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Recognizability of heterologous co-chaperones with *Streptococcus intermedius* DnaK and *Escherichia coli* DnaK

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Running Title: Heterologous co-chaperone recognition

List of Abbreviations

Ap: ampicillin

Cm: chloramphenicol

Ec: *Escherichia coli*

Hsps: heat shock proteins

IPTG: isopropyl β-D-1-thiogalactopyranoside

Km: kanamycin

OD: optical density

Si: *Streptococcus intermedius*

Sp: spectinomycin

ABSTRACTS

Streptococcus intermedius DnaK complements the temperature-sensitive phenotype of an *Escherichia coli dnaK* null mutant, only if co-chaperones DnaJ and GrpE are co-expressed. Therefore, we examined whether *S. intermedius* DnaK and *E. coli* DnaK could recognize heterologous co-chaperones *in vitro*. The addition of heterologous GrpE to DnaK and DnaJ partially stimulated ATPase activity, and almost completely stimulated the luciferase refolding activity. Addition of heterologous DnaJ to GrpE and DnaK also stimulated ATPase activity but significant luciferase refolding activity was not observed. Moreover, *E. coli* DnaJ had a negative effect on the luciferase refolding activity of the *S. intermedius* DnaK chaperone system. In *E. coli* chaperone mutants, with the exception of *E. coli* DnaJ higher expression of the heterologous co-chaperones partially or almost completely complemented the temperature-sensitive-phenotype. These results indicated that all heterologous co-chaperones could at least partially recognize DnaK of a distantly related species. A region of the ATPase domain that is present in the DnaK of gram-negative bacteria is absent in the DnaK of gram-positive bacteria. This region is believed to be important for the recognition of co-chaperones

from gram-negative bacteria. However, insertion of this segment into the *S. intermedius* DnaK failed to increase its ability to recognize *E. coli* co-chaperones. This implied that this region might be unnecessary or insufficient for the recognition of *E. coli* co-chaperones. Therefore, our data suggested that a basic structural similarity is conserved among the components of the *S. intermedius* and *E. coli* DnaK chaperone systems, which allow weak associations between heterologous components.

Key words: ATPase, DnaK chaperone system, Luciferase**,** *Streptococcus intermedius*

INTRODUCTION

Living organisms produce heat shock proteins (Hsps) under conditions of stress, such as exposure to heat and chemicals (1). Hsps include molecular chaperones (DnaK/Hsp70, ClpB/Hsp100, GroEL/Hsp60, and others) and several proteases (2, 3). Hsps are involved in the quality control of proteins, such as the folding of newly synthesized proteins and the refolding and degradation of denatured proteins (4). DnaK is a highly conserved molecular chaperone and is composed of an N-terminal nucleotide binding (ATPase) domain, a substrate binding domain, and a C-terminal lid domain (5). The DnaK chaperone system includes DnaK and two co-chaperones (DnaJ and GrpE). The co-chaperones are essential for chaperone activity and promote substrate binding and release of the denatured substrate from DnaK to stimulate ATP hydrolysis (6, 7).

It has been reported that the temperature-sensitive phenotype of *Escherichia coli dnaK* null mutant (Δ*dnaK*) is not complemented by DnaK from gram-positive bacteria (8, 9). *Streptococcus intermedius* is an opportunistic human pathogen that belongs to the anginosus group of streptococci (10, 11). *S. intermedius* Δ*dnaK* mutants exhibit a temperature-sensitive phenotype above 37°C, resulting in slow growth and accumulation

of GroEL (12). The amino acid sequence of *S. intermedius* DnaK is well conserved among the gram-positive bacteria (> 70% identity), conversely the amino acid sequence showed less homology (54% identity) to the DnaK of the gram-negative bacterium *E. coli* (12). *S. intermedius* DnaJ and GrpE show weak homology (48% and 26% identity, respectively) to *E. coli* DnaJ and GrpE (12). However, the J domain and the glycine- and phenylalanine-rich region (G/F region) of DnaJ as well as the C-terminal region of GrpE corresponding to the GrpE protein signature motif are significantly conserved between *S. intermedius* and *E. coli* (12). We previously reported that the temperature-sensitivity of the *E. coli* Δ*dnaK* mutant is not complemented by the expression of *S. intermedius* DnaK alone but that co-expression with the *S. intermedius* co-chaperones DnaJ and GrpE does complement the temperature-sensitive phenotype (12). The *S. intermedius* DnaK chaperone system shows a level of activity nearly equal to that of the *E. coli* DnaK chaperone system, which complements all the defects, including temperature-sensitivity, acid sensitivity, and constitutive heat-shock induction caused by the accumulation of the heat-shock transcription factor σ^{32}, as observed in the *E. coli* Δ*dnaK* mutant (12). In addition, the *S. intermedius* DnaK chaperone system is able to reduce the accumulation

of aggregated proteins observed in the *E. coli* σ^{32} deletion mutant (Δ*rpoH*) such that the level of the proteins is equal to that in the parental strain (12).

The DnaK protein from gram-negative bacteria has a conserved segment in the N-terminal ATPase domain that is characteristic of gram-negative bacteria (12, 13). This segment distinguishes gram-negative bacteria and eukarya from gram-positive bacteria and *Archaea* (14, 15). *S. intermedius* DnaK also lacks this segment, which includes amino acids 75–98 of the ATPase domain in *E. coli* DnaK. Based on the analysis of a deletion mutant of *E. coli* DnaK this segment is believed to have a crucial role in the cooperative function between the *E. coli* co-chaperones DnaJ and GrpE (13).

Only a few DnaK chaperone systems from gram-positive bacteria have been investigated with respect to this property. However, it has been observed that the activity of the DnaK chaperone system in gram-positive bacteria appears to vary with the species (8, 16-18). The *Clostridium acetobutylicum* DnaK chaperone system is able to refold chemically denatured luciferase, albeit with a lower yield than the *E. coli* DnaK chaperone system (16). *Tetragenococcus halophilus* DnaK does not show functional cooperativity with its own co-chaperones and is unable to refold chemically denatured

luciferase (8). For complementation of the *E. coli* Δ*dnaK* mutant temperature-sensitive phenotype, *S. intermedius* DnaK requires the co-expression of both of its own co-chaperones, DnaJ and GrpE, but *Bacillus subtilis* DnaK requires only the co-expression of its own DnaJ (9, 12). These data suggest that the chaperone activity of gram-positive bacterial DnaK and the degree of recognition of heterologous co-chaperones may vary, depending upon the individual species. Therefore, we compared the efficiency of stimulation of the ATPase activity and the luciferase refolding activities of *S. intermedius* DnaK and *E. coli* DnaK in the presence of their respective co-chaperones and with heterologous co-chaperone(s) in both *in vivo* and *in vitro* experiments.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* was grown in 0.5% NaCl (Lennox) or 1.0% NaCl (Miller) lysogeny broth (LB) at the indicated temperatures. Antibiotics were added at the following concentrations: 100 μg/mL ampicillin (Ap), 20 μg/mL chloramphenicol (Cm), 20 μg/mL kanamycin (Km),

and 50 μg/mL spectinomycin (Sp). The antibiotic concentrations were diluted two-fold when multiple antibiotics were added to the culture medium.

Construction of *S. intermedius* (Si) and *Escherichia coli* (Ec) expression plasmids of Si *dnaJ,* Si *grpE,* Si *dnaK-dnaJ*, Ec *dnaJ,* and Ec *grpE*

Genomic DNA (19) and genome sequence information of *S. intermedius* strain NCDO2227 (GenBank accession number: AP010969) was used for the design and implementation of polymerase chain reaction (PCR) amplification for the construction of expression plasmids for Si *dnaJ* and Si *grpE*. The Si *dnaJ* expression plasmid, pZAN43 Si*J*, was constructed, using the following procedure: Primers, Si dnaJ BamHI F and Si dnaJ HindIII R, were used to amplify Si *dnaJ* (Table 2). The amplified fragment was digested with restriction enzymes BamHI and HindIII and cloned into the corresponding sites in pBB535 (20). To construct the Si *grpE* expression plasmid pZE13 Si*E*, primers, Si grpE (ATG) BamHI F and Si grpE PstI R, were used to amplify Si *grpE* (Table 2). The amplified fragment was digested with BamHI and PstI and cloned into the corresponding sites in pZE13 (21). The Si *dnaK-dnaJ* expression plasmid, pZAN43 Si*KJ*, was created

as follows: the Si *dnaK-dnaJ* operon was excised from pZE13 Si*KJ* by BamHI and XbaI digestion (12), and the 3.5-kbp fragment containing the intact Si *dnaK-dnaJ* operon was cloned into the corresponding sites in pBB535.

To construct the Ec *dnaJ* expression plasmid, pZAN43 Ec*J*, the Ec *dnaK-dnaJ* operon in pBB535 was used as the template for PCR amplification. The Ec *dnaJ* gene was amplified using primers, Ec dnaJ BamHI F and Ec dnaJ PstI R (Table 2). The amplified fragment was digested with BamHI and PstI, and cloned into the corresponding sites in pBB535. The Ec *grpE* expression plasmid, pZE13 Ec*E*, was constructed by excising the Ec *grpE* gene from pBB530 (20) by XhoI and XbaI digestion, and the 1.7-kbp fragment containing the intact Ec *grpE* was cloned into the corresponding sites in pZE13.

Development of a strain expressing heterologous co-chaperones using the Ec co-chaperone mutant

To determine whether the over expression of Si *dnaJ* would complement the temperature-sensitive phenotype of the *E. coli* Δ*dnaJ*/Δ*cbpA* mutant AR7222 (22),

AR7222 was transformed with pZAN43 Si*J*. For negative and positive controls, AR7222 was also transformed with empty plasmid pBB529 (20) and pZAN43 Ec*J,* respectively. To determine whether the over expression of Si *grpE* would complement the temperature-sensitive phenotype of the *E. coli* Δ*grpE* strain DA259 (23), DA259 was transformed with pBB529 (which constitutively expresses Lac-repressor) and then transformed with pZE13 Si*E*. For negative and positive controls, DA259 pBB529 was also transformed with empty control plasmid pZE13 and pZE13 Ec*E,* respectively. The effect of complementation on the temperature-sensitive phenotype was estimated by spot test analysis (12).

Development of a strain expressing heterologous co-chaperones using the *E. coli* Δ*dnaK* strain

To determine whether the co-expression of Si *dnaK-dnaJ* and Ec *grpE* would complement the temperature-sensitive phenotype of Ec Δ*dnaK* strain BM271 (24), BM271 cells were co-transformed with pZAN43 Si*KJ* and pZE13 Ec*E*. For negative and positive controls, BM271 pZAN43 Si*KJ* was transformed with empty plasmid pZE13 or pZE13 Si*E*, respectively. The effect of complementation on the temperature-sensitive

phenotype was estimated by spot test analysis.

Construction of expression plasmids for His-tagged recombinants of *S. intermedius* and *E. coli dnaK*, *dnaJ*, and *grpE*

To construct the His-tagged Si *dnaK* expression plasmid, pHIS Si*K*, *S. intermedius* NCDO2227 genome sequence information was consulted and genomic DNA used to enable PCR amplification. The Si *dnaK* was amplified using primers, Si dnaK BamHI F and Si dnaK HindIII R (Table 2). The amplified fragment was digested with BamHI and HindIII, and cloned into the corresponding sites in the N-terminally His-tagged plasmid, pUHE212-1 (25). The His-tagged Si *dnaJ* expression plasmid, pHIS Si*J* was constructed according to the following procedure: The pZAN43 Si*J* was digested with BamHI and HindIII and then cloned into corresponding sites in pUHE212-1. The His-tagged Si *grpE* expression plasmid, pHIS Si*E* was constructed according to the following procedure: The pZE13 Si*E* was digested with BamHI and PstI and then cloned into corresponding sites in pUHE212-1.

The His-tagged Ec *dnaK* expression plasmid, pHIS E*K,* and the His-tagged Ec *dnaJ*

expression plasmid, pHIS Ec*J*, were constructed according to the following procedure: Both pZE13 Ec*K* (12) and pZE13 Ec*J* were digested with BamHI and HindIII, and then cloned into the corresponding sites in pUHE212-1 respectively. To construct the His-tagged Ec *grpE* expression plasmid pHIS Ec*E*, Ec *grpE* was amplified by PCR from the Ec W3110 (26) chromosome using primers, Ec grpE BglII F and Ec grpE PstI R (Table 2). The amplified fragment was digested with BglII and PstI, and then cloned into BamHI and PstI sites in pUHE212-1.

Construction of an expression plasmid for Si DnaK with an inserted segment characteristic of the gram-negative bacterial ATPase domain.

A gram-negative bacterial characteristic segment consists of Arginine 75 to Asparagine 98 of Ec DnaK (12, 13). An expression plasmid was constructed in which this segment was inserted into the corresponding region of Si DnaK (Si DnaK24) between Glycine 72 and Threonine 73, using the In-Fusion HD Cloning Kit (Takara Bio Inc., Shiga, Japan) as follows: The coding region of the gram-negative bacterial characteristic segment and a 15-bp extension (5′) complementary to the ends of the inserted region of

Si *dnaK* (102 bp) were amplified by PCR from pHIS Ec*K* using the primers Infusion 24AA F and Infusion 24AA R. The pHIS Si*K* (4760 bp) was linearized using inverse PCR with primers pHIS SiK F and pHIS SiK R (Table 2). Subsequently, the coding region of a gram-negative bacterial characteristic segment was cloned into the linearized plasmid using the In-Fusion reaction. To construct the negative control plasmid pHIS, the 935 bp coding region of Ec *dnaK* was removed from pHIS Ec*K* by digestion with BamHI and BglII, and the purified plasmid was self-ligated using T4 DNA ligase. The resulting plasmids, pHIS Si*K*24 and pHIS, were used in subsequent experiments.

Construction of a co-expression plasmid for Si GrpE, Si DnaK24, and Si DnaJ

To construct pZE13 Si*EK*24*J* for inducing co-expression of Si *grpE*, Si *dnaK*24, and Si *dnaJ*, the Si *dnaK*24 coding region (843 bp) was PCR amplified from pHIS Si*K*24 using primers Exchange SiK24 F and Exchange SiK24 R. The amplified fragment, which contained the coding region of the gram-negative bacterial characteristic segment, was digested with BsaBI and MluI, and then cloned into corresponding sites in pZE13 Si*EKJ* (12).

Transformation of *E. coli* Δ*dnaK* strain with pHIS Si*K*24 or pZE13 Si*EK*24*J*

To determine whether Si DnaK24 was able to recognize the Ec co-chaperones, *E. coli* Δ*dnaK* strain BM271 containing pDM1.1 (27) was transformed with pHIS Si*K*, pHIS Si*K*24, or control plasmid pHIS. As positive control, BM271 pDM1.1 was also transformed with pHIS Ec*K*. To determine whether Si DnaK24 was able to recognize the Si co-chaperones, BM271 pDM1.1 was transformed with pZE13 Si*EK*24*J*. Moreover, for positive and negative controls, BM271 pDM1.1 was also transformed with pZE13 Si*EKJ* and empty plasmid pZE13, respectively.

Purification of Si and Ec DnaK, GrpE, and DnaJ

Purification of Si and Ec DnaK and GrpE was performed as previously described with minor modifications (8). N-terminus His-tagged DnaK and GrpE were overproduced in *E. coli* using 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) as an inducer. The cells were harvested by centrifugation (8,000 × *g*, 20 min, 4°C) and resuspended in a spheroplast buffer containing 20 mM Tris-HCl (pH 7.6), 1 mM EDTA, 20 (w/v) %

sucrose and 1mg/mL lysozyme. Spheroplasts were disrupted by sonication, using an Astrason Ultrasonic Processor (model XL2020; MISONIX Inc., Farmingdale, NY, USA) with cooling on ice. Unbroken spheroplast cells were lysed by the addition of a 4-fold volume of lysis buffer containing 20 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, and 20 mM imidazole. Cellular debris was removed by centrifugation at 10,000 × *g* for 20 min, and the cell lysate was loaded onto a HisTrap FF column (5 mL; GE Healthcare, Buckinghamshire, UK). The column was washed with 10 volumes of washing buffer containing 20 mM Tris-HCl (pH 7.4), 300 mM NaCl, 50 mM KCl, 10 mM $MgCl_2$, and 20 mM imidazole, and then with 10 volumes of washing buffer supplemented with 1 mM ATP. Proteins were eluted using a linear gradient of 20–250 mM imidazole in 20 mM Tris-HCl (pH 8.0) containing 300 mM NaCl and 0.05 (v/v) % polyoxyethylene hexadecyl ether (Brij58). Brij58 was melted in hot water and then 10 % (v/v) stock solution was prepared by dissolving in ultra-pure water. Peak fractions were 10-fold diluted with 20 mM HEPES-KOH buffer (pH 7.6) containing 5 mM $MgCl_2$, and loaded onto a HiTrap Q HP (GE Healthcare). Proteins were eluted using a linear gradient of 0–1.0 M NaCl in 20 mM HEPES-KOH buffer (pH 8.0) containing 5 mM $MgCl_2$. The purified protein was

subjected to buffer exchange in a HiTrap Desalting column (GE Healthcare) using the buffer containing 20 mM HEPES-KOH (pH7.6), 50 mM KCl, 5 mM $MgCl_2$, and 20 (w/v) % glycerol. The final protein preparations were frozen at −80°C until use.

N-terminus His-tagged Si or Ec DnaJ were also overproduced in *E. coli* using 1 mM IPTG as an inducer. Because Ec and Si DnaJ tend to aggregate under low-ionic conditions (28), it was difficult to purify these proteins using the standard protocol described above. Therefore, we modified the purification method for Ec DnaJ and Si DnaJ, using Ni-NTA agarose and hydroxyapatite columns under high ionic conditions. The cells were harvested by centrifugation (8,000 × *g*, 20 min, 4°C), resuspended in spheroplast buffer, and incubated 30 min on ice. Then, four volumes of high ionic buffer containing 20 mM Tris-HCl (pH7.6), and either 0.3 M $(NH_4)_2SO_4$ for Si DnaJ or 1.0 M $(NH_4)_2SO_4$ for Ec DnaJ, was mixed with each spheroplast suspension. The spheroplasts were disrupted by sonication with cooling on ice. Cellular debris was removed by centrifugation at 10,000 × *g* for 20 min. The cell lysate was loaded onto a cOmplete™ His-Tag Purification Column (5 mL; Sigma-Aldrich Co., St. Louis, MO, USA). Proteins bound to the column were washed with five volumes of washing buffer containing 20

mM Tris-HCl (pH 7.6), 300 mM NaCl, 20 mM imidazole, and 0.05 (v/v) % Brij58P, and then eluted using a linear gradient of 20–250 mM imidazole in 20 mM Tris-HCl buffer (pH 8.0) containing 300 mM NaCl and 0.05 (v/v) % Brij58. The peak fractions were 4-fold diluted with 40 mM potassium phosphate buffer (pH 6.8) containing 5 mM dithiothreitol, 100 mM KCl, 10% glycerol, and 0.05 (v/v) % Brij58P, and loaded onto a hydroxyapatite column (Bio-Scale Mini CHT Type I: Bio-Rad Co., Hercules, CA, USA). Proteins bound to the column were washed with five volumes of washing buffer containing 100 mM potassium phosphate (pH 6.8), 5 mM dithiothreitol, 100 mM KCl, 10 (w/v) % glycerol and 0.05(v/v) % Brij58P, and then eluted by a single step gradient elution buffer containing 500 mM potassium phosphate (pH 6.8), 5 mM dithiothreitol, 100 mM KCl, 10 (w/v) % glycerol and 0.05 (v/v) % Brij58P. The purified proteins were subject to buffer exchange using a HiTrap Desalting column with the buffer containing 20 mM HEPES-KOH (pH7.6), 100 mM KCl, and 10 (w/v) % glycerol. The final protein preparations were frozen at −80°C until use.

ATPase activity assay

Measurement of the ATPase activity of DnaK was performed as previously described with minor modification (13, 29). The reaction mixture (50μL) containing 50 mM HEPES-KOH (pH 7.6), 5 mM $MgCl_2$, 50 mM KCl, 1 mM ATP, along with the indicated concentrations of the individual sets of chaperone proteins. After incubation at 30˚C for suitable times (up to 180 min), a 10 μL aliquot was mixed with 100 μL of BIOMOL Green™ Reagent (Enzo Life Sciences Inc., Farmingdale, NY, USA). The inorganic phosphate released by ATP hydrolysis was spectrophotometrically quantified by measuring the absorbance of 620 nm (A_{620}), according to the manufacturer's instructions. The background value derived from samples without incubation was subtracted from each observed value. The amount of released inorganic phosphate was determined using BIOMOL Green™ Reagent and phosphate standard (Enzo Life Sciences Inc). The velocity of ATP hydrolysis was calculated from the linear part of the reaction product concentration vs. time slope that was plotted using the linear regression method.

Luciferase refolding assays

Luciferase refolding assays were performed as previously described with minor modifications (13, 30). A portion of 100 μM firefly luciferase (Sigma-Aldrich Co., St. Louis, MO, USA) stock solution in 1 M glycylglycine (pH 7.4) was 100-fold diluted with the unfolding buffer containing 25 mM HEPES-KOH (pH 7.6), 50 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, and 6 M guanidinium-HCl, and denatured by incubation at room temperature for 1 h. The denatured luciferase was diluted in refolding buffer containing 28 mM HEPES-KOH (pH 7.6), 120 mM potassium acetate, 1.2 mM magnesium acetate, 2 mM DTT, 8.8 mM creatine phosphate, 7 units/ml creatine phosphokinase, 1 μM AgsA (30), and 1 mM ATP to make a final concentration of 10 nM, in the presence or absence of the indicated sets of chaperones. The refolding reaction was performed at 30˚C for 1 h, and then aliquots (5 μL) were withdrawn and diluted in 245 μL of assay buffer containing 25 mM glycylglycine (pH 7.8), 15 mM $MgSO_4$, and 5 mM ATP. 100 μL of diluted samples were mixed in an equal volume of Luciferase reaction buffer containing 25 mM glycylglycine (pH 7.8), 15 mM $MgSO_4$, 5 mM ATP, and 0.25 mM D-luciferin potassium salt (Wako Pure Chemical Co., Osaka, Japan). Bioluminescence activity was

immediately analyzed in a Junior LB 9509 Portable Tube Luminometer (Berthold Technologies, Bad Wildbad, Germany).

RESULTS

Comparison of ATPase activity and chaperone activity between *S. intermedius* DnaK and *E. coli* DnaK chaperone systems

It has been shown that the *S. intermedius* DnaK chaperone system has nearly equal chaperone activity in an *E. coli dnaK* null mutant, compared to its own chaperone system *in vivo* (12). Therefore, we first compared ATPase activities of *S. intermedius* DnaK and *E. coli* DnaK in the presence or absence of a range of concentrations of their respective co-chaperones (Fig. 1). DnaK from both microorganisms showed similar properties in that the addition of complete sets of their respective co-chaperones (DnaJ and GrpE) strongly stimulated ATPase activity. An increase in the concentration of *S. intermedius* co-chaperone DnaJ significantly stimulated the ATPase activity of Si DnaK in the absence of GrpE. *E. coli* DnaJ also stimulated Ec DnaK in the absence of GrpE, although the effect was lower than that observed for *S. intermedius* DnaJ. Addition of the respective GrpE

alone without DnaJ did not significantly stimulate ATPase activity in either *S. intermedius* DnaK or *E. coli* DnaK. ATPase activities of *S. intermedius* DnaK and *E. coli* DnaK were further stimulated by an increased concentration of their respective DnaJ (from 0.5 to 4.0 μM) in the presence of their respective GrpE (0.5 μM). *E. coli* DnaK was further stimulated in the presence of an increased concentration of its own GrpE (from 0.5 to 4.0 μM), in the presence of its own DnaJ. However, *S. intermedius* DnaK was not further stimulated by increased concentration of its own GrpE. These data suggest that a limiting factor for ATPase activity for both *S. intermedius* and *E. coli* DnaK chaperone systems is DnaJ and that *S. intermedius* GrpE exhibited a weaker effect on the activation of the ATPase activity of its own DnaK than did *E. coli* GrpE.

To compare chaperone activities of the *S. intermedius* DnaK chaperone system and the *E. coli* DnaK chaperone system we measured refolding activities using chemically denatured firefly luciferase, a model substrate used for evaluating DnaK chaperone activity (Fig. 2). In the presence of its own co-chaperones, *E. coli* DnaK restored >90% of the initial, undenatured luciferase activity and *S. intermedius* DnaK restored approximately 80% of luciferase activity. The *S. intermedius* DnaK chaperone system

required a 2-fold higher concentration of DnaK and 4-fold higher concentration of DnaJ for efficient refolding, compared to the *E. coli* DnaK chaperone system. Therefore, the *S. intermedius* DnaK chaperone system appeared to exhibit reduced chaperone activity than the *E. coli* system.

Activation of DnaK ATPase activity by heterologous co-chaperones

Since ATPase activity of both *S. intermedius* DnaK and *E. coli* DnaK was stimulated to similar levels by their respective co-chaperones (Fig. 1), we further examined whether heterologous co-chaperones would stimulate ATPase activity of DnaK (Fig. 3). ATPase activity of both *S. intermedius* DnaK and *E. coli* DnaK was significantly stimulated by higher amounts of heterologous DnaJ in the presence of their own GrpE. However, ATPase activity of *S. intermedius* DnaK was only weakly stimulated by the presence of 20.0 μM *E. coli* GrpE and 0.5 μM of its own DnaJ. In comparison, ATPase activity of *E. coli* DnaK was partially stimulated by 4.0 μM *S. intermedius* GrpE in the presence of 0.5 μM of its own DnaJ. These data indicated that heterologous co-chaperones could partially stimulate the ATPase activity of DnaK, although the degree of activation of DnaK was

dependent on the types (original source) of co-chaperones in the reaction systems.

Activation of DnaK chaperone activity by heterologous co-chaperone

Since heterologous co-chaperones stimulated ATPase activity of DnaK, we next examined whether heterologous co-chaperones could stimulate DnaK chaperone activity. The addition of heterologous GrpE to *S. intermedius* DnaK and DnaJ, or to *E. coli* DnaK and DnaJ, in both cases efficiently stimulated luciferase refolding activity (Fig. 2), although heterologous GrpE was not able to efficiently stimulate ATPase activity of DnaK of both microorganisms in the presence of their own DnaJ (Fig. 3). The addition of heterologous DnaJ to either *S. intermedius* DnaK and GrpE, or to *E. coli* DnaK and GrpE failed to significantly stimulate luciferase refolding activity (Fig. 2). Nevertheless, heterologous DnaJ could stimulate ATPase activity (Fig. 3). In addition, the presence of *E. coli* co-chaperone DnaJ with the Si DnaK chaperone system had a negative effect resulting in a significant reduction in the refolding activity of luciferase by the Si DnaK chaperone system (Fig. 2).

Complementation of the temperature-sensitive phenotype of the *E. coli* co-chaperone mutant by *S. intermedius* co-chaperones

Our *in vitro* data suggested that the heterologous co-chaperones could stimulate not only ATPase activity but also the chaperone activity of DnaK. Therefore, we expressed higher levels of the *S. intermedius* co-chaperone genes (Si *dnaJ*, Si *grpE*) in an *E. coli* co-chaperone null mutant using an IPTG-inducible plasmid (Fig. 4). First, we examined whether higher expression of Si *dnaJ* would complement the *dnaJ* and *cbpA* null mutant (Fig. 4A). It is known that *E. coli* has a *dnaJ* homologue (*cbpA*), which is able to substitute for the function of *dnaJ* (31). Therefore, the *dnaJ* and *cbpA* double (Δ*dnaJ*/Δ*cbpA*) mutant was used for this experiment. The temperature-sensitive phenotype of the Δ*dnaJ*/Δ*cbpA* mutant was complemented only by basal expression levels of *E. coli dnaJ* (Ec *dnaJ*) in the absence of IPTG. Higher amounts of IPTG (> 50 μM IPTG) were required to complement the Δ*dnaJ*/Δ*cbpA* mutation by Si *dnaJ*, although its complementation was only partial with smaller colonies compared to those in complementation of the Ec *dnaJ* strain. Next, we examined whether higher expression of Si *grpE* would complement the temperature-sensitive phenotype of the *E. coli grpE* null

(Δ*grpE*) mutant (Fig. 4B). The temperature-sensitive phenotype of the Δ*grpE* mutant was complemented only by basal expression levels of *E. coli grpE* (Ec *grpE*) in the absence of IPTG. Higher expression of Ec *grpE* had a negative effect, and lost the ability to complement the temperature-sensitive phenotype. While 50 μM IPTG was required to complement the Δ*grpE* phenotype by Si *grpE*, the addition of 100 μM IPTG caused a reduction in the ability to complement. These results indicated that the expression of *S. intermedius* co-chaperones in the *E. coli* co-chaperone mutant could stimulate the *E. coli* DnaK, although a higher concentration of IPTG was required to complement its temperature-sensitive phenotype. These results are consistent with our *in vitro* data shown in Fig. 2 and Fig. 3.

Complementation of the temperature-sensitive phenotype of *E. coli* Δ*dnaK* mutant by expression of *S. intermedius dnaK-dnaJ,* and *E. coli grpE*

We examined whether the expression of *E. coli* GrpE would stimulate *S. intermedius* DnaK *in vivo* in the presence of *S. intermedius* DnaJ (Fig. 5). For this purpose, *E. coli* Δ*dnaK* pZAN43 Si*KJ,* an *E. coli* Δ*dnaK* mutant able to express the *S. intermedius*

dnaK-*dnaJ* operon in an IPTG-inducible plasmid was constructed. Subsequently, the mutant was co-transformed with IPTG inducible plasmids containing *E. coli grpE* (Ec *grpE*) or *S. intermedius grpE* (Si *grpE*). Expression of Ec *grpE* following induction with 100 μM IPTG complemented the temperature-sensitive phenotype of *E. coli* Δ*dnaK* pZAN43 Si*KJ,* the same as the Si *grpE* expressing strain, although a lower concentration of IPTG (<50 μM) was not able to complement its temperature-sensitive phenotype. We also examined whether co-expression of *E. coli dnaJ*, *S. intermedius dnaK*, and *S. intermedius grpE* would complement *in vivo* the temperature-sensitive phenotype of *E. coli* Δ*dnaK*. For this purpose, *E. coli* Δ*dnaK* pZAN43 Si*EK,* an *E. coli* Δ*dnaK* mutant able to express the *S. intermedius grpE*-*dnaK* in an IPTG-inducible plasmid was constructed (data not shown). Transformation of this strain with the *S. intermedius* DnaJ-producing plasmid complemented its temperature-sensitive phenotype. However, transformation of the strain with the *E. coli dnaJ*-inducible plasmid has not thus far been successful (data not shown). Our data showed that *E. coli* DnaJ together with *S. intermedius* GrpE and DnaK was able to inhibit the refolding of luciferase, as shown in Fig. 2. This inhibition might be caused by unknown toxic effect(s) induced in this strain.

Contribution of a gram-negative bacterial characteristic segment in the recognition of *E. coli* co-chaperones

The *S. intermedius* DnaK ATPase domain lacks the 24 amino acids segment between amino acids 75–98 that is present in the *E. coli* DnaK, and is known to be a segment characteristic of the DnaK of gram-negative bacteria and eukarya. It is suggested that this segment has an important role in the recognition of the *E. coli* co-chaperones, since the removal of this segment from *E. coli* DnaK causes a loss of cooperativity with its own co-chaperones (13). Therefore, we inserted this segment into *S. intermedius* DnaK, and investigated the cooperativity with its own co-chaperones and *E. coli* co-chaperones *in vivo* (Fig. 6). Our data showed that co-expression of *S. intermedius dnaJ* and *S. intermedius grpE* with the mutated *S. intermedius dnaK*24 (Si*EK*24*J*) in *E. coli* Δ*dnaK* complemented its temperature-sensitive phenotype up to 41˚C (Fig. 6A). This complementation was only observed on LB Broth Miller (1.0% NaCl) agar plates but was not observed on LB Broth Lennox (0.5% NaCl) agar. Higher ionic concentrations may stabilize cellular proteins and/or the mutated DnaK at higher temperatures. However,

expression of the mutated *dnaK* (*K*24) alone in *E. coli* Δ*dnaK* could not complement the temperature-sensitive phenotype significantly on LB Broth Miller medium (Fig. 6B). We confirmed considerable induction and accumulation of Si DnaK24 in the Δ*dnaK* mutant with 100 μM IPTG, although this amount was less than that of Ec DnaK (supporting information online only‐Fig. S1). These results suggested that the 24 amino acid segment present in *E. coli* DnaK might not have a crucial function, or other regions also might be needed for the recognition of *E. coli* co-chaperones.

DISCUSSION

For previous investigations of DnaK and its co-chaperones, the gram-negative bacterium *E. coli* has been primarily used and therefore relatively few studies using gram-positive bacterial DnaK chaperone systems have been reported (8, 12, 16-18, 32, 33). For example, *Bacillus subtilis dnaK* operon mutants can grow within a temperature range of 16–52˚C and fail to form colonies above 52˚C, in contrast to *E. coli dnaK null* mutants (32). *Tetragenococcus halophilus* DnaK seems to lose most of its function and is unable to stimulate ATPase activity even in the presence of its own co-chaperones and cannot

refold chemically denatured luciferase (8). In contrast, the *Clostridium acetobutylicum* DnaK chaperone system was able to refold chemically denatured luciferase *in vitro*, although the yield was only 22% of that obtained with the *E. coli* DnaK chaperone system (16). The *S. intermedius* DnaK chaperone system also required a 2-fold and 4-fold concentration of DnaK and DnaJ, respectively, for efficient refolding of chemically denatured luciferase compared with the *E. coli* DnaK chaperone system (Fig. 2). These data indicate that the DnaK chaperone system may not equally contribute to the quality control of cellular proteins in gram-positive bacterial species.

Our *in vitro* experiment showed that heterologous DnaJ could significantly stimulate the ATPase activity of DnaK in the presence of its own GrpE, but could not stimulate refolding of chemically denatured luciferase (Fig. 2, 3). These data suggest that heterologous DnaJ may not efficiently deliver denatured luciferase to DnaK for refolding. Heterologous GrpE showed weaker activation than heterologous DnaJ; nevertheless, it was able to efficiently increase luciferase refolding activity of DnaK in the presence of its own DnaJ. Since it is suggested that the function of GrpE is not only as a nucleotide exchange factor, but also that its N-terminal region modulates substrate binding and

release (34-37), this function may also require the luciferase refolding activity of DnaK. Our *in vitro* data indicated that heterologous co-chaperones seem to conserve a basic structural similarity which allows weak association with DnaK despite the lack of strong sequence homology between the heterologous and native co-chaperones (12).

It is known that *T. halophilus* co-chaperones, which do not show strong homology with *E. coli* co-chaperones, are able to complement the temperature-sensitive phenotype, when expressed at high levels in *E. coli* co-chaperone mutants (8). Our *in vivo* data also showed that the expression of *S. intermedius* co-chaperones by IPTG could complement the temperature-sensitive phenotype of the *E. coli* co-chaperone mutants (Fig. 4). In addition, to examine the interaction between *S. intermedius* DnaK and *E. coli* co-chaperones, we developed assay systems using *E. coli* Δ*dnaK* that expressed *S. intermedius dnaK*-*dnaJ* and *E. coli grpE,* or *S. intermedius grpE*-*dnaK* and *E. coli dnaJ* (Fig. 5, data not shown). Higher expression of *E. coli grpE* and *S. intermedius dnaK*-*dnaJ* in *E. coli* Δ*dnaK* complemented the *E. coli* Δ*dnaK* temperature-sensitive phenotype (Fig. 5). On the other hand, the *E. coli dnaJ* expression plasmid was unable to transform *E. coli* Δ*dnaK* that was previously transformed with *S. intermedius grpE*-*dnaK* expression

plasmid (data not shown). Nevertheless, expression of these genes was under tight repressive control by the PA1lacO-1 promoter without IPTG induction (21). Interestingly our data showed that the addition of *E. coli* DnaJ to the *S. intermedius* DnaK chaperone system had a negative effect on luciferase refolding (Fig. 2). Therefore, dysfunction of *S. intermedius* DnaK in the *E. coli* Δ*dnaK* mutant by *E. coli* DnaJ may be toxic in this strain.

A characteristic amino acid segment exists in DnaK of gram-negative bacteria and eukarya (12, 13). Our *in vitro* and *in vivo* results showed that heterologous co-chaperones could partially recognize DnaK (Figs. 2, 3, 4). Insertion of the gram-negative characteristic segment into *S. intermedius* DnaK did not significantly enhance recognition of the latter by *E. coli* co-chaperones (Fig. 6). Therefore, this segment might not have a crucial function, or other regions also might be needed for the recognition of *E. coli* co-chaperones.

Gram-positive bacteria have no outer-membrane, therefore some species (*e.g. Bacillus brevis*) are used for the expression and purification of recombinant proteins that are secreted into the culture medium. It has been reported that not only SecB which is a secretion specific chaperone but also the DnaK chaperone system participates in protein

export in *E. coli* (38). This may be especially true for the secretion of unstable exogenous recombinant proteins, which seem to require higher activity of the DnaK chaperone system. Some data suggest that the DnaK chaperone system from gram-positive bacteria might have lower activity than the *E. coli* system (8, 16, 32). Therefore, overproduction of the exogenous DnaK chaperone system (*e.g. E. coli* DnaK chaperone system) in gram-positive bacteria may be an effective strategy to increase secretion of recombinant proteins.

Overall, our data suggested that a basic structural similarity was conserved among the components of the *S. intermedius* and *E. coli* DnaK chaperone systems, which allowed for a weak association between heterologous components, independent of the gram-negative bacterial characteristic segment.

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DISCLOSURE

The authors have no conflicts of interest associated with this study.

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FIGURE LEGENDS

Fig. 1. Stimulation of the ATPase activity of DnaK by its endogenous co-chaperones. ATP hydrolysis by *S. intermedius* DnaK or *E. coli* DnaK (each 0.5 μM) was assayed in the presence or absence of the indicated concentration (μM) of their own co-chaperones at 30˚C. The relative ATPase activity (Rel. ATPase activity) is indicated as relative value to that of the basal ATPase activity of the *S. intermedius* DnaK protein without co-chaperones which was set as 1.0. White bars, ATPase activity of *S. intermedius* DnaK; black bars, *E. coli* DnaK. Error bars represent the standard deviation for three experiments.

Fig. 2. Luciferase-refolding activity. Chemically denatured luciferase (10 nM) was renatured at 30˚C in the presence of ATP, with the indicated concentration (μM) of proteins: SiK, *S. intermedius* DnaK; SiJ, *S. intermedius* DnaJ; SiE, *S. intermedius* GrpE; EcK, *E. coli* DnaK; EcJ, *E. coli* DnaJ; EcE, *E. coli* GrpE. After refolding at 30˚C for 1 h, luciferase activity was measured as described in Materials and Methods. Refolding activity was calculated and expressed as a percentage of that observed for native luciferase without denaturation with 6M Gdn-HCl with the latter taken as 100%. White

Bars, Refolding activity of SiK containing own DnaJ and/or GrpE. Black Bars, Refolding activity of EcK containing own DnaJ and/or GrpE. Gray bars, Refolding activity of SiK or EcK containing a heterologous co-chaperone. Error bars represent the standard deviation of three experiments.

Fig. 3. Stimulation of the ATPase activity of DnaK by a heterologous co-chaperone.

ATP hydrolysis of 0.5 μM *S. intermedius* DnaK (SiK) or *E. coli* DnaK (EcK) was assayed in the presence of the indicated concentration (μM) of homologous co-chaperone(s) and/or a heterologous co-chaperone at 30˚C. Relative ATPase activity (Rel. ATPase activity) is indicated relative to the basal ATPase activity of *S. intermedius* DnaK protein without co-chaperones with the latter taken to be 1.0. White Bars, ATPase activity of SiK in the presence of its own DnaJ and/or GrpE. Black Bars, ATPase activity of EcK in the presence of its own DnaJ and/or GrpE. Gray bars, ATPase activity of SiK or EcK in the presence of a heterologous co-chaperone. Error bars represent the standard deviation of three experiments.

Fig. 4. Complementation of an *E. coli* co-chaperone mutant by overexpression of the *S. intermedius* co-chaperones.

Cells were cultured for 24 h at 30°C, and then standardized amounts (OD_{600} = 1.0) of cultures were diluted from 10^{-1} to 10^{-5} in LB medium. Two-microliter aliquots were spotted onto LB agar plates containing the indicated concentration of IPTG. The spotted plates were incubated for 24 h at the indicated temperatures. (A) Cont., *E. coli* Δ*dnaJ*/Δ*cbpA* mutant AR7222 transformed with empty control plasmid pBB529; Ec *dnaJ*, AR7222 transformed with pZAN43 Ec*J*; Si *dnaJ*, AR7222 transformed with pZAN43 Si*J*. (B) Cont., *E. coli* Δ*grpE* strain DA259 transformed with LacI expressing plasmid pBB529 and empty control plasmid pZE13. Ec *grpE*, DA259 pBB529 transformed with pZE13 Ec*E*; Si *grpE*, DA259 pBB529 transformed with pZE13 Si*E*.

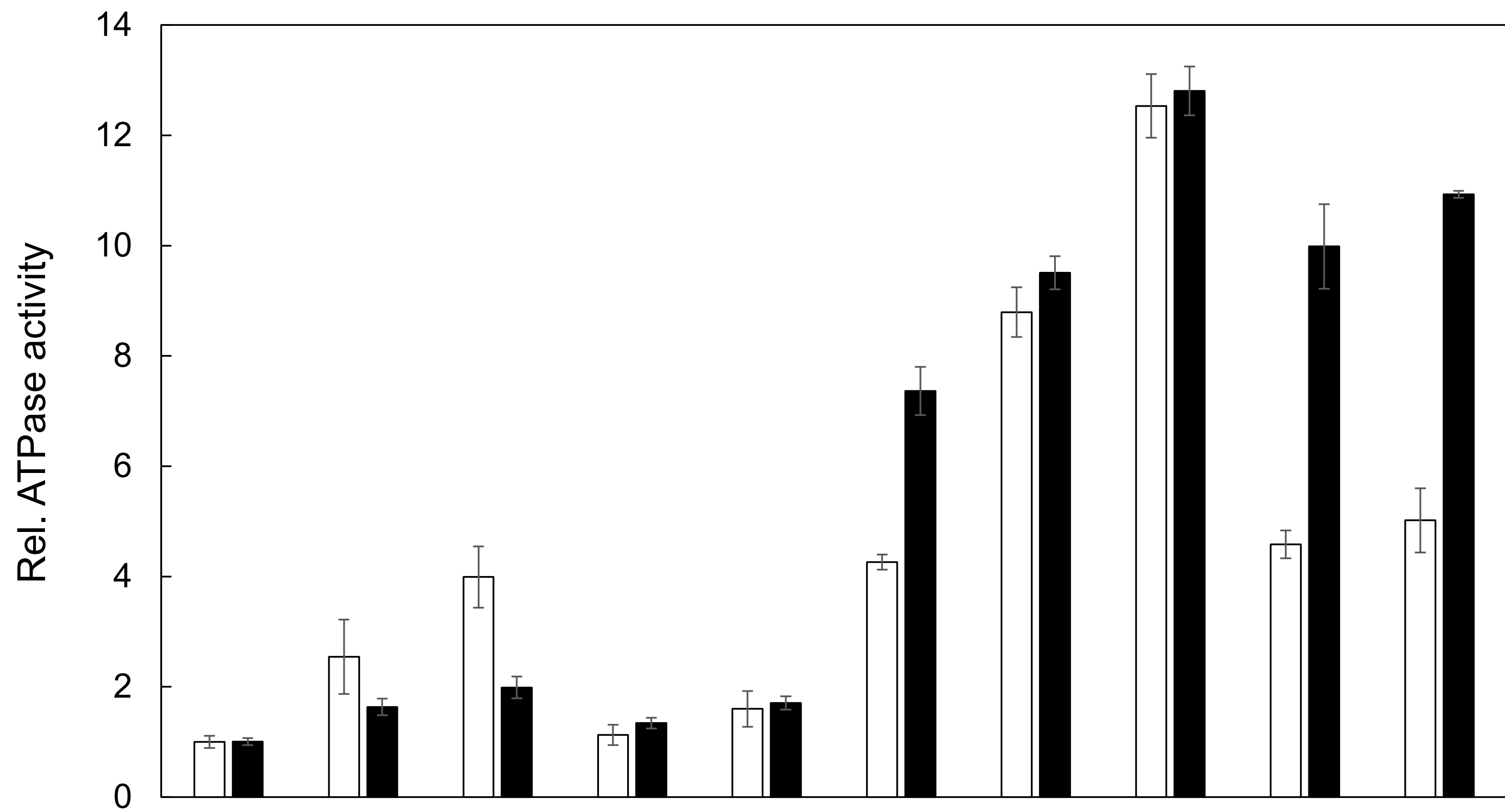
Fig. 5. Complementation of the *E. coli* Δ*dnaK* mutant by the co-overexpression of *S. intermedius* DnaK-DnaJ and *E. coli* GrpE.

Spotted plates were prepared as described in Fig. 4. Cont., Ec Δ*dnaK* strain BM271 transformed with pZAN43 Si*KJ* and empty control plasmid pZE13; Ec *grpE*, BM271

transformed with pZAN43 Si*KJ* and pZE13 Ec*E*; Si *grpE*, BM271 transformed with pZAN43 Si*KJ* and pZE13 Si*E*.

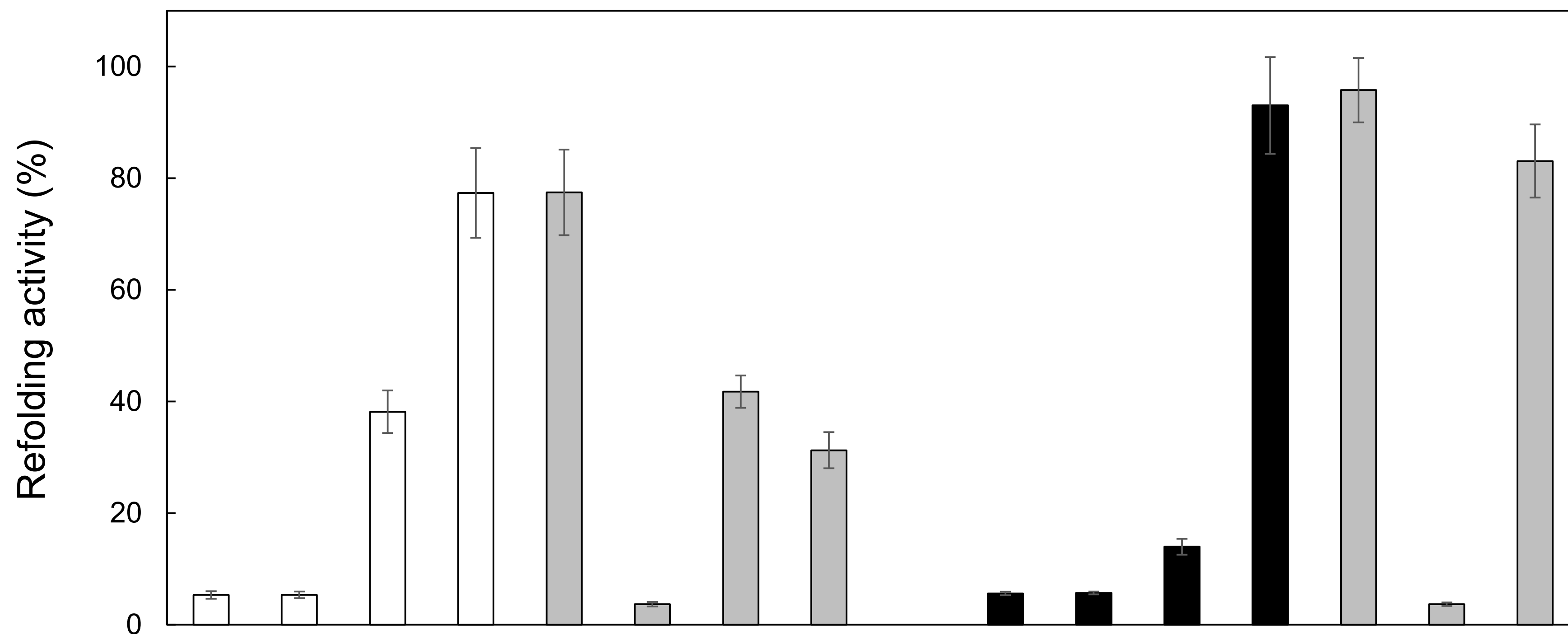
Fig. 6. Complementation of E. coli ΔdnaK by Si DnaK with the 24 amino acids (24AA) characteristic gram-negative bacterial segment (Si*K*24) inserted, with or without *S. intermedius grpE* (Si *grpE*) and *S. intermedius dnaJ* (Si *dnaJ*). Cells were cultured for 24 h at 30°C and then standardized amounts (OD_{600} = 1.0) of cultures were diluted from 10^{-1} to 10^{-5} in LB Miller medium. Two-microliter aliquots were spotted onto LB Miller agar plates containing 100 μM IPTG. (A) The upper illustration shows a schematic drawing of pZAN43 Si*EK*24*J*. ΔK+Vec, Ec Δ*dnaK* strain BM271 transformed with LacI expressing plasmid pDM1.1 and empty control plasmid pZE13; ΔK+Si*EKJ*, BM271 pDM1.1 transformed with pZAN43 Si*EKJ*; ΔK+Si*EK*24*J*, BM271 pDM1.1 transformed with pZAN43 Si*EK*24*J*. (B) The upper illustration shows a schematic drawing of pHIS Si*K*24. ΔK+Vec, Ec Δ*dnaK* strain BM271 pDM1.1 transformed with the negative control plasmid pHIS; ΔK+Ec*K*, BM271 pDM1.1 transformed with pHIS Ec*K*; ΔK+Si*K*, BM271 pDM1.1 transformed with pHIS Si*K*; ΔK+Si*K*24, BM271 pDM1.1 transformed with

pHIS Si*K*24.



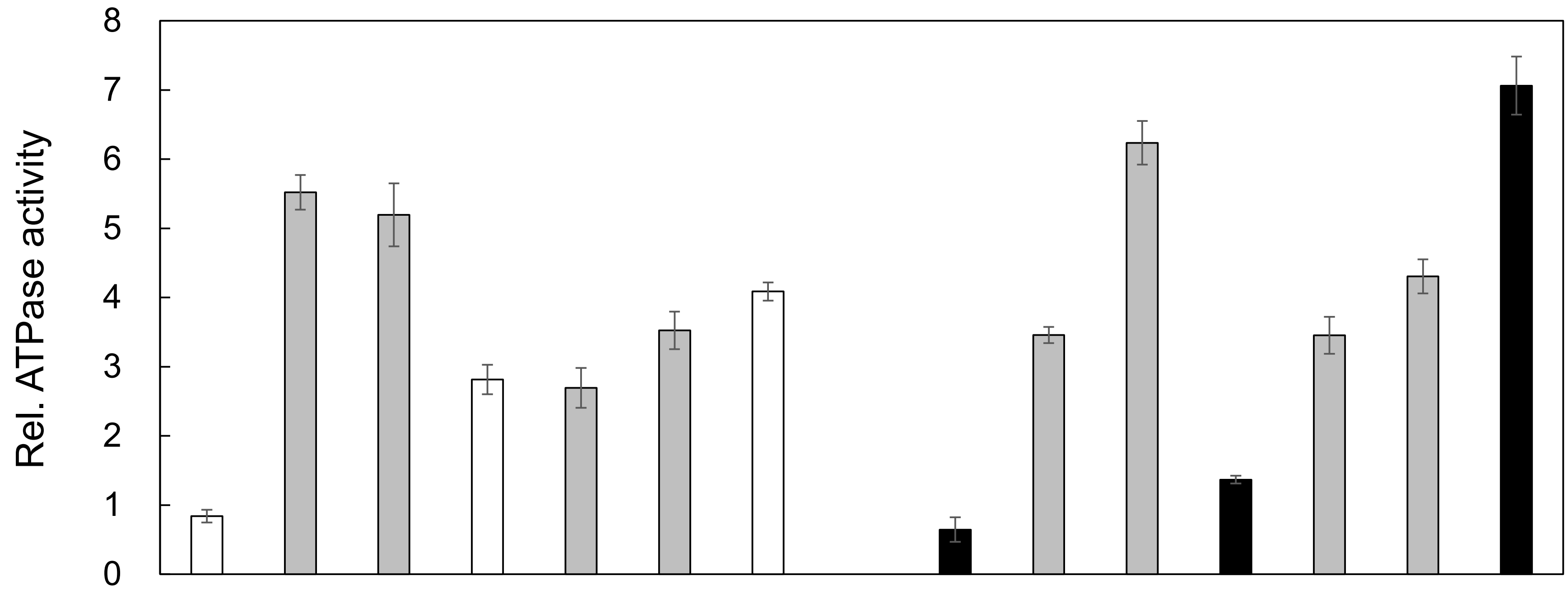
DnaJ	–	1.5	4.0	–	–	0.5	1.5	4.0	0.5	0.5
GrpE	–	–	–	1.5	4.0	0.5	0.5	0.5	1.5	4.0

Fig. 1



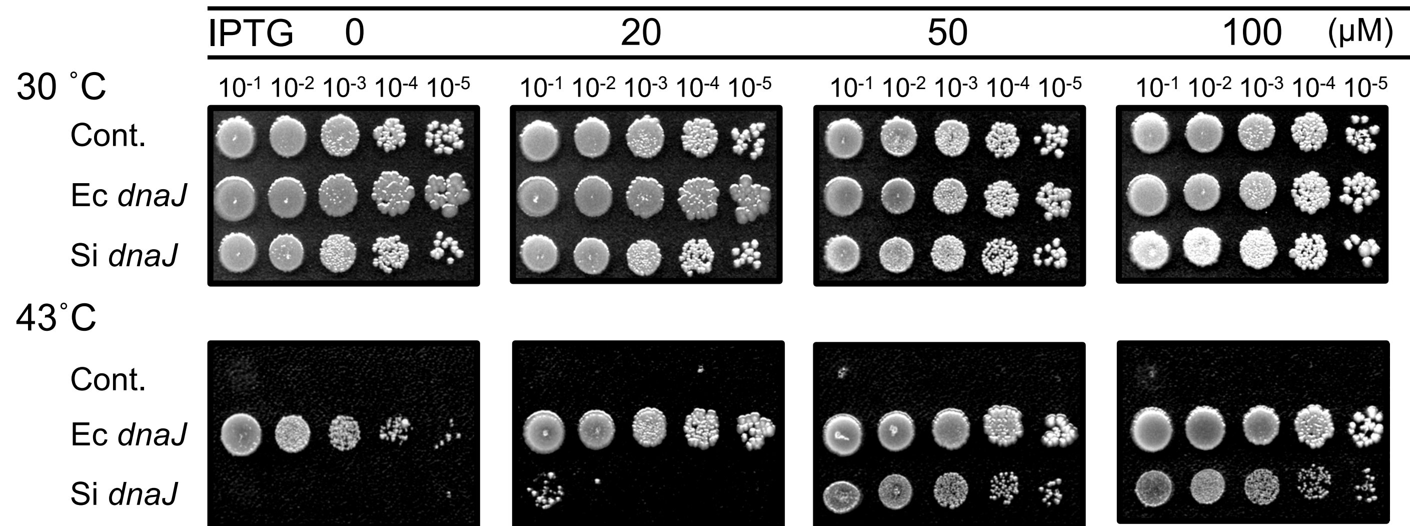
	SiK (4.0)								EcK (2.0)						
SiJ	–	–	0.8	0.8	0.8	–	0.8	0.8	–	–	–	–	–	–	–
SiE	–	0.8	–	0.8	–	0.8	0.8	0.8	–	–	–	–	0.8	0.8	0.8
EcJ	–	–	–	–	–	0.2	0.2	0.8	–	–	0.2	0.2	0.2	–	0.2
EcE	–	–	–	–	0.8	–	–	–	–	0.8	–	0.8	–	0.8	0.8

Fig. 2



	SiK (0.5)								EcK (0.5)						
SiJ	–	–	–	0.5	0.5	0.5	0.5		–	4.0	8.0	–	–	–	–
SiE	0.5	0.5	0.5	–	–	–	0.5		–	–	–	–	4.0	20	–
EcJ	–	4.0	8.0	–	–	–	–		–	–	–	0.5	0.5	0.5	0.5
EcE	–	–	–	–	4.0	20	–		0.5	0.5	0.5	–	–	–	0.5

Fig. 3



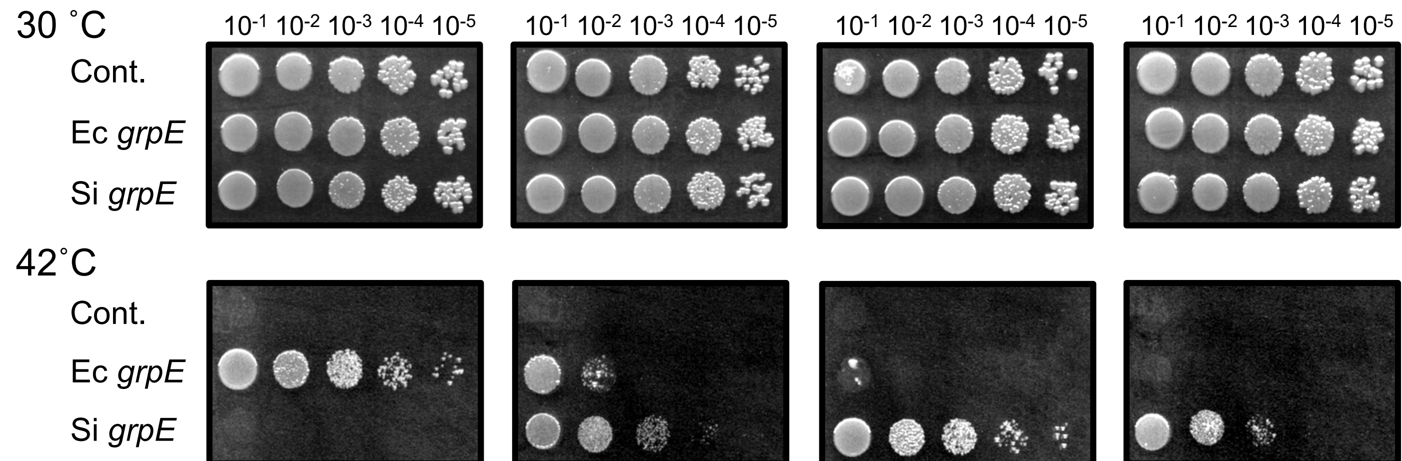


Fig. 4

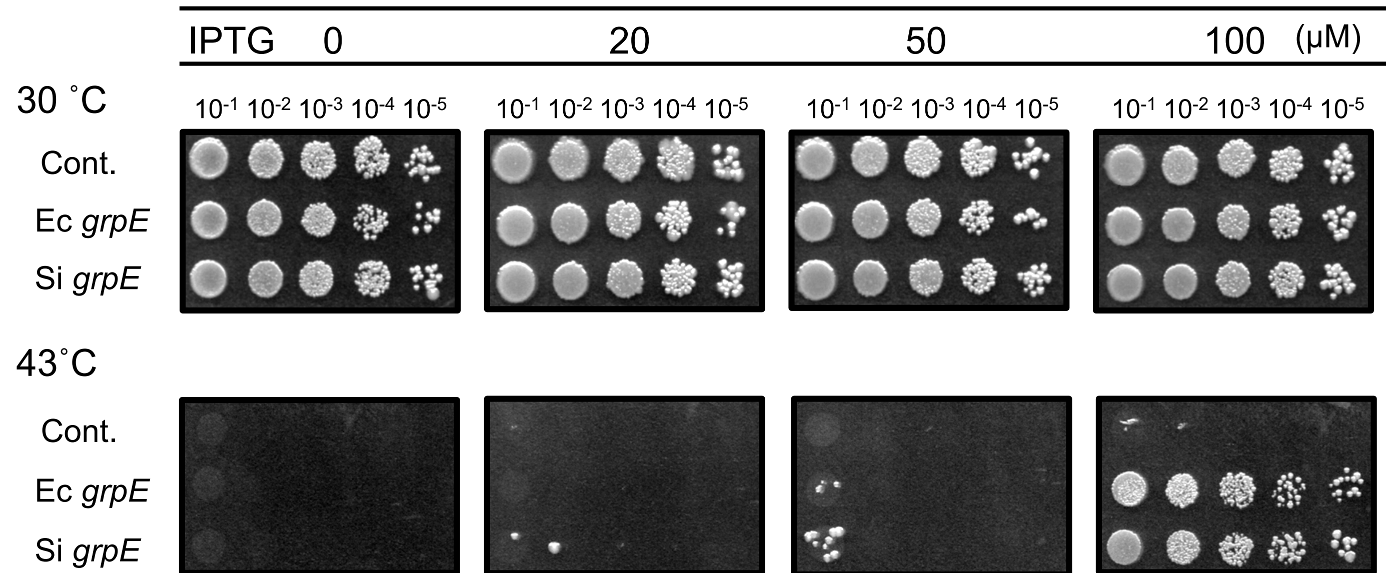
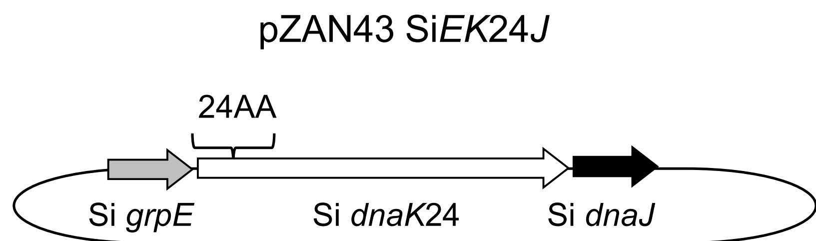
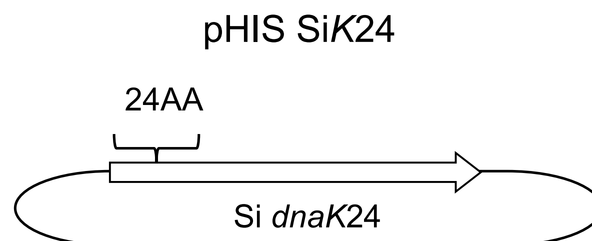


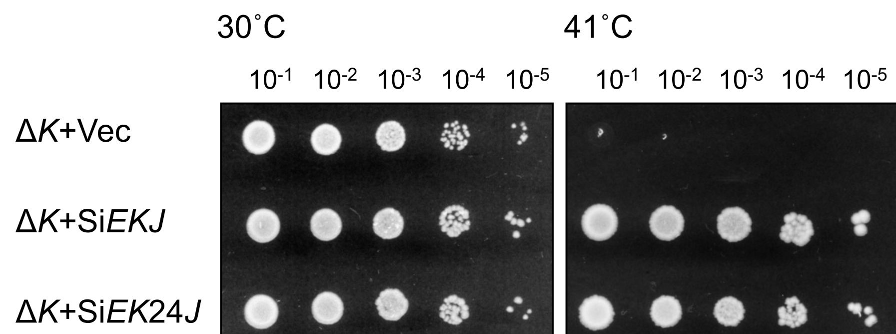
Fig. 5

(A)



(B)





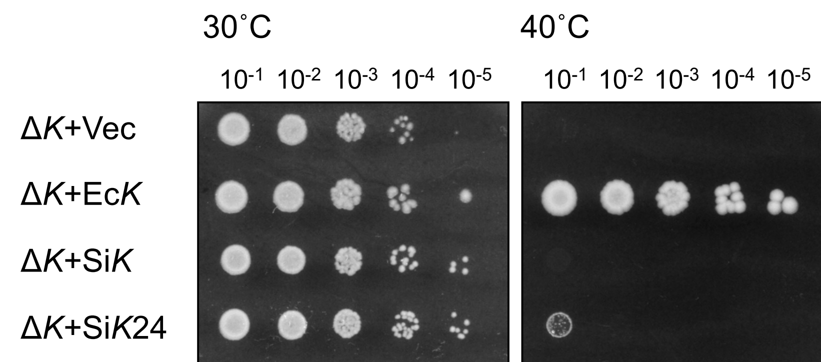


Fig. 6