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Cloning and Characterization of *dnaK* Operon of *Tetragenococcus halophila*

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We have cloned and characterized the *dnaK* operon of *Tetragenococcus halophila* JCM5888. The nucleotide sequence analysis of the cloned fragments showed that the *dnaK* operon consists of four open reading frames with the organization *hrcA–grpE–dnaK–dnaJ*. Two regulatory CIRCE (Controlling Inverted Repeat of Chaperone Expression) elements were identified in the region upstream of *hrcA*. The *T. halophila dnaK* encoded a protein of 618 amino acids with a calculated molecular mass of 67 kDa. The deduced amino acid sequence of *T. halophila* DnaK showed high similarities with the corresponding DnaK homologues of *Lactococcus lactis*, *Lactobacillus sakei* and *Bacillus subtilis*. Using a pET expression system, the *T. halophila* DnaK was overexpressed in *Escherichia coli* and the purified *T. halophila* DnaK was found to have ATPase activity. Northern hybridization analysis revealed that the transcription of *dnaK* gene was induced by heat shock, and several transcripts were detected including a maximum size of tetra–cistronic mRNA 4.9–kb which represents the transcript of complete *dnaK* operon. The amount of *dnaK* transcripts was also increased about 3.5–fold by high NaCl condition (3–4 M), but not by the same concentration of KCl. These results suggest that the cloned DnaK surely acts as the functional molecular chaperone and play an important role in the salinity adaptation.

INTRODUCTION

Heat shock proteins (HSPs) are temporarily overexpressed when cells are exposed to high temparature, high salinity and other various kinds of environmental stresses (Lindquist and Craig, 1988). HSPs, including members of the Hsp60 family (GroES/EL) and members of Hsp70 family (DnaK/DnaJ/GrpE), are known as highly conserved proteins. The Hsp70 molecular chaperone system in *Escherichia coli* as DnaK has been intensively characterized with regard to many functions such as preventing protein aggregation and acting as a role of protein stabilization factor to adapt stress condition (Gross *et al.*, 1990). Two other HSPs, DnaJ and GrpE have been found to stimulate chaperone activity of DnaK. In the presence of both DnaJ and GrpE, the ATPase activity of DnaK increases up to 50 times (Liberek *et al.*, 1991).

In Gram–negative bacterium *E. coli*, expression of almost the heat shock genes is

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mediated by the *rpoH*-encoded alternative sigma factor σ^H (Yura *et al.*, 1993). In contrast, Gram-positive bacterium *Bacillus subtilis* contains three different classes of heat shock genes (Hecker *et al.*, 1996). Class I heat shock genes, including DnaK/DnaJ/GrpE and GroES/GroEL systems, are regulated by the HrcA protein and the CIRCE (Controlling Inverted Repeat of Chaperone Expression) element (Schulz and Schumann, 1996). Class II heat shock genes possess σ^B dependent promoter which are expressed under several environmental stress conditions. The regulatory mechanism of class III heat shock genes is almost unknown.

The relation between DnaK functions and injury effects of high temperature has been studied in detail (Hartl, 1996). Recently, it has been reported that the *dnaK* mutant strain of *E. coli* failed to deplasmolyze and adapt to high salinity, and moreover mutant strain could not maintain intracellular concentration of K^+ (Meury and Kohiyama, 1991; Yaagoubi *et al.*, 1994). Therefore, DnaK seemed to be an indispensable factor to adapt *E. coli* to higher osmolarity. However little information is available about the function of DnaK homologue in halophilic eubacterium to adapt to high salinity condition.

Tetragenococcus halophila (formerly known as *Pediococcus halophilus*) is a halophilic Gram-positive lactic acid bacterium (LAB) used for brewing of Japanese soy sauce (Collins *et al.*, 1990). Based on 16S rDNA sequence studies, this bacterium shows close phylogenetic relationship to enterococci and lactobacilli. Unlike these genera of LAB, *T. halophila* can tolerate high salt concentrations (up to 4 M NaCl), and grows optimally in media containing 0.5 to 3.0 M of NaCl. When cultivated in a high salt concentration medium, *T. halophila* is known to accumulate intracellularly not only Na^+ but also much amount of K^+ and several organic substances as compatible solutes (Robert *et al.*, 2000). We have much interest in the functions and the expression behavior of *T. halophila* DnaK under the condition of high intracellular osmotic pressure and increased hydrophobic interactions in the protein structure. In this paper, we describe the cloning, expressing and transcriptional analysis of the *dnaK* of *T. halophila*. Data on the character of *T. halophila* DnaK contribute to better understanding of its adaptation mechanism to high salinity.

MATERIALS AND METHODS

Bacterial strains and growth conditions

T. halophila JCM5888 (ATCC33315^T) used in this study, was grown at 30 °C in MRS medium (Oxoid, Hampshire, England) containing 1 M NaCl. The medium was adjusted to pH 7.5 before sterilization. *Escherichia coli* JM109 (Toyobo, Osaka, Japan) and BL21 (DE3) were grown at 37 °C with shaking in Luria-Bertani (LB) broth. When the growing was appropriate for clonal selection, X-gal (5-bromo-4-isopropyl-β-D-4-chloro-3-indol-β-D-galactopyranoside), IPTG (isopropyl 1-thio-β-D-galactoside) and ampicillin were added at concentrations of 50, 40, 20 mg/l, respectively.

DNA isolation and manipulation

T. halophila chromosomal DNA was isolated by applying a combination of the two methods as described previously (Marmur, 1961; Berns and Thomas, 1965). Plasmid DNA, pUC18 vector, used for cloning of *T. halophila dnaK* operon, was purified from *E.*

coli with Mag extractor plasmid extraction kit (Toyobo). Restriction endonuclease digestions, analyses and ligations were performed according to the methods of Sambrook *et al* (1989). *E. coli* competent cells for electroporation were prepared according to the protocol recommended for the Gene Pulser apparatus (Bio–Rad, Hercules, CA, USA). Southern hybridization with nucleotide probes was performed by using the AlkPhos labeling system (Amersham Pharmacia Biotech, Uppsala, Sweden) according to the manufacture's protocol.

Cloning of *T. halophila dnaK* operon

Two degenerated oligonucleotides (dnaK–TS1 and dnaK–TA1) used as PCR primers were designed from DnaK conserved region of other Gram–positive bacteria (Table 1). PCR was performed in a 100–μl volume containing 1 μg genomic DNA, 50 mM KCl, 10 mM Tris–HCl (pH 9.0), 0.1% Triton X–100, 1.5 mM $MgCl_2$, 0.2 mM of each dNTPs, 100 pmol of each primer and 5 U of *Taq* polymerase. Amplification was carried out for 28 cycles (denaturation at 95 °C for 1 min, annealing at 48 °C for 20 sec, and polymerization at 72 °C for 1 min). Amplified fragment of an expected size (0.9–kb) was labeled with AlkPhos Direct System (Amersham Pharmacia Biotech) and used as a probe to screen a *T. halophila* genomic library. The 3.5–kb (pTX–H3) and 2.0–kb (pTXE–A1) fragments, which generated a strong hybridization signal with the probe, were sequenced with ALF express automated DNA sequencer (Amersham Pharmacia Biotech) and analyzed with the DNASIS program (Hitachi Software Engineering, Tokyo, Japan) and GENETYX–WIN (Software Development, Tokyo, Japan).

Immediately upstream region of partial *hrcA* gene which was cloned into pTXE–A1 was characterized by inverse PCR reactions (Innis *et al.*, 1990). *T. halophila* chromosomal DNA was digested completely with *Hin*dIII and religated for use as the template. Inverse PCR reaction, using the divergent primers hrc–IA1 and hrc–IS1 (Table 1), was performed with KOD DNA polymerase (Toyobo), which increased polymerization fidelity. The generated fragment was cloned into pUC18 and named pTIH–B1.

Table 1. Oligonucleotide primers used in this study

Names	Sequences	Notes
dnaK–TS1	5'–ATCAC(A/T)GT(A/T)CCTGCTTACTT–3'	The degenerative oligonucleotide primer corresponding to the 339 to 359 of the *L. lactis dnaK* gene.
dnaK–TA1	5'–ATATC(C/T)AATTGGAAACGACC–3'	The degenerative oligonucleotide primer corresponding to the 1262 to 1284 of the *L. lactis dnaK* gene.
hrcA–IS1	5'–CGGCGCTATCGATTGGACCTGATGTAAG–3'	The oligonucleotide primer corresponding to the 187 to 213 of the *T. halophila hrcA* gene.
hrcA–IA1	5'–CGATAACCCTTTAGCGAAGGGATACGACC–3'	The oligonucleotide primer corresponding to the 362 to 389 of the *T. halophila hrcA* gene.

Comparison of deduced amino acid sequence and phylogenetic analysis of *T. halophila* DnaK

The multiple alignments of the DnaK amino acid sequences were performed by the program ClustalW (Thompson *et al.*, 1990) and were adjusted manually. The phylogenetic tree based on the NJ (Neighbor–joining) method was constructed by Treeview (ver. 1.6) (Saitou and Nei, 1985). Accession numbers of other sequences used for the analysis are as follows; *Bacillus stearothermophilus dnaK*, X90709 (Herbort *et al.*, 1996); *B. subtilis dnaK*, X85182 (Wetzstein *et al.*, 1992); *Clostridium acetobutylicum dnaK*, M74561 (Narberhaus *et al.*, 1992); *E. coli dnaK*, K10420 (Cowing *et al.*, 1985); *Lactobacillus sakei dnaK*, AJ006274 (Schmidt *et al.*, 1999); *Lactococcus lactis dnaK*, X76642 (Eaton *et al.*, 1993); *Nitrosomonas europae dnaK*, AB018706 (Iizumi and Nakamura, 1997); *Listeria monocytogenes dnaK*, AB023064 (Hanawa *et al.*, 2000); *Pseudomonas syringae dnaK*, AF135163 (Keith *et al.*, 1999); *Rhodobacter capsulatus dnaK*, U57637 (Nickel *et al.*, 1997); *Staphylococcus aureus hsp70*, D30690 (Ohta *et al.*, 1994); *Streptococcus mutans dnaK*, U78296 (Jayaraman *et al.*, 1997); *Thermus thermophilus dnaK*, L57504 (Motohashi *et al.*, 1999); *Vibrio chorelae dnaK*, VCY14237 (Chakrabarti *et al.*, 1999).

Nucleotide sequence accession number

The nucleotide sequence reported in this article has been assigned GenBank accesion number AB070346.

Construction of the fusion plasmid and expression of *T. halophila dnaK* in *E. coli*

A 1.9–kb fragment that encoded the *T. halophila* DnaK protein was amplified by PCR with two oligonucleotide primers, 5'–GGG GTA GAG ATG ACT TTA AGA AAG C–3' and 5'–CAA CTT CAA TTG CTA CAG CAC GTT C–3', followed by digestion with *Xho*I and *Nde*I. The resultant fragment was ligated into the *Xho*I–*Nde*I site of pET14b (Takara, Tokyo, Japan). The resulting plasmid, named pTDnaK–His, was transformed into *E. coli* BL21 (DE3). Expression of the DnaK–poly His fusion was induced by the addition of IPTG to a final concentration of 0.3 mM. The *T. halophila* DnaK–poly His fusion protein was purified using the Mag Extracter–His tag kit (Toyobo). Protein samples were separated by SDS–PAGE using 10% (w/v) SDS–polyacrylamide gels and then stained with Coomassie Brilliant Blue.

Measurement of ATPase activity

ATPase activity was assayed in a reaction mixture (100 μl) containing 25 mM Tris–HCl, pH 7.5, 5 mM $MgCl_2$, 150 mM KCl, 1 mM ATP and 0.6 μg purified DnaK protein. After a 1–h incubation at 37 °C, the reaction was terminated by adding 25 μl 20% perchloric acid. The reaction mixtures were centrifuged at 15,000 rpm for 5 min at 4 °C, and the amount of released inorganic phosphate was measured using KH_2PO_4 as a standard (Lill *et al.*, 1990).

Northern blot hybridization

Total RNA was isolated from *T. halophila* cells using RNeasy Total RNA kit (Qiagen,

Chatsworth, CA, USA). RNA samples (5 μg) were denatured with formaldehyde and electrophoresed on a 1.5% agarose gel containing 20 mM MOPS (morpholine propane sulfonic acid) buffer, pH 8.0 and 2.2 M formaldehyde. After electrophoresis, capillary transfer to a nylon membrane (Hybond-N^+, Amersham Pharmacia Biotech) was carried out with 20× SSC (1× SSC: 0.15 M NaCl, 15 mM trisodium citrate, pH 7.0). The PCR-generated probe, used in cloning of the *T. halophila dnaK* operon described above, was labeled with ^{32}P-dCTP. Northern blot hybridization was carried out at 42 °C for 12 h. The reactive concentration of *dnaK* transcripts was estimated by autoradiograph densitometry with a BAS 2000 Bio-Imaging Analyzer system (Fuji Photo Film, Tokyo, Japan).

Slot-blot hybridization

Slot-blot hybridization method is less prone to pipetting errors compared to Northern blot hybridization, being more relevant in terms of exact quantification. Five microgram of alkaline-denatured total RNA was transferred to Zeta Probe blotting membranes (Bio-Rad) with a Bio-Dot SF microfiltration apparatus (Bio-Rad) as specific manufacturer, and treated with UV cross link. Prehybridization and hybridization were carried out by the same method as Northern hybridization described above.

RESULTS

Cloning and nucleotide sequence of the *dnaK*-operon from *T. halophila* JCM5888

Two oligonucleotide primers for degenerative PCR, dnaK-TS1 and dnaK-TA1 (Table1), were designed based on the amino acid sequence alignment of highly conserved regions of DnaK proteins from *Lactococcus lactis*, *B. subtilis* and *Clostridium acetobutylicum*. Amplified 900-bp fragment as expected size was cloned into *E. coli* JM109 using pUC18 and sequenced. The nucleotide sequence of the cloned PCR fragment showed high similarities to other bacterial *dnaK* homologues. This PCR fragment was labeled and used as a probe for Southern hybridization with chromosomal DNA of *T. halophila* JCM5888. The 3.5-kb *Xba*I and 2.0-kb *Xba*I-*Eco*RV digested fragments,

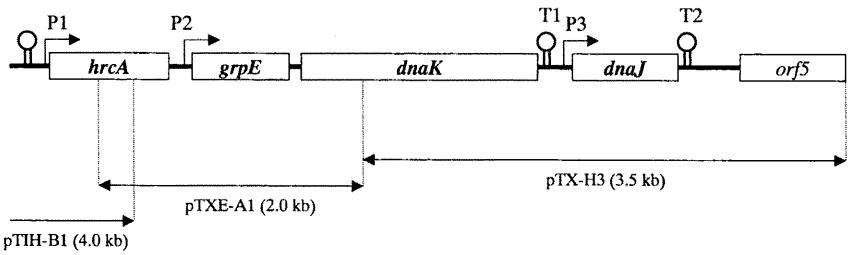

Fig. 1. Genetic organization of the *T. halophila dnaK* operon.
P indicates the putative promoter region, and potential stem-loop structure also indicated as T. The open reading frame *orf5* located immediately downstream of *dnaJ* is transcribed in the opposite direction to the *dnaK* operon.

hybridizing with the probe were purified from agarose gel, and cloned into pUC18 resulting in plasmids pTX–H3 and pTXE–A1. Nucleotide sequence revealed that these clones were covering several open reading frames (ORFs). Cloning of the gene encoding further upstream region of *hrcA* was achieved by inverse PCR as described in Materials and Methods. A 4.0–kb amplified fragment was generated from the *Hin*dIII–digested *T. halophila* chromosomal DNA. This fragment was cloned in *E. coli* JM109 using pUC18 and named as pTIH–B1. The complete nucleotide sequence of 6–kb revealed the presence of five ORFs. Four ORFs showed high degrees of similarity to *L. lactis* HrcA, GrpE, DnaK and DnaJ, respectively. ORF5 showed high similarity with the C–terminal end of *B. subtilis* ABC (ATP–binding cassette) transporter (Wey *et al.*, 1998) (48% identical). Thus, genetic organization of the *dnaK* operon of *T. halophila* is shown in Fig. 1.

Analysis of the nucleotide and deduced amino acid sequences of *T. halophila* DnaK

The *dnaK* gene encoded 618–amino acid protein with a predicted molecular mass of 66,783 Da. The similarities in amino acids between the *T. halophila* DnaK and the other bacterial DnaK were 79% (*L. lactis*), 76% (*Lactobacillus sakei*), 65% (*B. subtilis*), 53% (*E. coli*). The degree of similarity was consistently higher at the N–terminus and declined substantially toward the C–terminus. This particular feature could be shown by all the members of Hsp70. In front of *hrcA*, two inverted repeats were identified which corresponds to the consensus sequence of CIRCE element (Zuber and Schumann, 1994). A putative promoter region similar to the consensus Gram–positive vegetative promoter was found between two CIRCE elements (with a –35 sequence, 5'–TTAACA–3', and –10 sequence, 5'–TATATT–3') (Fig. 2). Immediately downstream of *dnaK*, stem–loop structure was found, which may be a rho–independent transcription terminator (T1). A rho–independent terminator was also found downstream of *dnaJ* (T2). DnaJ also exhibited high similarity with the DnaJ of *L. lactis* (67%), and had highly conserved region at the N–terminus.

Phylogenetic tree of 14 bacterial DnaK homologues, clearly showed that two distinct clusters existed (Fig. 3). The *T. halophila* DnaK was included in the cluster of gram–positive bacterium. Differed from the result of homology search, the *T. halophila* DnaK showed a close relation with DnaK of *L. sakei* more than that of *L. lactis*.

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1351  TGCTCCTTTAGCAAAGCATGTTTTGTCATTAGTTTACCATAGTTATTTATAAACTTAGACGAATTTCTAGCTATTTCTCCAGAATTAGCA  1440
                                                                                        >>>>>>
1441  CTCTAACATAAAGAGTGCTAATTTTTTGAGCATTTCCTCTTAACATTCTTGTGATTTGCGGTATATTAATAATCGTGTTAGCACTTAGAT  1530
      >>   CIRCE   <<<<<<<<              -35                -10          >>>>>>>   CIR
1531  ATAAGGAGTGCTAATTAAAGGGTGATAAAT TTGTTGACGCAAAGACAAGATAATATTTTGCATCAAATTATTCATAATTATACAAATTTA  1620
      CE   <<<<<<<<<   S.D.          M  L  T  Q  R  Q  D  N  I  L  H  Q  I  I  H  N  Y  T  N  L
                                  hrcA →
```

Fig. 2. Promoter region of the *T. halophila hrcA* gene.
The CIRCE elements and typical Shine–Dalgano sequence (S.D.) are indicated by arrowheads and is boxed, respectively. The putative promoter sequences, –35 and –10, are underlined. The alternative start codon (TTG) for *hrcA* is indicated by italics.

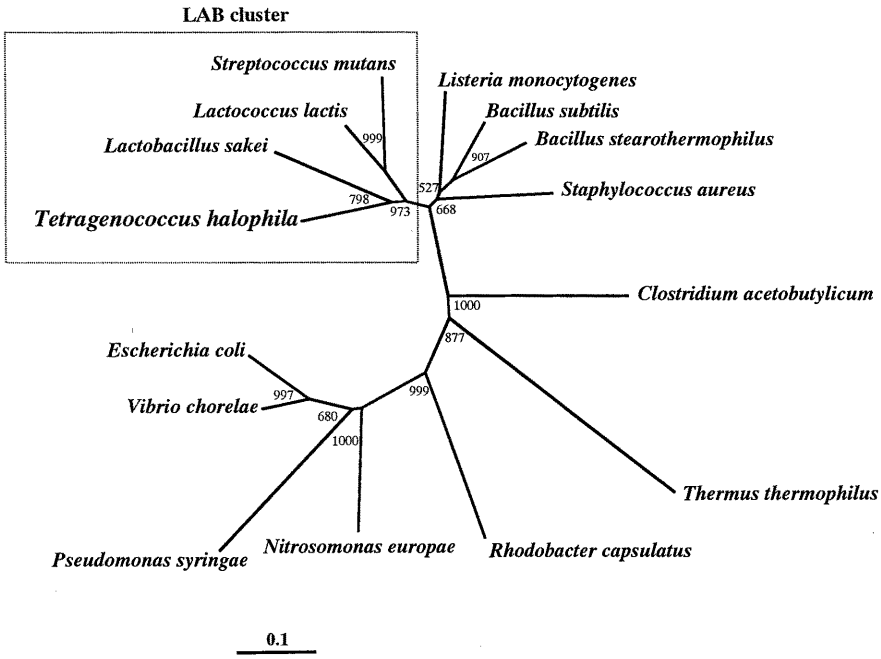

Fig. 3. Phylogenetic analysis of *T. halophila* DnaK and other bacterial DnaK (Hsp70) homologues.
A phylogenetic tree was constructed based on the NJ method as described in Materials and Methods.

Purification of the recombinant *T. halophila* DnaK

To confirm whether cloned *T. halophila dnaK* gene encodes a functional DnaK/Hsp70 homologue protein, the *T. halophila dnaK* gene expression plasmid pTHDnaK–His was constructed as described in Materials and Methods. After induction by the addition of IPTG, SDS–PAGE analysis revealed that a 75–kDa polypeptide was highly expressed in *E. coli* (Fig. 4). Purified recombinant *T. halophila* DnaK protein possessed ATPase activity. This indicates that the cloned *dnaK* gene from *T. halophila* chromosomal DNA encoded the protein which could act as a functional molecular chaperone.

Transcriptional analysis of *T. halophila dnaK* by heat shock

To clarify the expression behavior, and to confirm the function of cloned *T. halophila dnaK* as heat shock protein, we performed Northern blot hybridization and analyzed the effect of heat shock on the expression of *T. halophila dnaK*. Total RNA was purified from the cells and subjected to Northern analysis using specific probes for *dnaK*. The autoradiograph using *dnaK* probe revealed several bands of large transcripts 4.9, 3.8, 2.6 and 1.8–kb (Fig. 5), which may correspond to *hrcA–grpE–dnaK–dnaJ*, *hrcA–grpE–*

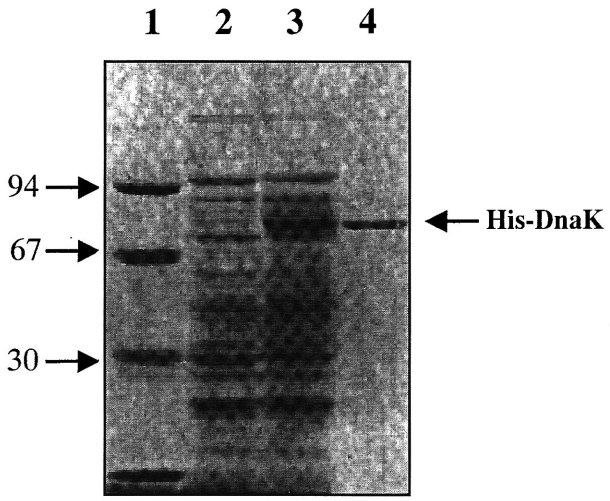

Fig. 4. SDS–PAGE of His-tagged *T. halophila* DnaK.
Lane 1, Molecular weight marker (kDa); Lane 2, before induction of the recombinant *T. halophila* DnaK; Lane 3, after overexpression of the recombinant *T. halophila* DnaK; Lane 4, the purified recombinant *T. halophila* DnaK.

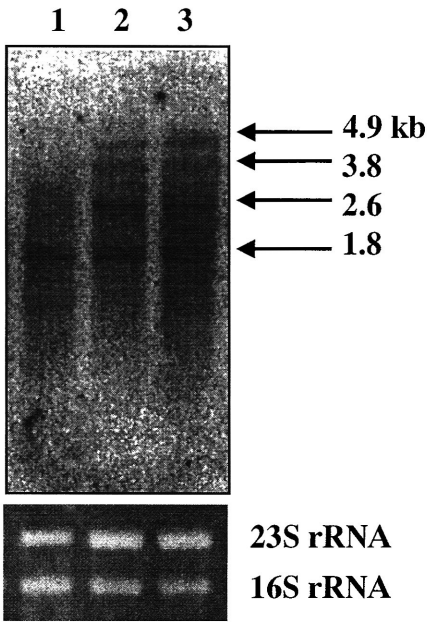

Fig. 5. Northern blot analysis of *dnaK* gene expression in *T. halophila*.
Each total RNA was isolated from the cells grown at 30 °C for 24 h in MRS medium containing 1 M NaCl (lane 1) and further incubated for 5 and 10 min after shifting to 45 °C (lanes 2 and 3). Ethidium bromide staining of the gel shows that equal amounts of RNA were loaded.

dnaK, *grpE–dnaK* and *dnaK* (see Fig. 1). To determine the induction level by heat shock more precisely, quantitative slot–blot hybridization was also performed. When cells were transferred from 30 °C to 45 °C, the *dnaK* mRNA level increased about 10–fold after 30 min (Fig. 6). These data suggested that cloned *T. halophila dnaK* surely belongs to a system of heat shock response.

Transcriptional induction of *T. halophila dnaK* by salt stress

In addition to heat shock, we analyzed the effect of high salinity on the induction of *T. halophila dnaK* by slot–blot hybridization. The cells grown in the presence of 1.0 M NaCl for 2 h were transferred to the same fresh medium containing the indicated NaCl concentrations, and incubated for further 2 h. Figure 7 indicates the amount of *dnaK* mRNA increased almost 3.5–fold with 3 M NaCl, which may be a limit concentration for growth. On the other hand, KCl showed no induction of the *dnaK* even at high concentrations (data not shown). These suggested that the salt induction of *T. halophila dnaK* is regulated at the transcriptional level, and is specific for sodium ion not osmolarities.

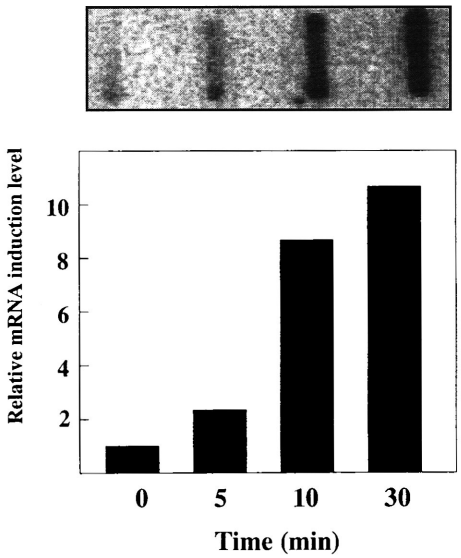

Fig. 6. Quantitative analysis of *T. halophila dnaK* gene expression by heat shock.
Transcript levels were quantified using the *dnaK* probe by slot-blot analysis. Each total RNA was isolated from the cells grown at 30 °C for 24 h in MRS medium containing 1 M NaCl and further incubated for 5, 10 and 30 min after shifting to 45 °C. The signal intensity before heat shock was defined as 1.0.

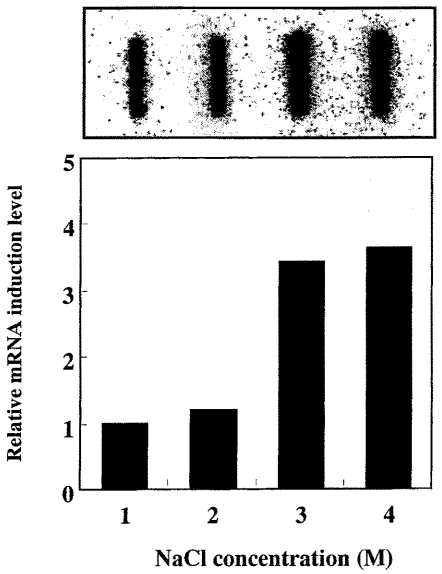

Fig. 7. Transcription of *T. halophila dnaK* gene under NaCl stress.
Transcript levels were quantified using the *dnaK* probe by slot-blot analysis. The cells were grown at 30 °C for 24 h in MRS nedium containing 1 M NaCl. Each total RNA was obtained from cells after transfer to the fresh MRS media containing the indicated concentrations of NaCl and 2 h of incubation. The signal intensity with 1 M NaCl was defined as 1.0.

DISCUSSION

In this paper, we describe the cloning and nucleotide sequence of *dnaK* operon of the halophilic lactic acid bacterium *T. halophila* JCM5888, as well as the expression analysis of *dnaK*. The *dnaK* operon of *T. halophila* consists of at least four open reading frames in the order *hrcA–grpE–dnaK–dnaJ*. This kind of genetic organization was also found in other several low G+C Gram-positive bacteria. In the case of *L. lactis*, the *dnaJ* gene is not part of *dnaK* operon, although the predicted amino acid sequence of *T. halophila* DnaK showed high similarity with that of *L. lactis* DnaK. Two of highly conserved inverted repeat sequence, termed CIRCE element, were found 101-bp and 17-bp upstream of *hrcA* start codon. This element is extensively studied in *B. subtilis* and *Staphylococcus aureus* (Zuber and Schumann, 1994; Ohta *et al.*, 1996). It has been recently shown that the HrcA interacts to the CIRCE element and regulates the expression of the *dnaK* operon in these bacteria. It can be concluded that in *T. halophila* the CIRCE element and HrcA protein also represent an important system to regulate the expression of class I heat shock genes.

Although amino acid sequence of *T. halophila* DnaK showed higher similarity with *L. lactis* DnaK more than that of *L. sakei*, phylogenetic analysis based on NJ method showed close relation between *T. halophila* and *L. sakei* DnaK and separated from *L. lactis* DnaK (Fig. 3). This result corresponded to the previous taxonomic analysis based

on 16S rDNA sequence. The deduced amino acid sequence of additional open reading frame (ORF5), immediately downstream of the *dnaJ*, showed high degree of similarity to that of ABC (ATP-binding cassette) transporter of *Bacillus subtilis* (48% identity). Since Northern analysis revealed that *orf5* was not induced by the heat shock on transcription level (data not shown), ORF5 is not part of the heat shock operon.

The cloned *T. halophila dnaK* gene was overexpressed in *E. coli* using pET system and the resulting DnaK-poly His fusion protein was puritied. SDS-PAGE profile revealed the molecular mass of 75 kDa, which was higher than the calculated one of *T. halophila dnaK* gene product. This discrepancy might be due to a feature of specific protein structure and the molecular mass of hexahistidine-tagged derivative. Purified protein of the recombinant *T. halophila* DnaK exhibited an apparent ATPase activity. Thus, the purifis protein corresponds to the functional molecular chaperone DnaK.

Northern hybridization analysis revealed polycistronic transcription manner with the maximum length 4.9-kb. This confirms that the *dnaK* operon of *T. halophila* is tetra-cistronic, and 4.9-kb transcript may encompass the complete *dnaK* operon. The more amount of 2.6-kb bi-cistronic transcript (*grpE-dnaK*) was detected than the amount of 4.9-kb (Fig. 5). This suggests that a certain level of transcription from P2 promoter might be occurred (Fig. 1). In *B. subtilis*, specific cleavage between *grpE* and *dnaK* was reported previously (Homuth *et al.*, 1999). The 1.8-kb transcript, which may encompass the *dnaK* gene, might be arisen due to the same reason.

Slot-blot analysis of total RNA confirmed the heat shock induction of the *T. halophila dnaK*. In addition to the heat shock induction, transcription of *T. halophila dnaK* was induced by 3 M NaCl. The increased expression of *dnaK* under high concentration of NaCl represents that DnaK acts as an important factor to adapt to environmental high salinity condition. Recently, several attempts to elucidate the role of DnaK upon osmotic adaptation have been performed with *E. coli*. Most microorganisms uptake or synthesize compatible solutes like potassium ion, glycine-betaine and choline in response to osmotic adaptation (Kempf and Bremer, 1998). *T. halophila* was also reported to uptake and accumulate glycine-betaine under high salinity condition, and imported compatible solutes regulate the intracellular Na^+ concentration. Thus, compatible solutes and DnaK may play complementary roles in the maintenance of cell structures by stabilizing protein and preventing the denaturation. Interestingly, similar induction behavior of *T. halophila* DnaK was not observed at high concentration of KCl. In the adaptation to hyperosmotic condition, accumulation of intracellular K^+ is a primary event for many bacteria (Palleros *et al.*, 1993). Since *T. halophila* is also known to accumulate much amount of K^+ intracellularly as compatible solute in the high NaCl concentration medium, environmental excess potassium ion may be allowed.

We are now investigating the ATPase and refolding activity of DnaK protein of *T. halophila* under halophilic condition. The halotolerant molecular chaperone, which possesses strong folding activity of denatured protein under high salinity might be useful for molecular biological application.

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