted temperature, high/low salinity, and high/low hyperosmotic pressure. Chaperonins are classified into two subfamilies, group I and group II, on the basis of sequence homology and structural differences (1). The thermosome is a group II chaperonin found in hyperthermophilic archaea, in which it plays a key role in thermal adaptation. Unlike bacterial chaperonins, which function as homotetradecamers, archaeal chaperonins form a heterohexadecamer comprising two back-to-back rings; each of these rings comprises eight heterogeneous chaperonin subunits. Like bacterial chaperonins, archaeal chaperonin monomers comprise three domains: the apical domain (which binds unfolded proteins), the intermediate domain (which acts as a hinge, thereby allowing the movement of the apical domain as well as the transition between *trans* and *cis* conformations needed for chaperonin function), and the equatorial domain (which is responsible for the ATPase and refolding activities in the central cavity of the ringed complex). However, thermosomes do not have cochaperonins, such as GroES. Rather, there is a conserved protrusion from the apical domain, which may play a role in guiding unfolded proteins. Phylogenetic analyses revealed that bacterial chaperonin genes have undergone many rounds of duplication to generate these diverse structures (2). The hyperthermophilic archaeon *Thermococcus kodakarensis* possesses two gene copies of a chaperonin: *cpkA* and *cpkB* (3, 4). CpkB is a classic thermosome, which is an essential heat-inducible factor that enables the cells to survive

TABLE 1 Strains and plasmids used for this study

Strain or plasmid	Relevant characteristic(s)	Source or reference
Strains		
E. coli		
DH5α	F⁻ φ80*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *recA1 endA1 hsdR17*(r_K^- m_K^-) *phoA supE44* λ⁻ *thi-1 gyrA96 relA1*	
BL21(DE3)pLysE	F⁻ *ompT hsdS*$_B$(r_B^- m_B^-) *gal dcm* (DE3)pLysE Camʳ	
T. kodakarensis		
KU216	*pyrF*	24
DA4	*pyrF cpkA-E530G*	This study
Plasmids		
pUD2-cpkAfr	Plasmid used to replace the *cpkA* gene in *T. kodakarensis*	5
pUD2-cpkA-E530G	pUD2-cpkAfr derivative; *cpkA-E530G*	This study
pCPAE	Expression vector for *cpkA* in *E. coli*	20
pCPAE-E530G	pCPAE derivative; carries *cpkA-E530G* mutation; expression vector for *E. coli*	This study
pCPAE-E530M	pCPAE derivative; carries *cpkA-E530M* mutation; expression vector for *E. coli*	This study
pCPAE-Q533G	pCPAE derivative; carries *cpkA-Q533G* mutation; expression vector for *E. coli*	This study
pCPAE-Q533M	pCPAE derivative; carries *cpkA-Q533M* mutation; expression vector for *E. coli*	This study
pCPAE-P538G	pCPAE derivative; carries *cpkA-P538G* mutation; expression vector for *E. coli*	This study
pCPAE-P538M	pCPAE derivative; carries *cpkA-P538M* mutation; expression vector for *E. coli*	This study
pCPAE-D545G	pCPAE derivative; carries *cpkA-D545G* mutation; expression vector for *E. coli*	This study
pCPAE-D545M	pCPAE derivative; carries *cpkA-D545M* mutation; expression vector for *E. coli*	This study

at high temperatures (>85°C). A relative of *T. kodakarensis*, *Pyrococcus* sp., has only one chaperonin, which is an orthologue of CpkB; therefore, this organism has a narrow but high growth temperature range (80 to 100°C). However, CpkA is an atypical archaeal group II chaperonin, which is cold inducible at the stationary phase of cell growth and is not found in *Pyrococcus* sp. A *cpkA* disruptant strain showed poor cell growth at 60°C but no significant growth defect at 85°C and 93°C (5). *T. kodakarensis* indole-3-glycerol-phosphate synthase (TrpC$_{Tk}$ or InGPS) was identified as one of the specific target proteins for CpkA in *T. kodakarensis*. The refolding of partially cold-denatured TrpC$_{Tk}$ was accelerated by the addition of CpkA but not by adding CpkB (6).

CpkA and CpkB are highly homologous (77%), except for their C-terminal regions. The C-terminal region of CpkA harbors tandem repeats of a GGM motif. Such coding tandem repeats are usually involved in protein-protein interactions (7). For example, in *Escherichia coli*, the C-terminal region of GroEL (which is also rich in GGM motifs) is involved in ATP hydrolysis and protein binding (8–12). Langer et al. found that removing the 50 C-terminal amino acid residues of GroEL resulted in a loss of ATPase activity (9). McLennan et al. found that although the 16 C-terminal residues of GroEL were dispensable for *E. coli* cell growth, the truncated protein could not complement the *groEL*(Ts) mutation in *E. coli* (13). Machida et al. reported that the 23 C-terminal residues of GroEL (which are hydrophilic) were not required for chaperonin function (10). However, when the last 6 residues (526-KNDAAD-531) were replaced by a neutral (neither hydrophobic nor hydrophilic) sequence (526-GGGAAG-531) or a hydrophobic sequence (526-IGIAAI-531), chaperonin function was defective both *in vitro* and *in vivo*. Suzuki et al. used a special microscopic method, called zero-mode waveguides, to show that the integrity of the C terminus of the chaperonin region facilitates the transition from the first to the second rate-limiting step (14).

Studies of the C-terminal regions of group II chaperonins have

just begun. Luo and Robb mutated the 15 to 25 C-terminal residues of the *Pyrococcus furiosus* chaperonin, which is highly homologous to CpkB, in an attempt to modify its thermostability and activity (15). An EK-rich motif (528-EKEKEKEGEK-537) was the key domain responsible for increased stability at around 100°C. Zhang et al. found that flexible interwoven termini determined the thermal stability of thermosomes, especially in the β subunit, which has more charged amino acids than the α subunit (16). However, the function of the CpkA C-terminal region remains unknown. In the present study, we found a clear correlation between the CpkA-type chaperonin gene copy number and growth temperature. We introduced single mutations into the CpkA C-terminal region to increase the number of GGM motifs to clarify its role during the evolution of *T. kodakarensis*.

MATERIALS AND METHODS

Microorganisms, media, and growth conditions. The microorganisms (strains) used in this study are listed in Table 1. *T. kodakarensis* strains KU216 (Δ*pyrF*) and DA4 (Δ*pyrF*; carries a *cpkA* variant which encodes Gly in place of Glu530 [*cpkA-E530G*]) were precultivated anaerobically overnight (18 h) at 85°C in 100 ml of MA-YT medium containing the following (per liter): 30.4 g of Marine Art SF reagent, 5 g of yeast extract (Nacalai Tesque, Kyoto, Japan), 5 g of tryptone (Nacalai Tesque), and 5 g of pyruvate (Wako Pure Chemical Industries, Ltd., Osaka, Japan). The culture was added to 20 ml of MA-2YTP medium (30.4 g/liter Marine Art SF reagent, 10 g/liter yeast extract, 10 g/liter tryptone, and 10 g/liter pyruvate) and incubated at a given temperature. All growth studies were carried out three times. For solid medium, 1% Gelrite (Wako, Osaka, Japan) was mixed with 2 ml/liter polysulfide solution (10 g of $Na_2S \cdot 9H_2O$ and 3 g of sulfur flowers in 15 ml of H_2O). *E. coli* DH5α strains were routinely cultivated at 37°C in lysogeny broth (LB) medium. Ampicillin (50 μg/ml) was added to the medium to select transformants.

Alignment of protein sequences and modeling of protein structure. All chaperonin (amino acid) sequences used for sequence alignment were identified and retrieved from the public National Center for Biotechnology Information (NCBI) database (17). The 20 C-terminal amino acids of

TABLE 2 Primers used for this study

Primer	Sequence (5′ to 3′)[a]
cpkA-E530G-Fw	CCCG<u>G</u>AGGCGGCCAGGGCGGCGGAATGCC
cpkA-E530G-Rv	GCCT<u>CC</u>GGGCTTGGTGGCCTTTGCAGCTATGACGTCGTC
cpkA-E530M-Fw	CCC<u>ATG</u>GGCGGCCAGGGCGGCGGAATGCC
cpkA-E530M-Rv	GCC<u>CAT</u>GGGCTTGGTGGCCTTTGCAGCTATGACGTCGTC
cpkA-Q533G-Fw	AGGCGGC<u>GGC</u>GGCGGCGGAATGCCCGGCGGAATGGGCGGAATGGACATGG
cpkA-Q533G-Rv	TCCGCCG<u>CCG</u>CCGCCGCCTTCGGGCTTGGTGGCCTTTGCAGCTATGA
cpkA-Q533M-Fw	GGC<u>ATG</u>GGCGGCGGAATGCCCGGCGGAATGGGCGGAATGGA
cpkA-Q533M-Rv	GCC<u>CAT</u>GCCGCCTTCGGGCTTGGTGGCCTTTGCAGCTATGACGTCGTCGAT
cpkA-P538G-Fw	ATG<u>GG</u>CGGCGGAATGGGCGGAATGGACATGGGCATGTG
cpkA-P538G-Rv	TTCCGCCG<u>CC</u>CATTCCGCCGCCCTGGCC
cpkA-P538M-Fw	ATG<u>AT</u>GGGCGGAATGGGCGGAATGGACATGGGCATGTG
cpkA-P538M-Rv	TTCCGCC<u>CAT</u>CATTCCGCCGCCCTGGC
cpkA-D545G-Fw	GGAATGCCCGGCGGAATGGG<u>C</u>GGAATGGGAATGGGCATGTGA
cpkA-D545G-Rv	CATTCCCATTCCG<u>C</u>CCATTCCGCCGGGCATTCCGCCGCCCTG
cpkA-D545M-Fw	GGAATGCCCGGCGGAATGGGCGGAATG<u>ATG</u>ATGGGCATGTGA
cpkA-D545M-Rv	CAT<u>CAT</u>CATTCCGCCCATTCCGCCGGGCATTCCGCCGCCCTG
cpkA-singlechecking	AATCCCGAAGACCCTCGCTGAAAACGCTGGCCTTGACACCGTCGAGATGCTCGTCAAAGT
cpkA-Fw	TTC**GAATTCGAATTC**TCGCTTCCCTTACG
cpkA-Rv	GAG**GAATTC**CTCCCTTGACCTGCTTATCG
SNP-E530-Fw	CGCCATAGAGAACTTCGCCGAT
SNP-E530-Rv	GGCATTCCGCCGCCCTGGCCGCC<u>AT</u>
SNP-E530G-Rv	GGCATTCCGCCGCCCTGGCCGCC<u>AC</u>

[a] The mutated nucleotides within the *cpkA* gene are indicated by underlining. Introduced restriction sites are indicated in bold.

each chaperonin were then aligned using ClustalW2, supported by the European Bioinformatics Institute.

A three-dimensional (3D) structural octamer of full-length CpkA was built in MODELLER 9.12 (18), using the chaperonin α subunit of *Thermococcus* strain KS-1 (PDB identifier [ID] 1Q2V) as the template. The protein sequence of CpkA was retrieved from the NCBI sequence database. The construction generated three models, and the one with the lowest discrete optimized protein energy score was selected.

DNA manipulation. DNA was isolated and handled according to the standard techniques described by Sambrook and Russell (19). A small-scale preparation of plasmid DNA was prepared from *E. coli* cells by using a GenElute plasmid miniprep kit (Sigma-Aldrich, St. Louis, MO). DNA sequencing was performed using a BigDye Terminator cycle sequencing ready reaction kit, version 3.1, and a model 3130 capillary DNA sequencer (Applied Biosystems, CA). The plasmids and primers used are listed in Tables 1 and 2, respectively.

Construction of expression plasmids encoding *cpkA* variants. pCPAE (20) was used as a template. Briefly, expression plasmids for the *cpkA* variants (encoding E530G, E530M, Q533G, Q533M, P538G, P538M, D545G, and D545M mutations) were generated by site-directed mutagenesis. To construct pCPAE-E530G, PCR was performed using primers cpkA-E530G-Fw and cpkA-E530G-Rv and the amplified DNA was ligated into pCPAE. *E. coli* DH5α cells were transformed with the ligated DNA, and plasmid DNA was extracted from positive transformants. Plasmid construction was confirmed by nucleotide sequencing with the primer cpkA-singlechecking (Table 2). Mutant plasmids pCPAE-E530M, -Q533G, -Q533M, -P538G, -P538M, -D545G, and -D545M were constructed using primers cpkA-E530M-Fw and cpkA-E530M-Rv, cpkA-Q533G-Fw and cpkA-Q533G-Rv, cpkA-Q533M-Fw and cpkA-Q533M-Rv, cpkA-P538G-Fw and cpkA-P538G-Rv, cpkA-P538M-Fw and cpkA-P538M-Rv, cpkA-D545G-Fw and cpkA-D545G-Rv, and cpkA-D545M-Fw and cpkA-D545M-Rv, respectively. The plasmids were then transformed into *E. coli* BL21(DE3) RIL cells.

Protein expression and purification. Recombinant CpkA and its variants were expressed in *E. coli* BL21(DE3) RIL at 25°C as previously described (20). CpkA and its variants remained in the soluble fraction after heat treatment at 85°C for 30 min. Further purification was carried out by anion-exchange chromatography (MonoQ HR 5/5; GE Health-

care, Tokyo, Japan) followed by gel filtration on a Superdex 200 column (GE Healthcare). Recombinant TrpC$_{Tk}$ (*T. kodakarensis* indole-3-glycerol-phosphate synthase; InGPS) was expressed and purified as previously described (21).

Thermostability of CpkA and its variants. The secondary structures of chaperonins were measured using a far-UV circular dichroism (CD) spectroscopy method in wavelength scan mode (220 nm to 260 nm) at 20°C in a J-820 spectrometer (Jasco, Japan), as described previously (6). The thermostability of CpkA and its variants was measured by far-UV CD spectroscopy using a J-820 spectrometer (Jasco, Japan) set in temperature scan mode. Chaperonins were dissolved in 25 mM Tris-HCl (pH 7.8) buffer containing 300 mM KCl (final concentration, 50 μg ml^{-1}), and the change in absorption at 222 nm was monitored from 30 to 100°C. The temperature scan rate was 1°C min^{-1}.

Enzyme assay to examine ATPase activity. ATPase activity was measured by monitoring the amount of phosphate released from ATP by chaperonins. The temperature dependency of the ATPase activity of CpkA and its variants was examined by incubating each chaperonin at 80 nM in HKM buffer (25 mM HEPES-NaOH, 100 mM KCl, 5 mM MgCl$_2$, pH 7.5) at different temperatures (20, 40, 60, 80, and 93°C). The reaction mixtures were preheated to the test temperature for 5 min prior to the addition of 2 mM ATP to trigger the reaction. After 10 min, the reaction was terminated by cooling on ice, and the amount of released phosphate was measured using a Biomol Green kit (Enzo Life Science, NY) (22, 23). One unit of ATPase activity was defined as the amount of enzyme that liberated 1 nmol of inorganic phosphate from ATP in 1 min.

Examination of chaperonin activity. Purified TrpC$_{Tk}$ was chemically unfolded and used as a substrate for chaperonins in a refolding assay as previously described (6). TrpC$_{Tk}$ (16 μM) was denatured on ice for 3 days in 50 mM Tris-HCl buffer (pH 8.0) containing 7 M urea. Next, 2 μl of unfolded TrpC$_{Tk}$ (8 μM) was added to 198 μl of HKM buffer (100-fold dilution) containing 2 mM ATP in the presence or absence of wild-type or variant CpkA. The solutions were incubated for 30 min at 50 or 60°C, and the refolding rates were determined by monitoring the amount of InGPS activity at 50 or 60°C as previously described (6). One unit of InGPS activity was defined as the amount of activity that resulted in the formation of 1 μmol of InGPS in 1 min.

Plasmids used to construct mutant strain DA4. The gene disruption strategy has been described previously (24). Briefly, plasmid pUD2-cpkA-E530G was first constructed by amplifying PCR fragments from the pUD2-cpkAfr plasmid by using primers cpkA-E530G-Fw and cpkA-E530G-Rv. The ligated PCR products were then used to transform *E. coli* DH5α cells. Plasmid DNA was extracted from transformants, and the nucleotide sequence of the *cpkA* region was confirmed by sequencing using the primer cpkA-singlechecking (Table 2). The resulting plasmid was named pUD2-cpkA-E530G and used to construct mutant strain DA4.

Transformation of *T. kodakarensis*. To construct mutant strain DA4 (*pyrF cpkA-E530G*), KU216 was used as the parental strain, and plasmid pUD2-cpkA-E530G was introduced into this strain. Transformation was performed as described in our previous work (6). Genotypes of positive candidates were confirmed by sequencing. The correctly constructed mutant was named strain DA4.

Growth profiles of DA4 and KU216. The growth of strains DA4 and KU216 was monitored by measuring the optical density at 600 nm (OD_{660}) in an automatic Bioshaker (BR-43FH; Taitec, Japan). Cells were first precultured overnight (18 h) at 85°C in MA-YT medium followed by culture in MA-2YTP medium, with a 1% inoculation ratio. The cells were then cultured at the indicated test temperature (50, 60, or 85°C) for several days.

Competitive cell growth assay. KU216 and DA4 were grown in MA-YT medium in separate cultures overnight (18 h) at 85°C. The two strains were then inoculated into fresh MA-2YTP medium (20 ml) at KU216/DA4 ratios of 1:1, 10:1, 10^2:1, and 10^4:1. The mixtures were then cultured at 50°C for 2 days. Each mixed culture was then inoculated into another 20 ml of fresh MA-2YTP medium and incubated at 50°C for another 2 days. Finally, the cells were spread on the MA-2YTP solid medium and incubated at 85°C overnight. Next, 106 colonies were picked at each KU216/DA4 ratio, and the genotypes of the cells were checked. The genotype of cells harboring wild-type *cpkA* was confirmed by PCR using primers SNP-E530-Fw and SNP-E530-Rv, whereas the genotype of cells harboring *cpkA-E530G* was confirmed using primers SNP-E530-Fw and SNP-E530G-Rv. To increase the specificity of the PCR, an extra mismatched nucleotide was introduced into the 3′ ends of primers SNP-E530-Rv and SNP-E530G-Rv (Table 2) according to previously described principles (25); this was done to avoid amplification of mismatches caused by the tandem repeats within the C-terminal region of the *cpkA* gene. The PCR conditions were as follows: initial denaturation for 2 min at 94°C followed by 20 cycles of denaturation at 94°C (15 s), annealing at 62°C (30 s), and extension at 68°C (1 min). GoTaq Green master mix (Promega, WI) was applied to perform the PCR described above. Genomic DNAs were extracted from 10 colonies randomly selected at each KU216/DA4 ratio to test the validity of the PCR described above. Briefly, the *cpkA* gene region was enriched in the first PCR amplification by using primers cpkA-Fw and cpkA-Rv. The genotypes were then confirmed by sequence analysis with the primer cpkA-singlechecking (Table 2).

RESULTS

Sequence alignment and temperature-dependent distribution of chaperonins. Although CpkA and CpkB share high overall sequence identity (77%), they harbor unique differences within the C-terminal region. To examine the role of the C-terminal region, we compared the last 20 amino acids within the C termini of chaperonins expressed by microorganisms at different growth temperatures (Fig. 1). The aligned sequences are shown in Fig. 1A. The C-terminal regions of chaperonins fell into three general clusters: CpkA type, CpkB type, and C type. The CpkA-type chaperonin had a mildly hydrophobic and flexible C terminus comprised primarily of GGM motifs, whereas the CpkB-type chaperonin had a more hydrophilic C-terminal region comprising glutamic acid (Glu [D]), serine (Ser [S]), lysine (Lys [K]), and aspartic acid (Asp

[E]). More interestingly, there were some "promiscuous" chaperonins that fell between these two clusters (C type) (Fig. 1A). C-type chaperonins contained more glycine (Gly [G]), alanine (Ala [A]), and methionine (Met [M]) residues but also retained a considerable number of charged or polar amino acids. In other words, two major types of C-terminal region were identified: one showing increased hydrophobicity and flexibility (CpkA type) and the other showing an increased electrical charge (CpkB type). Some hyperthermophiles, such as *Pyrococcus furiosus* and *Aeropyrum pernix*, harbor only CpkB-type chaperonins. CpkB-type chaperonins seem to be indispensable for the preferred high growth temperature of these organisms. The copy number of the CpkA-type chaperonin tended to increase as the optimal cell growth temperature decreased. This tendency was not unique to archaea (Fig. 1B). The extremely psychrophilic bacterium *Psychromonas ingrahamii*, which shows maximal growth at 15°C and minimal growth at 0°C or lower, harbored two CpkA-type chaperonins and one C-type chaperonin, whereas *Psychrobacter cryohalolentis*, which grows optimally at 22°C, harbored only a CpkA-type chaperonin. Therefore, the appearance of CpkA-type chaperonins appears to be related to adaptation to low temperatures. Microorganisms harboring the CpkA-type chaperonin might adapt to lower growth temperatures more readily than those in which it is absent.

Variant construction and protein stability. The C-terminal tail of GroEL is rich in GGM motifs; however, five of the residues in the tail region of CpkA are neither Gly nor Met (these residues are Pro529, Glu530, Gln533, Pro538, and Asp545). Therefore, to make this region resemble that of GroEL, we replaced Glu530, Gln533, Pro538, and Asp545 with Gly or Met. Pro529 was not replaced because mutation of this residue to Met or Gly would not result in successive GGM motifs. The variant proteins were then expressed in *E. coli* BL21(DE3) RIL and purified as described in Materials and Methods. The molecular masses of the chaperonins were around 60 kDa (see Fig. S1A in the supplemental material). CpkA and its variants showed almost identical CD profiles within the far-UV region (220 nm to 260 nm) at 20°C, suggesting that all possessed very similar secondary structures (see Fig. S1B). Next, we examined the thermostability of each purified protein. The thermal denaturation curves of the purified proteins were determined by monitoring the change in the CD value at 222 nm as the temperature increased from 20 to 100°C at a rate of $5°C \, min^{-1}$. All spectra were corrected to allow for the contribution of the buffer to the signal (Fig. 2A to H). All of the chaperonins showed a major unfolding event at around 90°C. The thermal unfolding plots for CpkA-E530M, -Q533M, -P538M, and -D545G were very similar to that for wild-type CpkA. CpkA-E530G and -Q533M showed slightly lower thermal stabilities, while CpkA-P538G and -D545M showed slightly higher stabilities than that of wild-type CpkA.

Effects of mutations on ATPase activity. Next, we examined the temperature dependency of the ATPase activity of each variant. The results are shown in Fig. 3. CpkA showed the highest ATPase activity between 60 and 70°C. Figure 3A shows that CpkA-E530G showed optimal ATPase activity at 50°C; the value was almost 4-fold higher than that of CpkA at the same temperature. CpkA-E530M showed the most activity at 70°C, although the value was almost half that of wild-type CpkA. The ATPase activity of CpkA-E530M at 50°C was a little lower than that of wild-type CpkA. CpkA-Q533G showed the most activity at 50°C, although it was similar to that of wild-type CpkA. The optimum activity of CpkA-Q533M was displayed at 80°C and was 4-fold higher than
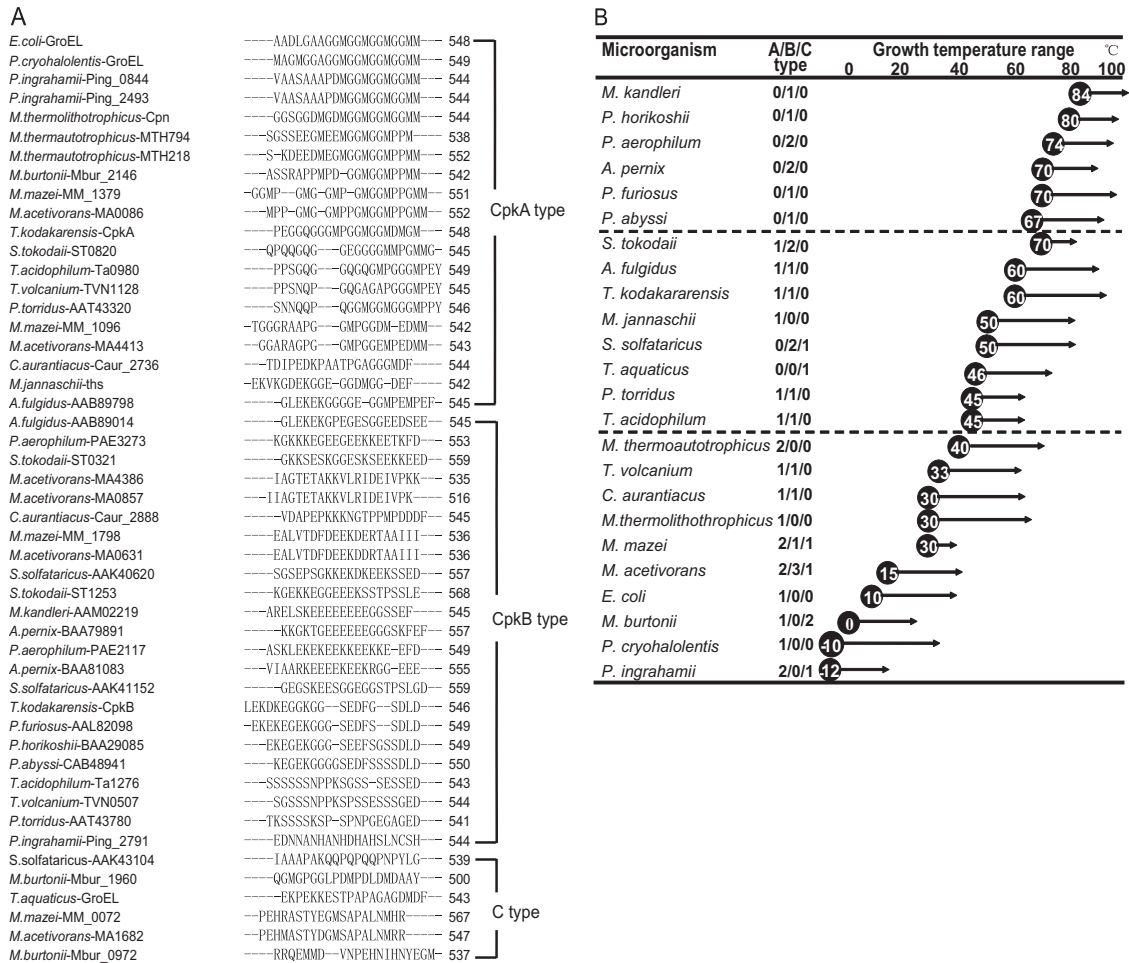
A

E.coli-GroEL	----AADLGAAGGMGGMGGMGGMM--	548
P.cryohalolentis-GroEL	--MAGMGGAGGMGGMGGMGGMM--	549
P.ingrahamii-Ping_0844	--VAASAAAPDMGGMGGMGGMM--	544
P.ingrahamii-Ping_2493	--VAASAAAPDMGGMGGMGGMM--	544
M.thermolithotrophicus-Cpn	--GGSGGDMGDMGGMGGMGGMM--	544
M.thermautotrophicus-MTH794	---SGSSEEGMEEMGGMGGMPPM--	538
M.thermautotrophicus-MTH218	--S-KDEEDMEGMGGMGGMPPMM--	552
M.burtonii-Mbur_2146	--ASSRAPPMPD-GGMGGMPPMM--	542
M.mazei-MM_1379	-GGMP--GMG-GMP-GMGGMPPGMM--	551
M.acetivorans-MA0086	---MPP-GMG-GMPGMGGMPPGMM--	552
T.kodakarensis-CpkA	----PEGGQGGGMPGGMGGMDMGM--	548
S.tokodaii-ST0820	--QPQQGQG--GEGGGGMMGPGMMG-	545
T.acidophilum-Ta0980	---PPSGQG--GQGQGMPGGGMPEY	549
T.volcanium-TVN1128	---PPSNQP--GQGAGAPGGGMPEY	545
P.torridus-AAT43320	---SNNQQP--QGGMGGMGGGMPPY	546
M.mazei-MM_1096	-TGGGRAAPG---GMPGGDM-EDMM--	542
M.acetivorans-MA4413	--GGARAGPG---GMPGGEMPEDMM--	543
C.aurantiacus-Caur_2736	---TDIPEDKPAATPGAGGGMDF----	544
M.jannaschii-ths	-EKVKGDEKGGE--GGDMGG-DEF----	542
A.fulgidus-AAB89798	-----GLEKEKGGGGE--GGMPEMPEF-	545
A.fulgidus-AAB89014	-----GLEKEKGPEGESGGEEDSEE--	545
P.aerophilum-PAE3273	----KGKKKEGEGEEKKEETKFD---	553
S.tokodaii-ST0321	----GKKSESKGGESKSEEKKEED--	559
M.acetivorans-MA4386	-----IAGTETAKKVLRIDEIVPKK----	535
M.acetivorans-MA0857	---IIAGTETAKKVLRIDEIVPK----	516
C.aurantiacus-Caur_2888	---VDAPEPKKKNGTPPMPDDDF--	545
M.mazei-MM_1798	----EALVTDFDEEKDERTAAIII---	536
M.acetivorans-MA0631	----EALVTDFDEEKDDRTAAIII---	536
S.solfataricus-AAK40620	----SGSEPSGKKEKDKEEKSSED--	557
S.tokodaii-ST1253	----KGEKKEGGEEEKSSTPSSLE--	568
M.kandleri-AAM02219	---ARELSKEEEEEEEGGSSEF----	545
A.pernix-BAA79891	-----KKGKTGEEEEEGGGSKFEF--	557
P.aerophilum-PAE2117	----ASKLEKEKEEKKEEKKE-EFD---	549
A.pernix-BAA81083	---VIAARKEEEEKEEKRGG-EEE---	555
S.solfataricus-AAK41152	-----GEGSKEESGGEGGSTPSLGD--	559
T.kodakarensis-CpkB	LEKDKEGGKGG--SEDFG--SDLD---	546
P.furiosus-AAL82098	-EKEKEGEKGGG-SEDFS--SDLD---	549
P.horikoshii-BAA29085	--EKEGEKGGG-SEEFSGSSDLD---	549
P.abyssi-CAB48941	----KEGEKGGGSEDFSSSSDLD--	550
T.acidophilum-Ta1276	----SSSSSSNPPKSGSS-SESSED--	543
T.volcanium-TVN0507	---SGSSSNPPKSPSSESSSGED--	544
P.torridus-AAT43780	---TKSSSSKSP-SPNPGEGAGED--	541
P.ingrahamii-Ping_2791	----EDNNANHAHDHAHSLNCSH---	544
S.solfataricus-AAK43104	----IAAAPAKQQPQPQQPNPYLG--	539
M.burtonii-Mbur_1960	----QGMGPGGLPDMPDLDMDAAY--	500
T.aquaticus-GroEL	----EKPEKKESTPAPAGAGDMDF--	543
M.mazei-MM_0072	--PEHRASTYEGMSAPALNMHR----	567
M.acetivorans-MA1682	--PEHMASTYDGMSAPALNMRR----	547
M.burtonii-Mbur_0972	-----RRQEMMD-VNPEHNIHNYEGM-	537

(CpkA type; CpkB type; C type)

B

Microorganism	A/B/C type	Lowest growth temp (°C)
M. kandleri	0/1/0	84
P. horikoshii	0/1/0	80
P. aerophilum	0/2/0	74
A. pernix	0/2/0	70
P. furiosus	0/1/0	70
P. abyssi	0/1/0	67
S. tokodaii	1/2/0	70
A. fulgidus	1/1/0	60
T. kodakararensis	1/1/0	60
M. jannaschii	1/0/0	50
S. solfataricus	0/2/1	50
T. aquaticus	0/0/1	46
P. torridus	1/1/0	45
T. acidophilum	1/1/0	45
M. thermoautotrophicus	2/0/0	40
T. volcanium	1/1/0	33
C. aurantiacus	1/1/0	30
M.thermolithothrophicus	1/0/0	30
M. mazei	2/1/1	30
M. acetivorans	2/3/1	15
E. coli	1/0/0	10
M. burtonii	1/0/2	0
P. cryohalolentis	1/0/0	10
P. ingrahamii	2/0/1	12

FIG 1 Three types of chaperonin and their distribution in prokaryotes. (A) Alignment of the C-terminal regions (last 20 amino acids) of chaperonins from *Pyrococcus, Thermococcus, Methanococcus, Sulfolobus, Thermus,* and *E. coli*. Chaperonins whose C-terminal regions do not belong to the CpkA or CpkB type are labeled "C type." (B) Distribution of chaperonins according to growth temperature. Prokaryotes examined included *Aeropyrum pernix* (37), *Archaeoglobus fulgidus* (38), *Chloroflexus aurantiacus* (39), *Escherichia coli* (40), *Methanosarcina acetivorans* (41), *Methanococcoides burtonii* (38), *Methanocaldococcus jannaschii* (38), *Methanopyrus kandleri* (38), *Methanococcus mazei* (42), *Methanothermobacter thermautotrophicus* (43), *Methanothermobacter thermolithotrophicus* (44), *Picrophilus torridus* (45), *Psychromonas ingrahamii* (46), *Pyrobaculum aerophilum* (38), *Pyrococcus abyssi* (38), *Psychrobacter cryohalolentis* (47), *Pyrococcus furiosus* (48), *Pyrococcus horikoshii* (38), *Sulfolobus solfataricus* (38), *Sulfolobus tokodaii* (49), *Thermus aquaticus* (50), *Thermococcus kodakarensis* (51), *Thermoplasma acidophilum* (38), and *Thermoplasma volcanium* (38). The numbers in the middle column represent the numbers of CpkA-type, CpkB-type, and C-type genes. The circled numbers indicate the lowest growth temperatures, and the arrows indicate the growth temperature ranges.

that of wild-type CpkA at this temperature (Fig. 3B). CpkA-P538G showed a temperature-dependent profile similar to that of CpkA, although its ATPase activity was almost 2-fold lower. CpkA-P533M showed optimal activity at 70°C, with a value similar to that of wild-type CpkA (Fig. 3C). Finally, CpkA-D545G showed its highest activity at 60°C, with a value equal to that of wild-type CpkA. The optimal activity of CpkA-D545M was detected at 70°C, with a value equal to that of wild-type CpkA (Fig. 3D).

Effects of mutations on chaperonin activity. InGPS from *T. kodakarensis* (TrpC$_{Tk}$) is a target protein for CpkA (6). Because the refolding of unfolded TrpC$_{Tk}$ requires the assistance of CpkA, we performed an *in vitro* refolding assay using chemically unfolded TrpC$_{Tk}$ as the substrate. Purified TrpC$_{Tk}$ (25.4 kDa) (see Fig. S1A in the supplemental material) was denatured in 7 M urea on ice and then refolded at either 50 or 60°C in the presence or absence of wild-type or mutant CpkA. The recovered InGPS activity is shown

in Fig. 4. At 60°C, the InGPS activities recovered by CpkA and CpkA-E530G were almost the same (0.66 and 0.67 U/mg, respectively), but they were only 28% of the native activity (2.39 U/mg). The InGPS activity recovered by CpkA-D545G (0.63 U/mg) was a little lower than that recovered by CpkA and CpkA-E530G. The InGPS activity of refolded TrpC$_{Tk}$ (assisted by these three chaperonins) was almost 7-fold higher than that of spontaneously refolded TrpC$_{Tk}$ (0.1 U/mg). CpkA-E530M, -Q533G, -Q533M, -P538G, -P538M, and -D545M were also able to assist in the refolding of TrpC$_{Tk}$, but the recovered InGPS activities were lower than those with CpkA, CpkA-E530G, and CpkA-D545G. There were significant differences between the chaperonin activities at 50°C. When TrpC$_{Tk}$ was refolded in the absence of CpkA, the specific activity of the refolded TrpC$_{Tk}$ was 0.06 U/mg (about 10% of the native activity [0.58 U/mg]). This increased to 0.25 U/mg (43% of native activity) in the presence of CpkA. Moreover, refolded TrpC$_{Tk}$ showed 1.5-fold higher activity (0.38 U/mg) in
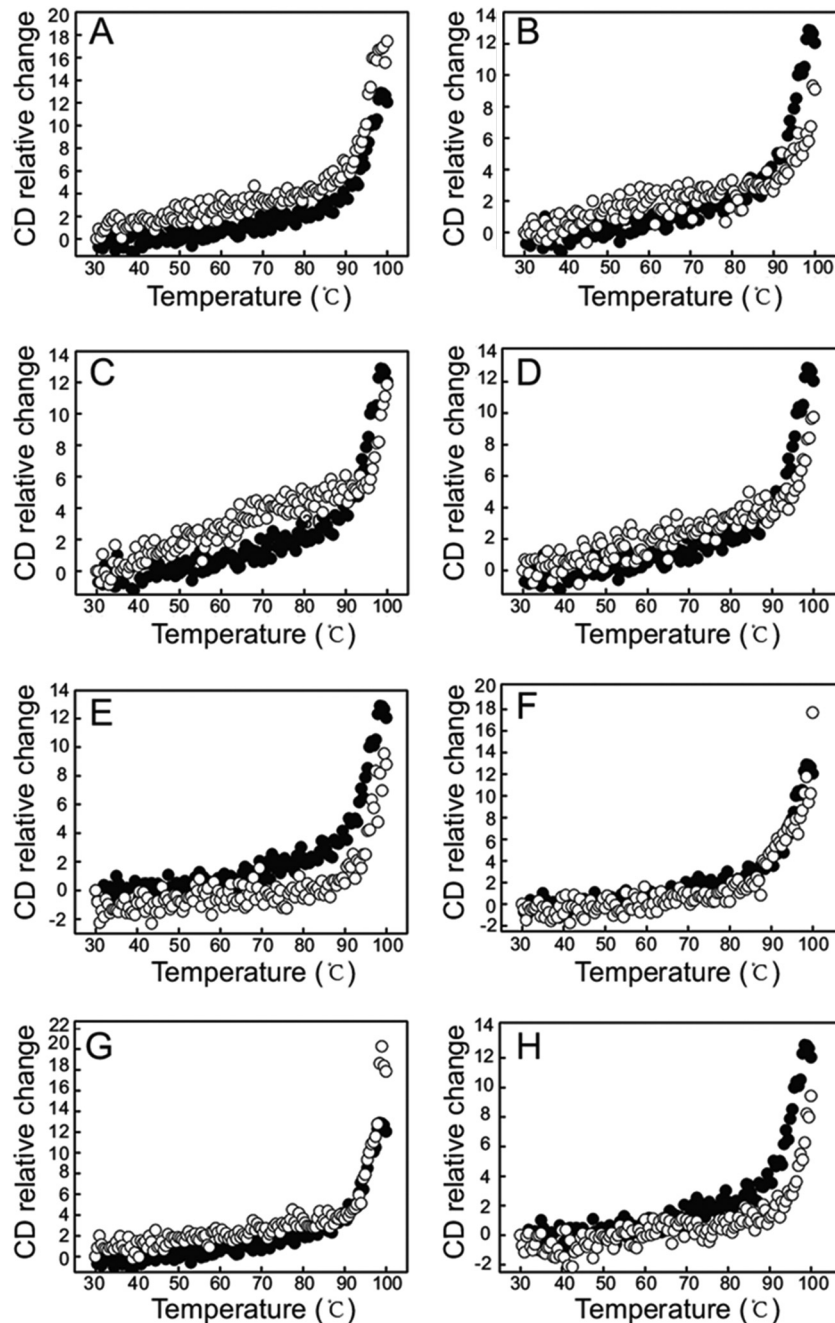
FIG 2 Thermostability of CpkA and its variants. Each sample contained 50 µg ml^{-1} of chaperonin dissolved in 25 mM Tris-HCl (pH 7.8) containing 300 mM KCl. Sample denaturation was monitored by measuring the change in absorption at 222 nm at temperatures from 30°C to 100°C. Data for each sample were plotted after subtracting data obtained at the indicated temperatures. The denaturation of CpkA is indicated by black dots in each frame, whereas that of mutants is indicated by white dots. (A) CpkA-E530G. (B) CpkA-E530M. (C) CpkA-Q533G. (D) CpkA-Q533M. (E) CpkA-P538G. (F) CpkA-P538M. (G) CpkA-D545G. (H) CpkA-D545M.

the presence of CpkA-E530G than in the presence of CpkA. Re-folded TrpC$_{Tk}$ also showed a slightly higher activity (0.3 U/mg) in the presence of CpkA-E530M than in the presence of CpkA. In general, the chaperonin activities of the variants were consistent with their ATPase activities (Fig. 3); i.e., a higher ATPase activity equated to a higher chaperonin activity and vice versa. The InGPS activity of refolded TrpC$_{Tk}$ was not significantly higher in the presence of CpkA-Q533G, -Q533M, -D545G, and -D545M than in the

presence of wild-type CpkA. Moreover, CpkA-P538G and -P538M were less effective than CpkA in terms of refolding of TrpC$_{Tk}$; their ATPase activities were also much lower than that of CpkA.

Growth profiles of strains KU216 and DA4 and growth competition assay results. To observe the effect of the E530G mutation on *T. kodakarensis in vivo*, we constructed strain DA4 (*pyrF cpkA-E530G*) and compared its growth profile with that of the
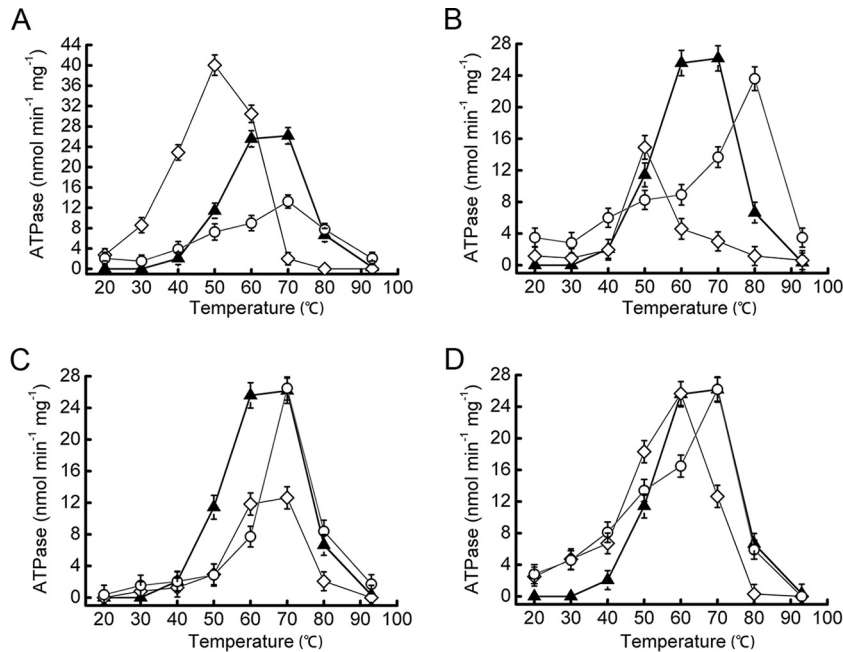
FIG 3 ATPase activities of CpkA and its variants. The temperature-dependent ATPase activities of CpkA (triangles) and its variants (glycine substitutions are indicated by diamonds, and methionine substitutions are indicated by circles) are shown in panels A to D. (A) CpkA, CpkA-E530G, and CpkA-E530M. (B) CpkA, CpkA-Q533G, and CpkA-Q533M. (C) CpkA, CpkA-P538G, and CpkA-P538M. (D) CpkA, CpkA-D545G, and CpkA-D545M.

parental strain KU216. At 85°C, DA4 showed a little higher growth rate during log phase than that of KU216 (Fig. 5A). At 60°C, DA4 showed a slightly shortened lag period in the growth curve (Fig. 5B). However, at 50°C, the lag period of DA4 was much shorter than that of KU216. Strain DA4 started to propagate 30 h earlier and reached the stationary growth phase nearly 80 h earlier than strain KU216 (Fig. 5C). These results indicate that strain DA4 has a growth advantage in a cold-stressed environment.

To examine how dominant the growth of DA4 was at 50°C, we performed a cocultivation experiment. KU216 and DA4 were cultured separately to an OD_{660} of ~0.6. The two cultures were then mixed at different KU216/DA4 ratios (1:1, 10:1, 10^2:1, and 10^4:1), and cell growth was monitored at 50°C. When growth entered log phase (after about 2 days), the cultures were inoculated into fresh medium and cultured for a further 2 days at 50°C. The cultures were then spread onto solid medium and incubated at 85°C overnight until colonies appeared. Finally, 106 colonies were picked and genotyped for each KU212/DA4 ratio. The final results are shown in Fig. 6. At a ratio of 1:1, DA4 was the dominant (~87%) population. However, the percentage of DA4 gradually decreased as the DA4/KU216 ratio decreased (83.5% at a ratio of 10^{-1}:1, 76.5% at 10^{-2}:1, and 64.5% at 10^{-4}:1, respectively). It is striking that DA4 was the dominant strain throughout, even when the starting population was 10^4-fold smaller than that of KU216.

DISCUSSION

The duplication and divergence of α and β chaperonin subunit genes, such as *cpkA* and *cpkB*, in *T. kodakarensis* were thought to have occurred in a common ancestor; the α subunit was subsequently lost from the *Pyrococcus* lineage (26). However, the results of the present study suggest another possibility. The natural habitat of *Thermococcus* organisms is a solfatara, which fluctuates between intermediate (around the solfatara; ~60°C) and high (at the center of the solfatara; ~100°C) temperatures; therefore, these thermophiles have evolved an extra chaperonin that allows them to adapt to a colder environment. Living in an environment that moves between temperature extremes facilitates natural selection; therefore, such thermophiles can evolve quickly (27). Thus, the *Thermococci* adapted to lower temperatures by duplicating the chaperonin α subunit (CpkA). CpkA acts as an "adaptive allele" and enables *T. kodakarensis* to adapt to its changing habitat. In the absence of the *cpkA* gene, it is difficult to grow *T. kodakarensis* at 60°C (5).

Cold tolerance and cold adaptation are related but different behaviors. Cold tolerance usually occurs in response to a temporary drop in temperature, whereupon cold shock proteins (CSPs) enable their hosts to survive. Cold adaptation refers to a longer period of low temperature, triggering a response not only by CSPs but also by many nonstress proteins. However, these nonstress proteins are unstable and incorrectly folded under cold-stress conditions. Chaperonin plays an important role in protein folding and refolding; however, CpkB is not suited to this task under cold-stress conditions, because it has low ATPase activity at temperatures below 60°C (6). Thus, CpkA evolved as a unique cold-inducible chaperonin. CpkA and CpkB differ at the C terminus, which forms part of the equatorial domain. The C-terminal region of CpkA has characteristics similar to those of *E. coli* GroEL in terms of amino acid sequence. CpkA, a cold-inducible thermosome, was classified as a CpkA-type chaperonin belonging to group I chaperonins (which include GroEL of *E. coli*) (Fig. 1A). In contrast, MM_1798 encodes a mesophilic chaperonin that was classified as a CpkB-type chaperonin, along with thermosomes from *Pyrococcus*. Moreover, the growth temperature-dependent distribution of chaperonins (Fig. 1B) and the growth profile of the *cpkA* disruptant (*T. kodakarensis* strain DA1) suggest that CpkA-type
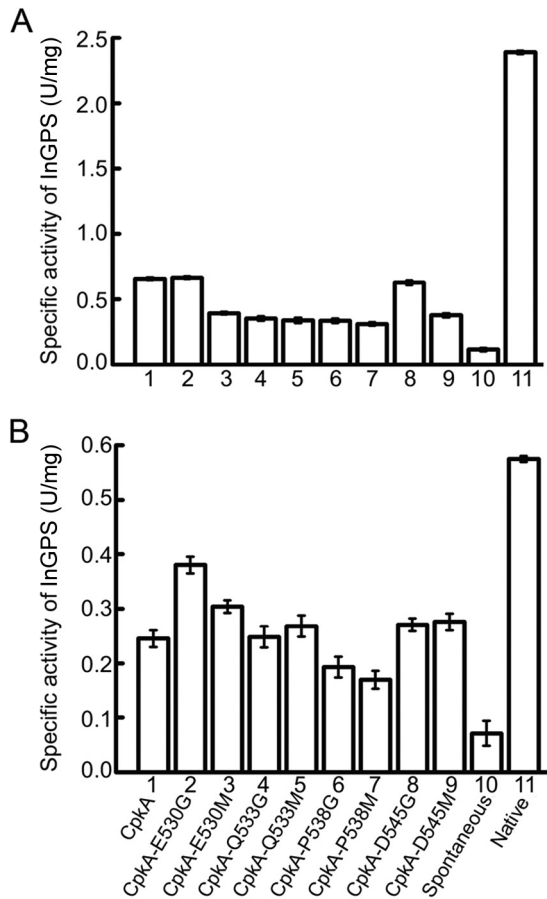
FIG 4 Effects of chaperonins on TrpC$_{Tk}$ refolding. The InGPS activity of refolded TrpC$_{Tk}$ was measured at 60°C (A) and 50°C (B) in the absence or presence of chaperonins. Columns 1 to 9 show the InGPS activities of refolded TrpC$_{Tk}$ in the presence of CpkA, CpkA-E530G, CpkA-E530M, CpkA-Q533G, CpkA-Q533M, CpkA-P538G, CpkA-P538M, CpkA-D545G, and CpkA-D545M, respectively. Column 10 shows the InGPS activity of spontaneously refolded TrpC$_{Tk}$ in the absence of chaperonin. Column 11 shows the activity of native TrpC$_{Tk}$. One unit of specific activity was defined as the amount of activity resulting in the formation of 1 μmol of InGPS in 1 min. All analyses were conducted in triplicate, and mean values are shown.
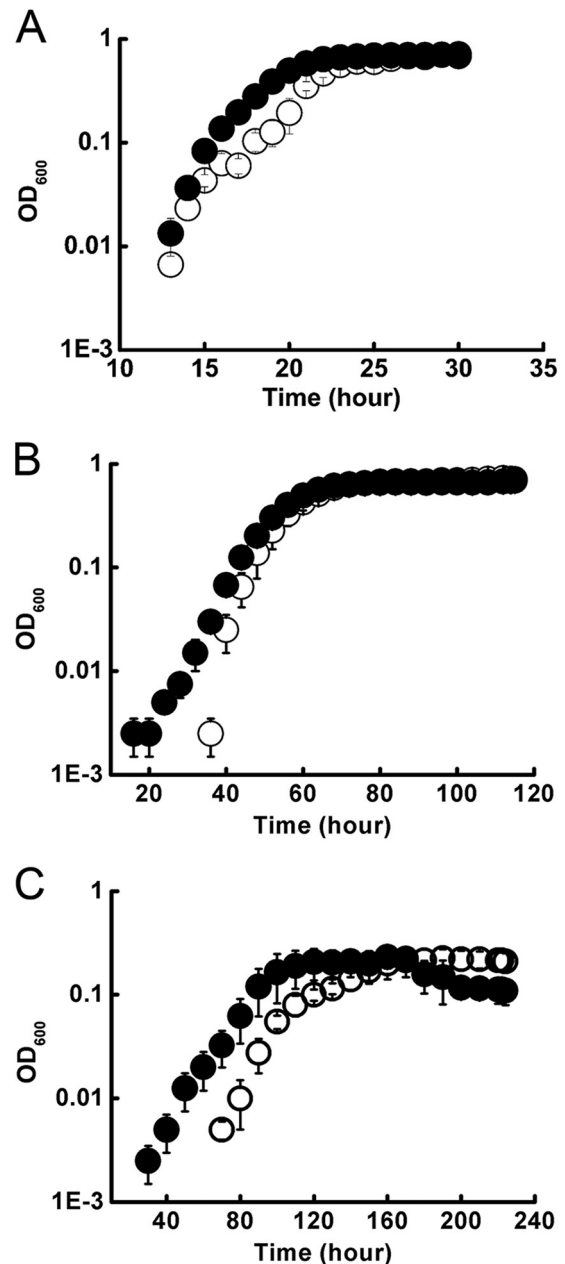

FIG 5 Growth profiles of KU216 and DA4. KU216 (○) and DA4 (●) were cultured in MA-2YTP medium at 85°C (A), 60°C (B), and 50°C (C), and their growth profiles were monitored by measuring the OD$_{660}$.

chaperonins play a key role in cold adaptation. An extra C-type chaperonin in microorganisms might respond to other situations; an example is the TCP1 (CCT)-containing chaperonin in eukaryotic cells, which has up to eight different subunits that interact with different substrates (28). The wide variation in the C-terminal regions of chaperonins enables microorganisms to adapt to changing environments.

In the present study, we constructed eight CpkA variants harboring single amino acid substitutions in the C-terminal region, leading to an increase in the number of GGM motifs. Purified CpkA-E530G showed increased ATPase and chaperonin activities at 50°C *in vitro*. Cold adaptation of *T. kodakarensis* is a multilocus-controlled phenomenon which is fine-tuned by chaperonins (5), transcription factors (29), the composition of the cell membrane (30), and DEAD-box RNA helicase (31). It is thought that beneficial mutations accumulate incrementally via each of these mechanisms and act synergistically to enable adaptation to cold environments. However, when we introduced *cpkA-E530G* into the

genome of *T. kodakarensis*, we were surprised to see that a single base pair substitution enabled *T. kodakarensis* to adapt to a temperature of 50°C; indeed, it showed a shorter lag phase than that of its parental strain (KU216) (Fig. 5C). We believe that the increased ability to adapt to colder conditions correlates with the improved chaperonin activity of CpkA-E530G at 50°C. The C-terminal region of GroEL also plays a role in ATPase activity (9) and substrate binding (12). CpkA has a substrate preference similar to that of GroEL (6). Here we showed that the ATPase and chaperonin activities of CpkA were dependent on the C-terminal region (Fig. 3 and 4). However, because the high flexibility of the C-terminal region means that it cannot be observed within the crystal struc-
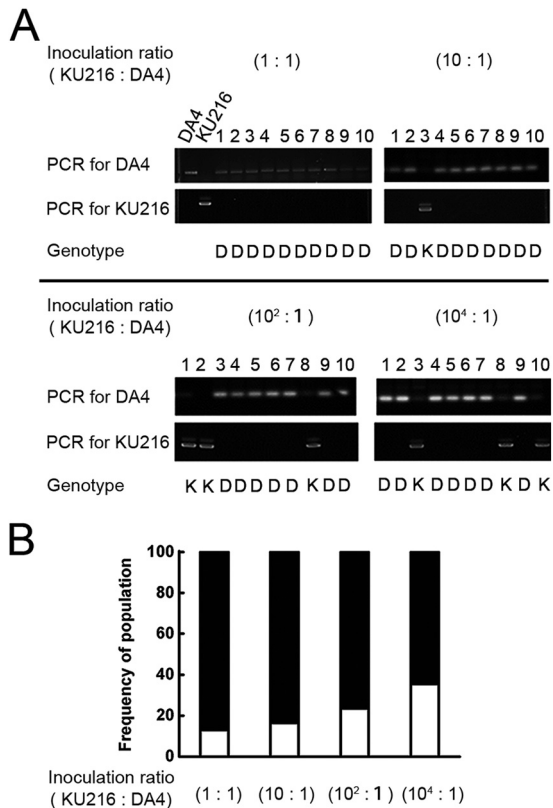
A

B

FIG 6 Dominant growth of DA4 at lower temperatures. KU216 and DA4 cells were precultured separately at 85°C. The two cultures were then mixed at different KU216/DA4 ratios (1:1, 10:1, 10^2:1, and 10^4:1) and cultured at 50°C for 2 days. The cultures were then added to fresh growth medium and cultured for another 2 days. The log-phase cultures were spread onto solid medium and cultured overnight at 85°C. Over 100 colonies for each KU216/DA4 ratio were randomly picked and genotyped by PCR, using primer pairs SNP-E530-Fw/SNP-E530-Rv and SNP-E530-Fw/SNP-E530G-Rv. (A) Genotypes of colonies confirmed by PCR and sequencing. The results for 10 representative colonies for each KU216/DA4 ratio are shown. The results were confirmed by sequencing (shown at the bottom of gels). K, KU216 genotype; D, DA4 genotype. (B) Proportions of DA4 and KU216 cells in cocultures at each KU216/DA4 ratio after culture at 50°C. DA4 and KU216 are indicated by black and white bars, respectively.

ture of the α subunit of the chaperonin from *Thermococcus* sp. strain KS-1 (PDB ID 1Q2V) (32), it is difficult to discuss the structure-function relationships of CpkA, which is an orthologue of the chaperonin in *Thermococcus* sp. KS-1 (99% identical). We did, however, construct a 3D structural model of a full-length CpkA octamer in MODELLER 9.12, using the 1Q2V structure as a template. The results showed that the C-terminal tail of CpkA is fully extended to form the floor of a cleft within the octamer structure (see Fig. S2A in the supplemental material) and that Glu530 faces a short loop between β1 and β2 which is adjacent to the catalytic residue Asp64 (see Fig. S2B). A cryo-electron microscopic study using chaperonins from *Acidianus tengchongensis* also revealed a flat-bottom shape of chaperonin (cpn-α complex) (16). According to the results presented herein, it is not arbitrary to say that the location of the amino acids within the C-terminal region is the primary factor affecting chaperonin function. Replacement of Glu530, which is ~20 Å from the functional ATPase domain of CpkA (see Fig. S2B), by either Gly or Met plays an important role

in increasing chaperonin activity at low temperatures. This distance was also an ideal mutation area in a directed evolution experiment (33). We also found that particular amino acids within the C-terminal region play another important role in cold adaptation. For example, Gly enhanced the ATPase activity at 50°C to a greater extent than that seen with Met. Pro, Ser, and Ala are the most common amino acids in tandem repeats, whereas Met, Ile, and Trp are largely absent (7). Replacing Gly with polar residues (E530G, Q538G, and D545G) increased the ATPase activity at 50°C, whereas replacing Met maintained the ATPase activity above 60°C. Functional movement of the catalytic core responsible for ATPase activity determines the catalytic rate and substrate binding of the chaperonin α subunit of *Thermococcus* KS-1 (32). The replacement of Glu530 with Gly in CpkA increased the ATPase activity and would remove any possible blemish between the intermediate domain and the equatorial domain, such that the functional movement would occur easily compared to that of the wild type (see Fig. S2B). Pro is usually thought of as a "turn maker" or "helix breaker," and it dictates the local structure of a protein by providing extra rigidity (i.e., resulting in lower entropy). Replacing Pro538 with either Gly or Met (CpkA-P538G or CpkA-P538M) would alter the structure of the peptide backbone and push the C-terminal tail in the wrong direction, resulting in reduced ATPase activity. We also tried to replace both Glu530 and Asp545 with Gly (CpkA-E530G/D545G); however, this double mutant (CpkA-E530G/D545G) did not show any additional increase in ATPase activity at 50°C. An alternative evolutionary path would be to increase the copy number of CpkA-type chaperonin genes, thereby increasing chaperonin activity at lower temperatures; indeed, this is the evolutionary pathway taken by some mesophiles and psychrophiles (Fig. 1B).

The final cell yields of DA4 and KU216 were almost similar when the growth entered the stationary phase (Fig. 5). However, DA4 showed a shorter lag phase than that of KU216 at 50°C, suggesting that CpkA-E530G in DA4 could perform its function on target proteins from the very start of the growth cycle, whereas KU216 would take much longer to accumulate correctly folded proteins.

In summary, we identified two growth temperature-dependent trends (correlation between the CpkA-type chaperonin gene copy number and growth temperature) in group II chaperonins, both of which are driven by the initial duplication and subsequent mutation of a chaperonin gene. The tandem repeats within the C-terminal region of CpkA are a mutational hot spot (34). Here we showed that a single base pair substitution (E530G) in the C-terminal region of *T. kodakarensis* CpkA allowed the organism to adapt to a lower growth temperature (50°C). Because chaperonins regulate protein-protein interactions within cells, especially under stress conditions (35), changes in the activity of chaperonins will lead to changes in cell properties, such as cold adaptation. GroEL has a marked effect on the refolding of proteins called mutators, which are involved in DNA repair (36); thus, GroEL might indirectly increase the mutation rate of the host cell. Because GroEL and CpkA have similar substrate biases (6), we expect the role of CpkA in *T. kodakarensis* to resemble that of GroEL. Further *in vivo* studies should aim to derive a mesophilic lineage from hyperthermophiles to better understand cold-adaptive mechanisms in archaea.

ACKNOWLEDGMENTS

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