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ORIGINAL PAPER

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Complementation studies of the DnaK–DnaJ–GrpE chaperone machineries from *Vibrio harveyi* and *Escherichia coli*, both in vivo and in vitro

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Abstract The marine bacterium *Vibrio harveyi* is a potential indicator organism for evaluating marine environmental pollution. The DnaK–DnaJ–GrpE chaperone machinery of *V. harveyi* has been studied as a model of response to stress conditions and compared to the *Escherichia coli* DnaK system. The genes encoding DnaK, DnaJ and GrpE of *V. harveyi* were cloned into expression vectors and *grpE* was sequenced. It was found that *V. harveyi* possesses a unique organization of the *hsp* gene cluster (*grpE–gltP–dnaK–dnaJ*), which is present exclusively in marine *Vibrio* species. In vivo experiments showed that suppression of the *E. coli dnaK* mutation by *V. harveyi* DnaK protein was weak or absent, while suppression of the *dnaJ* and *grpE* mutations by *V. harveyi* DnaJ and GrpE proteins was efficient. These results suggest higher species-specificity of the DnaK chaperone than the GrpE and DnaJ cochaperones. Proteins of the DnaK chaperone machinery of *V. harveyi* were purified to homogeneity and their efficient cooperation with the *E. coli* chaperones in the luciferase refolding reaction and in stimulation of DnaK ATPase activity was demonstrated. Compared to the *E. coli* system, the purified DnaK–DnaJ–GrpE system of *V. harveyi* exhibited about 20% lower chaperoning activity in the luciferase reactivation assay. ATPase activity of *V. harveyi* DnaK protein was at least twofold higher than that of the *E. coli* model DnaK but its stimulation by the cochaperones DnaJ and GrpE was significantly (10 times) weaker. These results indicate that, despite their high structural identity (approximately 80%) and similar mechanisms of action, the DnaK chaperones of closely related *V. harveyi* and *E.coli* bacteria differ functionally.

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Abbreviations KJE: DnaK, DnaJ, GrpE proteins · $DnaK_{Ec}$: DnaK protein of *E. coli* · $DnaJ_{Ec}$: DnaJ protein of *E. coli* · $GrpE_{Ec}$: GrpE protein of *E. coli* · $DnaK_{Vh}$: DnaK protein of *V. harveyi* · $DnaJ_{Vh}$: DnaJ protein of *V. harveyi* · $GrpE_{Vh}$: GrpE protein of *V. harveyi* · *gltP*: Glutamate transport protein gene · pfu: Plaque-forming unit · SBD: Substrate-binding domain of DnaK

Introduction

Chaperone proteins form a multiprotein network that governs protein folding, refolding, transport, degradation and regulation (Ellis 1999; Hartl and Hayer-Hartl 2002). Proper functioning of the network is particularly important under stress conditions, such as heat shock, but chaperones are also essential for normal viability of cells under physiological conditions. In the model organism *Escherichia coli*, the Hsp70 chaperone machinery, one of the major elements of the network, is composed of the DnaK(Hsp70)[K], DnaJ(Hsp40)[J] and GrpE(Hsp23)[E] proteins (reviewed by Hartl and Hayer-Hartl 2002; Bukau and Horwich 1998; Houry 2001). DnaK consists of an approximately 44-kDa NH_2-terminal ATPase domain and a 27-kDa COOH-terminal substrate-binding domain. The latter is divided into a subdomain with a peptide-binding cleft and an α-helical latch-like segment (Zhu et al. 1996). DnaK has weak ATPase activity, and it cycles through ATP- and ADP-bound stages, with its affinity for substrate (e.g., a partially denatured protein or a nascent peptide) being lower in the ATP-bound stage than in the ADP one (Żylicz et al. 1983; Liberek et al. 1991; Palleros et al. 1993; Buchberger et al. 1994; McCarty et al. 1995). In

the former, the α-helical latch over the peptide-binding cleft is in an open conformation and rapid peptide binding occurs. Stable holding of peptide involves closing of the latch, a conformational change that is achieved by hydrolysis of bound ATP to ADP. The cycling of DnaK between these states is regulated by the co-chaperones DnaJ and GrpE (the latter functions as a homodimer). The NH_2-terminal J domain binds to DnaK and accelerates hydrolysis of ATP by DnaK, thus facilitating peptide capture (Liberek et al. 1991; Karzai and McMacken 1996; Laufen et al. 1999; Mayer et al. 2000). The COOH-terminal domain of DnaJ functions as a chaperone in recognizing hydrophobic peptides and thus can recruit DnaK to substrates (Langer et al. 1992; Rudiger et al. 1999; Sha et al. 2000). GrpE induces release of ADP from DnaK, and upon rebinding of ATP the DnaK-peptide complex dissociates, completing the reaction cycle (Liberek et al. 1991; McCarty et al. 1995; Szabo et al. 1994; Jordan and McMacken 1995).

The KJE chaperone proteins are very well conserved in evolution in terms of structure and general mechanism of function (Gupta et al. 1997; Gribaldo et al. 1999; Hartl and Hayer-Hartl 2002), as documented by an impressive amount of experimental evidence. By contrast, little is known about the functional differences among the homologous chaperones from different species—especially regarding their species specificity. It has been shown that purified Hsp70 (DnaK) proteins other than DnaK of *E. coli* possess different affinities for model peptides, which might cause differences in substrate specificity in a cell (Levy et al. 1995; Jamsen et al. 1997; Silberg et al. 1998; Kluck et al. 2002). Despite these differences, most of the studied Hsp70 systems, representing all major groups of organisms, are able to refold thermally or chemically denatured firefly luciferase, which thus serves as a model protein to assay chaperone activity in vitro (Schröder et al. 1993; Terada et al. 1997; Lu and Cyr 1998; Żmijewski et al. 2004).

Vibrio harveyi is a free-living gram-negative γ purple bacterium found in diverse marine environments, in various geographic locations, at depths between surface and about 1000 m (Ruby et al. 1980; Baumann and Schubert 1984). Its use as a bioindicator of environmental pollution is under current investigation, especially for monitoring the levels of mutagenic substances. It has been shown that *V. harveyi* cells are highly sensitive to various mutagens and hypersensitive strains have been constructed for use in a modified Ames' test (Czyż et al. 2002; Wegrzyn and Czyż 2003). However, non-mutagenic toxic substances would not be detected in this type of a test. Heat-shock proteins have been proposed as general markers of cellular aggression and their use for environmental monitoring has often been suggested (Sanders and Martin 1993; Van Dyk et al. 1994; Ait-Aissa et al. 2000; Czyż et al. 2002), due to the fact that the heat-shock genes are up-regulated while cells are subjected to various stressors, such as heavy metals, oxidizing agents, organic solvents or UV irradiation. But in order to use *V. harveyi* heat-shock proteins as stress markers, a thorough knowledge of the heat-shock response in this bacterium is necessary.

Although *V. harveyi* is not pathogenic for humans, its close relatives, *Vibrio cholerae*, *Vibrio parahaemolyticus* and *Vibrio vulniformis*, cause devastating diseases; thus, *V. harveyi* is an important, very good and safe model for studying the biology of the *Vibrio* genus. It was shown for *V. cholerae* and *V parahaemolyticus* that heat-shock proteins may regulate synthesis of a pathogenic toxin (Chakrabarti et al. 1999; Wong et al. 2002); hence, studies on *Vibrio* heat-shock response seem highly pertinent. Moreover, *V. harveyi* is a model organism to study luminescence, DNA repair and bacterial quorum sensing (Czyż et al. 2002; Mok et al. 2003). Participation of chaperone proteins in regulation of both processes has been suggested, which again points to the necessity of heat-shock investigations in *V. harveyi*.

Based on the reasons outlined above, we studied the heat-shock response of *V. harveyi*, which led to identification of the major heat-shock proteins DnaK, DnaJ, GroEL, GroES, and IbpA/B (Klein et al. 1995, 2001), followed by cloning and characterization of their genes, including *dnaK–dnaJ* operon (Klein et al. 1998; Kuchanny et al. 1998; Kuchanny-Ardigo and Lipinska 2003). We found that the *V. harveyi dnaK–dnaJ* operon restored the thermoresistant phenotype of a *E. coli dnaJ* mutant but did not suppress the thermosensitivity of *E. coli dnaK* at 42°C. These results suggested that the DnaK chaperone may be more species-specific than the DnaJ co-chaperone.

In this work we provide further information regarding the DnaK–DnaJ–GrpE chaperone machinery of *V. harveyi* with respect to the species specificity of the DnaK chaperone. We presented sequence of the entire *grpE–gltP–dnaK–dnaJ* region and showed that *V. harveyi* DnaK, DnaJ and GrpE efficiently cooperate with the members of the *E. coli* DnaK–DnaJ–GrpE system in vitro. In addition, we have analyzed the suppression of mutations in *dnaK*, *dnaJ* and *grpE* of *E. coli* using homologous genes of *V. harveyi*.

Materials and methods

Bacterial strains, phage, plasmids, media, and reagents

The bacterial strains, bacteriophage, and plasmids used in this study are listed in Table 1. Luria–Bertani (L–B) medium and Luria agar (LA) were prepared according to the method of Sambrook et al. (1989) and were supplemented with appropriate antibiotics (when necessary): 100 μg ampicillin/ml , 68 μg chloramphenicol/ml , 10 μg tetracycline/ml and 30 μg kanamycin/ml. T-agar plates and T-top agar for phage titration were prepared according to a standard procedure (Sambrook et al. 1989). Boss medium and agar were prepared as described (Klein et al. 1998). Primers for DNA amplification and sequencing were purchased from Interactiva and all enzymes for DNA manipulations were from

Table 1 Bacterial strains, bacteriophages and plasmids used in this study

Strain, phage or plasmid	Relevant genotype	Source/Reference
Escherichia coli strains		
B178	W3110 galE sup$^+$	Our collection
MC4100	dnaK$^+$	B. Bukau/Casadaban (1976)
DH5α	supE44 ΔlacU169 (Φ80 lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1	Our collection/Sambrook et al. (1989)
BM271 (ΔK)	MC4100 ΔdnaK52::Cm^{R}ts	B. Bukau/Paek and Walker (1987)
CG1000 (ΔJ)	B178 ΔdnaJ Tn10 Tc^{R}ts	C. Georgopoulos/Sell et al. (1990)
DA259 (ΔG)	C600 ΔgrpE::Ω-Cm^R thr::Tn10	D. Ang Wu et al. (1994)
WKG190	MC4100 araD139 Δara714 ΔcbpA::kan dnaJ:: Tn10	A. Wawrzynów/Kelley and Georgopoulos (1997)
V. harveyi strain		
	Wild-type	Our collection/Lee et al. (1991)
Phages		
λ cIb2		Our collection
λ imm21		G. Węgrzyn
Plasmids		
pMOB45-dnaK$_{Ec}$$^+$	pMOB45-dnaK$_{Ec}$ Cm^R	Our collection/Żylicz and Georgopoulos (1984)
pDW19-dnaJ$_{Ec}$$^+$	pTTQ19-dnaJ$_{Ec}$ Amp^R	K. Liberek/Wall et al. (1994)
pRLM156-grpE$_{Ec}$	pRLM156-grpE$_{Ec}$ Amp^R	A. Wawrzynów, originally from R. McMacken
WKG190	MC4100 araD139 Δara714 ΔcbpA::kan dnaJ:: Tn10	A. Wawrzynów/Kelley and Georgopoulos (1997)
pGK1	pBR322-dnaKdnaJ$_{Vh}$ Amp^R	Our collection/Klein et al. (1998)
pMZ1	pT7-5-dnaJ$_{Vh}$ Amp^R	This work
pMZ20	pGEM3zF + -dnaK$_{Vh}$ Amp^R	This work
pMZ33	pGEM3zF + lacZ′-dnaK$_{Vh}$ Amp^R	This work
pMZ40	pUC18-grpE$_{Vh}$ Amp^R	This work
pBR322	Amp^R, Tet^R	Our collection/Klein et al. (1998)
PGEM3zf +	Amp^R	Our collection/Promega
pT7-5	Amp^R	Our collection/Tabor and Richardson (1985)
pGP1-2	T7pol Kan^R	Our collection/Tabor and Richardson (1985)
pUC18	Amp^R	Our collection/Sambrook et al. (1989)

Fermentas. All other reagents were purchased from Sigma or as indicated in the text. Molecular biology methods were done according to Sambrook et al. (1989).

Subcloning of *dnaK*$_{Vh}$ and *dnaJ*$_{Vh}$

The 1,250-bp *Xap*I DNA fragment of pGK1 *dnaK*$_{Vh}$*dnaJ*$_{Vh}$ plasmid, containing *dnaK*$_{Vh}$, was cloned into the *Eco*RI site of the pGEM Zf3 + vector. Subsequently, two plasmids, pMZ20 and pMZ33, which differed in orientation of the insert, were isolated. Orientation of the insert was determined by restriction analysis and sequencing of DNA. pMZ20 contained *dnaK*$_{Vh}$ under the control of the phage T7 ϕ10 promoter, while pMZ33 contained a *lacZ'–dnaK*$_{Vh}$ fusion. Both plasmids were used in in-vivo complementation experiments, and plasmid pMZ20 was used for overproduction of DnaK$_{Vh}$, according to Tabor and Richardson's system (1985). *V. harveyi dnaJ* coding sequence was amplified by PCR using *V. harveyi* chromosomal DNA as a template and primers: DnaJVhfor 5′-GGAATTCGATACAAACACCAAGCGG-3′ and DnaJVhrev 5′-CCTGCAGGTCGACTGGATTCAACTAACCAGC-3′. The primers were designed based on the known sequence of the *dnaKdnaJ* operon of *V. harveyi* (Klein et al. 1998). Both primers had artificially designed *Eco*RI or *Hin*dIII restriction enzyme digestion sites (underlined). The PCR fragment was digested with *Eco*RI and *Hin*dIII and ligated into corresponding sites of the pT7-5 vector plasmid. The quality of the resulting construct (pMZ1) was confirmed by restriction analysis and comparison to the known DNA sequence [AF055368]. pMZ1 was subsequently used for overproduction of DnaJ$_{Vh}$ using Tabor and Richardson's system (1985).

Cloning and sequencing of *grpE*$_{Vh}$

The gene coding GrpE$_{Vh}$ was amplified using PCR with chromosomal DNA of *V. harveyi* as template and the primers grpEVhfor 5′-TGAATTCTGGAGATATCATGAGCAACCAA-3′, containing the Shine-Dalgarno sequence and a start (ATG) codon, and grpEVhrev 5′-TTCTGCAGAATTATTTGGCAACCATCACCA-3′, containing a stop (TAA) codon and part of the intergenic (*grpE–gltP*) fragment. Sequences of the primers were designed based on the sequences of *grpE* genes of *Vibrio proteolyticus* [AF218211] and *Vibrio cholerae* [AF019558]. Additionally, sequences recognized by restrictions enzymes *Eco*RI or *Pst*I were introduced into the primers (underlined). Restriction analysis of the PCR product (∽650 bp) revealed that the coding sequence of the *V. harveyi grpE*, in contrast to the *grpE* genes of *V. proteolyticus* and *V. cholerae*, contained additional sites for both restriction enzymes (*Eco*RI and *Pst*I), so that these sites could not be used. Finally, *grpE* of *V. harveyi* was cloned into the *Sma*I site of pUC18 after creating blunt ends of the PCR product by Klenow polymerase, resulting in the plasmid pMZ40.

DNA of $grpE_{Vh}$ was sequenced; the sequence was deposited in GenBank [AY193779], and was later resubmitted as a part of the *grpE–gltP–dnaK–dnaJ* gene cluster [AY639008].

Analysis of the *grpE–gltP–dnaK–dnaJ* gene cluster composition and the *gltP* sequence

In order to determine the position of *grpE* with respect to the *dnaK–dnaJ* operon on the *V. harveyi* chromosome, a DNA fragment containing *grpE* and the 5′-region of *gltP* was amplified by PCR using the chromosomal DNA of *V. harveyi* as template and the primers: grpEVhfor (as above) and gltPVhI 5′-CCTTAAGCTTCTCAGTTAGCGTGC-3′. The resulting PCR fragment of 2,300 bp was sequenced using primers: gltPVhI (as above), gltPVhII 5′-CAGAGCAAATACACCGTAAGG-3′ and gltPVhIII 5′-CCAGATCACGAATCAAACACAG-3′. Combining the resulting sequence with those of $grpE_{Vh}$ [AY193779] and the $dnaK_{Vh}$–$dnaJ_{Vh}$ operon [AF055368] revealed a putative *gltP* gene between $grpE_{Vh}$ and $dnaK_{Vh}$. A new database entry was created for the entire $grpE_{Vh}$–$gltP_{Vh}$–$dnaK_{Vh}$–$dnaJ_{Vh}$ gene cluster [AY639008]. The sequences of *grpE* and *gltP* of *V. harveyi* were analyzed using the Internet programs: blastn, blastp, genome blast and blast 2 sequences on the NCBI server (www.ncbi.nih.gov). Multisequence comparisons were done using the ClustalW program on EBI server (www.ebi.ac.uk).

Suppression of the *E. coli* mutations by cloned *V. harveyi* genes

Mutants of *E. coli*: BM271 Δ*dnaK*, CG1000 Δ*dnaJ*, WKG190 Δ*cbpAdnaJ* and DA259 Δ*grpE* were transformed with plasmids carrying cloned *V. harveyi*: *dnaK*, *dnaJ*, *dnaKdnaJ*, or *grpE* genes and suppression of thermosensitive phenotype and bacteriophage λ growth were tested as described for the archaeon *Methanosarcina mazei* genes (Żmijewski et al. 2004). The $dnaK_{Vh}$ and $dnaJ_{Vh}$ genes from pMZ20 and pMZ1, respectively, were expressed with the help of pGP1-2 plasmid, encoding bacteriophage T7 polymerase (Tabor and Richardson 1985). Briefly, for the suppression of the thermosensitive phenotype of *E. coli* mutants, bacterial suspensions in 10 mM $MgSO_4$ (Sambrook et al. 1989) were prepared from appropriate transformants; 2 µl of serial dilutions from 10^{-1} to 10^{-6} of the suspensions were spotted onto LA plates and incubated at 30, 37, 42 and 45°C for 18 h. For the bacteriophage λ-tests, 3 ml of T-top agar (Sambrook et al. 1989) containing 100 µl of bacterial suspension in 10 mM $MgSO_4$, prepared as described above, was plated on a T-agar plate and allowed to dry. Two microliters of serial dilutions (10^{-1} to 10^{-9} in TM buffer) of bacteriophage λ *c*Ib2 [phage titer was at least 10^9 plaque-forming units (pfu) per ml] were spotted on the plates. Plates were incubated overnight at 30°C, plaques were counted, and the efficiency of plating (e.o.p.) was calculated as the ratio of pfu on the tested strain to the pfu on the wild-type reference strain (*E. coli* B178). All in vivo experiments were repeated at least three times.

Determination of $DnaK_{Vh}$ overproduction in *E. coli*

E. coli B178 (wt) and *E. coli* BM271 (Δ*dnaK*) were transformed with plasmids pGK1 $dnaK_{Vh}dnaJ_{Vh}$; pMZ33 *lacZ′*–$dnaK_{Vh}$; pMZ20 $dnaK_{Vh}$ and pGP1-2. In order to estimate the amount of $DnaK_{Vh}$ overproduction in transformed cells, overnight cultures were diluted (1:50) and incubated in L–B medium supplemented with proper antibiotic(s) at temperature of 30°C. At an optical density (OD) of 0.6, each culture was split: one part remained at 30°C while the other was subjected to heat shock at 42°C. After 1 h of incubation, 200 µl of each culture was harvested, resuspended in lysing buffer for SDS-PAGE (bromophenol blue was omitted) (Sambrook et al. 1989) and protein concentration was measured by the method of Bradford (1976). Cell lysates equivalent to 10 µg of protein were supplemented with bromophenol blue, analyzed by SDS-PAGE and visualized by Coomassie brilliant blue staining (Sambrook et al. 1989).

Overproduction, purification, and analysis of recombinant DnaK, DnaJ and GrpE

$DnaK_{Vh}$ and $DnaJ_{Vh}$ were overproduced using the Tabor and Richardson's (1985) system from plasmids pMZ20 $dnaK_{Vh}$ and pMZ1 $dnaJ_{Vh}$, in *E. coli* BM271 Δ*dnaK* and CG1000 Δ*dnaJ* cells, respectively. Both genes were under the control of the bacteriophage T7 φ10 promoter and their expression was supported by the presence of pGP1-2, encoding heat-inducible T7 phage RNA polymerase (Tabor and Richardson 1985). Cultures of *E. coli* cells overproducing $DnaK_{Vh}$ and $DnaJ_{Vh}$ were incubated oxically at 30°C to $OD_{595} = 0.6$ and then heat-shocked at 42°C for 2 h. $GrpE_{Vh}$ was expressed from the chromosomal gene in *V. harveyi* cells grown at 30°C to $OD_{595} = 1$ and then subjected to heat shock for 2 h at 39°C. $DnaK_{Ec}$, $DnaJ_{Ec}$ and $GrpE_{Ec}$ proteins were overproduced from plasmids pMOB45 $dnaK_{Ec}$, pDW19 $dnaJ_{Ec}$ and pRLM156 $grpE_{Ec}$ in an *E. coli* B178 background. $DnaK_{Ec}$ and $DnaJ_{Ec}$ were overproduced by induction of the genes with 1 mM IPTG at $OD_{595} = 0.6$, followed by incubation for an additional 2 h at 37°C. Cultures of the *E. coli* cells overproducing $GrpE_{Ec}$ protein were incubated oxically at 30°C to $OD_{595} = 0.6$ and then heat-shocked at 42°C for 2 h. All cultures were harvested by centrifugation (9,000*g* for 10 min at 4°C) and then resuspended in 50 mM Tris–HCl (pH 7.5), 10% (w/v) sucrose, 1 mM EDTA (pH 7.7) (1 ml per 1 g of cells). The suspensions were then frozen in liquid nitrogen and stored at −70°C until required. All

chaperone proteins of *V. harveyi* and *E. coli* were purified as described previously (Żmijewski et al. 2004). For preparation of DnaK free of the nucleoside-diphosphate kinase (Ndk), the purified protein was concentrated on a Millipore filter (cut-off 30 kDa), and then purified by HPLC chromatograph, using a Zorbax GF-250 column (Agilent Biotechnologies).

Protein concentration was measured by the method of Bradford (1976) and confirmed by densitometry of Coomassie-stained SDS-polyacrylamide gels. All proteins used in this study were purified to more than 95% as estimated by SDS-PAGE and were free of contamination with other chaperones as judged by Western blotting. In Western blotting experiments, rabbit polyclonal antibodies were used; anti-DnaK$_{Ec}$, anti-DnaJ$_{Ec}$, anti-GrpE$_{Ec}$ were prepared as described previously by Szewczyk and Summers (1998). The N-terminal amino acid sequence of the purified proteins was analyzed on a gas-phase sequencer (Model 491, Perkin Elmer-Applied Biosystems, Foster City, Calif., USA) at BioCenter (Jagiellonian University, Krakow, Poland).

Luciferase refolding assay

Luciferase refolding assays were performed as published previously (Żmijewski et al. 2004). Briefly, firefly luciferase was pre-incubated for 10 min at 25°C in the presence of various combinations of the chaperone proteins and subsequently denatured for 10 min at 42°C. Renaturation was started by addition of 5 mM ATP followed by 15–90 min incubation at 25°C. All experiments were done in luciferase-refolding buffer L (40 mM Tris–HCl, pH 7.4, 50 mM KCl, 5 mM DTT, 10 mM $MgCl_2$, 10% glycerol), with 1 μM DnaK, 0.2 μM DnaJ, 0.5 μM GrpE, and 80 nM luciferase. Control assays contained 0.5 mg bovine serum albumin (BSA)/ml. Modifications of this standard procedure are indicated in the figure legends, when pertinent. Luciferase activity was determined by using the Luciferase Assay System (Promega) and a Berthold luminometer. Initial activity of the native luciferase was set as 100%.

ATPase activity of DnaK

ATPase activity of the DnaK proteins was measured in the presence of various combinations of co-chaperones, as stated when pertinent. All reactions were performed at 25°C as described previously (Żmijewski et al. 2004) and free phosphate was assayed by the malachite green method (Lanzetta et al. 1979). The standard curve was obtained using H_3PO_4 or phosphate buffer, pH 6.8 (Sambrook et al. 1989). The enzymatic activity was calculated as pmol ATP/pmol DnaK/min.

All in vitro and in vivo tests were repeated at least three times, and mean values and standard deviations were calculated.

Results

Cloning of *V. harveyi dnaK, dnaJ* and *grpE*

dnaK

The coding sequence of *dnaK* of *V. harveyi* was cloned from the pGK1 *dnaK$_{Vh}$dnaJ$_{Vh}$* plasmid into expression vector pGEM Zf3+, as described in Materials and methods. Two plasmids were obtained: pMZ20 (orientation consistent with pT7 Φ10 promoter) and pMZ33 (orientation consistent with p *lac* promoter). SDS-PAGE of the lysates of *E. coli* BM271 Δ*dnaK* cells transformed with plasmid pMZ33 revealed that DnaK$_{Vh}$ migrated with lower mobility than expected (Figs. 1a, 3b). Theoretical examination of the pMZ33 nucleotide sequence showed a possible in-frame fusion of the *lacZ* fragment (120 nucleotides encoding 40 N-terminal amino acids) with *dnaK$_{Vh}$* (both genes in the same frame). This was confirmed by sequencing and, accordingly, the construct is referred to as pMZ33 *lacZ′*-dnaK$_{Vh}$. DnaK$_{Vh}$ expressed from the pMZ20 plasmid migrated in SDS-PAGE as predicted from the nucleotide sequence of *dnaK$_{Vh}$* [theoretical molecular mass of DnaK$_{Vh}$ is 69,076.6 Da, see Klein et al. 1998) [(Fig. 1a)]. Both

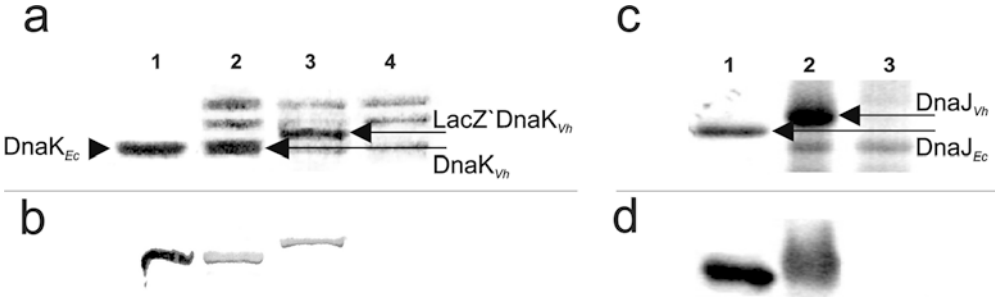

Fig. 1 Overproduction of *Vibrio harveyi* DnaK and DnaJ in *Escherichia coli* cells. **a**, **c** Electrophoretic analysis and **b**, **d** immunodetection of recombinant *V. harveyi* proteins synthesized in *E. coli* cells. **a** Purified DnaK$_{Ec}$ (*lane 1*); DnaK$_{Vh}$ overproduction from pMZ20 plasmid (in the presence of pGP1-2 plasmid) (*lane 2*) or from pMZ33 plasmid (*lane 3*) in *E. coli*Δ*dnaK* bacteria; *E. coli*Δ*dnaK* control cells (*lane 4*). **b** Western blotting analysis of DnaK$_{Vh}$ using anti-DnaK$_{Ec}$ polyclonal antibodies [*lanes* as in (**a**)]. **c** Purified DnaJ$_{Ec}$ (*lane 1*); DnaJ$_{Vh}$ overproduction from pMZ1 plasmid (in the presence of pGP1-2 plasmid) in *E. coli*Δ*dnaJ* bacteria (*lane 2*); *E. coli*Δ*dnaJ* control cells (*lane 3*). **d** Western blotting of DnaJ$_{Vh}$ using anti-DnaJ$_{Ec}$ polyclonal antibodies [*lanes* as in (**c**)]

proteins, $DnaK_{Vh}$ and LacZ′-$DnaK_{Vh}$, reacted with polyclonal antibodies raised against *E. coli* DnaK (Fig. 1b).

dnaJ

The coding sequence of *dnaJ* of *V. harveyi* was cloned by PCR using the chromosomal copy of the gene as template, as described in Materials and methods, and a plasmid, pMZ1 $dnaJ_{Vh}$, was obtained. The $dnaJ_{Vh}$ protein product migrated in SDS-PAGE with lower mobility than DnaJ of *E. coli*, as predicted from the nucleotide sequence [calculated molecular mass of $DnaJ_{Vh}$ is 41,618.8 Da (Klein et al. 1998) and of $DnaJ_{Ec}$ is 41,100.3 Da (Bardwell et al. 1986) (Fig. 1c). The $DnaJ_{Vh}$ protein reacted with polyclonal antibodies raised against $DnaJ_{Ec}$ (Fig. 1d).

grpE

We previously reported the sequence of the *dnaKdnaJ* operon of *V. harveyi* (Klein et al. 1998) but a sequence of the third gene of the chaperone triad—*grpE*—remained unknown. In order to fill that gap, the gene coding *V. harveyi* GrpE was cloned, sequenced (see Materials and methods for details) and the sequence of the $grpE_{Vh}$ was deposited in GenBank (accession number AY193779). $grpE_{Vh}$ consists of 597 base pairs and encodes a hypothetical protein of 198 amino acids with a calculated molecular mass of 22,307 Da and isoelectric point pI = 4.61; a model GrpE of *E. coli* is a 21,812 Da protein with pI = 4.68.

Analysis of the $grpE_{Vh}$ DNA sequence and of hypothetical $GrpE_{Vh}$ revealed a high level of identity (80–90%) to genes and proteins of other members of *Vibrio* genus (Table 2). Interestingly, the identity of *grpE* genes was higher among the marine *Vibrios* (*V. harveyi*, *V. parahaemolyticus*, *V. vulnificus* and *V. proteolyticus*) than to the human pathogen, *V. cholerae*. Protein identity decreased very rapidly when the sequence of $GrpE_{Vh}$ was compared to other bacterial GrpE sequences, for example that of *E. coli* or *Bacillus subtilis* (Table 2). Comparison of the sequence of $GrpE_{Vh}$ to those from *Archaea* and *Eukarya* resulted in 29 and 27% identity, respectively (Table 2). Our results confirm that in the DnaK–DnaJ–GrpE system GrpE is the least conserved in evolution (Gupta et al. 1997; Cheetham and Caplan 1998; Gribaldo et al. 1999).

Cluster of the *V. harveyi* heat-shock genes

We have previously reported that directly upstream of the $dnaK_{Vh}$ coding sequence there is an open reading frame, *orf136* (Klein et al. 1998). Further investigation (sequencing of *V. harveyi* chromosomal DNA and database searching) resulted in extending the known sequence to 317 nucleotides (not shown), which revealed that this fragment shared about 80% of identity with the *gltP* genes of other *Vibrios*, encoding the glutamate transport proteins. The presence of the *gltP* homolog upstream of the *V. harveyi dnaK* gene (in the same orientation) suggested a similar arrangement of the *V. harveyi* genes to that of *V. parahaemolyticus* (accession number AP005075) and *V. proteolyticus* (accession number AF218211), namely *grpE–gltP–dnaK–dnaJ*, in contrast to *V. cholerae*, in which *gltP* is situated separately on the chromosome (accession number AE004170). The order of the genes on *V. harveyi* chromosome was confirmed by PCR and the primers: grpEVhfor and gltPVhI, followed by sequencing of the 2,300-bp PCR product (see Materials and methods for details). A sequence of the region between *grpE* and *orf136* was obtained, which enabled us to compose a contiguous sequence of the *grpE–gltP–dnaK–dnaJ* region of *V. harveyi*. The entire sequence was deposited in GenBank under accession number AY639008. The sequence of the putative *gltP* gene of *V. harveyi* shares a very high degree of identity with the *gltP* genes of other *Vibrio* species and very low identity with the *gltP* genes of other Bacteria, Archaea or Eukarya (Table 2). The existence of the additional gene in the *grpE–dnaK–dnaJ* gene cluster in marine *Vibrio* species is very unique and suggests an involvement of *gltP* in their heat-shock response. However, we were able to find only a putative

Table 2 Comparison of the sequences of *V. harveyi grpE* and *gltP*, and their products to selected bacterial, eukaryotic and archaeal sequences. It should be noted that all organisms presented here possess more than one gene encoding proton/glutamate or sodium/dicarboxylate symporter (*gltP*). The sequence of the protein/gene with the highest blastp/blastn score was chosen for the final comparison. The human *hmge* sequence was used for comparison

Organism	Accession number	DNA identity (%)		Protein identity (%)	
		grpE	*gltP*	GrpE	GltP
V. parahaemolyticus	AP005075	88	80	95	92
V. vulnificus YJ016	NC_005128	75	77	79	93
V. proteolyticus	AF218211	77	74	79	91
*V. cholerae*O1 biovar eltor str. N16961	AE004170	73	54	71	42
E. coli O157:H7	NC_002128	58	< 5	46	31
Bacillus subtilis subsp. *subtilis* str. 168	NC_000964	40	< 5	34	28
Methanosarcina mazei	NC_003901	27	< 5	29	28
Homo sapiens	XM_380013	3	< 5	27	23

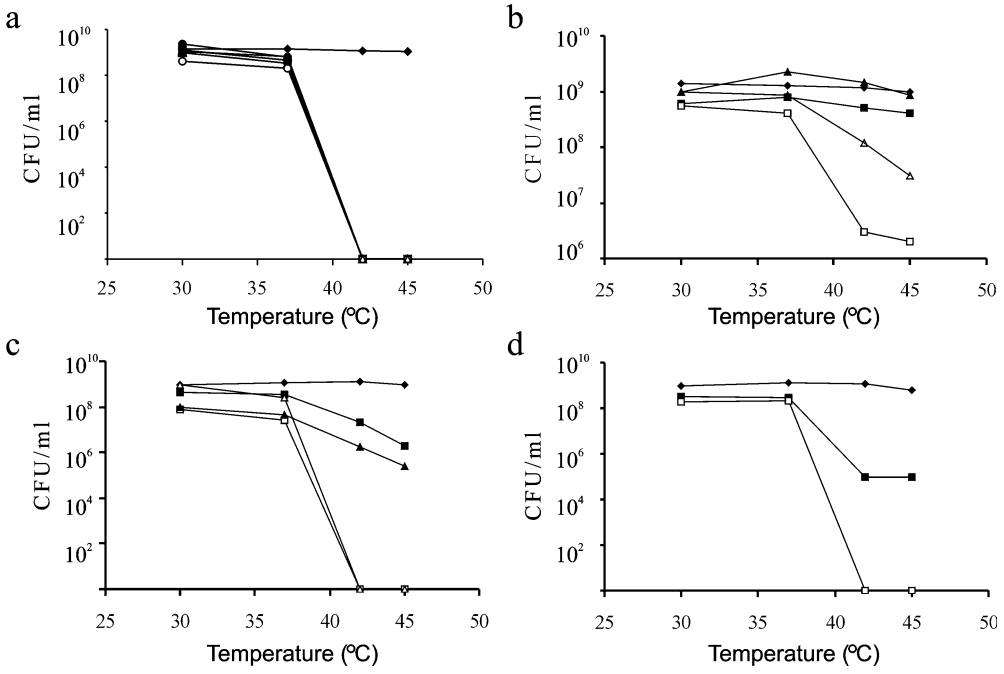

Fig. 2 Complementation experiments of *E. coli* mutations with *V. harveyi* genes. *E. coli* strains: **a** Δ*dnaK*, **b** Δ*dnaJ*, **c** Δ*cbpAdnaJ*, **d** Δ*grpE* were transformed with plasmids carrying relevant *V. harveyi* genes or vector DNA, as a control, and the ability of transformants to grow at different temperatures was tested as indicated. *Filled diamonds*: **a**, **b**, **c**, **d** *E. coli* B178 wild-type; **a**, **b**, **c** *filled triangles*: plasmid pGK1$dnaK_{Vh}dnaJ_{Vh}$ (pBR322 derivative); **a**, **b**, **c** *open triangles*: pBR322 vector; *filled squares*: plasmid carrying the relevant *V. harveyi* gene: **a** pMZ20$dnaK_{Vh}$; **b**, **c** pMZ1$dnaJ_{Vh}$ and **d** pMZ40$grpE_{Vh}$; **a** *filled circles*: pMZ33*lacZ′*-dnaK$_{Vh}$; *open squares*: vector control: **b**, **c** pT7-5; **d** pGEM3zf+ (see Materials and methods for details). Additionally, cells bearing pMZ1, pMZ20 and corresponding vector controls were co-transformed with pGP1-2 plasmid. Results are presented as a number of colony forming units (CFU)

vegetative (sigma 70) promoter sequence before the *gltP* coding sequence—no typical heat-shock promoter could be identified (data not shown).

Cooperation of the KJE chaperone proteins of *V. harveyi* and *E. coli* in vivo

Mutants of *E. coli* carrying a deletion of *dnaK*, or *dnaJ*, or *grpE* are thermosensitive and resistant to bacteriophage λ infection (Georgopoulos et al. 1990). The *E. coli* mutant strains BM271 Δ*dnaK*, CG1000 Δ*dnaJ*, WKG190 Δ*cbpAdnaJ* and DA259 Δ*grpE* were transformed with plasmids carrying the relevant *V. harveyi* gene, and the transformants were tested for growth at high temperatures (42 and 45°C), as well as for their ability to support growth of λ phage (see Materials and methods).

DnaK of *V. harveyi* was not able to suppress thermosensitivity of *E. coli* mutant (Figs. 2a, 3a) when the protein was expressed from high-copy-number plasmids pMZ33 *lacZ′*–dnaK or pMZ20 *dnaK* (both pGEM3zf + derivatives). The lack of rescue by the *V. harveyi dnaK* was not due to the absence of $dnaK_{Vh}$ expression, because DnaK$_{Vh}$ was present at high levels in the transformed *E. coli dnaK* mutant cells at 42°C (Fig. 3b). The expression of the *lacZ′*–dnaK$_{Vh}$ gene from pMZ33 was high even without IPTG induction (Fig. 3b).

It has been previously reported that overexpression of DnaK$_{Ec}$ might be toxic for *E. coli* (Rockabrand and Blum 1995). To rule out the possibility that non-physiologically high levels of DnaK$_{Vh}$ had a lethal effect on *E. coli* growth, the growth of *E. coli* MC4100 $dnaK^+$ strain transformed with pMZ20, pMZ33, or pGK1 plasmids was monitored; all transformants were able to grow at both 30 and 42°C (Fig. 3a).

According to Mogk et al. (1999), lack of suppression of the *E. coli dnaK* mutation by a foreign *dnaK* gene expressed from a plasmid might be due to an insufficient level of DnaJ$_{Ec}$ in the *E. coli*Δ*dnaK* strain (Mogk et al. 1999). It has been previously reported that, in the case of plasmid pGK1 carrying a complete $dnaK_{Vh}dnaJ_{Vh}$ operon, both DnaK and DnaJ were expressed in *E. coli* cells (Klein et al. 1998). However, pGK1 did not support growth of *E. coli* Δ*dnaK*, similar to plasmids expressing only $dnaK_{Vh}$ (Figs. 2a, 3a and Klein et al. 1998); thus, we concluded that *V. harveyi* DnaK is unable to function efficiently in *E. coli* cells. In order to gain additional information concerning in vivo activity of DnaK$_{Vh}$, the ability of pMZ33 carrying *lacZ′*–dnaK$_{Vh}$ fusion to support bacteriophage λ growth in *E. coli* BM271 Δ*dnaK* cells was tested. To some extent, *V. harveyi* DnaK was able to support bacteriophage λ growth: the e.o.p. for mutant cells transformed with pMZ33 was 3.8×10^{-4} compared to an e.o.p. $<10^{-8}$ obtained for mutant cells transformed with vector plasmids only. These results were similar to those reported previously for $dnaK_{Vh}dnaJ_{Vh}$ cloned in pGK1 (Klein et al. 1998).

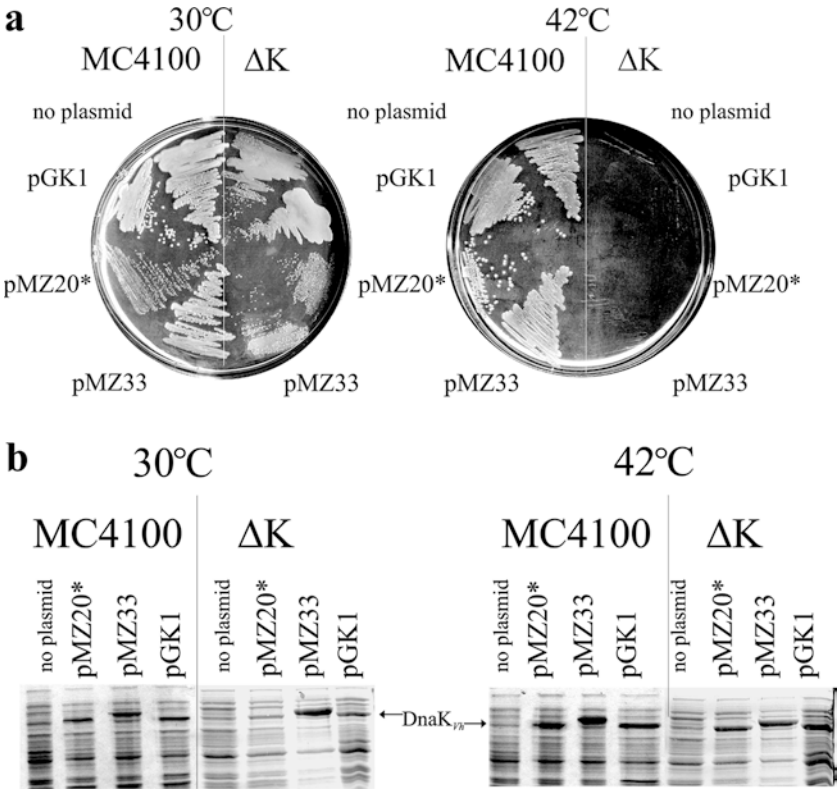

Fig. 3 Overexpression of *V. harveyi* DnaK is insufficient for growth of *E. coli*Δ*dnaK* bacteria under heat-shock conditions (42°C) and is not toxic for *E. coli* cells. **a** The *E. coli* MC4100 (wild-type) [(*left side of a plate*)] or *E. coli*Δ*dnaK* (*right side of a plate*) cells transformed with various plasmids carrying $dnaK_{Vh}$ were spread on LA plates and incubated at 30°C (*left plate*) or 42°C (*right plate*) for 18 h. **b** Electrophoretic analysis of the whole-cell lysates of *E. coli* MC4100 (wild-type) and *E. coli* Δ*dnaK* bacteria transformed with plasmids carrying $dnaK_{Vh}$ (as in Fig. 2a). The following plasmids were used: pMZ20 $dnaK_{Vh}$, pMZ33 *lacZ′*-dnaK$_{Vh}$, pGK1 $dnaK_{Vh}dnaJ_{Vh}$. Cells were grown to optical density $OD_{595} = 0.6$ at 30°C and then shifted to 42°C or incubated at 30°C for an additional hour. See Materials and methods for details. **E. coli* cells bearing pMZ20 also carried pGP1-2

Cloned *dnaJ* and *grpE* of *V. harveyi*, in contrast to $dnaK_{Vh}$, rescued the growth defect of the *E. coli* mutants at 42 and 45°C (Fig. 2b,d). Complementation by *V. harveyi dnaJ* was also very efficient in the double knock-out mutant Δ*cbpAdnaJ*, deprived not only of DnaJ but also of its homolog, CbpA, as well (Fig. 2c). The *V. harveyi* DnaJ is a type I DnaJ according to the classification of Cheetham and Caplan (1998), similarly as *E. coli* DnaJ, while CbpA belongs to class II. A single *cbpA* mutation does not cause thermosensitivity but the double mutant Δ*cbpAdnaJ* is more thermosensitive than *E. coli dnaJ* bacteria and commonly used for complementation tests (Ueguchi et al. 1995; Kelley and Georgopoulos 1997). Moreover, both DnaJ and GrpE of *V. harveyi* were able to support bacteriophage λ growth in *E. coli* mutant cells. The complementation efficiency was significantly higher than in the case of DnaK$_{Vh}$ (e.o.p was 0.05 for *E. coli*Δ*dnaJ* cells transformed with pMZ1$dnaJ_{Vh}$, 1.0 for *E. coli*Δ*cbpAdnaJ* and 0.8 for *E. coli*Δ*grpE* transformed with pMZ40$grpE_{Vh}$). These results show that *V. harveyi* DnaJ and GrpE are able to function efficiently in *E. coli* cells. It should be noted that phage plaques formed on *E. coli*Δ*dnaK* bacteria transformed with pMZ33 or pGK1 coding $dnaK_{Vh}$ were significantly smaller than those formed on the *E. coli*Δ*dnaJ* or Δ*grpE* bacteria transformed with relevant *V. harveyi* genes (data not shown). Results obtained for the single *dnaJ* of *V. harveyi* expressed from pMZ1 were similar to those previously reported for pGK1 $dnaK_{Vh}dnaJ_{Vh}$ (Klein et al. 1998). Since the expression of $dnaJ_{Vh}$ from pMZ1 was significantly higher than from pGK1 (data not shown), it could be concluded that the level of DnaJ$_{Vh}$ had a minor effect on the results of the in vivo tests. Although the level of GrpE$_{Vh}$ expressed from pMZ40 was very low (data not shown), GrpE$_{Vh}$ was able to function efficiently in the DnaK$_{Ec}$–DnaJ$_{Ec}$–GrpE$_{Vh}$ system in vivo.

These results indicate that DnaK is more species-specific in vivo than DnaJ and GrpE, despite the fact that DnaK is the best conserved in evolution among the DnaK–DnaJ–GrpE chaperone proteins.

Purification of *V. harveyi* DnaK, DnaJ and GrpE

The positive complementation tests in vivo indicated that functional DnaJ$_{Vh}$ and GrpE$_{Vh}$ were expressed in *E. coli* cells from the cloned genes. In addition, DnaK$_{Vh}$ and DnaJ$_{Vh}$ expressed from pMZ20 and pMZ1, respectively, could be detected in bacterial lysates analyzed by SDS-PAGE as additional bands with the expected mobility, similar to those of the homologous *E. coli* molecules (Figs. 1a,c, 3b), and could be detected using antibodies against DnaK and DnaJ of *E. coli* (Fig. 1b,d). DnaK$_{Vh}$ and DnaJ$_{Vh}$ were overexpressed from pMZ20 $dnaK_{Vh}$ and pMZ1 $dnaJ_{Vh}$, respectively, in relevant *E. coli* mutants, using the Tabor and Richardson's expression system (see Materials and methods for details). While the *E. coli*Δ*dnaK* mutant transformed with pMZ20 and pGP1-2 plasmids was not able to form colonies on LA plates at 42°C (Figs. 1a, 2a) and did not survive prolonged incubation at 42°C in L–B medium

(data not shown), efficient overproduction of $DnaK_{Vh}$ was achieved after 2 h of incubation under heat-shock conditions. Although the fusion protein LacZ′–DnaK encoded by pMZ33 was highly overproduced in *E. coli* MC4100 (wild-type) and BM271 Δ*dnaK*, and seemed to possess similar activity as $DnaK_{Vh}$, judging from the results of in vivo tests (Fig. 2a), it was not used for the in vitro experiments.

GrpE of *V. harveyi* did not cross-react with antibodies raised against the *E. coli* GrpE homolog and, consequently, it was not possible to detect its presence in *V. harveyi* wild-type cells or *E. coli*Δ*grpE* cells transformed with DNA of pMZ40 $grpE_{Vh}$ (data not shown). Since the expression of the cloned *V. harveyi grpE* in *E. coli* was not evident on Coomassie-stained gels (data not shown), the GrpE of *V. harveyi* was purified directly from *V. harveyi* (wild-type) cells subjected to heat shock, as described in Materials and methods.

The identity of $GrpE_{Vh}$ was confirmed, after purification of the candidate protein on the $DnaK_{Ec}$-Sepharose affinity column, by sequencing of its N-terminus. The N-terminal amino acids of $GrpE_{Vh}$ (SNEENKVTEEELDQI) were as predicted from gene sequencing, except that purified protein lacked the initial methionine.

DnaK, DnaJ and GrpE of *V. harveyi* were purified to homogeneity using a procedure similar to that previously described for the *E. coli* homologues (Żmijewski et al. 2004); the proteins migrated in SDS-PAGE as 70, 41 and 24 kDa proteins, respectively (Fig. 4). The observed mobilities were consistent with the predicted molecular mass of $DnaK_{Vh}$ (69,076.6 Da), $DnaJ_{Vh}$ (41,618.8 Da) and $GrpE_{Vh}$ (22,307 Da).

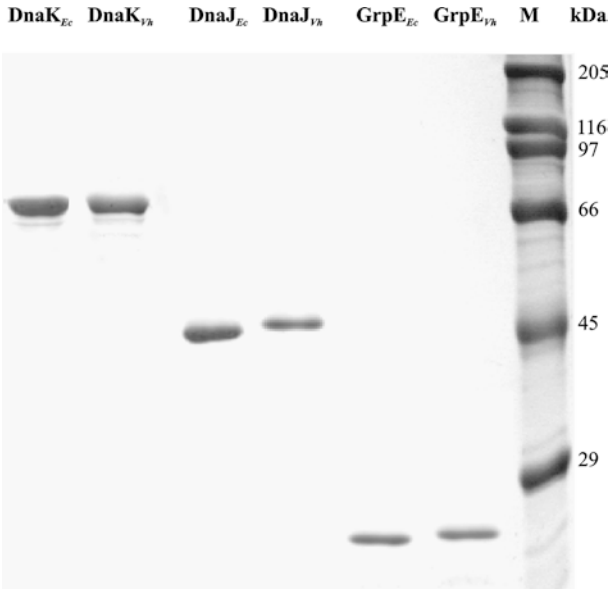

Fig. 4 Electrophoretic analysis of purified chaperone proteins of *V. harveyi* and *E. coli*. Purified chaperones were resolved by SDS-PAGE performed in a 12.5% gel and stained with Coomassie blue. *Lane M* Ladder of molecular mass standards

Renaturation of luciferase by the KJE system of *V. harveyi*

Chaperone activity of the *V. harveyi* DnaK–DnaJ–GrpE system was studied by measuring the ability of the system to reactivate thermally denatured firefly luciferase. As a reference model of the KJE chaperone machinery, the chaperone proteins of *E. coli* were used. The level of luciferase activity after reactivation by the *E. coli* system was about 80% (Fig. 5a) and was similar to that previously published (Laufen et al. 1999).

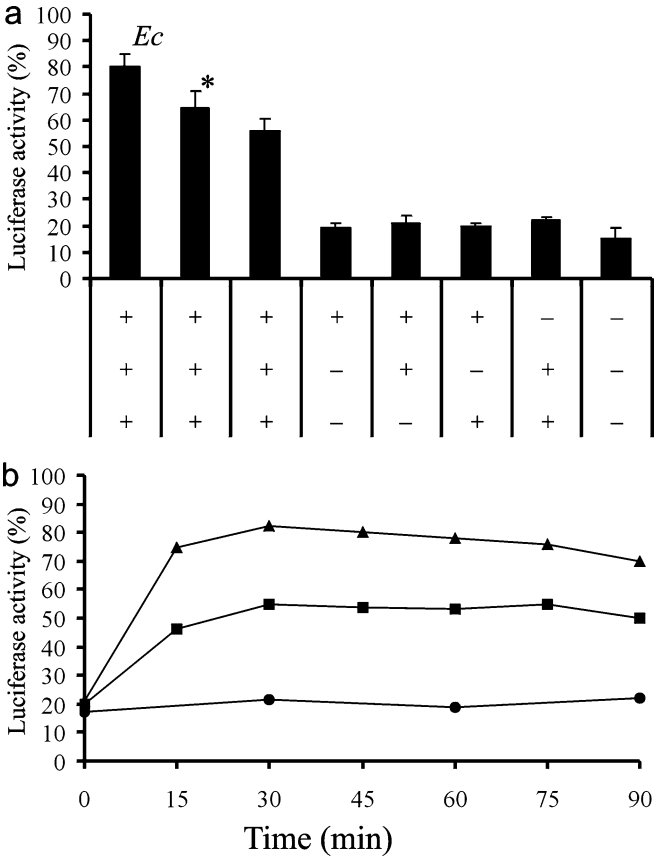

Fig. 5 Luciferase refolding by the *V. harveyi* DnaK chaperone system. Thermally denatured luciferase (80 nM) was renatured in the presence of ATP by various combinations of chaperones, and luciferase activity was then measured. **a** Reactivation (expressed as percentage of the initial activity; absolute value was 5×10^6 relative light units) after 60 min of incubation with chaperones as indicated and 5 mM ATP, at 25°C. *V. harveyi* chaperones were used in all experiments except that of the reference, in which *E. coli* proteins were employed (bar marked *Ec*). **b** Kinetics of luciferase refolding in the presence of: $DnaK_{Ec}$, $DnaJ_{Ec}$ and $GrpE_{Ec}$ (*filled triangles*); $DnaK_{Vh}$, $DnaJ_{Vh}$ and $GrpE_{Vh}$ (*filled squares*). In these experiments, ATP (l mM) was present, both during the denaturation and the renaturation steps. As a control, (*filled circles*) luciferase, without ATP, was denatured and renatured in the presence of $DnaK_{Vh}$, $DnaJ_{Vh}$, and $GrpE_{Vh}$. In standard experiments, the protein concentrations were: DnaK 1 μM, $DnaJ_{Ec}$ 0.2 μM, and GrpE 0.5 μM. In one case marked with an *asterisk*, $DnaK_{Vh}$ was 2 μM. *Error bars* Standard deviation for at least three determinations. For the sake of clarity in **b**, the standard variations, which did not exceed 10%, have not been drawn

The K–J–E chaperones from *V. harveyi* reactivated luciferase less efficiently than the *E. coli* system under standard conditions (DnaK 1 μM) (Fig. 5a). Increasing the concentration of $DnaK_{Vh}$ to 2 μM improved the process and reactivation of luciferase to 65% was achieved (Fig. 5a); a further increase of the $DnaK_{Vh}$ concentration did not result in higher reactivation (not shown). Similar results were achieved when DnaK was purified directly from *V. harveyi* cells (data not shown), which excluded the possibility that the recombinant DnaK differed from the cellular DnaK. There results indicate that the *V. harveyi* DnaK system has a lower ability to refold luciferase than the *E. coli* chaperone system, under conditions optimal for the *E. coli* system. Since *V. harveyi* is a marine bacterium, the optimal conditions for its chaperone proteins' function might be different from those for the *E. coli* chaperones. In order to explore this possibility, various experimental conditions (modifications of the reaction buffer) were tested as follows: (1) Mops-based buffer (pH 7.2) instead of Tris–HCl (pH 7.4) was used (Minami et al. 1996); (2) since optimal growth of *V. harveyi* demands 3% (510 mM) NaCl in the medium, increasing concentrations of NaCl (50, 100, 200, 300 mM) instead of KCl in the reaction buffer were used; (3) magnesium acetate was substituted for magnesium chloride, as published for the eukaryal Hsp70 machineries (Terada et al. 1997); (4) various concentrations of ATP were used since we found that the ATPase activity of $DnaK_{Vh}$ was two-fold higher than that of $DnaK_{Ec}$ (see below). It has been shown that DnaJ loses its activity when Zn atom is removed from the protein (during extensive dialysis) but addition of $ZnCl_2$ reverses the inactivation (Banecki et al. 1996). To ensure high-quality *V. harveyi* DnaJ, the protein was incubated in a reaction buffer supplemented with 5 mM $ZnCl_2$ for 30 min prior to the refolding reaction. None of the presented modifications of the luciferase refolding reaction yielded higher luciferase activity (data not shown). Additionally, it was found that at increased concentrations of NaCl (200 mM and higher) reactivation of luciferase by the *V. harveyi* K–J–E system was inhibited.

As shown in Fig. 5a, refolding of luciferase by *V. harveyi* DnaK was strictly dependent on the presence of GrpE, which is typical for bacterial systems, and it was ATP-dependent, like all Hsp70 systems (Fig. 5b) (for recent review see Hartl and Hayer-Hartl 2002). Kinetics of reactivation of the heat-shock-denatured luciferase was measured and it was found that reactivation in both systems (*V. harveyi* and *E. coli*) reached maximum after 30 min (Fig. 5b).

Cooperation of DnaK, DnaJ and GrpE of *E. coli* and *V. harveyi*

The observed lack of $DnaK_{Vh}$ function in *E. coli* cells in vivo might have been caused by its inability to cooperate with endogenous co-chaperones. We therefore tested reactivation of luciferase using different combinations of *E. coli* and *V. harveyi* DnaK, DnaJ and GrpE. Reactivation was efficient in all tested variants, although a significant decrease in refolding efficiency was observed when DnaK from *V. harveyi* was incubated with two cochaperones of *E. coli* and vice versa (15–25% decrease of luciferase reactivity) [(Fig. 6)]. Our results showed that the yield of active luciferase depended mainly on DnaK activity but not on DnaJ or GrpE activity. Firstly, the KJE chaperone machinery of *E. coli* exhibited higher refolding activity (80%) than that of *V. harveyi* proteins (65%) (Fig. 5). Secondly, replacing of one co-chaperone (DnaJ or GrpE) or both DnaJ and GrpE always resulted in a decrease of refolding efficiency, compared to the homologous system from which a DnaK was derived (Fig. 6). If the activity of the heterologous system was limited by DnaJ or GrpE activities, cooperation of $DnaK_{Vh}$ with DnaJ and/or GrpE of *E. coli* should elevate the amount of active luciferase.

Stimulation of ATPase activity of DnaK proteins

To further understand the functional similarities and differences of the bacterial DnaK chaperone machineries, the ATPase activity of $DnaK_{Vh}$ was compared to that of $DnaK_{Ec}$, alone and in the presence of various co-chaperones. The basic level of ATPase activity of $DnaK_{Vh}$ was more than two times higher than that of $DnaK_{Ec}$ (0.05 ± 0.02 compared to 0.11 ± 0.4) when DnaK proteins were purified using an additional gel

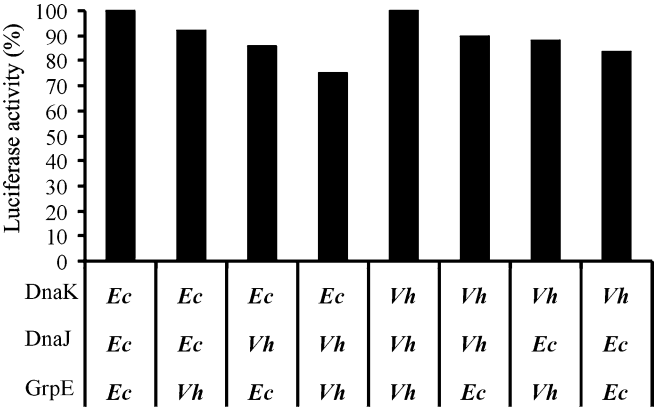

Fig. 6 Cooperation of DnaK–DnaJ–GrpE chaperones from *V. harveyi* and *E. coli* in luciferase refolding. Thermally denatured luciferase (80 nM) was renatured in the presence of 5 μM ATP by various combinations of chaperones at 25°C, and luciferase activity was then measured after 60 min. For the sake of clarity, luciferase reactivation for each homologous system (all *E. coli* and all *V. harveyi*) was set as 100% and data obtained in each experiment were recalculated. Mean values of at least three experiments are shown; the standard deviations, which did not exceed 10%, have not been drawn. Absolute value of the initial activity of luciferase was 5×10^6 relative light units. Protein concentrations were as in standard experiments presented in Fig. 5a. Reactivation in all *E. coli* and all *V. harveyi* was 80% and 55%, respectively

filtration step, removing exogenous ATPase activity (NDP kinase), which usually co-purifies with DnaK (Barthel and Walker 1999). It should be noted that the additional purification step had no influence on the chaperone activity of either DnaK in the luciferase refolding assay (data not shown) but significantly decreased the ATPase activity of the DnaK preparations (almost three-fold for $DnaK_{Vh}$).

The ATPase activity of $DnaK_{Vh}$ could be stimulated by the presence of the *V. harveyi* and also *E. coli* co-chaperones, although stimulation by both DnaJ and GrpE co-chaperones of the *E. coli* origin was less efficient—approximately 50% of that caused by the *V. harveyi* co-chaperones (Fig. 7a,c). The presence of GrpE was essential for efficient stimulation of ATPase activity of *V. harveyi* DnaK by DnaJ proteins (Fig. 7a,c), similar to the case of the model DnaK of *E. coli* (Fig. 7b,d). Stimulation of the ATPase activity of $DnaK_{Vh}$ by co-chaperones (DnaJ and GrpE) was at least 10 times weaker than stimulation of $DnaK_{Ec}$ (compare Fig. 7a,c with b). The stimulation of $DnaK_{Vh}$ ATPase activity was more efficient at high, non-physiological concentrations of DnaJ (1 μM and higher) [(Fig. 7a,c)], similar to the case of $DnaK_{Ec}$ (Fig. 7b); under such conditions, DnaJ probably serves both as co-chaperone and substrate for DnaK (Laufen et al. 1999).

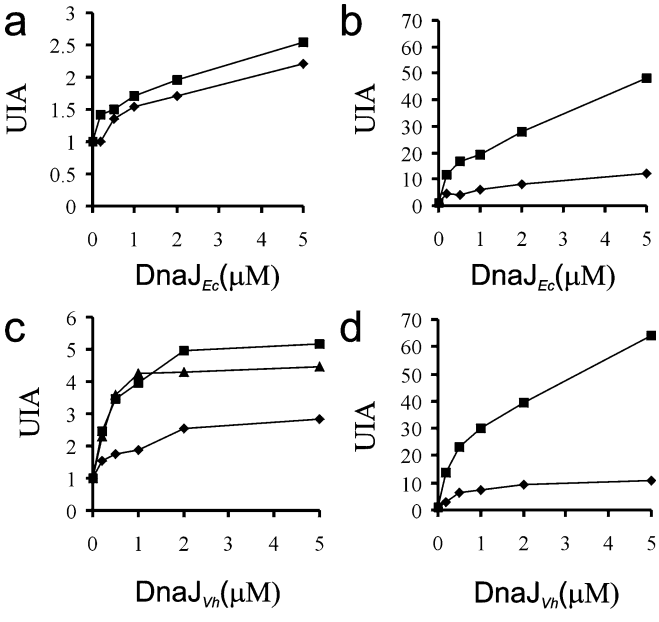

Fig. 7 DnaJ-dependent stimulation of *V. harveyi* DnaK ATPase activity in the presence of GrpE. **a**, **c** ATPase activity of $DnaK_{Vh}$ and **b**, **d** $DnaK_{Ec}$ was measured in the presence of increasing concentrations of DnaJ (0–5 μM), either with GrpE (0.5 μM), or without GrpE. **a** *Filled diamonds* $DnaK_{Vh}$ + $DnaJ_{Ec}$, *filled squares* $DnaK_{Vh}$ + $DnaJ_{Ec}$ + $GrpE_{Ec}$; **b** *filled diamonds* $DnaK_{Ec}$ + $DnaJ_{Ec}$, *filled squares* $DnaK_{Ec}$ + $DnaJ_{Ec}$ + $GrpE_{Ec}$; **c** *filled diamonds* $DnaK_{Vh}$ + $DnaJ_{Vh}$, *filled squares* $DnaK_{Vh}$ + $DnaJ_{Vh}$ + $GrpE_{Ec}$, *filled triangles* $DnaK_{Vh}$ + $DnaJ_{Vh}$ + $GrpE_{Vh}$; **d** *filled diamonds* $DnaK_{Ec}$ + $DnaJ_{Vh}$, *filled squares* $DnaK_{Ec}$ + $DnaJ_{Vh}$ + $GrpE_{Ec}$. All values are expressed in units of increased activity (UIA in the ordinate), and were calculated as the ratio of the $DnaK_{Vh}$ ATPase activity in the presence of the co-chaperones to the activity without co-chaperones (ATPase activity was calculated as described in Materials and methods). Mean values of at least three independent experiments are presented. The standard deviations did not exceed 10%

Discussion

In this work, the *V. harveyi* chaperone system, composed of the DnaK–DnaJ–GrpE proteins, was characterized and compared to the model DnaK system of *E. coli* both in vitro and in vivo. Purified DnaK of *V. harveyi* was able to cooperate well with DnaJ and GrpE co-chaperones of *E. coli* in protein refolding (in reactivation of thermally denatured luciferase), and also in stimulation of DnaK ATPase activity. Protein refolding by the *V. harveyi* DnaK system was, similar to *E. coli* (and other bacteria), GrpE-dependent and occurred with similar kinetics, although with lower efficiency. These findings indicate that the mechanisms of action of the DnaK chaperone machineries of *V. harveyi* and *E. coli* are similar. This is in agreement with the high identity of the participating proteins, 81, 71 and 46% for DnaK, DnaJ and GrpE, respectively, and with the fact that *V. harveyi* and *E. coli* belong to the same systematic group (γ-proteobacteria). Despite the described similarities, DnaK of *V. harveyi* was not able to suppress the thermosensitivity of the *E. coli* mutant while cloned *dnaJ* and *grpE* of *V. harveyi* rescued the growth defect of the *E. coli* mutants at 42 and 45°C. Moreover, both DnaJ and GrpE of *V. harveyi* were able to support bacteriophage λ growth in *E. coli* mutant cells. The in vivo results suggest that—notwithstanding the higher sequence conservation of DnaK as compared with those of DnaJ and GrpE—the DnaK chaperone is more species-specific than are its co-chaperones.

Detailed comparison of separate domains of DnaK proteins of *V. harveyi* and *E. coli* revealed that the degree of conservation differs among the domains. The identity and similarity of the ATPase domains are 86 and 92%, and of the substrate-binding domains (SBD) 81 and 91%. Moreover, modeling of the $DnaK_{Vh}$ SBD domain and analysis of its sequence revealed that all the amino acids shown to be involved in the model peptide-binding by $DnaK_{Ec}$, as well as those responsible for the salt bridges and H-bond formation between the SBD and the latch (Zhu et al. 1996), were preserved in $DnaK_{Vh}$ (not shown). The most variable part, by contrast, is located at the COOH-terminus, in the latch region (residues 537–638 of $DnaK_{Ec}$), with 60% identity and 75% similarity. It is possible that this least conserved region might be involved in creating DnaK species specificity.

Similar results, pointing to DnaK species specificity in vivo were obtained for the DnaK system of the archaeon *M. mazei* (Żmijewski et al. 2004) and for the Hsp70 chaperone machinery of the yeast *Saccharomyces cerevisiae*, consisting of the mitochondrial proteins Ssc1p–Mdj1p–Mge1p (Deloche et al. 1997). In these

cases, the inability of the DnaK(Hsp70) to function in *E. coli* cells might be explained by the fact that *E. coli*, *M. mazei* and *S. cerevisiae* represent different kingdoms and their chaperones are less similar than those of *V. harveyi* and *E. coli*. Interestingly, in both studies mentioned above exogenous DnaJ and GrpE were able to complement the corresponding *E. coli* mutations. Lack of complementation of *E. coli dnaK* mutations by the cloned DnaK genes has been shown previously for *Bacillus megaterium* (Sussman and Setlow 1987), *Borrelia burgdorferi* (Tilly et al. 1993), *Bradyrhizobium japonicum* (Minder et al. 1997) and *Mycobacterium tuberculosis* (Mehlert and Young 1989). However, Mogk et al. (1999) reported that DnaK of *B. subtilis* was able to substitute the lack of DnaK in an *E. coli* mutant (BM271) at 40°C when DnaJ of *E. coli* was co-expressed, restoring the physiological level of that protein, and it has been shown that *dnaK* genes of *Brucella ovis* (Cellier et al. 1992), *Francisella tularensis* (Zuber et al. 1995) and *Zymomonas mobilis* (Michel 1993) rescued growth of *E. coli dnaK* mutants. Interestingly, there is no simple correlation between the identity level of these DnaK proteins and their ability to function in *E. coli* cells. It should be admitted that the results of these studies are rather difficult to compare, because in the cited experiments at least four different mutants of *E. coli* were used and experimental conditions were not identical. A general conclusion, however, might be drawn that exogenous DnaK is much less likely to function in *E. coli* cell than the DnaJ and GrpE chaperones.

Our in vitro experiments showing efficient cooperation of $DnaK_{Vh}$ with *E. coli* DnaJ and GrpE suggest that it was not the inability of a foreign DnaK to interact with endogenous co-chaperones that caused the lack of $DnaK_{Vh}$ function in *E. coli* cells.

Nonetheless, we have shown that *V. harveyi* DnaK was less active as a chaperone and its ATPase activity was significantly less stimulated by co-chaperones than *E. coli* DnaK. This indicates that, despite a similar mechanism of action, the DnaK chaperones of closely related *V. harveyi* and *E. coli* bacteria differ functionally. It is possible that these differences of quantitative nature are responsible, at least partially, for the observed species specificity of DnaK.

There are at least two more possible scenarios that could explain DnaK specificity. (1) It is well established that DnaK(Hsp70) proteins from different organisms recognize model peptides with slightly different affinities (Levy et al. 1995), which can be explained by the discrete amino acid substitutions within the DnaK substrate-binding cavity (Mayer et al. 2000). It could be speculated that chaperone proteins evolved such that they better recognized unfolded proteins of the same origin. This would cause lower affinity/efficiency of a chaperone system with heterologous DnaK. Since the KJE system participates in multiple cellular processes, even a minor impairment of its function would have a cumulative final effect of serious consequences for the cell as a whole, particularly under stress conditions (heat shock, phage infection). However, while this problem should be overcome by overexpression of a heterologous DnaK, this did not occur in the experiments described in this work or in experiments with overexpression of *M. mazei* DnaK in *E. coli* cells (Żmijewski et al. 2004). (2) It is also possible that some of the chaperone substrates in *E. coli* cells are not recognized by $DnaK_{Vh}$. If these were key proteins, probably native, essential for cell survival and their proper function under stress conditions depended on efficient chaperone actions, the effects of the lack of recognition could be lethal. The best known example of the native *E. coli* protein recognized by DnaK is the heat-shock transcription factor σ^{32}. It is well established that σ^{32} is negatively regulated by DnaK–DnaJ–GrpE chaperone machinery (Morita et al. 2000); thus, the different affinity of DnaK for σ^{32} could destabilize its turnover. Bukau and coworkers have shown that DnaK of *Bacillus subtilis* was able to suppress mutations of *dnaK* of *E. coli* under certain conditions but failed to substitute $DnaK_{Ec}$ in negative regulation of σ^{32} (Mogk et al. 1999). An increase in σ^{32} level is not likely to be lethal; however, there may be other proteins whose deregulation would be fatal for the cell. Species specificity of DnaK/Hsp70 proteins is still far from being understood but we believe that the presented work is a good starting point for further investigations of this phenomenon.

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References

Ait-Aissa S, Porche J, Arrigo A, Lambre C (2000) Activation of the hsp70 promoter by environmental inorganic and organic chemicals; relationships with cytotoxicity and lipophilicity. Toxicology 145:147–157

Banecki B, Liberek K, Wall D, Wawrzynów A, Georgopoulos C, Bertoli E, Tanfani F, Żylicz M (1996) Structure–function analysis of Zinc Finger region of DnaJ molecular chaperon. J Biol Chem 271:1–8

Bardwell JC, Tilly K, Craig E, King J, Zylicz M, Georgopoulos C (1986) The nucleotide sequence of the *Escherichia coli* K12 *dnaJ+* gene. A gene that encodes a heat shock protein. J Biol Chem 261:1782–1785

Barthel TK, Walker GC (1999) Inferences concerning the ATPase properties of DnaK and other HSP70s are affected by the ADP kinase activity of copurifying nucleoside-diphosphate kinase. J Biol Chem 274:36670–36678

Baumann P, Schubert RMW (1984) Vibrionaceae. In: Krieg NR, Holt JG (eds) Bergey's manual of systematic bacteriology, vol I. Williams&Wilkins, Baltimore, pp 516–517

Bradford MM (1976) A rapid and sensitive method for quantition of proteins utilizing the principles of protein-dye binding. Anal Biochem 72:248–254

Buchberger A, Valencia A, McMacken R, Sander C, Bukau B (1994) The chaperone function of DnaK requires the coupling

of ATPase activity with substrate binding through residue E171. EMBO J 13:1687–1695
Bukau B, Horwich AL (1998) The Hsp70 and Hsp60 chaperone machines. Cell 92:351–366
Casadaban MJ (1976) Transposition and fusion of the *lac* genes to select promoters in *Escherichia coli* using bacteriophage lambda and Mu. J Mol Biol 104:541–555
Cellier MF, Teyssier J, Nicolas M, Liautard JP, Marti J, Sri Widada J (1992) Cloning and characterization of the *Brucella ovis* heat shock protein DnaK functionally expressed in *Escherichia coli*. J Bacteriol 174:8036–8042
Chakrabarti S, Sengupta N, Chowdhury R (1999) Role of DnaK in in vitro and in vivo expression of virulence factors of *Vibrio cholerae*. Infect Immun 67:1025–1033
Cheetham ME, Caplan AJ (1998) Structure, function and evolution of DnaJ, conservation and adaptation of chaperone function. Cell Stress Chaperones 3:28–36
Czyż A, Szpilewska H, Dutkiewicz R, Kowalska W, Biniewska-Godlewska A, Wegrzyn G (2002) Comparison of the Ames test and a newly developed assay for detection of mutagenic pollution of marine environments. Mutat Res 519:67–74
Deloche O, Kelley WL, Georgopoulos C (1997) Structure–function analyses of the Ssc1p, Mdj1p, and Mge1p *Saccharomyces cerevisiae* mitochondrial proteins in *Escherichia coli*. J Bacteriol 179:6066–6075
Ellis RJ (1999) Molecular chaperones: pathways and networks. Curr Biol 9:R137–R139
Georgopoulos C, Liberek K, Żylicz M, Ang D (1990) Properties of the *Escherichia coli* heat shock proteins and their role in bacteriophage λ growth. In: Morimoto RI, Tissieres A, Georgeopoulos C (eds) The biology of the heat shock proteins and molecular chaperones. Cold Spring Harbor Laboratory, New York, pp 209–224
Gribaldo S, Lumia V, Creti R, de Macario EC, Sanangelantoni A Cammarano P. (1999) Discontinuous occurrence of the *hsp70 (dnaK)* gene among *Archaea* and sequence features of HSP70 suggest a novel outlook on phylogenies inferred from this protein. J Bacteriol 181:434–443
Gupta RS, Bustard K, Falah M, Singh D (1997) Sequencing of heat shock protein 70 (DnaK) homologs from Deinococcus proteolyticus and Thermomicrobium roseum and their integration in a protein-based phylogeny of prokaryotes. J Bacteriol 179:345–357
Hartl FU Hayer-Hartl M (2002) Molecular chaperones in the cytosol: from nascent chain to folded protein. Science 295:1852–1858
Houry WA (2001) Chaperone-assisted protein folding in the cell cytoplasm. Curr Prot Pep Sci 2:244–227
Jamsen P, Pfund C, Craig EA (1997) Functional specificity among Hsp70 molecular chaperone. Science 275:387–389
Jordan R, McMacken R (1995) Modulation of ATPase activity of the molecular chaperone DnaK by peptides and the DnaJ and GrpE heat shock proteins. J Biol Chem 270:4563–4569
Karzai AW McMacken R (1996) A bipartite signaling mechanism involved in DnaJ-mediated activation of the *Escherichia coli* DnaK protein. J Biol Chem 271:11236–11246
Kelley WL Georgopoulos C (1997) The T/t common exon of simian virus 40, JC, and BK polyomavirus T antigens can functionally replace the J-domain of the *Escherichia coli* DnaJ molecular chaperone. Proc Natl Acad Sci USA 94:3679–3684
Klein G, Walczak R, Krasowska E, Błaszczak A, Lipińska B (1995) Characterization of heat-shock response of the marine bacterium *Vibrio harveyi*. Mol Microbiol 16:801–811
Klein G, Żmijewski M, Krzewska J, Czeczatka M Lipińska B (1998) Cloning and characterization of the dnaK heat shock operon of the marine bacterium *Vibrio harveyi*. Mol Gen Genet 110:145–165
Klein G, Laskowska E, Taylor A, Lipinska B (2001) IbpA/B small heat-shock protein of marine bacterium *Vibrio harveyi* binds to proteins aggregated in a cell during heat shock. Mar Biotechnol (NY) 3(4):346–354
Kluck CJ, Patzelt H, Genevaux P, Brehmer D, Rist W, Schneider-Mergener J, Bukau B, Mayer MP (2002) Structure–function analysis of HscC, the *Escherichia coli*. Member of a novel subfamily of specialized Hsp70 chaperones. J Biol Chem 277:41060–41069
Kuchanny D, Klein G, Krzewska J, Czyz A, Lipinska B (1998) Cloning of the groE operon of the marine bacterium *Vibrio harveyi* using a lambda vector. Acta Biochim Pol 45(1):261–270
Kuchanny-Ardigo D, Lipinska B (2003) Cloning and characterization of the *groE* heat-shock operon of the marine bacterium *Vibrio harveyi*. Microbiology 149(Pt 6):1483–1492
Langer T, Lu C, Echols H, Flanagan J, Hayer MK (1992) Successive action of DnaK DnaJ and GroEL along the pathway of chaperone-mediated protein folding. Nature 356:683–689
Lanzetta PA, Alvarez LV, Reinach PS, Candia OA (1979) An improved assay for nanomole amounts of inorganic phosphate. Anal Biochem 100:95–97
Laufen T, Mayer MP, Beisel C, Klostermeier D, Mong A, Reinstein J, Bukau B (1999) Mechanism of regulation of Hsp70 chaperones by DnaJ cochaperones. Proc Natl Acad Sci USA 96:5452–5457
Lee J, Wang YY, Gibson BG (1991) Electronic excitation transfer in the complex of lumazine protein with bacterial bioluminescence intermediates. Biochemistry 30:6825–6835
Levy EJ, McCarty J, Bukau B, Chirico WJ (1995) Conserved ATPase and luciferase refolding activities between bacteria and yeast Hsp70 chaperones and modulators. FEBS Lett 368:435–440
Liberek K, Marszałek J, Ang D, Georgopoulos C, Żylicz M (1991) *Escherichia coli* DnaJ and GrpE heat shock proteins jointly stimulate ATPase activity of DnaK. Proc Natl Acad Sci USA 88:2874–2878
Lu Z, Cyr DM (1998) Protein folding activity of Hsp70 is modified differentially by the hsp40 co-chaperones Sis1 and Ydj1.J Biol Chem 273:27824–27830
Mayer MP, Schröder H, Rüdiger S, Paal K, Laufen T, Bukau B (2000) Multistep mechanism of substrate binding determines chaperone activity of Hsp70. Nat Struct Biol 7:586–593
McCarty JS, Buchberger A, Reinstein J, Bukau B (1995) The role of ATP in the functional cycle of the DnaK chaperone system. J Mol Biol 249:126–137
Mehlert A, Young DB (1989) Biochemical and antigenic characterization of the *Mycobacterium tuberculosis* 71 kD antigen, a member of the 70 kD heat-shock protein family. Mol Microbiol 3:125–130
Michel GP (1993) Cloning and expression in *Escherichia coli* of the *dnaK* gene of *Zymomonas mobilis*. J Bacteriol 175:3228–3231
Minami Y, Hohfeld J, Ohtsuka K, Hartl FU (1996) Regulation of the heat-shock protein 70 reaction cycle by the mammalian DnaJ homolog, Hsp40. J Biol Chem 271:19617–19624
Minder AC, Narberhaus F, Babst M, Hennecke H, Fischer HM (1997) The dnaKJ operon belongs to the sigma32-dependent class of heat shock genes in *Bradyrhizobium japonicum*. Mol Gen Genet 254:195–206
Mogk A, Bukau B, Lutz R, Schumann W (1999) Construction and analysis of hybrid *Escherichia coli-Bacillus subtilis dnaK* genes. J Bacteriol 181:1971–1974
Mok KC, Wingreen NS, Bassler BL (2003) *Vibrio harveyi* quorum sensing: a coincidence detector for two autoinducers controls gene expression. EMBO J 22:870–881
Morita MT, Kanemori M, Yanagi H, Yura T (2000) Dynamic interplay between antagonistic pathways controlling the sigma 32 level in *Escherichia coli*. Proc Natl Acad Sci USA 97(11):5860–5865
Paek KH, Walker GC (1987) *Escherichia coli* dnaK null mutants are inviable at high temperature. J Bacteriol 169:283–290
Palleros DR, Reid KL, Shi L, Welch WJ, Fink AL (1993) ATP-induced protein–Hsp70 complex dissociation requires K^+ but not ATP hydrolysis. Nature 365:664–666
Rockabrand D, Blum P (1995) Multicopy plasmid suppression of stationary phase chaperone toxicity in *Escherichia coli* by phosphogluconate dehydratase and the N-terminus of DnaK. Mol Gen Genet 249:498–506

Ruby G, Greenberg EP, Hastings JW (1980) Planctonic marine luminous bacteria—species distribution in the water column. Appl Environ Microbiol 39:302–306

Rudiger S, Roder D, Langen A, Bukau B (1999) Identification of thermolabile *Escherichia coli* proteins: prevention of aggregation by DnaK and ClpB. EMBO J 18:6934–6949

Sambrook J, Frisch EF, Maniatis T (1989) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor

Sanders BM, Martin LS (1993) Stress proteins as biomarkers of contaminant exposure in archived environmental samples. Sci Total Environ 139–140:459–470

Sha B, Soojin L, Cyr DM (2000) The crystal structure of the peptide-binding fragment from the yeast Hsp40 protein Sis1. Structure 8:799–807

Schröder H, Langer T, Hartl FU, Bukau B (1993) DnaK, DnaJ, GrpE form a cellular chaperone machinery capable of repairing heat-induced protein damage. EMBO J 12:4138–4144

Sell SM, Eisen C, Ang D, Zylicz M Georgopoulos C (1990) Isolation and characterization of dnaJ null mutants of *Escherichia coli*. J Bacteriol 172:4827–4835

Silberg JJ, Hoff KG, Vickery LE (1998) The Hsc66-Hsc20 chaperone system in *Escherichia coli*: chaperone activity and interactions with the DnaK–DnaJ–GrpE system. J Bacteriol 180:6617–6624

Sussman MD, Setlow P (1987) Nucleotide sequence of a *Bacillus megaterium* gene homologous to the dnaK gene of *Eschrichia coli*. Nucleic Acids Res 15:3923

Szabo A, Langer T, Schröder H, Flanagan J, Bukau B Hartl FU (1994) The ATP hydrolysis-dependent reaction cycle of the *Escherichia coli* Hsp70 system—DnaK, DnaJ, and GrpE. Proc Natl Acad Sci USA 91:10345–10349

Szewczyk B, Summers DF (1998) Use of proteins blotted to polyvinylidene difluoride membranes as immunogens. Methods Mol Biol 80:81–85

Tabor S, Richardson CC (1985) A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes Proc Natl Acad Sci USA 82:1074–1078

Terada K, Kanazawa M, Bukau B, Mori M (1997) The human DnaJ homologue dj2 facilitates mitochondrial protein import and luciferase refolding. J Cell Biol 139:1089–1095

Tilly K, Hauser R, Campbell J, Ostheimer GJ (1993) Isolation of *dnaJ*, *dnaK*, and *grpE* homologues from *Borrelia burgdorferi* and complementation of *Escherichia coli* mutants. Mol Microbiol 7:359–369

Ueguchi C, Shiozawa T, Kakeda M, Yamada H, Mizuno T (1995) A study of the double mutation of *dnaJ* and *cbpA*, whose gene products function as molecular chaperones in *Escherichia coli*. J Bacteriol 177:3894–3896

Van Dyk TK, Majarian WR, Konstantinov KB, Young RM, Dhurjati PS, LaRossa RA (1994) Rapid and sensitive pollutant detection by induction of heat shock gene-bioluminescence gene fusions. Appl Environ Microbiol 60:1414–1420

Wall D, Żylicz M, Georgopoulos C (1994) The NH_2-terminal 108 amino acid of the *Escherichia coli* DnaJ protein stimulate the ATPase activity of DnaK and are sufficient for lambda replication. J Biol Chem 269:5446–5451

Wegrzyn G, Czyż A (2003) Detection of mutagenic pollution of natural environment using microbiological assays. J Appl Microbiol 95:1175–1181

Wong HC, Peng PY, Lan SL, Chen YC, Lu KH, Shen CT, Lan SF (2002) Effects of heat shock on the thermotolerance, protein composition, and toxin production of *Vibrio parahaemolyticus*. J Food Prot 65:499–507

Wu B, Ang D, Snavely M, Georgopoulos C (1994) Isolation and characterization of point mutations in the *Escherichia coli grpE* heat shock gene. J Bacteriol 176:6965–6973

Zhu X, Zhao X, Burkholder WF, Gragerov A, Ogata CM, Gottesman ME, Hendrickson WA (1996) Structural analysis of substrate binding by the molecular chaperone DnaK. Science 272:1606–1614

Żmijewski MA, Macario AJL, Lipińska B (2004) Functional similarities and differences of an archaeal Hsp70(DnaK) stress protein with the homologue from the bacterium *Escherichia coli*. J Mol Biol 336(2):539–549

Zuber M, Hoover TA, Dertzbaugh MT, Court DL (1995) Analysis of the DnaK molecular chaperone system of *FraSncisella tularensis*. Gene 164:149–152

Żylicz M, Georgopoulos C (1984) Purification and properties of the *Escherichia coli* DnaK replication protein. J Biol Chem 259:8820–8825

Żylicz M, LeBowitz JH, McMacken R, Georgopoulos C (1983) The *dnaK* protein of *Escherichia coli* possesses an ATPase and autophosphorylating activity and is essential in an in vitro DNA replication system. Proc Natl Acad Sci USA 80:6431–6435