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ORIGINAL PAPER

Role of *Streptococcus intermedius* DnaK chaperone system in stress tolerance and pathogenicity

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Abstract *Streptococcus intermedius* is a facultatively anaerobic, opportunistic pathogen that causes purulent infections and abscess formation. The DnaK chaperone system has been characterized in several pathogenic bacteria and seems to have important functions in stress resistance and pathogenicity. However, the role of DnaK in *S. intermedius* remains unclear. Therefore, we constructed a *dnaK* knockout mutant that exhibited slow growth, thermosensitivity, accumulation of GroEL in the cell, and reduced cytotoxicity to HepG2 cells. The level of secretion of a major pathogenic factor, intermedilysin, was not affected by *dnaK* mutation. We further examined the function and property of the *S. intermedius* DnaK chaperone system by using *Escherichia coli* Δ*dnaK* and Δ*rpoH* mutant strains. *S. intermedius* DnaK could not complement the thermosensitivity of *E. coli* Δ*dnaK* mutant. However, the intact *S. intermedius* DnaK chaperone system could complement the thermosensitivity and acid sensitivity of *E. coli* Δ*dnaK* mutant. The *S. intermedius* DnaK chaperone system could regulate the activity and stability of the heat shock transcription factor σ^{32} in *E. coli*, although *S. intermedius* does not utilize σ^{32} for heat shock transcription. The *S. intermedius* DnaK chaperone system was also able to efficiently eliminate the aggregated proteins from Δ*rpoH* mutant cells. Overall, our data showed that the *S. intermedius* DnaK chaperone system has important functions in quality control of cellular proteins but has less participation in the modulation of expression of pathogenic factors.

Keywords *Streptococcus intermedius* · *Escherichia coli* · DnaK chaperone system · Thermosensitivity · Intermedilysin

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Introduction

Streptococcus intermedius is a facultative anaerobe that belongs to the Anginosus group of streptococci, forming part of the normal flora of the human oral cavity as well as the upper respiratory, gastrointestinal, and female urogenital tracts (Whiley et al. 1990, 1992). *S. intermedius* is an opportunistic human pathogen and a leading cause of deep-seated purulent infections, including brain and liver

abscesses (Whiley et al. 1990, 1992; Jacobs et al. 1995; Jerng et al. 1997; Claridge et al. 2001). This pathogen secretes a human-specific cytolysin, intermedilysin (ILY), which is a member of the cholesterol-dependent cytolysin family (Nagamune et al. 1996). ILY is a major virulence factor in *S. intermedius*, which is essential for invasion and cytotoxicity to human cells (Nagamune et al. 2000; Sukeno et al. 2005).

Various Gram-negative and Gram-positive intracellular pathogens have been shown to adapt to stress conditions, such as those set by an effective immune system and fever in the host organism, by producing stress proteins (HSPs) such as molecular chaperones (DnaK chaperone system, GroEL and GroES, etc.) and several proteases (Henderson et al. 2006). The ensemble of HSPs constitutes a cellular system for de novo folding and quality control of proteins that relies on the ability of chaperones and proteases to refold or degrade misfolded proteins under stress conditions (Ben-Zvi and Goloubinoff 2001; Dougan et al. 2002). The DnaK chaperone system is composed of DnaK and co-chaperones (DnaJ and GrpE). Co-chaperones are necessary for operation of the DnaK chaperone cycle (Mayer et al. 2000; Ben-Zvi and Goloubinoff 2001; Genevaux et al. 2007); DnaJ binds the substrate (newly synthesized protein, denatured protein, etc.) and interacts with DnaK by transferring its bound substrate to the substrate-binding domain of DnaK and simultaneously stimulating ATP hydrolysis by DnaK. Consequently, DnaK is transformed to the ADP-bound state, which exhibits high affinity and low exchange rate for its substrate. GrpE is a nucleotide exchange factor of DnaK that helps release ADP from DnaK. The nucleotide-free DnaK immediately binds ATP. The ATP-bound state of DnaK exhibits low affinity to the substrate, release of substrate, and resetting of the DnaK chaperone cycle.

DnaK null mutant was originally isolated from the Gram-negative bacterium *Escherichia coli* (Bukau and Walker 1989a). This mutant has been reported to exhibit pleiotropic defects in growth, cell division, plasmid replication, and thermal and chemical stress resistance (Bukau and Walker 1989a, b; Genevaux et al. 2007). In addition, the DnaK chaperone system negatively controls the activity and stability of the heat shock transcription factor σ^{32} in *E. coli* (Arsène et al. 2000; Yura and Nakahigashi 1999). Hence, constitutive induction of heat shock proteins and accumulation of σ^{32} are observed in the *E. coli* Δ*dnaK* mutant. The phenotypes of the DnaK mutants from most Gram-positive bacteria are similar to the phenotypes of the *E. coli* Δ*dnaK* mutant in showing sensitivity to several stresses (Koch et al. 1998; Hanawa et al. 1999; Köhler et al. 2002; Singh et al. 2007).

It has been reported that the DnaK chaperone system is required not only for stress tolerance but also for the pathogenicity of bacteria; DnaK of the Gram-negative intracellular pathogen *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) positively controls the expression of *Salmonella* pathogenicity islands 1 and 2 and the *dnaK* null mutant was non-pathogenic (Takaya et al. 2004). Similar phenotypes were also reported in Gram-positive pathogens: a *dnaK* null mutant of *Staphylococcus aureus* showed reduced survival in a mouse host during systemic infection (Singh et al. 2007). Constitutive and lower-level expression of DnaK induced by promoter replacement of the *dnaK* gene in the intracellular pathogen *Brucella suis* resulted in non-proliferation of the organism within the macrophage-like U937 cells (Köhler et al. 2002) and in *Listeria monocytogenes* DnaK, although not largely involved in intracellular growth within macrophages is nevertheless required for efficient phagocytosis by macrophage-like J774.1 cells (Hanawa et al. 1999). In contrast to the examples described above, the exact role of the DnaK chaperone system in stress resistance and pathogenicity control of the facultatively anaerobic opportunistic pathogen *S. intermedius* remains unclear. In order to investigate this we constructed a *dnaK* null mutant of *S. intermedius*. Our data show that *S. intermedius* DnaK chaperone system has the same chaperone activity in vivo as observed with the *E. coli* DnaK chaperone system plays an important role in key functions including growth, thermoresistance, and heat shock regulation but has less involvement in the modulation of pathogenic factor expression.

Materials and methods

Bacterial strains, plasmids, and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *S. intermedius* was cultured at the indicated temperature under anaerobic conditions. Brain–heart infusion (BHI) broth (Becton-Dickinson, Palo Alto, CA, USA) was used for the liquid culture of *S. intermedius*. *E. coli* was grown in Luria–Bertani (LB) medium at the indicated temperature under aerobic conditions. Antibiotics were added at the following concentrations: ampicillin (Ap) at 100 μg/mL for culturing *E. coli*, chloramphenicol (Cm) at 20 μg/mL for *E. coli* and 2 μg/mL for *S. intermedius*, erythromycin (Em) at 1 μg/mL for *S. intermedius*, and kanamycin (Km) at 20 μg/mL for *E. coli*.

Databases and multiple sequence alignment

Nucleotide and protein sequences of the DnaK chaperone system from *Streptococcus pneumoniae* R6 (DnaK: GenBank Acc. No. NP_358049, DnaJ: NP_358050, GrpE:

Table 1 Bacterial strains and plasmids used in this study

Strains	Relevant characteristics	Reference or source
S. intermedius		
NCDO2227	Type strain	Nagamune et al. 2000
UNS38	High intermedilysin-producing strain from human brain abscess	Sukeno et al. 2005
UNS38 B3	As UNS38 except for Δ*ily*::Em	Sukeno et al. 2005
UNS38 Δ*dnaK*	As UNS38 except for Δ*dnaK*::Em	Present study
UNS38 Δ*dnaK* R37	As UNS38 Δ*dnaK*, but able to grow to 37°C by an unknown suppressor mutation	Present study
E. coli		
DH5αZ1	F^- Φ80Δ(*lacZ*)M15 Δ(*lacZYA-argF*)U169 *deoR recA1 endA1 hsdR17*($r_K^- m_K^+$) *phoA supE44* λ^- *thi-1 gyrA96 relA1 tetR lacI*^{q} *aadA*$^+$	Lutz and Bujard 1997
MC4100	F^- *araD139* Δ(*argF-lac*)U169 *deoC1 thiA flbB5301 ptsF25 relA1 rpsL150 tonA21*	Bukau and Walker 1990
BB1554	As MC4100 except for Δ*dnaK52*::Cm *sidB2*	Bukau and Walker 1990
BB1554 *lacI*^{q}	BB1554 carrying pDMI, I	Present study
BB7224	As MC4100 except for Δ*rpoH*::Km *suhX401 araD*$^+$	Tomoyasu et al. 2001
CS5262	BB7224 carrying pBB528	Tomoyasu et al. 2010b
Plasmids		
pDMI, I	Lac repressor–producing plasmid, p15A ori, Km^r	Lanzer and Bujard 1988
pBB528	Lac repressor–producing plasmid, pSC101 ori, Cm^r	Tomoyasu et al. 2001
pSETN1	Reported as pSET1 Δ*lac* p15A, Cm^r	Tomoyasu et al. 2010a, b
pSETN1 CP25	pSETN1-carrying CP25 promoter	Present study
pSETN1 *EKJ*	pSETN1-carrying *grpE, dnaK*, and *dnaJ* operon	Present study
pZE13	IPTG-inducible expression vector, pMB1 ori, Ap^r	Lutz and Bujard 1997
pZE13 Ec*K*	pZE13-carrying *E. coli dnaK*	Present study
pZE13 Si*K*	pZE13-carrying *S. intermedius dnaK*	Present study
pZE13 Si*EK*	pZE13-carrying *S. intermedius grpE* and *dnaK*	Present study
pZE13 Si*KJ*	pZE13-carrying *S. intermedius dnaK* and *dnaJ*	Present study
pZE13 Si*EKJ*	pZE13-carrying *S. intermedius grpE, dnaK* and *dnaJ*	Present study

NP_358048), *Lactococcus lactis* subsp. *lactis* II1403 (DnaK: NP_267110, DnaJ: NP_268381, GrpE: NP_267109), *Bacillus subtilis* subsp. *subtilis* str. (DnaK: NP_390425, DnaJ: NP_390424, GrpE: NP_390426), and *E. coli* str. K-12 substr. W3110 (DnaK: AP_000678, DnaJ: AP_000679, GrpE: AP_003194) were obtained from GenBank by an Entrez cross-database search at the National Center for Biotechnology Information (NCBI; National Institutes of Health, Bethesda, MD, USA). The nucleotide sequence of the *hrcA-grpE-dnaK-dnaJ* operon of *S. intermedius* type strain NCDO2227 has been submitted to the DDBJ database and given a specialized accession number: AB608790. Sequence similarity in the DnaK chaperone systems of these bacteria was analyzed using the NCBI BLAST Needleman–Wunsch Global Sequence Alignment Tool. Multiple sequence alignment was performed using the CLUSTAL W (1.81) program and the sequence motif search was performed using the PROSITE Pattern and PROSITE Profile databases (Kyoto University Bioinformatics Center, Japan; http://www.bic.kyoto-u.ac.jp/).

Generation of *dnaK* knockout mutants of *S. intermedius*

dnaK knockout mutants (Δ*dnaK*) were produced by homologous recombination. DNA fragments of *S. intermedius* type strain NCDO2227 genomic DNA were amplified by PCR. The 5′ region of the *dnaK* DNA fragment (1,009 bp) includes a 315-bp coding region of *dnaK* that was amplified by using *dnaK Eco*RI F and internal primer Si *dnaK Bam*HI R (Table 2), and then digested with *Bam*HI. The 3′ region of the latter (1,058 bp) DNA fragment includes a 429-bp coding region of *dnaK* that was amplified using internal primers, Si *dnaK Sal*I F and Si *dnaK Sph*I R (Table 2), and then digested with *Sal*I. The erythromycin-resistance cassette (*erm* cassette) was amplified from genomic DNA from an *ily* knockout mutant UNS38 B3 (Sukeno et al. 2005) using the primers, *erm* (*Bam*HI) F and *erm* (*Sal*I) R (Table 2) and the *erm* cassette ligated to the *Bam*HI-digested 5′ region and *Sal*I-digested 3′ region of the *dnaK* ORF. This ligated fragment was amplified by PCR with primers, Si *dnaK Eco*RI and Si *dnaK Sph*I R (Table 2). UNS38 Δ*dnaK* was produced by transformation of competence-stimulating peptide (CSP:

Table 2 Oligonucleotides used in this study

Purpose	Name	Sequence (5′–3′)
Disruption of *dnaK*		
	Si *dnaK Eco*RI F	ACAGAATTCGAACTAGCTAATGAGCGTG
	Si *dnaK Sph*I R	TTGCATGCCGCTCAAGGTTTCATAAGCC
	Si *dnaK Sal*I F	ATTGTCGACCAATCTAACAGCGGCCTGAC
	Si *dnaK Bam*HI R	CTGGATCCCCAAGATATTCTTCTGCATAG
Erythromycin-resistant cassette		
	erm (*Bam*HI) F	AATGGATCCCCCGATAGCTTCCGCTATTG
	erm (*Sal*I) R	CAGTAGTCGACCTAATAATTTATCTAC
Construction of a constitutive promoter in *S. intermedius*		
	CP25 oligo1 F	CGCCCGGGCTTTGGCAGTTTATT
	CP25 oligo1 R	GTCAAGAATAAACTGCCAAAGCCCGGGCG
	CP25 oligo2 F	CTTGACATGTAGTGAGGGGGCTGGT
	CP25 oligo2 R	GATTATACCAGCCCCCTCACTACAT
	CP25 oligo3 F	ATAATCACATAGTACTGTTGAGCTCGC
	CP25 oligo3 R	GCGAGCTCAACAGTACTATGT
	RBS-F (*Sac*I)	AACGAGCTCAAAGGAGAACGTTGGATCCAC
	CP25 *Pst*I	CGCTGCAGCTTTGGCAGTTTATTCTTGAC
	CP25 *Bam*HI	GGGGATCCAACGTTCTCCTTTGAGCTCAAC
Complementation of Δ*dnaK* mutant, conformation of *dnaK* disruption and complementation		
	Si *grpE Bam*HI F	GGAGGATCCTTGTGGCAAAACATAAACAAGAAGAAC
	Si *dnaK Bam*HI F	GAAGGATCCATGTCTAAAATTATCGGTATTGAC
	Si *dnaK* R	CTCTCTAGATTACTTTTCCGTAAACTCTCCGTC
	Si *dnaJ* R	CGATCTAGACCCGGGCGTGCAACACATCATTACAAG
	Si 3′ *hrcA* F	GATATCTGAGTAGTAATCACTATGAAGTCC
	Si 5′ *dnaK* R	GCTGAGTTTGTTGTACCTAAGTCAATACCG
	5′ *erm* R	GCAAACATATAACCGAGGAACAAAGGTATG
	Si 3′ *dnaK* F	GTGATGATGTCGTAGACGGAGAGTTTACGG
	pSETN1 R	CCCAGTCACGACGTTGTAAAACGACGGCC

DSRIRMGFDFSKLFGK)-treated strain UNS38 with the PCR amplicon. Colonies were selected and isolated on BHI agar-containing erythromycin at 30°C. Disruption of *dnaK* was confirmed by immunoblotting using anti-*Tetragenococcus halophilus* DnaK rabbit antiserum (Sugimoto et al. 2008) and by PCR.

Complementation of *S. intermedius* UNS38 Δ*dnaK* strain

For complementation of the UNS38 Δ*dnaK* mutant, the *Streptococcus suis–E. coli* shuttle vector pSETN1 (previously reported as pSET1 Δ*lac* p15A) was modified (Tomoyasu et al. 2010a). For constitutive expression of the *grpE-dnaK-dnaJ* operon in the *ΔdnaK* mutant, a DNA fragment (CGCTGCAGCT TTGGCAGTTT ATTCTT GACA TGTAGTGAGG GGGCTGGTAT AATCACATAG TACTGTTGAG CTCAAAGGAG AACGTTGGAT CCAC) containing synthetic promoter CP25 (Jensen and Hammer 1998) and Shine–Dalgarno sequence was synthesized by PCR, using synthetic primers (Table 2) and multiple cloning steps (data not shown). This fragment was amplified using primers, CP25 *Pst*I and CP25 *Bam*HI, digested with *Pst*I and *Bam*HI, and cloned into the corresponding sites in pSETN1 and transformed into *E. coli* DH5αZ1 (Lutz and Bujard 1997). The resultant plasmid (pSETN1 CP25) was used for the construction of the plasmid for complementation of the Δ*dnaK* mutant. The *grpE-dnaK-dnaJ* operon was amplified from *S. intermedius* NCDO2227 genomic DNA by PCR using primers, Si *grpE Bam*HI F and Si *dnaJ* R (Table 2). The amplified fragment was digested with *Bam*HI and *Sma*I and cloned into the corresponding sites in pSETN1. The resultant plasmid (pSETN1 *EKJ*) and pSETN1 CP25 were digested with *Sph*I and *Bam*HI. A 9.24-kbp fragment from pSETN1 *EKJ* and a 0.92-kbp fragment from pSETN1 CP25, which contains the CP25 promoter, were recovered and then ligated. The ligated plasmid was transformed into a CSP-treated UNS38 Δ*dnaK* mutant (Δ*dnaK* R37). Transformants were selected and isolated on a BHI agar plate containing 2 μg/mL chloramphenicol. Complementation of Δ*dnaK* was

confirmed by immunoblotting using anti-*T. halophilus* DnaK rabbit antiserum, recovery of thermosensitive phenotype, and PCR.

PCR analysis

To confirm disruption of the *dnaK* gene by the *erm* cassette in the Δ*dnaK* mutant and to analyze the status of the plasmid in a complemented strain, chromosomal DNA from the parental (UNS38), Δ*dnaK* mutant, and complemented strains was applied to PCR using the primers listed in Table 2. The primer sets used to amplify each gene fragment were as follows: Si *dnaK Bam*HI F and Si *dnaK* R to amplify the *dnaK* gene; Si 3′ *hrcA* F and Si 5′ *dnaK* R to amplify a fragment from the 3′ region of *hrcA* to the 5′ region of *dnaK*; Si 3′ *hrcA* F and 5′ *erm* R to amplify a fragment from the 3′ region of *hrcA* to the *erm* cassette: Si 3′ *hrcA* F and Si *dnaK* R to amplify a fragment from the 3′ region of *hrcA* to the 3′ region of *dnaK*: Si 3′ *dnaK* F and pSETN1 R to amplify a fragment from the 3′ region of *dnaK* to pSETN1 CP25; *erm* (*Bam*HI) F and pSETN1 R to amplify a fragment from the *erm* cassette to pSETN1 CP25; Si *dnaK Bam*HI F and pSETN1 R to amplify a fragment from the 5′ coding region of *dnaK* to pSETN1 CP25; and CP25 *Pst*I and Si *dnaK* R to amplify a region from the CP25 promoter region to the 3′ region of *dnaK*.

Hemolysis assay

S. intermedius cells were grown in BHI broth at 37°C for 24 h under anaerobic conditions. The culture supernatant was separated by centrifugation (5,000×*g*). Hemolysis was assayed as described by Nagamune et al. (1996), with minor modifications. Human blood was obtained from healthy Japanese volunteers and stored in sterilized Alsever solution at 4°C. Erythrocytes were washed three times with PBS at 4°C by centrifugation (1,000×*g*) before use. Chilled PBS containing 5×10^7 erythrocytes per milliliter and standardized amounts of the culture supernatants (optical density at 600 nm; OD_{600}=1.0), which was diluted 200- to 6,400-fold with PBS, were mixed in microcentrifuge tubes (total of 0.5 mL). Hemolysis was performed at 37°C for 1 h. After the reaction, non-lysed erythrocytes were removed by centrifugation (1,000×*g*) at 4°C for 5 min. Absorbance at 540 nm (A_{540}) of 200 μL of supernatant was measured in a Microplate Reader Model 550 (Bio-Rad, Hercules, CA, USA). The percent hemolysis was calculated as follows: hemolysis (%)=[(A_{540} of supernatant from the sample containing diluted culture supernatant−A_{540} of supernatant from the sample containing no diluted culture supernatant)/(A540 of supernatant from the sample completely hemolyzed by hypotonic processing−A_{540} of supernatant from the sample containing no diluted culture supernatant)]×100.

Construction of isopropyl β-D-1-thiogalactopyranoside-regulated *S. intermedius dnaK*, *grpE-dnaK*, *dnaK-dnaJ*, *grpE-dnaK-dnaJ*, or *E. coli dnaK* expression plasmids

To construct *S. intermedius* DnaK-, GrpE-DnaK-, DnaK-DnaJ-, or GrpE-DnaK-DnaJ producing plasmids (pZE13 Si*K*, pZE13 Si*EK*, pZE13 Si*KJ*, pZE13 Si*EKJ*), *S. intermedius* NCDO2227 genomic DNA was used for PCR amplification of (1) the *dnaK* gene was amplified using primers, Si *dnaK Bam*HI F and Si *dnaK* R (Table 2); (2) the *grpE-dnaK* operon amplified using primers, Si *grpE Bam*HI F and Si *dnaK* R (Table 2); (3) the *dnaK-dnaJ* operon was amplified using primers, Si *dnaK Bam*HI F and Si *dnaJ* R (Table 2) and (4) the *grpE-dnaK-dnaJ* operon amplified using primers, Si *grpE Bam*HI F and Si *dnaJ* R (Table 2). Each fragment was digested with *Bam*HI and *Xba*I, and then cloned into the corresponding sites in pZE13 (Lutz and Bujard 1997). *E. coli* DnaK-producing plasmid (pZE13 Ec*K*) was also created as follows: *E. coli dnaK* gene was excised by *Bam*HI and *Hin*dIII digestion in pBB535, and a 2.17-kbp fragment containing intact *E. coli dnaK* was cloned into the corresponding sites in pZE13. Then, *E. coli* Δ*dnaK* strain BB1554 *lacI*^{q} (Table 1) was transformed by the plasmids constructed above to analyze the complementation of this mutant.

In vivo chaperone activity of *S. intermedius* DnaK chaperone system in *E. coli* Δ*rpoH* mutant

The chaperone activity of *S. intermedius* DnaK chaperone system was determined by measuring and comparing the amount of aggregated proteins in both the Δ*rpoH* mutant (CS5262) and the pZE13 Si*EKJ* transformed CS5262 (Table 1). Cells were grown in 10 mL of LB medium containing the indicated concentrations of β-D-1-thiogalactopyranoside (IPTG) for 4 h at 30°C and then shifted to 42°C for 1 h. Isolation of total and aggregated proteins was performed as previously described with minor modifications (Tomoyasu et al. 2001, 2010b). After heat treatment, bacterial cultures were rapidly cooled on ice and centrifuged for 10 min at 5,000×*g* at 4°C to harvest cells. Pellets were resuspended in 80 μL of buffer A (10 mM potassium phosphate buffer [pH 6.5] containing 1 mM EDTA, 20% [*w*/*v*] sucrose, 1 mg/mL lysozyme) and incubated for 30 min on ice. Spheroplasts were destroyed by the addition of 720 μL of buffer B (10 mM potassium phosphate buffer [pH 6.5] containing 1 mM EDTA) and sonication with microtip (level 3, 50% duty ratio, for 10 s) in an Astrason Ultrasonic Processor (model XL2020; MISONIX Inc., Farmingdale, NY, USA) while

cooling on ice. The insoluble cell fraction from the total proteins was isolated by centrifugation at 17,000×*g* for 5 min at 4°C. The pellet fractions were frozen, resuspended in 800 μL of buffer B by sonication, and centrifuged (17,000×*g*, 5 min, 4°C). The washed pellet fractions were again resuspended in 640 μL of buffer B by brief sonication; then, 160 μL of 10% (*v*/*v*) Nonidet-P40 (NP40) was added, and the aggregated proteins were isolated by centrifugation (17,000×*g*, 5 min, 4°C). This washing procedure was repeated for the complete removal of contaminating membrane proteins. NP40-insoluble pellets were washed with 800 μL of buffer B and resuspended in 200 μL of buffer B by brief sonication. Quantification of the amount of total and aggregated proteins was performed using the Bradford assay reagent (Bio-Rad, Hercules, CA, USA) with bovine serum albumin as a standard.

Infection assay

S. intermedius cells were grown in BHI broth at 37°C for 48 h under anaerobic conditions. The infection assay was performed as previously described with minor modifications (Sukeno et al. 2005). HepG2 (1×10^5 cells per well) in 350 μL of DMEM containing 10% fetal bovine serum (FBS) without antibiotics was dispensed into 48-multiwell tissue culture plates and cultured overnight at 37°C in the presence of 5% CO_2. For cell infection, bacterial cultures were centrifuged at 13,000×*g* for 1 min, and cells were suspended to a density of 3×10^5 cells in 350 μL of DMEM containing 10% FBS without antibiotics. The bacterial suspension was added to HepG2 cells, and infection was allowed to proceed for 3 h in the 48-multiwell tissue culture plates. The supernatant was completely removed, and cells were washed three times with PBS. Infected cells were then cultured in 350 μL of fresh medium. A portion of the culture medium (200 μL) was replaced with fresh medium every 12 h to avoid accumulation of ILY. The viability of infected cells was determined using the neutral red (NR) method (Borenfreund and Puerner 1985). After infection, the medium was removed at the indicated time point, and the cells were incubated with 350 μL of NR solution (50 μg/mL) in DMEM for 3 h at 37°C. The cells were subsequently washed three times with PBS and then fixed with 200 μL of formaldehyde (1.0%, *v*/*v*) containing 1 mM HEPES-KOH (pH 7.3), 0.85% NaCl, and 1.0% $CaCl_2$. To extract the dye, viable cells were lysed with 1% acetic acid in 50% (*v*/*v*) ethanol. The absorbance was then measured at 540 nm (A_{540}). The 0% viability control consisted of cells exposed to 1 M HCl and the 100% viability control consisted of cells incubated in DMEM without bacteria. The level of cytotoxicity was calculated as follows: Viability (%)=(A_{540} of extract from the infected cells−A_{540} of extract from the 0% control)/(A_{540} of extract from the 100% control−A_{540} of extract from the 0% control)×100.

Spot tests for thermosensitivity and acid tolerance

The thermosensitivity and acid tolerance of *S. intermedius* cells were determined as follows: Cells were grown in BHI medium for 48 h at 30°C under anaerobic conditions, and the OD_{600} of the cultures was measured. Standardized cultures (OD_{600} adjusted to 1.0) were diluted from 10^{-1} to 10^{-5} in BHI medium. To analyze thermosensitivity, 5 μL aliquots were spotted onto BHI agar plates and incubated at 37°C or 42°C for 48 h. For acid tolerance, testing aliquots of the standardized (OD_{600}=1.0) cultures were diluted to 10^{-2} in pH-conditioned glycine-buffered BHI medium (pH 3.5 or pH 7.0) and cultured for 1 h at room temperature. Aliquots of each incubated culture were further diluted from 10^{-3} to 10^{-5} in BHI medium and 5 μL spotted onto BHI agar plates. Plates were incubated at 30°C for 72 h.

To analyze thermosensitivity, *E. coli* cells were grown in LB medium overnight at 30°C. Two microliters of aliquots were spotted onto LB agar plates containing from 0 to 100 μM IPTG, and plates were incubated at 30°C or 42°C for 24 h. To analyze acid tolerance, *E. coli* cells were grown in LB medium for 3 h at 30°C in the presence of 50 μM IPTG, and the OD_{600} of the cultures was measured. Standardized amounts (OD_{600}=1.0) of cultures were diluted to 10^{-2} in pH-conditioned glycine-buffered LB medium (pH 3.5 or pH 7.0) and then cultured for 1 h at room temperature. Aliquots of each culture were further diluted from 10^{-3} to 10^{-5} in LB medium. Five microliters of aliquots were spotted onto LB agar plates containing 50 μM IPTG and incubated at 30°C for 48 h.

Gel electrophoresis and immunoblotting

S. intermedius cells were grown in BHI broth at 37°C under anaerobic conditions. The culture supernatant and cells were separated by centrifugation (5,000×*g*). The cells were washed three times with PBS and resuspended in 1 or 0.5 mL of 20 mM Tris–HCl (pH 8.0) containing 100 mM NaCl and 1 mM EDTA. Samples were then added to Lysing Matrix B tubes (Qbiogene Inc., Carlsbad, CA, USA) and lysed in a FastPrep cell disruptor (Savant Instruments, Holbrook, NY, USA). To obtain the soluble protein fraction, samples were centrifuged at 17,400×*g* for 30 min, and the supernatants were retained. The total protein (10 μg) of the supernatants or standardized amounts of the culture supernatants were analyzed by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (Laemmli 1970). *E. coli* cells were grown in 10 mL of LB medium

containing the concentrations of IPTG indicated for 4 h at 30°C. The OD_{600} of the cultures was measured, and the cells were harvested by centrifugation (17,400×*g*). The cell pellets were lysed by adding sample buffer (Laemmli 1970), and then standardized amounts (10 μL) of the cell lysate at OD_{600}=5.0 were analyzed by 12% SDS–PAGE. For immunoblotting analysis, the resolved proteins were transferred to a poly(vinylidene difluoride) membrane (Millipore, Bedford, MA, USA), incubated with anti-*T. halophilus*, anti-*E. coli* GroEL (Kusukawa et al. 1989), or anti-σ^{32} (Gamer et al. 1992) rabbit antiserum, a 2nd then developed with a 5-bromo-4-chloro-3′-indolyl phosphate/nitro-blue tetrazolium chloride, using alkaline phosphatase-conjugated anti-rabbit immunoglobulin G as the secondary antibody.

Results

Comparison of the amino acid sequence of *S. intermedius* DnaK chaperone system with those of other DnaK chaperone systems in Gram-positive and Gram-negative bacteria

The genetic organization of the DnaK chaperone system of *S. intermedius* was similar to that found in other Gram-positive bacteria with genes of this system localized at the *hrcA-grpE-dnaK-dnaJ* operon. A highly homologous region with the CIRCE operator sequence, which is the binding site for the HrcA heat shock repressor, was detected in the *hrcA* promoter region (data not shown). We compared the sequence homology of each component of the *S. intermedius* DnaK chaperone system to the corresponding component of the DnaK chaperone systems of *S. pneumoniae*, *L. lactis*, *B. subtilis*, and *E. coli* (Table 3). *S. intermedius* DnaK sequence was well conserved among the Gram-positive bacteria, and >70% amino acid homology was observed. However, *S. intermedius* DnaK showed weak homology and 54% identity to the DnaK of the Gram-negative bacterium *E. coli*. *S. intermedius* GrpE showed less amino acid homology (26%) to *E. coli* GrpE. We surmised that since a nucleotide exchange factor GrpE for DnaK does not have chaperone activity, it might allow more amino acid substitutions than DnaK and DnaJ. We therefore further characterized a sequence feature of *S. intermedius* DnaK, DnaJ and GroE by multiple sequence search analysis of these proteins among *B. subtilis* and *E. coli* (Fig. 1). This analysis indicated that *S. intermedius* DnaK has features typical of DnaK of a Gram-positive bacterium, i.e. lacking segments 75–98 and 211–214 in the ATPase domain that are present in almost all proteobacteria (Fig. 1a). The J domain in *S. intermedius* DnaJ was well conserved (Fig. 1b). However, the glycine- and phenylalanine-rich region (G/F region) was variable among these bacteria. Although *S. intermedius* GrpE showed only weak amino acid homology to *E. coli* GrpE, the C-terminal region corresponding to the GrpE protein signature motif was significantly conserved (Fig. 1c)

Table 3 Sequence identity between the *S. intermedius* DnaK chaperone system and those of other Gram-positive and Gram-negative bacteria

	GrpE (%)	DnaK (%)	DnaJ (%)
S. pneumoniae	79	96	86
L. lactis	66	85	71
B. subtilis	38	75	56
E. coli	26	54	48

Sequence identity was examined by the Needleman-Wunsch alignment.

Construction of *S. intermedius dnaK* null mutants and its complemented strain

To analyze the in vivo function of the DnaK chaperone system in *S. intermedius*, we disrupted the *dnaK* gene by insertion of the *erm* cassette between the *dnaK* coding sequences. In addition, a *dnaK*-complemented strain was also constructed by transformation of a Δ*dnaK* mutant (Δ*dnaK* R37) with the expression vector pSETN1 CP25 carrying the *grpE-dnaK-dnaJ* operon to exclude the possibility of *dnaK* null phenotypes resulting from other mutations in the chromosome. We obtained chloramphenicol (selective marker for pSETN1 CP25)-resistant colonies, although we did not recover the plasmid from the independent colonies. Therefore, the status of the plasmid in a chloramphenicol-resistant strain was analyzed by PCR (Supplementary Fig. 1A and B). When the primers for amplifying the *dnaK* gene were used, we detected two fragments from the chloramphenicol-resistant strain. The shorter fragment (1.9 kbp) corresponded to the *dnaK* gene and the longer fragment (2.9 kbp) corresponded to the *erm* cassette-inserted *dnaK* gene. These data confirmed that the chloramphenicol-resistant strain obtained actually complemented the *dnaK* gene disrupted with the *erm* cassette through the presence of intact *dnaK*. Subsequently, we analyzed the status of the *hrcA-grpE-dnaK-dnaJ* operon in the complemented strain. PCR using primers to amplify from the 3′ region of *hrcA* to the 5′ region of *dnaK* produced a 0.9-kbp fragment from all strains examined. PCR using primers to amplify from the 3′ region of *hrcA* to the *erm* cassette produced a 1.3-kbp fragment from both Δ*dnaK* R37 and its complemented strain. The primers from the 3′ region of *hrcA* to the 3′ region of *dnaK* produced a

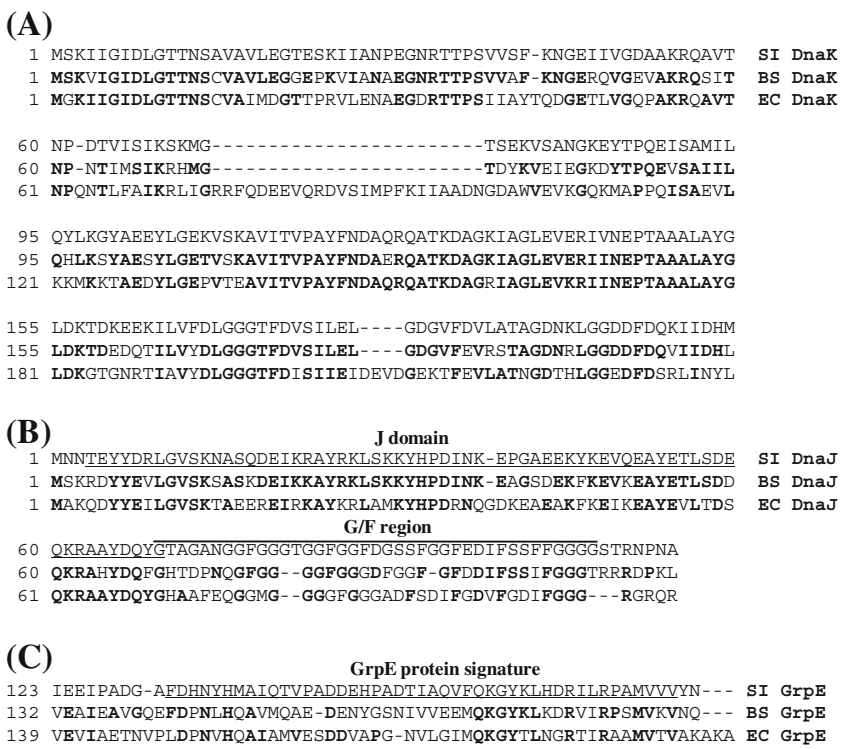

Fig. 1 Multiple sequence alignment of DnaK, DnaJ, and GrpE. **a** Part of the ATPase domains of DnaK from *S. intermedius* (SI DnaK), *B. subtilis* (Bs DnaK), and *E. coli* (Ec DnaK) were aligned with the CLUSTAL W program. **b** The N-terminal regions of DnaJ including the J domain (*underlined*) and G/F region (*overlined*) from *S. intermedius* (SI DnaJ), *B. subtilis* (Bs DnaJ), and *E. coli* (Ec DnaJ) were aligned. **c** The C-terminal regions of GrpE from *S. intermedius* (SI GrpE), *B. subtilis* (Bs GrpE), and *E. coli* (Ec GrpE) were aligned. The GrpE protein signature motif is *underlined*. Identical amino acid residues to *S. intermedius* DnaK, DnaJ, or GrpE are shown in *bold*

2.7-kbp fragment from UNS38 or a 3.7-kbp fragment from both Δ*dnaK* R37 and the complemented strain. These results suggest that *dnaK* in the *hrcA-grpE-dnaK-dnaJ* operon was disrupted by the *erm* cassette in the complemented strain. We further analyzed the plasmid localization in the *S. intermedius* chromosome. PCR amplification using primers to amplify from the 3′ region of *dnaK*, *erm* cassette, or 5′ coding region of *dnaK* to pSETN1 CP25 produced 1.7-, 4.3-, or 4.6-kbp fragments respectively only from the complemented strain. These results suggest that pSETN1 CP25 was integrated downstream of the *hrcA-grpE-*Δ*dnaK-dnaJ* operon (Supplementary Fig. 1A). In addition, we tried to amplify the fragment between the CP25 promoter and the 3′ region of *dnaK* and obtained 2.7-kbp fragments only from the complemented strain indicating that *grpE* and intact *dnaK* were localized downstream of the CP25 promoter. Overall, our results suggest that the plasmid was recombined with the *hrcA-grpE-*Δ*dnaK-dnaJ* operon of Δ*dnaK* R37 (Supplementary Fig. 1A). Thus far, we have obtained only the plasmid-integrated strain. Since a *S. suis-E. coli* shuttle vector, pSET1, was not stable in *S. intermedius* (data not shown), the integrated strain might be preferentially selected.

Because the homology between *S. intermedius* DnaK and *E. coli* DnaK (Table 3) was weak, we could not detect *S. intermedius* DnaK by immunoblotting analysis using anti-*E. coli* DnaK antiserum (data not shown). On the other hand, *T. halophilus* DnaK (Sugimoto et al. 2008) has high homology with *S. intermedius* DnaK with 77% amino acid identity. Therefore, immunoblotting analysis was carried out using anti-*T. halophilus* DnaK antiserum to confirm the amount of DnaK in Δ*dnaK* R37 and its complemented strain (Fig. 2a). The disappearance of the band corresponding to DnaK was confirmed in the cell extract from Δ*dnaK* R37, while on the other hand, obvious recovery of DnaK was observed in the complemented strain. In addition, accumulation of a 60-kDa protein was observed in Δ*dnaK* R37, which cross-reacted with anti-*E. coli* GroEL antibody. The complemented strain showed a significant reduction of the accumulated protein to the level of the parental strain.

Thermosensitivity and acid sensitivity of *S. intermedius dnaK* null mutants

As observed for other Gram-negative and Gram-positive *dnaK* null mutants, Δ*dnaK* R37 showed a thermosensitive phenotype and did not form colonies at 42°C, while UNS38 and the complemented strain formed colonies at both 37°C and 42°C (Fig. 2b). We have isolated four independent *dnaK* null mutants from UNS38 thus far. Three *dnaK* null mutants (Δ*dnaK* S37 1–3) did not grow at 37°C (data not shown), although one mutant (Δ*dnaK* R37) grew at this temperature. To investigate the cytotoxicity of *S. intermedius* to human-derived culture cells, the infection assay was carried out at 37°C. Therefore, since the Δ*dnaK* mutant, which can grow at 37°C, was necessary for the assay, Δ*dnaK* R37 was used for these experiments. *E. coli* Δ*dnaK*

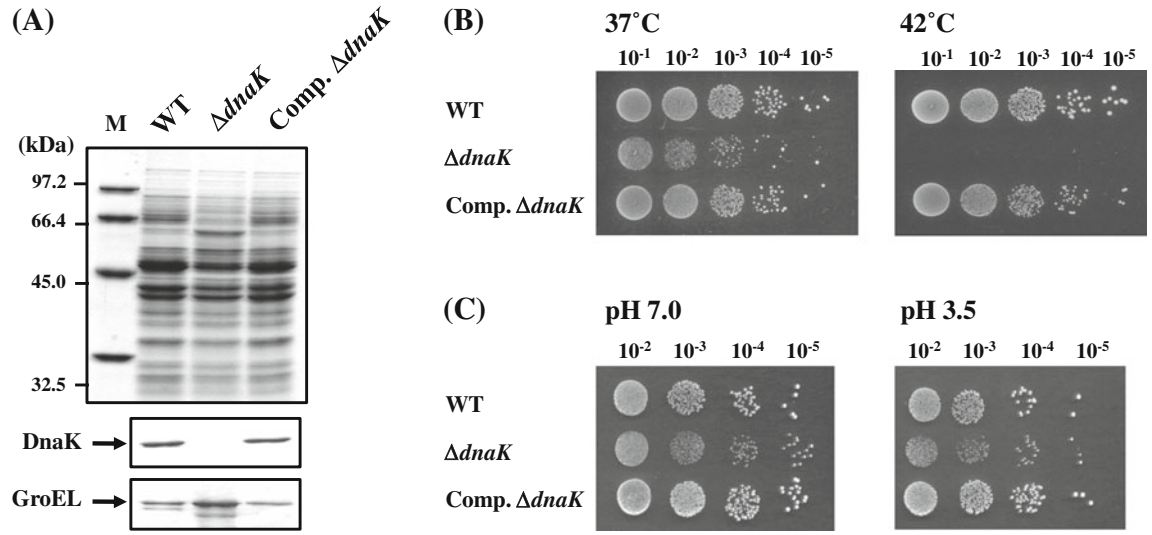

Fig. 2 Immunoblotting analysis and stress-sensitivity of *S. intermedius* Δ*dnaK mutant*. **a** Immunoblotting analysis of Δ*dnaK* R37 and its complemented strain. Whole-cell extracts (10 μg) were separated by 12% SDS–PAGE. Immunodetection was carried out with anti-*T. halophilus* DnaK antiserum or anti-GroEL antiserum. **b** Spot test for the examination of thermosensitivity. Spotted plates were incubated for 48 h at the indicated temperature. **c** Spot test for the examination of acid tolerance. Spotted plates were incubated for 48 h at 30°C. *M* molecular weight marker; *WT* UNS38; Δ*dnaK*, Δ*dnaK* R37; *Comp.* Δ*dnaK* the complemented strain of Δ*dnaK*

mutant is known to have an acid-sensitive phenotype. Then, the acid sensitivity of Δ*dnaK* R37 was examined (Fig. 2c). The viability of Δ*dnaK* R37 was the same as UNS38 and the complemented strain under acidic conditions. Unlike *E. coli* Δ*dnaK* mutant, *S. intermedius* Δ*dnaK* R37 could not show significant acid sensitivity.

Effect of *dnaK* null mutation on growth and secretion of pathogenic factors

The growth rate of UNS38, Δ*dnaK* R37, and the complemented strain in BHI medium at 37°C were examined (Fig. 3a). The growth curves of UNS38 and the complemented strain were similar (i.e., both cells enter the stationary phase within 16 h). On the other hand, Δ*dnaK* R37 exhibited a significantly slower growth rate and entered the stationary phase within 20 h. Although OD_{600} of Δ*dnaK* R37 at the stationary phase was slightly higher than UNS38 and the complemented strain.

We determined the amount of ILY secreted in the culture supernatant at 37°C from UNS38, Δ*dnaK* R37, and the complemented strain by the hemolysis assay (Fig. 3b). The results showed no large difference in hemolytic activity due to ILY between these strains. The level of ILY in the culture

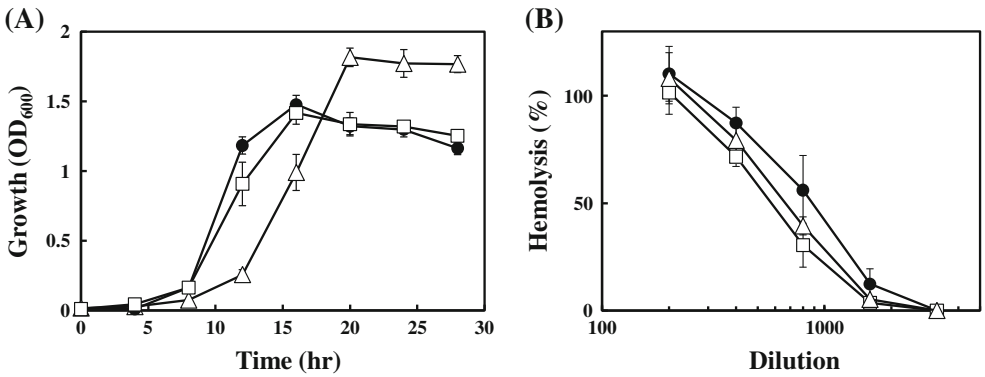

Fig. 3 Effect of *dnaK* null mutation on cell growth and secretion of ILY. **a** Growth curves of UNS38, Δ*dnaK* R37, and its complemented strain. Strains were cultured in BHI medium and the OD_{600} measured at the indicated time points. The graphical data are the mean values± standard deviation of at least four replicated independent experiments. **b** Hemolytic activity in the culture supernatant. Strains were cultured in BHI medium 24 h at 37°C, and the culture supernatants collected. Culture supernatant standardized at OD_{600} was diluted from 200- to 3,200-fold by 2-fold serial dilutions, and the cytolytic activity of ILY in the diluted culture supernatant was estimated by hemolysis assay. *Solid circle* UNS38; *open triangle* Δ*dnaK* R37; *open square* the complemented strain

supernatant was estimated by immunoblotting using anti-ILY antibody and showed that Δ*dnaK* R37 could secrete ILY at levels similar to UNS38 and the complemented strain (data not shown). We further examined the amount of the secreted hyaluronidase in the culture supernatant at 37°C from UNS38, Δ*dnaK* R37, and the complemented strain by the substrate-agarose plate assay using hyaluronan as substrate. No significant difference in hyaluronidase activity was observed among these strains (data not shown). In addition, although we also examined the hemolytic and hyaluronidase activities in the culture supernatant at 30°C from Δ*dnaK* S37 1–3 and Δ*dnaK* R37, they showed the same activities as UNS38 (data not shown).

Cytotoxicity of *S. intermedius dnaK* null mutant on HepG2 cells

Several studies on pathogenic bacteria have reported that mutation of *dnaK* causes attenuation of cytotoxicity and pathogenicity. Therefore, we examined the cytotoxicity of Δ*dnaK* R37 and the complemented strain using HepG2 human cells (Fig. 4). The viability of HepG2 was significantly reduced after 3 days of infection with UNS38 or the complemented strain, and all HepG2 cells were killed at 4 days post-infection. However, the Δ*dnaK* mutant showed only slight cytotoxicity to HepG2 compared to UNS38 or the complemented strain, with HepG2 cells surviving even after 4 days post-infection. These data show that disruption of the *dnaK* gene of *S. intermedius* causes attenuation of cytotoxicity despite ILY secretion being unaffected by this mutation.

Complementation of *E. coli* Δ*dnaK* by *S. intermedius* DnaK chaperone system

To examine the functions and characteristics of the *S. intermedius* DnaK chaperone system, *S. intermedius dnaK* (Si*K*), *grpE-dnaK* (Si*EK*), *dnaK-dnaJ* (Si*KJ*), or *grpE-dnaK-dnaJ* (Si*EKJ*) was cloned on the *E. coli* IPTG-inducible vector pZE13 and these plasmids used to transform *E. coli* Δ*dnaK* mutant (Ec Δ*dnaK*). *E. coli dnaK* (Ec*K*) was also cloned on the same vector as a positive control. The thermosensitivity of these transformants was examined using the spot test (Fig. 5a). These strains were spotted onto LB agar plate containing the concentrations of IPTG indicated and then cultured at 30°C or 42°C. The results showed that all strains grew at 30°C in the presence or absence of IPTG. However, only the pZE13 Ec*K*- and pZE13 Si*EKJ*-transformed Ec Δ*dnaK* grew at 42°C. These strains grew well in the presence of 20 or 50 μM IPTG, although a slight growth defect was observed in the absence of IPTG (pZE13 Si*EKJ*-transformant) or in the presence 100 μM IPTG (pZE13 Ec*K*-transformant). In contrast, pZE13 Si*K*-, pZE13 Si*EK*-, or pZE13 Si*KJ*-transformed Ec Δ*dnaK* did not grow at 42°C indicating that *S. intermedius* DnaJ and GrpE were not compatible with *E. coli* DnaJ and GrpE. However, the intact DnaK chaperone system of *S. intermedius* could complement the thermosensitivity in *E. coli*.

E. coli Δ*dnaK* mutant is known to be acid sensitive, and we have also confirmed this phenotype. The *E. coli* Δ*dnaK* mutant showed lower viability than the wild strain (MC4100) when exposed to low pH conditions (pH 3.5) for 1 h. The *S. intermedius* DnaK chaperone system could also complement acid sensitivity in the *E. coli* Δ*dnaK* mutant, and pZE13 Si*EKJ*-transformed Ec Δ*dnaK* showed nearly equivalent acid tolerance compared to the wild strain (Fig. 5b).

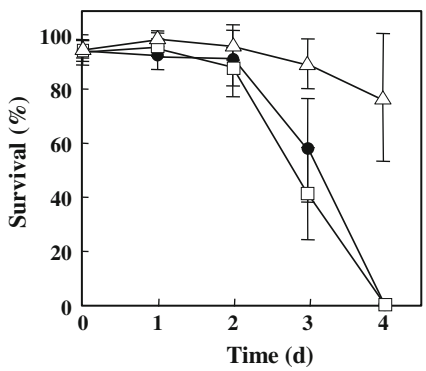

Fig. 4 Cytotoxic effect on HepG2 cells of Δ*dnaK* R37 and its complemented strain. Cytotoxic effects were observed over 4 days post-bacterial infection. *Solid circle* UNS38; *open triangle* Δ*dnaK* R37; *open square* the complemented strain

Function of *S. intermedius* DnaK chaperone system in *E. coli*

Several studies have reported that negative regulation of the *hrcA-grpE-dnaK-dnaJ* and *groES-groEL* operons of many Gram-positive bacteria including streptococci are carried out by the HrcA repressor (Lemos et al. 2001; Woodbury and Haldenwang 2003; Kim et al. 2008), and this activity seemed to be controlled by the DnaK chaperone system and GroESL (Mogk et al. 1997; Koch et al. 1998). On the other hand, heat shock response in many Gram-negative bacteria, including *E. coli* whose heat shock response is known to be regulated by the heat shock transcriptional factor σ^{32}, differs from that of streptococci. Because the activity and stability of σ^{32} is controlled by the DnaK chaperone system, constitutive induction of heat shock response and accumulation of σ^{32} were observed in Ec Δ*dnaK* (Fig. 6a). Although the control mechanism of heat shock response differs between *S. intermedius* and *E. coli*, we examined whether *S. intermedius* DnaK chaperone system could regulate the *E. coli* heat shock response by controlling the

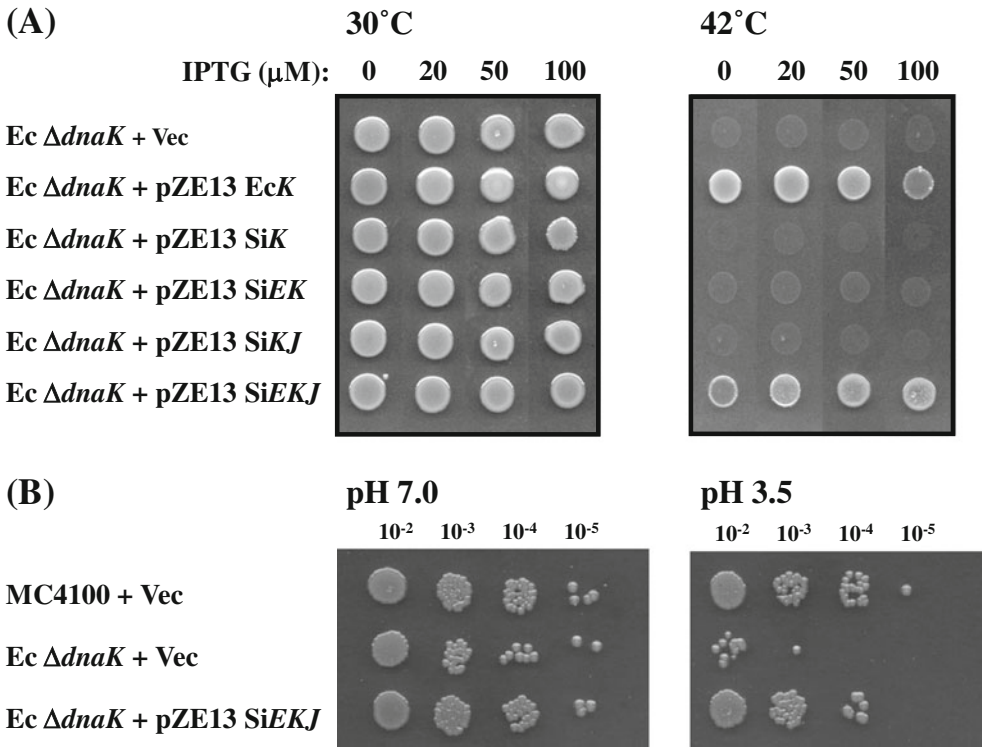

Fig. 5 Complementation of *E. coli* Δ*dnaK* by *S. intermedius* DnaK chaperone system. **a** Spot test for determining thermosensitivity. Cells were cultured for 24 h at 30°C and then spotted on LB agar plates containing the indicated amounts of IPTG. Spotted plates were incubated 24 h at the indicated temperatures. **b** Spot test for the detection of acid tolerance. Spotted plates were incubated for 48 h at 30°C. *MC4100+ Vec* MC4100 transformed with plasmid pZE13; *Ec ΔdnaK+ Vec E. coli ΔdnaK* mutant transformed with plasmid pZE13; *Ec ΔdnaK+pZE13 EcK E. coli ΔdnaK* mutant transformed with plasmid pZE13 Ec*K*; *Ec ΔdnaK+pZE13 SiK E. coli ΔdnaK* mutant transformed with plasmid pZE13 Si*K*; *Ec ΔdnaK+pZE13 SiEK E. coli ΔdnaK* mutant transformed with plasmid pZE13 Si*EK*; *Ec ΔdnaK+pZE13 SiKJ E. coli ΔdnaK* mutant transformed with plasmid pZE13 Si*KJ*; *Ec ΔdnaK+pZE13 SiEKJ E. coli ΔdnaK* mutant transformed with plasmid pZE13 Si*EKJ*

stability and activity of σ^{32}. Our data showed that *S. intermedius* DnaK chaperone system could reduce the accumulation of σ^{32} and GroEL in Ec Δ*dnaK* up to the level of the wild-type.

Since the σ^{32}-encoding gene is *rpoH*, *E. coli* Δ*rpoH* mutant (Ec Δ*rpoH*) is largely devoid of all the major cytosolic chaperones, except for GroEL/GroES, and has lower levels of proteases (Tomoyasu et al. 2001). The lack of chaperones and proteases results in a large accumulation of aggregated proteins by heat treatment, and approximately 9% of the total proteins were aggregated (Fig. 6b). Therefore, Ec Δ*rpoH* could be used to estimate the in vivo chaperone activity of *S. intermedius* DnaK chaperone system. When *S. intermedius* DnaK chaperone system was expressed in Ec Δ*rpoH* by adding 20 or 50 μM IPTG (Fig. 6b), aggregated proteins dramatically decreased to less than 1% of the total proteins at 42°C. Thus, it was concluded that *S. intermedius* DnaK chaperone system could efficiently eliminate the aggregated proteins from Δ*rpoH* mutant cells.

Discussion

DnaK is a major cytosolic chaperone and has an important function in quality control of cellular protein in bacteria, in cooperation with DnaJ and GrpE (Mayer et al. 2000; Ben-Zvi and Goloubinoff 2001; Genevaux et al. 2007). Therefore, the construction and isolation of a *dnaK* null mutant is believed to be difficult and only a few knockout null mutants have been reported thus far. However, it has been reported in studies on *dnaK* knockout mutants that the requirement and importance for quality control of the cellular protein DnaK differs among bacterial species. For example, the *dnaK* null mutants of Gram-negative *E. coli* and *S.* Typhimurium showed pleiotropic phenotypes, such as thermosensitivity, cell elongation, and constitutive heat shock induction (Bukau and Walker 1989a, b, 1990; Takaya et al. 2004). In contrast, Gram-positive *B. subtilis* DnaK mutant showed no apparent phenotype and indicated that DnaK was not essential for normal growth (Schulz et al. 1995). Attempts to isolate a *dnaK* null mutant of *Streptococcus mutans* have been

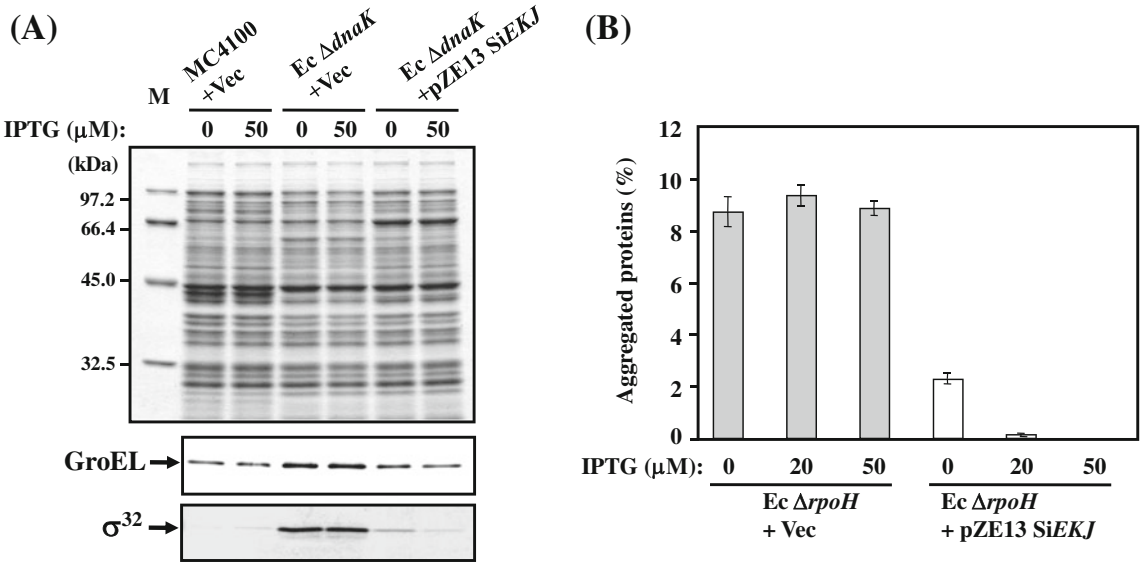

Fig. 6 Function of *S. intermedius* DnaK chaperone system in *E. coli*. **a** Cellular level of GroEL and σ^{32} in *E. coli ΔdnaK* mutants in the presence or absence of the *S. intermedius* DnaK chaperone system. Cells were grown in LB medium containing the indicated concentrations of IPTG for 4 h at 30°C, the OD_{600} of the cultures measured and standardized amounts of the cell lysates analyzed by 12% SDS–PAGE. Immunodetection was carried out with anti-*E. coli* GroEL or anti-σ^{32} antiserum. *M* molecular weight marker; *MC4100+Vec* MC4100 transformed with a plasmid pZE13; *Ec ΔdnaK+Vec E. coli ΔdnaK* mutant transformed with a plasmid pZE13; *Ec ΔdnaK+pZE13 SiEKJ E. coli ΔdnaK* mutant transformed with a plasmid pZE13 Si*EKJ*. **b** Amounts of aggregated protein in *E. coli ΔrpoH* mutants in the presence or absence of the *S. intermedius* DnaK chaperone system. Cells were grown in LB medium containing the indicated concentrations of IPTG for 3 h at 30°C and further cultured at 42°C for 1 h. Aggregated proteins were isolated as described in "Materials and methods." The amount of aggregated protein was quantified by the Bradford assay reagent and calculated in relation to total protein content (set at 100%). Each value is the average from at least three different experiments. *Ec ΔrpoH+Vec E. coli ΔrpoH* mutant transformed with a plasmid pZE13; *Ec ΔrpoH+pZE13 SiEKJ E. coli ΔrpoH* mutant transformed with a plasmid pZE13 Si*EKJ*

unsuccessful thus far and only a *dnaK* downregulated strain has been reported (Lemos et al. 2007). Therefore, we tried to create *dnaK* null mutants of *S. intermedius* to examine the cellular function of the DnaK chaperone system in detail. Since *S. intermedius dnaK* null mutants showed a thermosensitive phenotype, as observed in the *dnaK* mutants of other Gram-positive cocci (Koch et al. 1998; Lemos et al. 2007; Singh et al. 2007), the DnaK chaperone system seemed to have important function in the maintenance of cellular proteins at high temperatures among these bacteria.

Moreover, since constitutive heat shock induction and accumulation of heat shock proteins such as GroEL in Gram-positive bacteria were reported in *L. lactis dnaK* null mutants and *S. mutans dnaK* downregulated strain, the DnaK chaperone system seemed to be required to maintain the DNA-binding activity of HrcA in these strains (Koch et al. 1998; Lemos et al. 2007). Accumulation of GroEL was also observed in a *S. intermedius dnaK* null mutant, *ΔdnaK* R37 (Fig. 2a). The DnaK chaperone system of this bacterium might require the activity of HrcA. However, this does not exclude the possibility that the accumulated misfolded proteins caused by *dnaK* null mutation could promote depletion of GroEL, which is also known to be required to maintain HrcA activity. On the basis that (1) *ΔdnaK* R37 did not show obvious acid sensitivity, in contrast to *E. coli dnaK* mutant and *S.* Typhimurium *dnaK* null mutant (Figs. 2c, 5b, and our unpublished result), and (2) a *S. mutans dnaK* downregulated strain exhibited a slightly higher acid tolerance than the parental strain (Lemos et al. 2007), the activity control or folding of protein(s) which is involved in acid tolerance by the DnaK chaperone system in streptococci might be less important than in *E. coli dnaK* and *S.* Typhimurium.

It has been reported that not only the stress-inducible chaperone (DnaK) but also the stress-inducible proteases (ClpXP, Lon) can regulate the pathogenicity of several Gram-negative pathogenic bacteria including *S.* Typhimurium, enterohemorrhagic *E. coli*, and *Yersinia pestis* (Yamamoto et al. 2001; Takaya et al. 2002; Jackson et al. 2004; Tomoyasu et al. 2005). Therefore, these bacteria are believed to control the expression of virulence factors and possess the ability to sense stresses such as those presented by an effective immune system and accompanying fever in the host organism. Therefore, we investigated whether such the stress-inducible proteins participated in the expression control of virulence factors of *S. intermedius* as well as in the Gram-negative bacteria. ILY is a major virulence factor of *S. intermedius*, which is essential for the invasion and

cytotoxicity to human cells (Nagamune et al. 2000; Sukeno et al. 2005). Compared to the wild strain, *S. intermedius dnaK* null mutation did not cause reduction in hemolytic (ILY) activity and hyaluronidase activity in the culture medium; the attenuation of cytotoxicity in this mutant might be caused indirectly by slightly reduced viability and the slower growth phenotype at 37°C (Figs. 2b and 3a). We also examined the contribution of stress-inducible ClpP peptidase, which is a catalytic subunit of ClpXP protease, to the expression of virulence factors and cytotoxicity of *S. intermedius*. For this purpose, a *clpP* null mutant was constructed, and its phenotype was analyzed. Our result also showed that *clpP* null mutation did not cause reduction of hemolytic and hyaluronidase activities and cytotoxicity to HepG2 cells (unpublished result).

It has been reported that many streptococcal virulence factors are controlled by the carbon catabolite repression (CCR) sensing the extracellular amount of utilizable carbohydrates (Abranches et al. 2008; Shelburne et al. 2008a, b; Kietzman and Caparon 2010). One of the important factors for CCR is catabolite control protein A (CcpA), which can control the expression not only of carbon catabolite genes but also of streptococcal virulence factors. Recently, we have shown that *ily* expression is also controlled by CcpA (Tomoyasu et al. 2010b). Thus, the expression of virulence factors and cytotoxicity of *S. intermedius* seemed to be mainly regulated by the amount of extracellular utilizable carbohydrates rather than extracellular stress conditions.

Previous studies have demonstrated that DnaK of Gram-positive bacteria was unable to complement the viability of *E. coli dnaK* null mutants (Sussman and Setlow 1987; Tilly et al. 1993; Minder et al. 1997; Mogk et al. 1999; Sugimoto et al. 2008). Multiple sequence alignment by CLUSTAL W shows that DnaK of *S. intermedius* is typical of Gram-positive bacteria, lacking segment 75–98 in the ATPase domain that is conserved in almost all proteobacteria (Fig. 1a). This segment seems to play a crucial role in the cooperative function with *E. coli* DnaJ and GrpE co-chaperones as shown in the analysis using a segment deletion mutant of *E. coli* DnaK (Sugimoto et al. 2007). These data also support our results that the expression of *S. intermedius* DnaK could not be functionally activated by DnaJ and GrpE in the *E. coli* Δ*dnaK* mutant (Fig. 5a). Interestingly, the intact *S. intermedius* DnaK chaperone system was active in *E. coli*, was able to complement the thermosensitive phenotype of *E. coli* Δ*dnaK* mutant, and could eliminate aggregated proteins in the Δ*rpoH* mutant (Figs. 5a and 6b). Although the heat shock response of many Gram-positive bacteria including streptococci is regulated by the HrcA repressor, the *S. intermedius* DnaK chaperone system could also regulate the stability and activity of σ^{32} (Fig. 6a). It has been reported that binding with *E. coli* DnaK and DnaJ induces conformational changes of σ^{32}, and consequently, in vivo, could modulate stability and activity (Rodriguez et al. 2008). Similarly, *S. intermedius* DnaK and DnaJ might bind to σ^{32} and induce their conformational changes.

Overall, our results show that the DnaK chaperone system of the Gram-positive bacterium, *S. intermedius*, has the same function as the system in *E. coli* and plays a fundamental role in vital functions such as growth, thermoresistance, and heat shock regulation, but not in the modulation of expression of pathogenic factors.

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