

Allelic variations in *dnaK* of thermotolerant bacilli inhabiting thermal springs

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Abiotic stresses including high or low temperature are known to affect productivity in plants as well as microflora, and often heat shock proteins (Hsps) are induced in cells experiencing such heat stress. Hsp 70 which belongs to one of the five major families of Hsps, DnaK family, plays a vital role in the protection and recovery of cells damaged by heat stress. Further, the role of *dnaK* gene encoding a protein related to Hsp70 in thermoregulation has been established already. In this study, we analyzed a set of thermotolerant bacilli for variation in the *dnaK* gene that codes for DnaK/Hsp70 protein. A part of the *dnaK* was amplified from seven different bacilli isolated from Manikaran hot springs, Himachal Pradesh, India. Phylogenetic analyses of *dnaK* in the set of strains generated four clusters, three representing highly thermotolerant strains and one, a moderately thermotolerant strain. However, analyses of deduced amino acid sequences generated only three clusters, two representing highly thermotolerant strains and one representing moderately thermotolerant strain. Comparison of DnaK of highly thermotolerant strain (M5) and moderately thermotolerant strain (M36) revealed polymorphism for 26 amino acids in the ATPase domain and for one amino acid in the substrate binding domain. It can be hypothesized that polymorphism in the DnaK could be linked to the difference in the temperature tolerance limit of the two strains.

Keywords: Abiotic stress, Extremophiles, Heat shock protein (Hsp), Heat stress, Hot springs, Polymorphism, Sequencing

Abiotic stress such as high or low temperature, salinity, drought, or even those induced by chemicals play a major role in reducing the productivity of plant, animal and microflora. A lot of research has focussed on major stress tolerance mechanisms, including ion transporters, signalling cascades, osmoprotectants, free radical scavengers, transcription factors and other proteins/chaperone proteins¹⁻⁴. Heat shock proteins (Hsps) are class of proteins that are induced in cells experiencing abiotic stress, which were first discovered in *Drosophila*⁵. The archetypal stress response is a sudden rise in the outside temperature, called heat shock⁶. Five major families of Hsps are recognized; Hsp70 (DnaK) family; the chaperonins (GroEL and HSP60); Hsp90 family; Hsp100 (Clp) family and the small Hsp (sHsp) family^{2,7}. A gene coding for small heat shock protein Hsp22 was isolated from an Indian isolate *Chaetomium globosum*. This gene was shown to increase resistance

to NaCl and Na₂CO₃ in a heterologous host, *E. coli* (BL21)⁸. Hsp70 proteins are involved in de novo protein folding, membrane translocation, formation and disassembly of protein complexes and degradation of misfolded proteins⁹. Hsp70 consists of a NH₂-terminal ATPase domain, COOH-terminal substrate binding domain and an α -helical domain. They play a role in the protection and recovery of cells from the ill effects of many physiological stresses¹⁰. The gene encoding a protein related to Hsp70 in the domain bacteria is called *dnaK*¹¹. The role of *dnaK* in thermoregulation is well established by gene expression studies at mRNA level¹² and deletion mutation studies¹³. Enhancement of thermotolerance in the heterologous system has been reported in various organisms such as *Escherichia coli*^{6,14}, tobacco¹⁰, *Arabidopsis thaliana*¹⁵ and rice¹⁶. The *dnaK* gene from *Trichoderma harzianum* has been found to enhance drought and freezing stress tolerance in poplar¹⁷, and the *dnaK* gene from *Alicyclobacillus acidoterrestris* improved resistance of *E. coli* against heat and acid stress¹⁸.

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In our earlier studies on diversity of culturable thermotolerant bacteria from Indian hot springs, many *Bacillus* strains capable of tolerating growth temperatures up to 70°C were identified^{19,20}. Such microbial diversity in extreme environments provides an opportunity to mine novel genes and metabolites²¹. Allele/gene mining is one such novel approach which exploits the nucleotide sequence of one organism to identify useful alleles from other different organisms²². The present study looks for allelic variation in the gene *dnaK* of different thermotolerant bacilli isolated from thermal springs in Manikaran, Himachal Pradesh, India. A moderately thermotolerant strain is also used in the study for comparing *dnaK* between highly thermotolerant and moderately thermotolerant bacteria.

Materials and Methods

Bacterial strains and growth conditions

The bacterial strains used in the study were isolated from Manikaran hot springs, Himachal Pradesh, India (32° 02' 00" N 77° 20' 48" E)¹⁴. A list of seven strains used with their NCBI accession number and temperature tolerance are given in Table 1. Nutrient broth (Peptone 0.5%, Beef extract 0.3% and NaCl 0.5%) was used to grow the cultures at an incubation temperature of 37°C for 16 h.

Genomic DNA preparation

Bacterial strains were grown overnight in a shaking incubator at 37°C and cells were pelleted down from 5 mL culture. Pellets were then washed thrice with TE buffer (10 mM Tris and 1 mM EDTA, pH 8.0) and resuspended in 750 µL of TE buffer. Genomic DNA was isolated from the suspended pellet using ZR Fungal/Bacterial DNA MicroPrepTM following the manufacturer protocol (Zymo Research, The Epigenetics companyTM).

Table 1 — Strains used and their temperature tolerance

Strains	16S rRNA sequence NCBI Accession No.	Temperature tolerance (°C)
M4 <i>Bacillus icheniformis</i>	JX312615	70
M5 <i>Bacillus pumilus</i>	JX312616	70
M6 <i>Bacillus subtilis</i>	KC315773	70
M7 <i>Bacillus pumilus</i>	KC315774	70
M8 <i>Bacillus</i> sp.	KC315775	70
M36 <i>Brevibacillus</i> sp.	JX312626	45
M55 <i>Bacillus megaterium</i>	JX312633	70

PCR amplification of *dnaK* region

A partial fragment of *dnaK* gene (650 bp) was amplified using a forward primer (5'-CCGGCGACAACCGTCTGGGTGGGGA-3') and reverse primer (5'-GGGCGCTCACCTTGAAGAACATGGA-3')²³.

Amplification was carried out in a 25 µL reaction volume containing 50-100 ng of template DNA, primers (100 ng each), dATP, dCTP, dTTP and dGTP (200 µM each), Advantage2 Polymerase reaction buffer (10X) 2.5 µL and 1.0 U Advantage2 Polymerase. PCR products were resolved by electrophoresis at 60 V for 1 h in 1.2 % agarose gel in 1X TAE buffer. Gels were then stained with ethidium bromide and visualized on a gel documentation system (Alpha Imager).

Sequencing and analysis of *dnaK* gene

PCR amplified *dnaK* region was purified and sequenced using both forward and reverse primers and sequencing was done employing a dideoxy cycle with fluorescent terminators and run in a 3130xl Applied Biosystems ABI prism automated DNA sequencer. Amino acid sequence from nucleotide sequences were deduced using ExpPASy Translate tool from ExpPASy Bioinformatics Resource Portal (<http://web.expasy.org/translate/>). Both nucleotide sequence and amino acid sequence were aligned using the multiple sequence alignment tool CLUSTAL W2²⁴ with default parameters. Dendrogram was constructed on aligned data sets using Neighbour joining (NJ) method²⁵ using the program MEGA 4.0.2²⁶. Bootstrap analysis was performed on 1000 random samples taken from multiple alignments²⁷.

Results and Discussion

Gene or allele mining for abiotic stress tolerance is among the major goals of research worldwide in the light of climate change and the resultant reducing crop yields. Recognizing the importance of the gene *dnaK* in the thermoregulation and its potential role in the management of abiotic stress in crop plants^{10,15,16} and the possibility of recombinant overexpression of particular protein of interest²⁸, an attempt was made to explore polymorphisms in the gene *dnaK* of seven bacilli strains isolated from Manikaran hot springs in Himachal Pradesh, India¹⁹.

Among the seven strains, six were tolerant to incubation temperatures up to 70°C and one strain (M36) was tolerant only up to 45°C. Amplification with the primers yielded a PCR product of 650 bp from all the seven strains (Fig. 1). Sequencing of the

purified PCR product followed by BLAST search showed that the sequence had an overall similarity to well described *dnaK* homologues (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Liang *et al.*⁹ identified and characterized a heat shock protein 70 (Hsp70/DnaK) in *Bacillus licheniformis*, which was 1839 bp in length and coded for a protein containing 612 amino acids; however they did not undertake comparisons with the available homologues.

In the present investigation, sequencing and analysis by CLUSTAL W2 revealed that nucleotide sequences of the gene *dnaK* from these seven *Bacillus* strains were aligned into 4 clusters; Cluster I (M5, M6 and M55), Cluster II (M7 and M8), Cluster III (M4), and Cluster IV (M36) (Fig. 2A). Alignment of the deduced amino acid sequences revealed that the protein DnaK from these seven strains were distributed into three clusters; Cluster I (M5, M6, M7, M8 and M55), Cluster II (M4) and Cluster III (M36) (Fig. 2B). A pairwise alignment between the Cluster I and Cluster II of amino acid sequence revealed a variation in only single amino acid; Glutamic acid in Cluster I and aspartic acid in Cluster II. Interestingly, the strains in these two clusters (M4, M5, M6, M7, M8 and M55) are all tolerant to temperatures up to 70°C. Hence, this single amino acid substitution in the DnaK of these strains may not be playing a major role in the tolerance to high temperatures. In order to look for the differences in the amino acid sequence in the DnaK of the highly thermotolerant strain M5 and moderately thermotolerant strain M36, a pairwise alignment was done for 175 amino acids from position 211 to 385 (211 to 354; ATPase domain and 355-385; substrate binding domain). This revealed that DnaK of M5 and M36 are polymorphic for 27 amino acids (Fig. 3) from which 26 are in the N-terminal ATPase domain and one in the substrate binding domain. Nucleotide binding and ATP hydrolysis, activities of ATPase are important steps in the chaperonic activity of DnaK¹¹. *In vitro* studies have shown that Hsp70/DnaK proteins bind both denatured proteins and some short peptides, and release these substrates in response to the addition of ATP^{29,30}. Polymorphism between the DnaK of M5 and M36 in the ATPase domain may be the reason for the difference in the thermotolerance limits between these two strains. Although in the present study, not much of polymorphism was observed in the analysed DnaK protein, among the highly thermotolerant strains, it may exist in the other regions. Future research needs

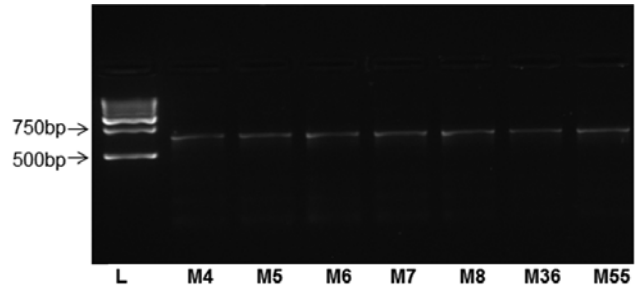


Fig. 1 — Amplification of *dnaK* from selected thermotolerant *Bacilli* inhabiting Manikaran hot springs. [Lane 1, Marker; and Lanes 2-8, Amplicons of *dnaK*

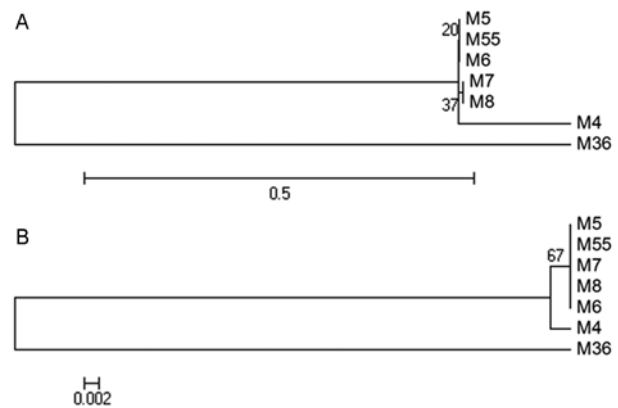


Fig. 2 — Unrooted dendrogram based on comparison of (A) *dnaK* nucleotide sequences of seven thermotolerant *Bacilli* isolated from Manikaran hot springs; and (B) DnaK amino acid sequences of seven thermotolerant *Bacilli* isolated from Manikaran hot springs. [Dendrogram was constructed based on aligned datasets using Neighbour joining (NJ) method using the program MEGA 4.0.2. Numbers on the tree indicates percentage of bootstrap sampling derived from 1000 random samples]

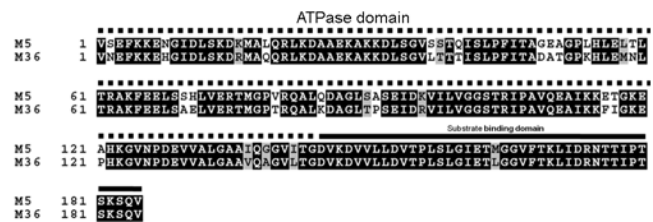


Fig. 3 — Pairwise alignment of DnaK proteins of M5 (highly thermotolerant strain) and M36 (moderately thermotolerant strain) showing variations in the amino acid sequence.

to be pursued towards extensive screening using different primers to clone full length genes from all the highly thermotolerant strains.

Heat shock proteins such as Hsps act as molecular chaperones and represent integral components of stress alleviation machinery of the cell, which helps to maintain homeostasis under both normal and adverse conditions. With the recent advances, especially in the

development of transgenics, genetic engineering of plants for abiotic stress tolerance is becoming a useful approach. Overexpression of *dnaK* from a halotolerant cyanobacterium is known to enhance the high temperature tolerance of tobacco seeds in the germination and early growth stages¹⁰. It was also reported in our earlier study that a gene *dnaK* from *Bacillus pumilus* when cloned and transformed in *E. coli* host increased the thermotolerance of transformant up to 60°C³¹. It may be plausible to utilize the promising *dnaK* from our study for developing stress tolerant transgenics in future.

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