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Cloning and expression of *dnaK* gene from *Bacillus pumilus* of hot water spring origin ☆

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ABSTRACT

A set of thermotolerant strains isolated from hot springs of Manikaran and Bakreshwar (India) were selected with an aim to isolate *dnak* gene which encodes DnaK protein. The gene *dnaK* along with its flanking region was successfully amplified from 5 different strains (4 from Bakreshwar and one from Manikaran). Restriction fragment length polymorphism (RFLP) revealed that amplicons were almost identical in sequence. The *dnak* gene from one representative, *Bacillus pumilus* strain B3 isolated from Bakreshwar hot springs was successfully cloned and sequenced. The *dnaK* gene was flanked by gene *grpE* on one side. The *dnaK* gene was 1842 bp in length encoding a polypeptide of 613 amino acid residues. Calculated molecular weight and pI of the protein were 66,128.36 Da and 4.72 respectively. The deduced amino acid sequence of this gene shared high sequence homology with other DnaK proteins and its homologue Hsp 70 from other microorganisms, but possessed 36 substitutions and two insertions, as compared to DnaK protein of *Bacillus subtilis*. The *dnaK* gene of *B. pumilus* was successfully expressed in *Escherichia coli* BL 21 (DE3) using pET expression systems. Heterologous expression of *dnaK* of *B. pumilus* in *E. coli* BL 21 (DE3) allowed for the growth of *E. coli* up to 50 °C and survival up to 60 °C for 16 h, suggesting that *dnak* from *B. pumilus* imparts tolerance to host cells under high temperature. This novel gene can be an important component for possible utilization in abiotic stress management of plants.

1. Introduction

Agriculture, one of the most vulnerable sectors to climate change is posed with the threat to sustainability due to the ever increasing global temperature, and other abiotic and biotic stresses. Among the principal abiotic stresses to crop plants in India like soil moisture, high temperatures, soil salinity/alkalinity, low pH and metal toxicity (Grover, 2011), management of high temperature stress is important in the context of global warming. Although crop plants continue to evolve to cope up with the temperature fluctuations, this capacity might not keep pace with global warming. Technological solutions like glasshouse cultivation and controlled atmosphere cultivation are not economically viable option. A more practical solution is to alter the plant genetic machinery such that the plants can grow and reproduce normally under increased ambient temperature (Grover et al., 2013) for which the availability of diverse genetic material is a pre-requisite.

Physiological stresses like sudden change in temperature, an increased salt or acid concentration and oxidative stress induces the synthesis of class of proteins called heat shock proteins (Hsps). The first report on such heat shock protein was in *Drosophila* induced by heat shock (Ritossa, 1962). Five major families of Hsps are recognized; Hsp 70 (DnaK) family; the chaperonins (GroEL and Hsp 60); Hsp 90 family; Hsp 100 (Clp) family and the small Hsp (sHsp) family (Wang et al., 2004). DnaK proteins are involved in de novo protein folding, membrane translocation, formation and disassembly of protein complexes and degradation of misfolded proteins (Liang et al., 2009). DnaK consists of a highly conserved NH_2-terminal ATPase domain, COOH-terminal substrate binding domain and a α-helical domain. They are believed to play a role in the protection and recovery of cells from ill effects of many physiological stresses (Ono et al., 2001). The gene encoding a protein related to Hsp 70 or DnaK in the domain Bacteria is called *dnaK* (Ward-Rainey et al., 1997). The role of *dnak* in thermoregulation is well established by gene expression studies at mRNA level (Wetzstein et al., 1992) and deletion mutation studies (Singh et al., 2007). Enhancement of thermotolerance in the heterologous system has been reported in various organisms like *Escherichia coli* (Liang et al., 2009), tobacco (Ono et al., 2001), *Arabidopsis thaliana* (Montero-Barrientos et al., 2010) and Rice (Uchida et al., 2008). *dnaK* gene from *Trichoderma harzianum* has been found to enhance tolerance to drought and freezing stress in poplar (Takabe et al., 2008).

Abbreviations: IPTG, isopropyl β-D-1-thiogalactopyranoside; Hsp, heat shock protein.

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In our earlier studies on the diversity of culturable thermotolerant bacteria from Indian hot springs, members of the genera *Bacillus*, *Exiguobacterium*, and *Pseudomonas* were found to tolerate temperatures up to 70 °C The present study is designed with an aim to isolate gene *dnaK* from these thermotolerant strains. In the domain Bacteria, the *dnaK* gene is part of an operon called *dnaK* operon which in addition to gene *dnaK* also includes *dnaJ* and *grpE*. The genes *dnaJ* and *grpE* which code for Hsp 40 and GrpE respectively in bacteria modulate the activities of DnaK by acting as co-chaperones (Singh et al., 2007). Whole genome sequence of different *Bacillus*, *Exiguobacterium* and *Pseudomonas* genera (http://www.ncbi.nlm.nih.gov/genome) allowed for the designing of primers in the region flanking *dnaK* gene. The native strain of *Bacillus pumilus* B3 in the *E. coli* host exhibiting enhanced thermotolerance is a Gram-positive spore forming bacterium isolated from Bakreshwar hot springs which is phylogenetically related to *B. pumilus* SAFR-032, for which the whole genome sequence is available in the NCBI database (Gioia et al., 2007). In the present study, we have demonstrated the successful cloning and expression of *dnaK* from this *B. pumilus* B3 strain.

2. Materials and methods

2.1. Materials, bacterial strains, vectors and growth conditions

Restriction endonucleases and T4 DNA ligase were purchased from New England Biolabs Inc. (USA). Advantage 2 proof reading polymerase mixes were from Clonetech Laboratories Inc. (CA, USA). DNA and protein molecular markers were from BR Biochem Life Sciences Pvt. Ltd. (New Delhi, India). Reagents for SDS-PAGE electrophoresis were from Sigma-Aldrich (New Delhi, India). The bacterial strains used in the study were isolated from the Manikaran and Bakreshwar hot springs (Kumar et al., 2013). Strains M4, M5, M6, M7, M8, M46, M47 and M55 were tolerant up to 70 °C and strains B3, B5, B12 and B34 were tolerant up to 60 °C. *E. coli* DH5α from Novagen (Madison, WI, USA) was used for the preparation of recombinant plasmids. *E. coli* BL21 (DE3) from Novagen (Madison, WI, USA) was employed for T7 RNA polymerase-mediated over expression of recombinant proteins. The plasmids pGEM®-T and pET vector systems were purchased from Promega Life Sciences (Madison, WI, USA). *E. coli* strains were grown in LB medium with rotary shaking (180 rpm) at 37 °C for 16 h. Ampicillin and kanamycin were used at a final concentration of 100 and 25 μg/ml, respectively. Other bacterial strains used in the study were cultivated in nutrient broth at 37 °C for 16 h.

2.2. Genomic DNA preparation, PCR amplification, restriction analysis, sequencing and analysis of dnaK gene

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 Zymo Research Fungal/Bacterial DNA MicroPrep™ following the standard protocol prescribed by the manufacturer. Primers were designed in the region flanking *dnaK* of different species. A list of primers used to amplify *dnaK* from different strains is given in Table 1. 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 (10×) 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 1× TAE buffer. Gels were then stained with ethidium bromide and visualized on a gel documentation system (Alpha Imager). Restriction digestion of the purified PCR products was undertaken with three restriction endonucleases — *Alu*I, *Hha*I and *Hae*III — in a 30 μl reaction volume, using recommended buffers at 37 °C. The digested PCR products were resolved by electrophoresis at 45 V for 1.5 to 2 h in 2.5% agarose gels in 1× TAE buffer. Gels were then stained with ethidium bromide to visualize the profiles. Strong and clear bands were scored as binary data (presence and absence of bands). DNA sequencing was performed by Scigenome Labs Pvt. Ltd. (Cochin, India) employing primer walking technique. The sequence was then analyzed using ORF finder available in the National Centre for Biotechnological Information (NCBI) tools. The deduced amino acid sequence was analyzed with the program BLAST-P from NCBI. Molecular weight and pI were calculated using ExPASy tools (web.expasy.org/compute_pi/). Alignment of amino acid sequence was done by T-Coffee alignment method (Notredame et al., 2000).

2.3. Cloning expression of dnaK of B. pumilus B3 in E. coli

Competent cells of *E. coli* were generated using the methods of Dagert and Ehrlich (1979). Restriction enzyme digests and other conventional DNA manipulation techniques were done as per Sambrook and Russell (2001). The open reading frame of *dnaK* of *B. pumilus* was amplified using the primers BKEF (5′CGCGGATCCATGAGTAAAATCATTGGTATTGAC 3′) and BKER (5′CGAGCTCGTTATTTTTTCTCTTGATCGTCG 3′). The PCR product was cloned into the pGEM-T easy vector to get a plasmid pGEM-BpdnaK in the cloning host *E. coli* DH5α. This plasmid was isolated

Table 1
List of primers used to amplify *dnaK* from different strains.

Strains	Probable species	16S rRNA sequence NCBI accession number	Primers
M4	*Bacillus licheniformis*	GQ280087	5′GCAGGTTGAGGATGAAGCGT3′ 5′ CTCATAGTAATCACGCTTAC3′
M5	*Bacillus pumilus*	JQ435673	5′CGATCCAAACCTTCATCAAGCC3′
M7	*Bacillus pumilus*	EF442670	5′CTCATCTCTTCACACTCCCG3′
M8	*Bacillus* sp.	HF536558	
B3	*Bacillus pumilus*	KC121051	
B5	*Bacillus pumilus*	JQ782895	
B12	*Bacillus pumilus*	JX307688	
B34	*Bacillus pumilus*	EU931553	
M6	*Bacillus subtilis*	KC492102	5′ GTCGAAGCCATCGAAGCTGT3′ 5′ ATAGTAATCACGCTTACTCA3′
M55	*Bacillus megaterium*	KC596003	5′ TGTTGAAGCAATTGAATCAG3′ 5′ ATCTCGCTTACTCATCAATA3
M46	*Pseudomonas psychrophila*	KC596004	5′ CGCCTTCGATTGACGAGAAG3′ 5′ TTGGTTCATGCGGTATTTGC3
M47	*Exiguobacterium acetylicum*	KC492105	5′CGTCCAAGTATGGTCAAAGTCGC3′ 5′GACATGGAAAGAGCCAAAGC3

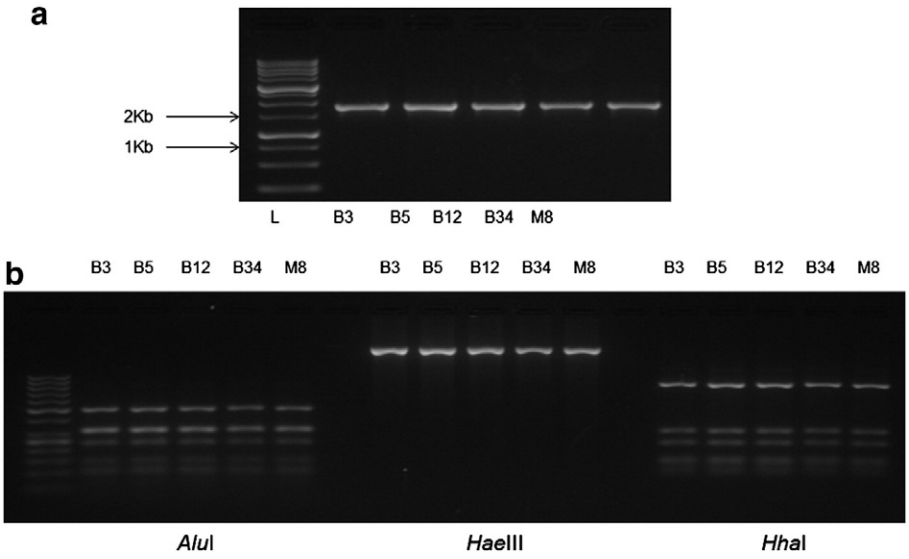

Fig. 1. a) Amplification of *dnaK* in selected thermotolerant strains from extreme habitats. Lane 1 — Marker; lanes 2 to 6 — Amplicons of *dnaK* with flanking region. b) Restriction patterns of amplified dnaK region of thermotolerant isolates generated using the restriction enzymes *Alu*I, *Hae*III and *Hha*I. Lane 1 — DNA marker, lanes 2 to 6 — Restriction patterns of amplified dnaK region of thermotolerant isolates generated using the restriction enzymes-*Alu*I, lanes 8 to 12 — Restriction patterns of amplified dnaK region of thermotolerant isolates generated using the restriction enzymes *Hae*III, lanes 14 to 18 — Restriction patterns of amplified dnaK region of thermotolerant isolates generated using the restriction enzymes *Hha*I.

and the gene was digested out from this plasmid using the restriction enzymes *Bam*HI and *Sac*I and cloned into pET 29 a(+) vector to generate a plasmid pET 29-BpdnaK which was used for the transformation and expression in the host *E. coli* BL21 (DE3). *E. coli* BL21 (DE3) transformed with pET29-BpdnaK plasmid was grown in LB medium with kanamycin (25 μg/ml) at 37 °C for 16 h. An aliquot (20 μl) of overnight grown culture was used to inoculate a fresh LB medium (20 ml in 100 ml flasks) and maintained at 37 °C with shaking (180 rpm) until the $O.D_{600\,nm}$ reaches up to 0.6. Recombinant protein expression was induced with IPTG to a final concentration of 0.2 mM and cultivated further. One milliliter of culture was harvested from induced culture by centrifugation at 6000 rpm at 4 °C for 10 min. Two controls were also set up simultaneously, one with *E. coli* BL21 (DE3) transformed with pET 29 a(+) plasmid backbone and other with *E. coli* BL21 (DE3) transformed with pET29-BpdnaK plasmid but without induction.

2.4. SDS-PAGE of cell lysates and MALDI-TOF analysis of expressed protein

Cell pellets harvested were suspended in SDS-PAGE loading buffer (5% 2-mercaptoethanol, 2% SDS, 0.1% bromophenol blue, and 10% glycerol in 50 mM Tris–HCl buffer; pH 6.8) and fractioned with 12% separating gel. After electrophoresis, the gels were stained with 0.25% Coomassie brilliant blue dissolved in 50% methanol–10% acetic acid and then destained in a 30% methanol–10% acetic acid solution. The band corresponding to 70 kDa from the SDS-PAGE gel was excised from the gel and subjected to MALDI-TOF mass spectrometry (Sandor Proteomics Private Limited, Hyderabad, India). Protein identity from peptide mass fingerprints was determined by the MASCOT program (Matrix Science Inc., Boston, MA; http://www.matrixsciende.com/search-form-select.html).

2.5. Heat stress experiments

To check for the tolerance to heat stress at 50 °C and 60 °C, aliquots of transformants both with pET29-BpdnaK and with pET 29 a(+) which were under IPTG induction for 12 h were inoculated in to a fresh 50 ml of LB medium. The transformants were inoculated at a volume equivalent to 0.6 $OD_{600\,nm}$. Growth was monitored by measuring the optical density at 600 nm at regular intervals (0, 2, 4, 8, 16 and 20 h after inoculation).

3. Results and discussion

3.1. PCR amplification and restriction analysis of dnaK region

Amplification with primers designed using *B. pumilus* SAFR-032 as template (primers designed in the region flanking *dnaK* gene) yielded a product of 2.2 kb for the strains B3, B5, B12, B34 and M8 (Fig. 1a). Out of these five strains, four (B3, B5, B12 and B34) had been earlier identified as *B. pumilus* and strain M8 as *Bacillus* sp. based on 16S rRNA gene based identification (manuscript submitted). All the other primers evaluated did not give amplification of *dnaK*, however failure to attain amplification of *dnaK* from these strains cannot be considered as non-availability of the gene in the organism as *dnaK* or its homologue is known to be present across the domain from Archaea to Eubacteria and from plants to animals (Gupta and Golding, 1993; Boorstein et al., 1994; Falah and Gupta, 1997) and would be attributed to non-

ATCGTTGTAGAAGAATTGCAAAAGGGCTATAAACTCAAAGACCGAGTTATTCGTCCATCAATGGTAAAAGTAA
ATCAATAACTACATAATGGGAGGTCATCGAATATGAGTAAAATCATTGGTATTGACTTAGGAACAACAAACT
CATGCGTTGCAGTACTCGAAGGCGGCGAGCCAAAAGTTATTGCAAACGCTGAAGGAGCACGTACAACA
CCATCTGTTGTCGCTTTTAAAAATGGAGAGCGCCAAGTAGGTGAAGTAGCGAAACGTCAATCAATTACAA
ACCCGAACACCATCATGTCTGTTAAAAGACATATGGGTACAGATTATAAAGTAGAAGTTGAAGGCAAGAA
CTACACGCCGCAGGAAATCTCTGCAATTATTCTTCAACACCTTAAATCTTATGCTGAAGCTTATCTTGGCG
AAGAAGTAACAAAAGCGGTTATTACAGTTCCTGCTTACTTCAACGATGCAGAGCGCCAAGCAACAAAAG
ATGCTGGTAAAATTGCTGGTCTTGAAGTAGAACGTATCATCAACGAACCAACAGCAGCTGCACTTGCTTA
TGGTTTAGATAAAACAGATGAAGATCAAACGATCCTTGTATATGACCTTGGCGGCGGTACATTTGACGTAT
CAGTCCTTGAGCTTGGAGACGGCGTCTTTGAAGTACGCTCAACTGCTGGGGACAACCGTCTAGGTGGA
GACGATTTTGACCAAGTCATCATCGATCATCTAGTGGCTGAGTTCAAAAAAGAAAACGGCATTGATCTTT
CTAAAGATAAAATGGCGCTTCAGCGTTTAAAAGATGCTGCTGAAAAAGCGAAAAAAGATCTTTCTGGTGT
ATCTTCTACACAAATCTCACTGCCATTTATCACAGCTGGAGATGCAGGTCCTCTTCACCTAGAATTAACGC
TAACGCGTGCTAAATTCGAAGAGCTTTCTGCAGACCTTGTAGAGCGTACAATGACACCTGTACGTCAATC
ACTGAAAGATGCTGGTCTATCTGCTAGCGAGATTGATAAAGTTATCCTTGTTGGTGGATCAACTCGTATTC
CTGCAGTACAAGAAGCAATCAAAAAAGAAACAGGTAAAGAGCCTCATAAAGGCGTAAACCCTGATGAAG
TGGTTGCACTTGGTGCGGCAATTCAAGGTGGAGTTATCACAGGAGATGTCAAAGACGTTGTTCTTCTTGA
CGTCACACCACTTTCTTTAGGAATTGAAACAATGGGCGGCGTATTCACAAAGCTGATTGAACGTAATACA
ACCATTCCAACAAGTAAATCTCAAGTATTCTCAACGGCTGCTGACAACCAAACTGCTGTAGACATTCACG
TCTTACAAGGTGAGCGTCCAATGGCAGCAGACAACAAAACATTAGGTCGTTTCCAATTGACTGATATCCC
GCCAGCACCACGCGGCGTACCACAAATCGAAGTATCTTTTGATATCGATAAAAACGGTATTGTCAACGTA
CGTGCGAAAGATATGGGTACTGGAAAAGAACAAAACATCACAATCAAATCTTCTTCAGGTCTTTCTGATG
ATGAGATCGAAAAAATGGTCAAAGAAGCAGAAGAAAATGCTGAAGCAGATGCAAAGAAAAAAGAAGAA
ATCGAAGTGCGCAATGAAGCAGATCAATTAGTGTTTACAACAGAAAAAACATTAAAAGATCTTGAAGGCA
AAATCGATGAAGAGCAAGTGAAAAAAGCGAATGATGCAAAAGATGCGCTAAAAGCTGCGATCGAAAAA
GGCGAGCTTGAAGACATCAAAGCGAAAAAAGATGAGCTACAAACAATCGTTCAAGAATTAACGACAAAG
CTCTATGAAGAAGCTGCAAAACAAGCACAAGCTCAGCAAGAAGGCGGCGCTGAAGGTGCTCAAAAAGC
AGATGACAATGTAGTGGATGCAGAATACGAAGAAGTAAACGACGATCAAGAGAAAAAATAACTCACCTTT
ACTTTTGAAAAAGACTGCTGAAACGTACTCGTCAGAAAGTCAAAGTCAGGATCTCTTGGCTTTGACTTTTTT
CTTTTCAACGATGAAAAGGAATGAAAAAGATCAATTAAAAT

Fig. 2. Nucleotide sequence showing amplified *dnaK* region of *Bacillus pumilus* strain B3. Open reading frame is marked in a different color (red) and start and stop codons are marked in different colors (green).

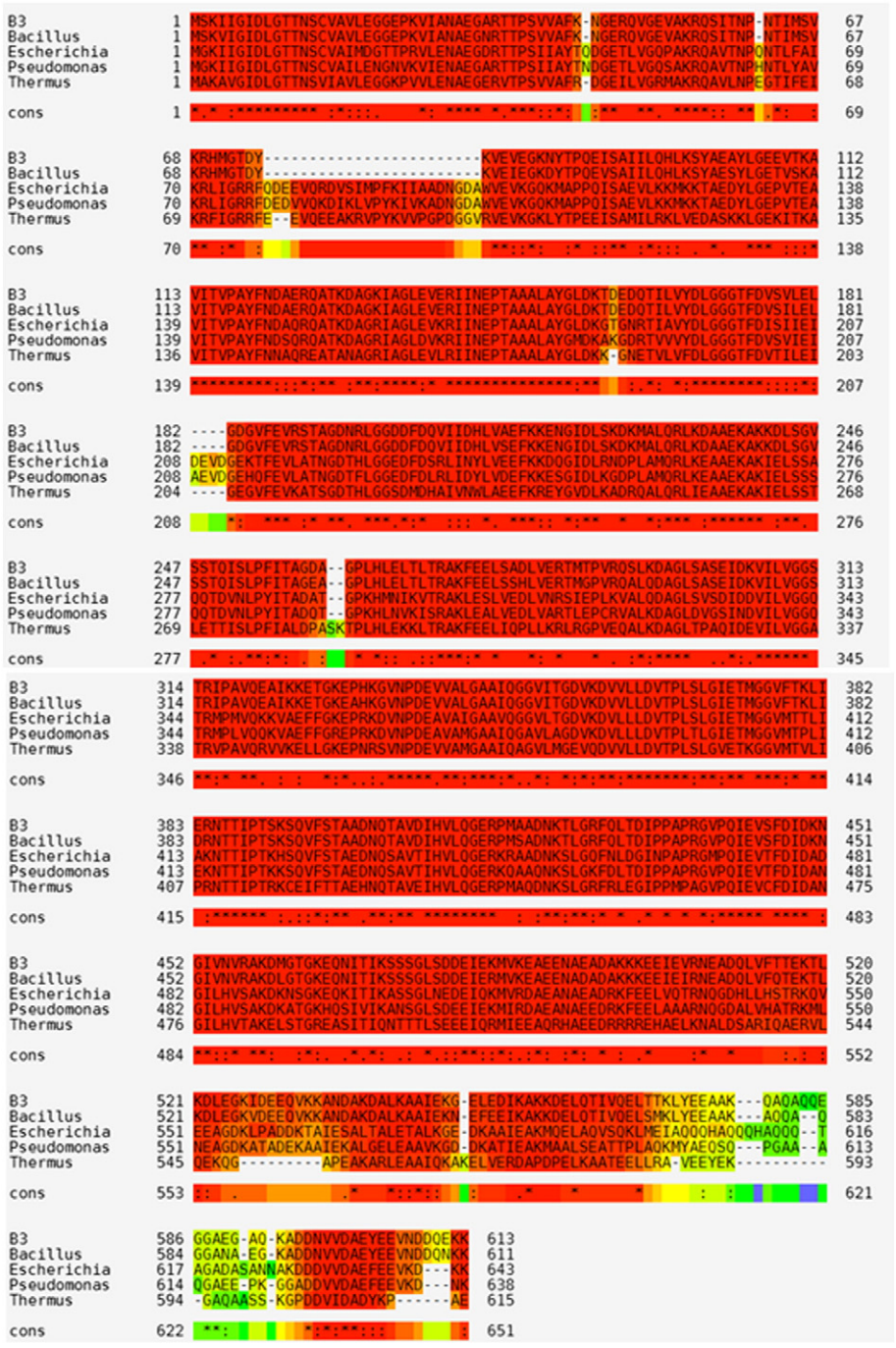

Fig. 3. Comparative analyses of DnaK proteins through alignment of amino acid sequences. The deduced amino acid sequence of DnaK *from Bacillus pumilus* (B3), *Bacillus subtilis* (*Bacillus*), *Escherichia coli* (*Escherichia*), *Pseudomonas thermotolerans* (*Pseudomonas*) and *Thermus thermophilus* DnaK(*Thermus*) are shown. Gaps in the aligned sequences (dashes) are introduced to maximize similarities.

availability of binding region for the primers designed in the respective templates. Future research needs to be pursued towards extensive screening with different primers. Restriction profiling is generally done to look for variants in the sequence and ARDRA (Amplified Ribosomal DNA Restriction Analysis) has been used by many groups to avoid any redundancy while sequencing 16S rRNA genes (Yadav et al., 2010; Sahay et al., 2011). In the present study restriction fragment length polymorphism (RFLP) was employed to look for any variant in the gene *dnaK* from among the five strains under study. Restriction digestion of purified PCR products with three restriction enzymes (*Alu*I, *Hha*I and *Hae*III) separately, yielded a similar restriction patterns (Fig. 1b) revealing that there are no variations in the *dnaK* among the five strains. This suggests that the sequence of *dnaK* is near identical in all the five strains that yielded amplification. Hence we selected *dnaK* from one representative culture (B3) for further characterization and expression analysis.

3.2. Sequencing and analysis of B3 dnaK

Sequencing of the purified PCR product through primer walking and analysis allowed for identification of an open reading frame of 1842 bp (Fig. 2) which codes for the protein DnaK/Hsp 70. Derived amino acid sequence predicted for a protein of 613 amino acids. Based on the available information in the database (http://tw.expasy.org/Swiss-Prot/

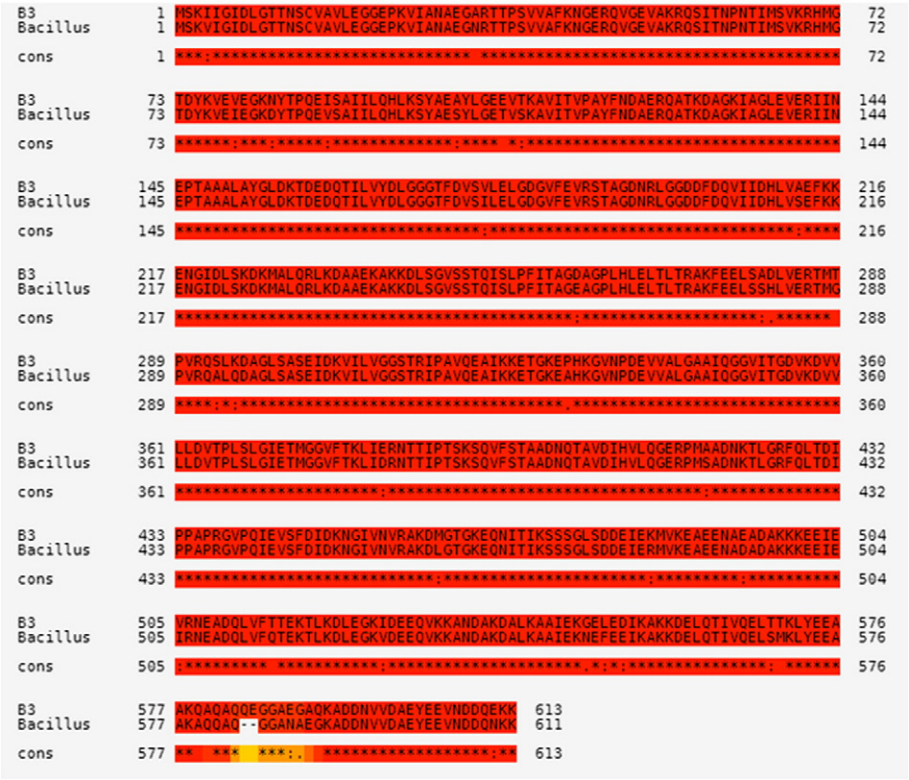

Fig. 4. Pairwise alignment of DnaK proteins of *Bacillus pumilus* B3 (B3) and *Bacillus subtilis* (*Bacillus*) showing substitutions and insertion of amino acids.

TrEMBL) DnaK proteins contain an amino acid length ranging from 596 to 656 (Liang et al., 2009). Molecular weight and pI of the protein were calculated using Expasy tool which yielded a size of 66128.36 Da and pI of 4.72. Blast search of the translated amino acids in the protein data base showed that the DnaK of *B. pumilus* B3 share an overall similarity to the well described homologues. The DnaK of *B. pumilus* B3 shared 93%, 58% 56% and 56% amino acid sequence identity with DnaK of *Bacillus subtilis*, *Pseudomonas thermotolerans*, *Thermus thermophilus* and *E. coli* respectively. The alignment of DnaK of *B. pumilus* B3 with DnaK of *B. subtilis*, *P. thermotolerans*, *T. thermophilus* and *E. coli* revealed a deletion of about 24 amino acids in the N-terminal region of DnaK of *B. pumilus* B3 as compared to DnaK of *E. coli* and *P. thermotolerans* (Fig. 3). Alignment of DnaK of *B. pumilus* B3 with the closest match-DnaK of *B. subtilis* revealed an insertion of two amino acids Glutamine and Glutamic acid in the α-helical domain. Alignment also revealed a total of 36 substitutions, out of which 18 were in the NH_2-terminal ATP binding domain, 2 in the substrate binding domain and 16 in the α-helical domain (Fig. 4). Nucleotide binding and ATP hydrolysis activities of ATP binding domain are important steps in the chaperonic activity of DnaK (Liang et al., 2009). In vitro studies have shown that DnaK proteins bind both denatured proteins and some short peptides, and release these substrates in response to the addition of ATP (Flynn et al., 1989; Gragerov et al., 1994). In this context, the 18 amino acid substitution in the ATP binding domain of DnaK of *B. pumilus* from its mesophilic counterpart, DnaK of *B. subtilis* can lead to a different secondary structure that enhances the chaperonic activity of DnaK of *B. pumilus*.

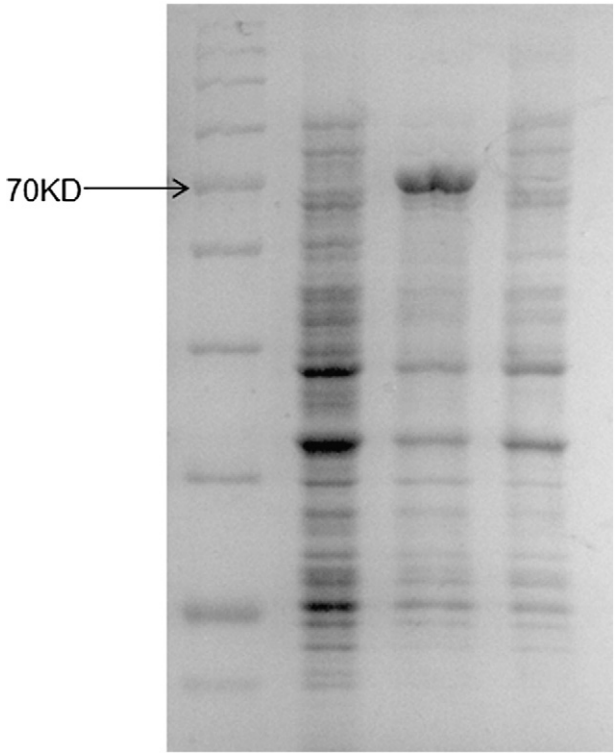

Fig. 5. SDS-PAGE of crude cell extracts of transformants. Lane 1 — Protein molecular mass marker, lane 2 — *E. coli* transformant (uninduced), lane 3 — *E. coli* transformant induced (cell lysates collected 1 h after induction), lane 4 — *E. coli* transformant induced (cell lysates collected immediately after induction).

3.3. Expression of B3 dnaK

For the expression of *dnaK* of *B. pumilus* B3, the *E. coli* BL21 (DE3) harboring pET29-BpdnaK was grown in liquid broth up to an $OD_{600\,nm}$ of 0.6. Once the required growth was achieved, the expression of recombinant protein was induced with IPTG at a final concentration of 0.2 mM. Cells were harvested 1 h after induction and SDS-PAGE of crude cell extracts of IPTG induced *E. coli* showed one predominant band of approximately 70 kDa. The same predominant band was not seen with crude cell extracts of *E. coli* BL21 (DE3) transformed with pET 29 a(+) plasmid backbone and *E. coli* BL21 (DE3) transformed

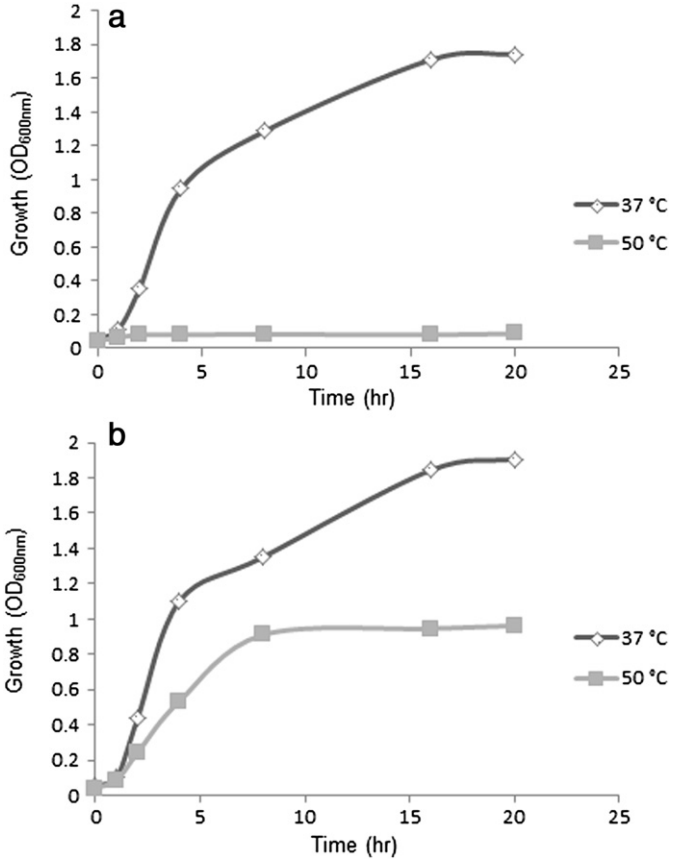

Fig. 6. High temperature/thermal stress tolerance of *E. coli* cells carrying *Bacillus pumilus dnaK*. a) The *E. coli* cells with pET 29(a) plasmid and b) with pET29-BpdnaK plasmid; cultured in LB medium at 37 °C and 50 °C. Cell growth was monitored by measuring the OD at 600 nm.

with pET29-BpdnaK plasmid but without induction (Fig. 5). MALDI-TOF analysis of the 70 kDa protein revealed matches with well described homologues of DnaK of bacteria. Hence, *dnaK* of *B. pumilus* B3 was successfully expressed in *E. coli* BL21 (DE3).

3.4. Enhanced tolerance of E. coli BL21 (DE3) carrying B3 dnaK to heat stress

The ability of the transformant with pET29-BpdnaK to grow at temperature 50 °C was studied by growing the transformants which were under IPTG induction for 12 h. Transformant with pET 29(a) plasmid alone was included in the study as control. Cells were inoculated with $OD_{600\ nm}$ equivalent to 0.6. The growth curves of *E. coli* cells with the pET29-BpdnaK and pET 29(a) grown at incubation temperatures of 37 °C and 50 °C are shown in Fig. 6a and b. At 37 °C, there was no difference between the growth of transformant with pET29-BpdnaK and transformant with pET 29(a) plasmid. At 50 °C the transformant with pET29-BpdnaK showed less growth with respect to growth at 37 °C, however much better cell growth was observed in the transformant with pET 29-BpdnaK as compared to transformant with pET 29(a) plasmid. A similar experiment at 60 °C did not show any growth even up to 16 h. But the transformants with pET29-BpdnaK when shifted to 37 °C started showing growth which was not observed in case of pET 29(a) plasmid containing transformant. This suggests that *dnaK* of *B. pumilus* B3 imparts thermal stress tolerance in *E. coli*. *dnaK* from various organisms have been shown to play a role in thermoregulation and enhance thermotolerance in *A. thaliana* (Montero-Barrientos et al., 2010), tobacco (Ono et al., 2001) and Rice (Uchida et al., 2008). However the cellular mechanism of DnaK function under stress conditions are not fully understood (Wang et al., 2004).

Enhancement of thermotolerance in the *E. coli* cells expressing *dnaK* gene in this study provides experimental evidence of the protective function of DnaK against protein denaturation, since protein denaturation occurs at higher temperature (Liang et al., 2009). It is well known that each class of Hsps has its own function in stress response, but the co-operation between different Hsps appears to be a central principle of the integrated Hsp machinery. We are still far from understanding the co-operation of different Hsp machineries operating in the stressed cell (Wang et al., 2004). Although the specific mechanism of action of DnaK is not known from previous studies (Ono et al., 2001; Uchida et al., 2008; Montero-Barrientos et al., 2010), it can be suggested that the gene *dnaK* plays an essential role in thermoregulation and could be used in the production of transgenics for abiotic stress tolerance. DnaK is known to bring about enhancement in the recombinant over-expression of particular protein of interest and have a potential role in biotechnology to enhance the production of high value recombinant proteins in *E. coli* (Schlicker et al., 2002). In depth analysis of co-operation of DnaK with other Hsps/stress induced genes in the promising bacterium is required, before exploring its possible role in developing abiotic stress tolerant crop plants.

Acknowledgments

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