JOURNAL OF BIOSCIENCE AND BIOENGINEERING
Vol. 99, No. 2, 136–142. 2005
DOI: 10.1263/jbb.99.136

DnaK from *Vibrio proteolyticus*: Complementation of a *dnaK*-Null Mutant of *Escherichia coli* and the Role of Its ATPase Domain

KAZUAKI YOSHIMUNE,[1§] ANDREY GALKIN,[1] LJUDMILA KULAKOVA,[1] TOHRU YOSHIMURA,[1§§] AND NOBUYOSHI ESAKI[1]*

Institute for Chemical Research, Kyoto University, Uji, Kyoto 611-0011, Japan[1]

Received 30 September 2004/Accepted 15 November 2004

We cloned the 4.8-kbp DNA fragment containing the *dnaK* gene from the chromosomal DNA of *Vibrio proteolyticus*. It contained four genes arranged unidirectionally in the order of *grpE*, *gltP*, *dnaK* and *dnaJ*. The DnaK gene of *V. proteolyticus* (VprDnaK) allowed a *dnaK*-null mutant of *Escherichia coli* (Δ*dnaK52*) to propagate λ phages but not to grow at 43°C. However, a chimeric DnaK gene comprising the regions corresponding to the N-terminal ATPase domain of *E. coli* DnaK (EcoDnaK) and the C-terminal region of VprDnaK including the substrate-binding domain, enabled the mutant to grow at 43°C. The temperature dependence for the ATPase activity of the chimeric DnaK was similar to that of EcoDnaK. Fluorometric analyses showed that the chimeric DnaK is much more thermostable than EcoDnaK and VprDnaK. These findings indicate that the thermal stability of the ATPase domain of DnaK is responsible for its chaperone action at high temperatures such as 43°C.

[**Key words:** DnaK, *Vibrio proteolyticus*, *Escherichia coli*, ATPase, ATPase domain, substrate binding domain, cloning]

The 70 kDa heat-shock protein (Hsp70) family of molecular chaperones is highly conserved among many organisms (1). Hsp70 plays an essential role in the repair, folding, and assembly of proteins in cells under normal physiological conditions as well as under various stresses (2). Hsp70 consists of an N-terminal ATPase domain, a substrate-binding domain, and a C-terminal variable domain. Many organisms contain multiple Hsp70s with distinct and non-overlapping functions, and their physiological functions are not interchangeable. However, domain-shuffling experiments among different Hsp70s indicated that some physiological functions of Hsp70s are chiefly governed by their ATPase domains but not by substrate-binding domains (3, 4). For example, *Saccharomyces cerevisiae* contains different kinds of Hsp70s such as Ssb1 and Ssb2 (5), and James *et al*. (4) showed through a domain-shuffling experiment that the ATPase domain of Ssb1 determines the ability of *S. cerevisiae* to grow at low temperatures such as 18°C.

Deletion of the *dnaK* gene of *Escherichia coli* causes defects in growth at low and high temperatures and propagation of λ phages (6–8). The structures of DnaKs are highly conserved, but the defects of the *E. coli dnaK* deletion mutant were not fully complemented by other DnaKs from other bacterial species (9, 10). *Vibrio proteolyticus* belongs to the same group as *E. coli*: γ-subdivision proteobacteria. However, its upper-limit growth temperature (about 40°C) is lower than that of *E. coli* (49°C) (11). We have cloned the *dnaK* gene from *V. proteolyticus* and characterized its gene product (VprDnaK) in order to clarify the function of its ATPase domain and compare it with that of DnaK from *E. coli* (EcoDnaK).

MATERIALS AND METHODS

Enzymes, chemicals, bacterial strains and plasmids *V. proteolyticus* (ATCC 15338) was obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA). Plasmids pNRK416 encoding the EcoDnaK and pKV8001 encoding the EcoDnaK and DnaJ of *E. coli* were given by Dr. Y. Akiyama of the Institute for Virus Research, Kyoto University. A DnaK-null mutant of *E. coli* MC4100 (Δ*dnaK52*) was provided by Dr. C. Wada of the same institute. An expression vector pKK223-3 was purchased from Amersham Pharmacia Biotech, Buckinghamshire, UK. pUC118 cloning vectors, restriction enzymes and other DNA-modifying enzymes were purchased from Takara Shuzo, Kyoto, or Toyobo, Osaka. The synthetic oligonucleotides were purchased from Biologica, Nagoya. Gelatin-agarose gel was obtained from Sigma, St. Louis, MO, USA. All other chemicals were from Wako Pure Chemicals, Osaka.

DNA manipulation and sequence analysis Plasmid DNAs and PCR fragments were sequenced with an Applied Biosystems Automated DNA Sequencer model 377B and a dye-labeled terminator sequencing kit (Applied Biosystems, Foster, CA, USA). The

* Corresponding author. e-mail: esaki@scl.kyoto-u.ac.jp
phone: +81-(0)774-38-3240 fax: +81-(0)774-38-3248
Present address: §Department of Applied Chemistry, Faculty of Engineering, Oita University, Oita, Oita 870-1192, Japan and §§Department of Applied Molecular Biosciences, Graduate School of Bioagricultural Sciences, Nagoya University, Chikusa-Ku, Nagoya, Aichi 464-8601, Japan.

N-terminal amino acid sequence was determined by automated Edman degradation with a Shimadzu PPSQ10-S protein sequencer (Shimadzu, Kyoto).

Gene cloning and plasmids construction The chromosomal DNA of *V. proteolyticus*, prepared by lysozyme and proteinase K digestions followed by phenol-chloroform extraction, was digested with *Hin*dIII, and the fragments obtained were ligated into pUC118 previously treated with the same enzyme. *E. coli* JM109 transformed with the resulting plasmids was subjected to colony hybridization with DNA probes labeled with digoxigenin (Roche Diagnostics, Mannheim, Germany). The probes specific to DnaK genes were obtained by PCR with the following primers designed on the basis of the DNA sequence of the conserved regions of *dnaK*s: 5′-GGTATCGATCTAGGTACAACTAACTCTTGTGT-3′ (corresponding to the residues 6–16 of EcoDnaK); 5′-TTCGATA CCTAGTGATAAAGGCGTTACGTC-3′ (residues 392–402 of EcoDnaK). The plasmid obtained from the selected positive clone was named pVprDK1. For the construction of the expression vector, the VprDnaK gene was amplified by PCR with the primers, 5′-TTTATTCGGAGATCCCGGGATGGGTAAAATC-3′ (*Sma*I site, underlined) and 5′-GCCACCTGTCAGGAAGCTTTTACTT CTTG-3′ (*Hin*dIII site, underlined) and pVprDK1 as a template. The VprDnaK gene obtained was digested with *Sma*I and *Hin*dIII, and the fragment obtained was ligated into the plasmid pKK223-3 treated with the same enzymes. The resulting plasmid was named pVprDK2.

pVprDK1 was digested with *Cla*I and *Nco*I, and the resultant 862-bp fragment encoding the C-terminal 235 amino acids of VprDnaK was ligated into pKV8001 treated with *Acc*I and *Nco*I. Thus, pEVPDKJ encoding EvpDnaK was constructed.

A plasmid pDNAJ, an expression vector for *E. coli* DnaJ, was constructed by the introduction of a 2-kbp *Sal*I-*Pst*I fragment of pDNAJ-A (12) into a plasmid pMPM-T3 (13) previously digested with the same enzymes.

Protein purification EcoDnaK was purified from *E. coli* JM109 transformed with pNRK416. To avoid contamination by EcoDnaK, VprDnaK and EvpDnaK were purified from Δ*dnaK52* cells transformed with pVprDK2 or pEVPDKJ. Purifications of DnaKs were carried out with a gelatin-agarose column as described previously (14). The homogeneity of the final DnaK preparations was confirmed by SDS–PAGE.

ATPase assays The ATPase activity of DnaK was determined by the measurement of ADP formed from ATP by reverse-phase HPLC (15). Reaction mixtures (18 μl) containing a 0.1 M HEPES–NaOH buffer (pH 7.5), 150 mM KCl, 5 mM $MgCl_2$, 1 mM dithiothreitol (DTT), and 2 μM protein were pre-incubated for 3 min before the addition of ATP. The reaction, which was started by addition of ATP (2 μl) at a final concentration of 5 mM, was carried out at the same temperature for 30 min and stopped by the addition of 2 μl of 20% perchloric acid. In a blank, perchloric acid was added before the addition of ATP.

Fluorescence measurements Fluorescence of DnaK was measured at various temperatures with a Hitachi F-3000 fluorescence spectrophotometer with an excitation wavelength at 290 nm and an emission wavelength at 340 nm. The excitation and emission slits were 6 and 10 nm, respectively. The protein concentration was 1 μM in 0.1 M HEPES–NaOH buffer (pH 7.5), 150 mM KCl, 5 mM $MgCl_2$, 1 mM ADP and 1 mM DTT. Temperature was increased from 20°C at a rate of 2.5°C/min and data were collected every 1 min with a time constant of 4 s.

Compensation for the defects of *E. coli* Δ*dnaK52* cells by expression of EcoDnaK, VprDnaK, and EvpDnaK *E. coli* Δ*dnaK52* transformed with pNRK416, pVprDK2, or pEVPDKJ was cultured on LB agar plates containing 0.1 mM IPTG at 15°C, 30°C, or 43°C. The transformants were also grown in an LB liquid medium supplemented with 50 μg/ml of ampicillin at 30°C. An 100-μl portion of the culture was mixed with λ phage (10 μl, 10^3 PFU) and incubated at 30°C for 15 min. The mixture was then mixed with 3 ml of λ top agar containing 0.1 mM IPTG and poured onto LB agar plates containing 50 μg/ml of ampicillin. Titers of λ phage were assayed after the plates were incubated at 30°C for about 12 h.

RESULTS AND DISCUSSION

Gene cloning of DnaK from *V. proteolyticus* *V. proteolyticus* is a gram-negative marine bacterium whose cultivation temperature is recommended to be 26°C by ATCC. However, we observed that the cells grow at 40°C but not at 45°C (data not shown). Thus, the upper-limit growth temperature for *V. proteolyticus* is similar to that for *V. harveyi* and lower than that for *V. parahaemolyticus* (Table 1). We obtained a fragment of the *dnaK* gene by PCR with the genomic DNA from *V. proteolyticus* as a template and synthetic primers designed from the regions conserved in DnaKs from various bacterial sources. The primary structure of the PCR product was highly homologous to that of EcoDnaK.

TABLE 1. Comparison of the amino acid sequences of GrpE, Gltp, DnaK and DnaJ from *V. proteolyticus* with those from other species

	Genes cloned from *V. proteolyticus*				Upper-limit growth temperature (°C)	Reference	Accession no.
	ORF1	ORF2	ORF3	ORF4			
Coding protein	GrpE	Gltp	DnaK	DnaJ		This study	GenBank AF218211
Nucleotides (bp)	615	1278	1914	73[a]			
Amino acids	204	425	6372	24[b]			
Molecular mass (Da)	22983	44378	68992				
Sequence identity (%) with those from							
V. proteolyticus	100	100	100 (100:100)[c]	100	40	This study	GenBank D10483
E. coli	[41][d]	[28][d]	82 (87:75)[c]	63	49	11, 26	GenBank D10483
V. harveyi	78	91	94 (95:93)[c]	100	39	9, 27	EMBL AY639008
V. parahaemolyticus	78	91	98 (99:96)[c]	100	44	28, 29	EMBL AP005075

[a] Partial sequence was determined.
[b] N-terminal 24 amino acids.
[c] The first and second numbers in the parentheses represent sequence identity with the ATPase domain (residues 1–394) and the C-terminal region (residues 395–638) of VprDnaK, respectively.
[d] *grpE* (EMBL X07863) and *gltP* (EMBL M32488) genes are not located in the *dnaK* locus.