गर्म पानी के झरनों में जीवाणु विविधता एवं उच्चताप सहनशीलता वाले जीन का खनन

Bacterial diversity of thermal springs and mining of gene(s) for high temperature tolerance

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Bacterial diversity of thermal springs and mining of gene(s) for high temperature tolerance

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CERTIFICATE

This is to certify that the thesis entitled **“Bacterial diversity of thermal springs and mining of gene(s) for high temperature tolerance”** submitted to the faculty of the Post-Graduate School, Indian Agricultural Research Institute, New Delhi in partial fulfilment of **DOCTOR OF PHILOSOPHY** in Microbiology, embodies the results of *bonafide* research work carried out by **Mr. Kumar M** under my guidance and supervision, and that no part of this thesis has been submitted for any other degree or diploma.

The assistance and help availed during the course of investigation as well as source of information have been duly acknowledged by him.

Date:
Place: New Delhi

(Anil Kumar Saxena)
Chairman

Affectionately Dedicated to
My Parents and Teachers

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Introduction

1. INTRODUCTION

Microorganisms are fundamentally important to the history and function of life on earth. They carry out a diverse array of metabolic activities, several of which were instrumental in creating conditions for the evolution of life and its sustenance thereafter. Through their colonization of diverse and extreme environments, involvement in the geochemical cycling of matter, and interactions among themselves and with all other organisms, microbes define the limits of the biosphere and perform functions essential for ecosystem development and health (Dunlap 2001). Microorganisms have made the environment conducive for other life forms on earth and it is not unworthy to say, without microorganisms life on earth would cease to sustain. A significant development in microbial ecology and evolution has been the realization that microbial life, primarily prokaryotic life, is extremely hardy and indeed thrive in the environments previously thought uninhabitable on Earth. Microbial life is found in every conceivable ecological niche: from tropics to the poles, from underground mines to stratosphere and mountain ranges, from deserts to Dead sea and hot springs (Cangenella and Wiegel 2011). The endless combination of terrestrial, aquatic and other unusual habitats and enormous potential of microbes inhabiting there provides us with tremendous resource at hand for the discovery of commercially valued products. Man has long exploited this metabolic wealth to his benefits in food, health and industrial applications (Hunter-Cevera 1998).

The conservation and use of microbial genetic resources is an important subject; a subject about which the United Nations Environment Programme (UNEP) has long been concerned, “the conservation of all kinds of living organisms comprising the microbial gene pool, with the aim of improving human environment and welfare through prudent utilization of the full potential of living resources”. Twenty years after the UN Conference on the Human Environment in Stockholm it is timely now to take stock on where we stand. This is especially relevant in the light of the recently concluded Convention on Biological Diversity and UNCED's increased demands on the role of biodiversity in improving human welfare and sustainable development, to exchange views and decide and recommend appropriate strategies which could assist us in nudging the world towards its current goal of sustainable development. Both the Convention and UNCED have reconfirmed the concern of world governments to protect the earth's biological diversity.

The success and potential of biotechnology relies on the diversity of microorganisms and the biodiversity of the molecules they produce as a result of primary and secondary metabolism and on the conservation of the genetic resources they provide. However, 20 years after Stockholm, we can say that the world community has been concerned for some time about the conservation of diversity in higher plants and vertebrates. Equal attention was not given to microorganisms irrespective of their importance. There is a need to persuade policy-makers and researchers to be more concerned than they currently are about the conservation of diversity in microorganisms.

Microorganisms from extreme habitats like thermal springs constitute an important component of largely unexplored biological potential. Thermal springs are manifestation of geological activity and represents aquatic microcosms that are produced by the emergence of geothermally heated ground water from earth’s crust. They represent extreme niches whose pristine quality is maintained over a period of time. Many terrestrial hot springs exist on earth which represent hot spots for unusual forms of life, genes and metabolites. In the Indian subcontinent, thermal springs roughly numbers to 400 in the seven major geothermal provinces (Cinti et al. 2009). Vast information has been generated on geochemical characteristics of various Indian hot springs (Chandrasekharam et al. 2005; Cinti et al. 2009). However, only a countable number of these hot springs have been explored microbiologically (Ghosh et al. 2003; Kumar et al. 2004; Dwivedi et al. 2012). These investigations lack entirety as no comparisons were made between hot springs and mostly focus was on a particular group of microorganisms. The vast bacterial diversity expected in these hot springs provides us with a broad genetic base to harvest novel ideas (Kuddus and Ramtekke 2012).

Culturing techniques for analyzing microbial diversity, however, have several limitations; they outweigh culture independent analyses in the advantages offered by the generation of valuable germplasm and immense possibilities for exploring them in due course for potential biotechnological applications (Acharya and Chaudhary 2012). Hence, a comprehensive approach is needed to bring bacteria from hot springs into culture, analyze their diversity and explore them for possible utilization. The current approach used for defining bacterial species is based on phenotypic and genomic properties. Traditional and novel DNA-based molecular methods are improving our knowledge of bacterial diversity in nature. Advances in molecular biology have been important for studies of diversity, considerably improving our knowledge of morphological, physiological, and ecological features of bacterial taxa. Woese and other researchers have developed a novel tool to identify bacteria and evaluate phylogenetic relationships among bacteria in the early 1980s. Among more than 20 candidate genes available for phylogenetic analysis, 16S rRNA was found to be the best evolutionary chronometer. PCR-based methods investigating 16S rRNA gene sequences, and other approaches, such as metagenomics, have been used to study the physiology and diversity of bacteria and to identify novel genes with potential pharmaceutical and other biotechnological applications.

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, such as soil moisture, high temperatures, soil salinity/alkalinity, low pH and metal toxicity (Grover et al., 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 propagation and controlled atmosphere are not economically viable options. A more practical solution is to alter the plant genetic machinery such that the plants can grow and reproduce normally under increased ambient temperature for which the availability of diverse genetic material is a pre-requisite. Allele mining is a research area aimed at identifying sequence based variation of relevant traits within the genetic resource collections. Identification and access to allelic variation that affects the phenotype and genotype of an organism is of the utmost importance for the utilization of genetic resources. Allele mining for abiotic stress tolerance in general and thermotolerance in particular is of major importance in the light of decreasing crop yield due to climate change. Identifying naturally occurring genetic variants that regulate gene expression is an important route for connecting genotype to phenotype based on changes in gene expression rather than changes in the encoded protein.

Variations in gene expression are manifested as an increase or decrease in a set of proteins. Bacteria use adaptive networks by triggering programs of specific gene expression to survive under extreme conditions. Heat shock proteins (Hsps) are the class of proteins that are induced in cells experiencing abiotic stress, which were first discovered in *Drosophila* (Ritossa 1962). The archetypal stress response is a sudden rise in the outside temperature, called heat shock. The five major classes of heat shock proteins; 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). Proteomic approaches have been used to identify stress related proteins in different studies. Identification of new biomolecules and relevant genes in these bacteria from hot springs can be a boon to Indian agriculture as these genes could be utilized to develop transgenics tolerant to abiotic stress.

Based on the available information, the present study was designed with the following objectives:

1. Characterization of bacterial diversity from thermal springs based on 16S rRNA gene sequences.
2. Understanding the mechanism(s) of tolerance to high temperature in selected bacteria in relation to presence of HSP.
3. Identification of gene(s) responsible for imparting tolerance to high temperature.

Background

2. BACKGROUND

2.1 Terminologies

2.1.1 Bacterial diversity

It is defined as the range of different kinds of bacteria. Characteristics such as cellular metabolism, physiology, morphology, ecological distribution and activities, distinct genomic structure, expression and evolution distinguish different kinds of bacteria (Dunlap 2001). For centuries, the focus of biologists and ecologists was on animal and plant diversity studies, at continental scales. Until recently, similar studies were impossible for microorganisms (Fierer and Jackson 2006); the advent of new tools in molecular biology permits the study of microbial diversity in general and bacterial diversity in particular rapidly.

2.1.2 Thermal springs

Thermal springs or hot springs are manifestation of geological activity and represent aquatic bodies that are produced by the emergence of geothermally heated groundwater from the earth’s crust (Kumar et al. 2004). They are of considerable significance to microbiologists worldwide as they are analogous to primitive earth. Some of the thermal springs that are best known and most investigated in terms of their biota are those in the Yellowstone National Park in USA, Kamchtaka peninsula in Russia, Iceland hot springs and Naples area in Italy (Hobel 2004).

2.1.3 16S ribosomal RNA gene

16S ribosomal RNA is a component of 30S ribosomal subunit of prokaryotes and the gene coding is called 16S ribosomal RNA gene or simply 16S rRNA gene or 16S rDNA. 16S rRNA gene and 16S rDNA are both used synonymously. This small subunit rRNA gene is the recognized gold standard for estimating phylogenetic diversity among bacteria and archaea (Vos et al 2012).

2.1.4 Heat shock proteins

Organisms respond to heat stress and cytotoxic stress by inducing a set of proteins called heat shock proteins (HSPs), many of which function as molecular chaperones. The response is highly conserved from archaebacteria to eubacteria and from animals to plants (Lindquist 1988). Heat shock proteins can be classified into three categories according to their molecular weight: (I) high-molecular weight (69–120 kDa), (II) medium-molecular weight (39–68 kDa), and (III) low molecular weight (below 38 kDA) (Liu et al. 2007).

2.1.5 Gene or Allele Mining

Allele mining is defined as the identification and isolation of novel allelic variants associated with the phenotype of interest that exists within large germplasm collections. Identification of allelic variation and access to the same is of utmost importance for connecting the traits with genotype (Varshney et al. 2011).

2.2 Extremophiles

Humans and a majority of higher organisms thrive in the environments where the pH is near neutral, temperatures between 4 °C to 40 °C, air pressure 1 atm and adequate levels of available water, nutrients and salts. Environments possessing these conditions are termed moderate environments. Any environment that deviates from these conditions and thereby are not compatible for growth of humans and most of their commensals, are designated as extreme environments (Satyanarayana et al. 2005). The organisms thriving in such extreme environments and/or require extreme conditions are called extremophiles. The anthropocentric term, extremophiles was first used by MacElroy (1974) who described these organisms as comprising, mainly prokaryotes. Some of the important extremophiles and their typical habitats are tabulated in Table 2.1.

Intellectual curiosities aside, interest in studying the microbiology of extreme environments arise from the potential industrial applications. The various industrial applications of extremophiles are tabulated in Table 2.2.

2.3 Thermal springs in India

Indian subcontinent is home to several thermal springs, a rough estimate numbers to 400 thermal springs that are part of the following seven major geothermal provinces 1) the tectonic belts of Himalaya, 2) the Sohna fault zone, 3) Cambay, 4) the Son–Narmada–Tapi lineament (namely, SONATA), 5) the West Coast fault zone, 6) Godavari and 7) Mahanadi (Cinti et al. 2009). The major ones gathering attention are in Yamunotri, Gangnani, Kedarnath and Rudraprayag in Uttrakhand, Bakreshwar and Bhuri in West Bengal, Tantloi in Jharkand, Gangtok in Sikkim, Manikaran and Vashist in Himachal Pradesh, Ganeshpuri and Vajreshwari in Maharastra, Puttur in Karnataka and Chummathang in Leh. Geochemical studies on these thermal springs showed that these waters are generally associated with tectonic belts, midcontinental rifts, cretaceous–tertiary volcanism and regional fault zones (Gupta 1996; Alam et al. 2004; Walia et al. 2005). Although vast information has been generated on geochemical characteristics of various Indian hot springs, only a countable number of these hot springs have been explored microbiologically (Jha 1992; Nawani and Kaur 2000; Ghosh et al. 2003; Kumar et al. 2004; Dwivedi et al. 2012). It will be challenging to bring the microbes from these hot springs into culture avoiding the limitations of traditional artificial media and laboratory conditions (Ranjard et al. 2000), analyse their diversity and explore them for useful traits.

2.4 Access to microbial diversity in environments

Life on earth depends on microbial processes. The role of life in forming the physiochemical environments of our planet and its importance in maintaining the environment in its current state were emphasized by Gaia hypothesis (Gaia means Earth goddess) formulated by

Table 2.1 Categories of extremophiles and their growth habitats

Extremophiles	Conditions			Common habitats	Examples
	Minimal	**Optimal**	**Maximal**		
I Temperature extremes					
a) Themophiles	45 °C	60 °C	70 °C	Hot springs, Hydrothermal vents	*Thermus aquaticus* *Clostridium thermocellum*
b) Hyperthermophiles	60 °C	80 °C	> 100 °C	-do-	*Pyrolobus furiosus*, *Pyrococcus*, *Thermococcus*
c) Psychrophiles	ambiguous	> 15 °C	20 °C	Permafrost, Arctic and Antarctic ice	*Frigoribacterium*, *Maribacter antarcticus*
II pH extremes					
a) Acidophiles	pH close to 0	pH 2-4	pH 5	Coal mines, Soils of volcanic origin	*Acidithiobacillus thiooxidans*, *Thiobacillus prosperus*
b) Alkaliphiles	Near neutral pH	pH > 9	ambiguous	Soda lakes, Tanneries, Paper mills	*Ectothiorhodospira*, *Halomonadaceae*
III Salinity extremes					
a) Slightly halophiles	-	2-5%	-	Soda lakes	*Halobacillus*
b) Moderate halophiles		5-20%		-do-	*Haloanaerobium*, *Haloanaerobacter*, and *Haloincola*
c) Extreme halophiles		20-30%		-do-	*Halorhodospira halophila*, *Halorhodospira halochloris*

Cangenella and Wiegel (2011)

Table 2.2 Applications of major groups of extremophiles

Extremophiles	**Enzymes/organic compounds**	**Organisms**	**Application**	**Reference**
Thermophiles	Protease	*Bacillus stearothermophilus*	Amino acid production from various proteinaceous material, baking, brewing and detergents	Synowiecki 2010
	α-Amylase	*Pyrococcus woessi*	Starch conversions and liquefaction	Antranikian 1991
	Xylanase	*Bacillus pumilus*	Paper bleaching	Ashapoorna and Prema 2007
	Cellulase	*Geobacillus* sp.	Production of ethanol from agricultural residues	Zambare et al. 2011
	Thermostable DNA polymerase Taq	*Thermus aquaticus*	Polymerase chain reactions	Chien et al. 1976
Psychrophiles	Metalloprotease	*Sphingomonas paucimobilis*	Food and detergent	Cavicchioli et al. 2002
	Cellulase	*Fibrobacter succinogens*	Animal feeds and textiles	Cavicchioli et al. 2002
	Lipases	*Psychrobacter okhotskensis*	Additive in detergents	Yumoto et al. 2003
Halophiles	α-Amylase	*Micrococcus halobius*	Starch conversions and liquefaction	Onishi and Kamekura 1972
	Compatible solutes	*Halobacillus*, *Rhodothermus* and various other halophiles and Alkaliphiles	Pharmaceuticals	Lentzen and Schwarz, 2006

Lovelock. The hypothesis states that Earth acts like a superorganism and through the biochemical activities of its biota, its physicochemical properties are self-regulated, so that they are maintained in a favourable range for life (Lovelock 1972). If Lovelock's hypothesis is correct, Gaia exercises her powers principally through microbial processes (Atlas and Bartha 1998). Microbes through their colonization in various environments, their geochemical cycling of matter and their biological interactions among themselves and with all other organisms define the limits of biosphere and perform essential functions for ecosystem sustenance (Dunlap 2001). Microbes grow across a broad range of temperarture, pH, salinity and oxygen, and play an essential role in our routine life including maintaining the biosphere and improving our lifestyles (Hunter-Cevera 1998). Changes in either microbial community composition or the activity of microbial communities affect the ecosystem function in the long-term (Perry et al. 1989). Hence, microbial diversity analysis is therefore important in order to (i) increase the knowledge of the diversity of genetic resources in microbial communities, (ii) understand patterns in the relative distribution of microorganisms, (iii) increase knowledge base on the functional role of the diversity, and therefore, (iv) understand the regulation of biodiversity, as well as assessing to what extent the function and sustainability of an ecosystem depend on maintaining a certain level of genetic diversity (Ovreas 2000). The various methods to analyse bacterial diversity in environments are reviewed here.

2.4.1 Culture dependant methods of diversity analysis

Pure culture techniques perfected by Robert Koch and the invention of Petridish by Richard Petri in the late nineteenth century marked the new beginning for microbiology by supplying tools for the development of fields like microbial taxonomy, soil microbiology, industrial and food microbiology, microbial genetics and other related disciplines.

2.4.1.1 Dilution plating and culturing methods

Analysis of microbial communities in environments relied traditionally on culturing microbes on various culture media under different incubation conditions to get maximum number of recoverable microbes in any particular environment (Hill et al. 2000). Many different phenotypic and genotypic features have been used to analyse the diversity among the microorganisms isolated from natural environments (Vandamme et al. 1996). The advantages of dilution plating and culturing methods lay in its inexpensive nature and rapidity. The major disadvantages of this technique are; i) uncultured microorganisms are not detected ii) bias towards fast growing microbes (Kirk et al. 2004).

Despite these disadvantages culturing methods have been used to analyse diversity from various environments like soil, freshwater, thermal environments and others.

Kumar et al. (2004) studied the microbial diversity of soil in Uttarkhand by culture based method, employing different media and incubation conditions. They reported the presence of diverse groups of bacteria from all physiological groups like aerobic and anaerobic, thermotolerant, thermophiles, hyperthermophiles, halophiles, alkaliphiles and acidophiles. Diversity of culturable bacteria was analysed by Kohler et al. (2011) in the soils of south western Pennsylvania. They reported the presence of over 40 genera spanning 30 families. Phyla Firmicutes and Proteobacteria dominated the culturable bacteria from the soil samples.

Culturable bacterial diversity analysis by Vaz-Moreira et al. (2011) in fresh water samples from Portugal revealed the presence of different genera of bacteria like *Ralstonia*, *Chryseobacterium*, *Chitinophaga*, *Bacillus*, *Exiguobacterium*, *Delfia* and *Lactococcus*. Cerritos et al. (2011) analysed the diversity of culturable thermotolerant bacteria in the extreme oligotrophic pools of Mexico and found a diverse group of bacteria belonging to different phyla like Firmicutes, Actinobacteria and Proteobacteria.

Diversity analysis of culturable bacteria from a permafrost sample in Canada resulted in the isolation and cultivation of bacteria belonging to different genera like *Bacillus*, *Sporosarcina*, *Paenibacillus*, *Arthrobacter*, *Micrococcus*, *Kocuria*, *Rhodococcus* and *Pseudomonas*. Most of the isolates were psychrotolerant and only one true psychrophile could be brought in to culture (Steven et al. 2007). Bacterial diversity analyses of soil in the vicinity of Pindari glacier (India) showed the presence of diverse groups of organisms belonging to the genera *Arthrobacter*, *Bacillus, Sporosarcina*, *Rhodococcus*, *Lysinibacillus*, *Pseudomonas* and *Viridibacillus* (Shivaji et al. 2011).

Studies on bacterial diversity of Jordanian hot springs employing culture dependant methods by Malkawi and Al-Omari (2010) resulted in isolation of 132 thermophilic isolates which were able to grow up-to 75 °C. Majority of the isolates belonged to the genus *Bacillus*. Adiguzel et al. (2009) studied the diversity of thermophilic bacilli isolated from four hot springs of Turkey and reported the presence of genera like *Geobacillus*, *Bacillus*, *Anoxybacillus* and *Brevibacillus*.

2.4.1.2 Community level physiological profiles

Community level physiological profiling is one of the widely used culture-dependant methods for analyzing microbial communities in natural environments. Traditional methods of bacterial taxonomy in which bacterial species are identified based on carbon source utilization, is the basis for community level physiological profiling (Hill et al. 2000). A redox based technique for testing the utilization of different carbon sources by bacterial isolates was developed by Biolog, Inc in the early 90’s. Color produced from the reduction of tetrazolium violet is used as an indicator of respiration of sole carbon sources. Commercially available microplates allow for simultaneous testing of 95 separate carbon sources. Direct incubation of whole environmental samples in Biolog plates, therefore, may produce patterns of metabolic response suitable for the rapid classification of heterotrophic microbial communities (Garland and Mills 1991). The challenge in using this technique lies in the analyses of vast data generated. Multivariate statistical analyses are generally used for interpretation of microbial community level studies using this technique (Hackett and Griffiths 1997). The major advantages of this technique are that, it is rapid, reproducible and relatively inexpensive besides providing options for taxon specific plates. The major disadvantages of this technique are that, it represents only culturable fraction of the community and within the culturable community only organisms capable of utilizing available carbon sources and fast growers are represented (Kirk et al. 2004).

Community level physiological profiling provides useful information for the assessment of microbial diversity in natural environments, but they too suffer from the same bias problems encountered with culture plating methods, making data interpretation problematic. Although culture based techniques are not ideal for the studies of the composition of natural microbial communities when used alone, they provide useful regarding the growth habit, development and potential function of microorganisms in natural habitats (Liesack et al. 1997).

2.4.1.3 Use of 16S rDNA in culture dependent techniques

In the early 1980s, a new tool was developed in the laboratories of Woese and other researchers for identifying bacteria and evaluating the phylogenetic relationships among bacteria (Claridge 2004). Results revealed that all life forms could be identified by comparing a stable part of the genetic code. Candidate genes used for these evolutionary studies are as many as 20 (Nelson et al. 1999). A few of them are 5S rRNA (Sun and Caetano-Anolles 2009), 16S rRNA (Janda and Abbot 2007), 23S rRNA (Hunt et al. 2006) and 16-23S rRNA internal transcribed spacer (ITS) region (Boyer et al. 2001). 16S rRNA gene is considered to be the best evolutionary chronometer (Patel 2001). Identification of bacteria based on 16S rRNA gene sequencing involves extraction of DNA from bacteria and PCR amplification of 16S rRNA genes using universal primers. The different universal primers used for amplification of 16S rRNA gene are tabulated in Table 2.3. Different forward and reverse primers in the list can be used in combination to get product of appropriate size for specific purpose. Once the 16S rRNA gene is amplified, it can be sequenced and analysed by comparing with the sequence in database for identification and phylogenetic studies.

In the early 1990s the availability of DNA sequencers in terms of cost, methodologies, and technology improved dramatically, such that many centres can now afford such instrumentation. Hence sequencing of amplified 16S rRNA gene for describing

Table 2.3 Universal primers used for amplication of bacterial 16S rRNA gene

Primer	**Sequence (5’-3’)**	**Reference**
8F	AGAGTTTGATCCTGGCTCAG	Turner et al. 1999
27F	AGAGTTTGATCMTGGCTCAG	Lane 1991
CC [F]	CCAGACTCCTACGGGAGGCAGC	Rudi et al. 1997
357F	CTCCTACGGGAGGCAGCAG	Turner et al. 1999
515F	GTGCCAGCMGCCGCGGTAA	Turner et al. 1999
533F	GTGCCAGCAGCCGCGGTAA	Weisburg et al. 1991
16S.1100.F16	CAACGAGCGCAACCCT	Turner et al. 1999
1237F	GGGCTACACACGYGCWAC	Turner et al. 1999
pA	AGAGTTTGATCCTGGCTCAG	Edwards et al. 1989
pH	AAGGAG GTGATCCAGCCGCA	Edwards et al. 1989
519R	GWATTACCGCGGCKGCTG	Turner et al. 1999
CD [R]	CTTGTGCGGGCCCCCGTCAATTC	Rudi et al. 1997
907R	CCGTCAATTCMTTTRAGTTT	Lane 1991
1100R	AGGGTTGCGCTCGTTG	Turner et al. 1999
1391R	GACGGGCGGTGTGTRCA	Turner et al. 1999
1492R	GGTTACCTTGTTACGACTT	Turner et al. 1999

new species has become a common practice worldwide. (Janda and Abbot 2007). It has been demonstrated that 16S rRNA gene sequence data on an individual strain with a nearest neighbour exhibiting a similarity score of < 97% represents a new species. A further improvement in 16S rRNA gene based diversity study is amplified ribosomal DNA restriction analysis which circumvents sequencing for identification upto genus level (Divya et al. 2010).

2.4.1.4 Amplified Ribosomal DNA Restriction Analysis (ARDRA)

ARDRA is an extension of RFLP (Restriction Fragment Length Polymorphism) for analysing sequence diversity in the gene encoding 16S rRNA. The technique involves PCR amplification of 16S rRNA gene using universal primers followed by digestion using a restriction enzyme. The pattern obtained is said to be representative of species analysed (Vaneechoutte et al. 1993). Originally developed to identify *Mycobacterium*, this technique is now used extensively in diversity analyses by both culture dependent and culture independent techniques. Several groups of scientists reported ARDRA to be an efficient technique for discriminating microbes at the genus level (Steingrube et al. 1997; Laurent et al. 1999; Harvey et al. 2001). Yadav et al. (2010) used ARDRA to cluster plant growth promoting bacilli into different groups to avoid any redundancy while sequencing the 16S rRNA genes. They were able to group 49 isolates into 13 ARDRA clusters employing three different restriction enzymes. ARDRA has also been used to screen clone libraries in culture independent studies for grouping the clones into phylogenetic clusters (Sklarz et al. 2009). Apart from contributing to the exploration of microbial diversity, ARDRA also minimizes experimental costs by clustering different isolates into phylotypes and avoiding redundancy while sequencing (Sahay 2011).

2.4.1.5 Housekeeping genes

Conserved genes other than 16S rRNA used in bacterial diversity and phylogeny studies are RNA polymerase β subunit (*rpoB*), gyrase β subunit (*gyrB*) and recombinase (*recA*). All these housekeeping genes are generally used in strain typing within a species and genus. *rpoB* has been used in the phylogenetic studies on *Anoxybacillus* (Inan et al. 2011) and *gyrB* has been used in the phylogenetic studies of *Geobacillus* (Tourova et al. 2010). Costechareyre et al. (2010) have explored diversity of *recA* gene for rapid typing of *Agrobacterium* strains.

2.4.2 Culture independent methods for diversity analysis

The inherent limitations of culture based techniques coupled with the scientific advancements led the ecologist to shift their focus on culture independent techniques for analysing diversity in natural environments. Understanding the microbial communities based on culture independent techniques rely upon extraction, quantification and identification of molecules specific to certain microorganisms or microbial groups. Molecules may be either nucleic acids or phospholipid fatty acids.

2.4.2.1 Phopholipid fatty acid analysis (PLFA)

Phospholipid fatty acids are components of the membranes of all organisms and each species has a characteristic fatty acid pattern. In microorganisms, phospholipids are found exclusively in cell membranes and not in other parts of the cell as storage products. Following cell death, cell membranes are rapidly degraded and the component phospholipid fatty acids are rapidly metabolized. So phospholipids can serve as important indicators of active microbial biomass as opposed to non-living microbial biomass (Marschner 2007). The analyses of phospholipid fatty acids from natural environments have been used in studies on microbial diversity by various researchers (Jiang et al. 2007; Bell et al. 2009; Osburn et al. 2011). The advantages of PLFA are; i) Extraction procedure is relatively simple and quick, ii) Phospholipids are rapidly dephosphorylated and fatty acids decomposed in soil; therefore PLFAs are considered to be derived mainly from living organisms. iii) Signature fatty acids and their ratios can be used as indicators for biomass of certain microbial groups. The sum of microbial signature fatty acids in a given sample can also be used as a measure of microbial biomass, iv) Peak patterns represent community composition and can be compared using multivariate analyses. With some multivariate analysis methods, such as canonical correspondence analysis or multidimensional scaling, it is possible to relate community composition to environmental factors, v) Certain fatty acids can be used to assess the physiological status of microbial communities. Major limitations of this technique are i) Only a small number of fatty acids are truly characteristic for certain groups, many are ubiquitous and may be derived from other soil organisms. Hence, the background of unspecific fatty acids may mask differences in microbial community structure. ii) FAME patterns may also contain fatty acids from dead microorganisms/other plant residues in natural environments (Marschner 2007).

2.4.2.2 Nucleic acid techniques

Among the nucleic acid techniques used for analyses of microbial community composition and diversity in complex habitats, the most useful is the determination of the sequences of 16S ribosomal RNA (rRNA) genes (i.e. encoded by rDNA) in prokaryotes and 5S or 18S rRNA genes in eukaryotes (Hill et al. 2000). These rDNA molecules are widely used in such studies for the following reasons. i) They are universal to all the three domains of life; Bacteria, Archaea and Eukarya, ii) they have both highly conserved regions and highly variable regions. Because of these differential rates of sequence evolution, phylogenetic relationships at several hierarchical levels can be measured from comparative sequence analyses, iii) they are relatively large in size and have secondary structure which further enhances the phylogenetic information iv) they are easy to amplify using polymerase chain reaction (Patel 2001).

The greatest advantage of analysing small subunit (SSU) rDNA is that the microorganisms can be characterized without culturing. Such a culture independent approach based on SSU rDNA analysis is used to study microbial communities from various environments like hot springs (Hugenholtz et al. 1998; Ferris et al. 2001; Ghosh et al. 2003; Tomova et al. 2010; Mackenzie et al 2013), soil (Borneman and Triplett 1997; Faoro et al. 2010) cold environments (Shivaji et al. 2011) and salt lakes (Pagaling et al. 2009). All these studies employed extraction of DNA from natural samples followed by PCR amplification. The PCR products are analysed primarily by 1) clone library method, 2) genetic fingerprinting, 3) DNA microarrays, or by a combination of these techniques.

2.4.2.2.1 Clone Library Method

The most widely used method to analyze PCR products amplified from an environmental sample is to clone and then sequence the individual gene fragments. The obtained sequences are compared to known sequences in a database such as GenBank and Ribosomal Database Project. Typically, cloned sequences are assigned to phylum, class, order, family, subfamily, or species at sequence similarity cut-off values of 80, 85, 90, 92, 94, or 97%, respectively (DeSantis et al. 2007). While clone libraries of 16S rRNA genes permit an initial survey of diversity and identify novel taxa, studies have shown that environmental samples may require over 40,000 clones to document 50% of the richness (Dunbar et al. 2002). However, typical clone libraries of 16S rRNA genes contain fewer than 1,000 sequences and therefore reveal only a small portion of the microbial diversity present in a sample. Despite its limitations, clone libraries are still considered the “gold standard” for preliminary microbial diversity surveys (DeSantis et al. 2007). With the advent of newer and inexpensive sequencing methods, great progress is expected in this method of microbial diversity analysis.

2.4.2.2.2 Genetic Fingerprinting Techniques

In the recent years, techniques complementary to cloning procedures called denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) are increasingly been used to separate amplicons using electrophorectic technique (Ferris and Ward 1997; Muyzer and Smalla 1998). This technique allows separation of mixtures of PCR products that are of the same length but differ only in sequence. The separation power of this technique rests with the melting behaviour of the double stranded DNA molecule. As DNA molecules are electrophoresed in an increasing gradient of denaturant or temperature, it remains double-stranded until it reaches the denaturant concentration or temperature that melts the double-stranded molecule. As the DNA melts, it branches, thus reducing its mobility in the gel. Since the melting behaviour is largely dictated by the nucleotide sequence, the separation resolves individual bands, each corresponding to a unique sequence. Theoretically, any SSU rRNA gene found in the mixed template DNA extracted from soils could be specifically amplified and resolved on a DGGE gel. Once the amplicons are separated, they can then be sequenced and analysed (Hill et al. 2000). Diversity studies based on denaturing gradient gel electrophoresis (DGGE) of 16S rDNA have been carried out in various environments like salt lakes (Rees et al. 2004) and hydrothermal springs (Sahm et al. 2013).

2.4.2.2.3 DNA Microarrays

DNA microarrays have been used primarily to provide a high-throughput and comprehensive view of microbial communities in environmental samples. The PCR products amplified from total environmental DNA is directly hybridized to known molecular probes, which are attached on the microarrays (Gentry et al. 2006). After the fluorescently labelled PCR amplicons are hybridized to the probes, positive signals are scored by the use of confocal laser scanning microscopy. The microarray technique allows samples to be rapidly evaluated with replication, which is a significant advantage in microbial community analyses. In general, the hybridization signal intensity on microarrays is directly proportional to the abundance of the target organism. Cross hybridization is a major limitation of microarray technology, particularly when dealing with environmental samples. In addition, the microarray is not useful in identifying and detecting novel prokaryotic taxa. The ecological importance of a genus could be completely ignored if the genus does not have a corresponding probe on the microarray. DNA microarrays used in microbial ecology could be classified into two major categories depending on the probes: 1) 16S rRNA gene microarrays and 2) functional gene arrays (FGA).

2.4.2.2.4 Whole Community Analysis Approaches

Whole-genome molecular techniques offer a more comprehensive view of genetic diversity compared to PCR-based molecular approaches that target only a single or few genes. These techniques attempt to analyze all the genetic information present in total DNA extracted from an environmental sample or pure culture.

Exploring microbial systems through whole-genome analysis is a comprehensive and integrated approach to understand microbial ecology and function. Whole microbial genomes are sequenced using a shotgun cloning method that involves 1) extraction of DNA from pure cultures, 2) random fragmentation of obtained genomic DNA into small fragments of ~2 kb, 3) ligation and cloning of DNA fragments into plasmid vectors, and 4) bidirectional sequencing of DNA fragments.

Once the sequences are obtained, they are aligned and assembled into finished sequences using specialized computer programs such as MEGAN (MEtaGenome ANalyzer) (Huson et al. 2007). The sequences are annotated in open reading frames (ORFs) to predict the encoded proteins (functions). Whole-genome sequencing has provided unprecedented insights into microbial processes at the molecular level and has potential applications in individual and community ecology, bioenergy production, bioremediation, human and plant health, and various industries (Ikeda et al. 2003). Recent developments in short-read sequencing techniques such as pyrosequencing have dramatically reduced the time and cost needed for whole-microbial-genome sequencing projects (Metzker 2010). The enormous amount of data gathered from genome sequencing programs is deposited in searchable databases that could be mined with various powerful bioinformatic tools available at the Integrated Microbial Genomes (IMG) Web server (Markowitz et al. 2010) for evolutionary studies, comparative genomics, and proteomics.

The usefulness of nucleic acid techniques does not come without limitations. The major limitations associated with nucleic acid techniques for microbial diversity analyses are; a) Storage of samples prior to processing can result in bias. Differing conditions while storage from natural habitats can also result in shifts in the ratio of active functional groups, b) Extraction efficiency differs among the microorganisms and the type of environments, some are easy to lyse and others are difficult to lyse and incomplete lysis of some species can result in underestimates of diversity or specific activity, c) Sequence amplification and detection depends on the primers and probes used, which show an apparent bias towards certain groups of organisms (Hill et al. 2000).

Understanding the advantages and limitations of both culture dependent and culture independent techniques provides us with an opportunity to complement culture independent techniques with culture dependent techniques, for a complete understanding of microbial diversity in any natural environments. Such complementary approach have been used by various groups of researchers to study microbial diversity in soil (Lopes et al. 2011), hot springs (Malkawi and Al-Omari 2010) and fresh water samples (Vaz-Moreira et al. 2011).

2.5 Microbial diversity in thermal environments

Strains of *Thermus aquaticus*, a non sporulating thermophile, were first isolated from thermal springs in Yellowstone National Park and California (Brock and Freeze 1969). This was immediately followed by the isolation of strains of *Sulfolobus*, a thermoacidophile with a temperature and pH optima of 70-75 °C and 2-3 respectively. *Thermus* spp. have also been isolated from various environments (Brock et al. 1972). In the last 40 years, since that magical moment, scientific community all over the world were fascinated by the diversity of microbes in thermal environments for reasons of fundamental interest, biotechnological applications and economic benefits (Charlier and Droogmans 2005). Since that breakthrough by Thomas Brock (Brock and Freeze 1969; Brock et al. 1972), this field continues to explode so rapidly that a comprehensive review on microbial diversity in thermal environments encompassing all those accomplishments is literally impossible. Some of the major investigations on microbial diversity in thermal environments are reviewed here. The review is focused mainly on prokaryotes (Bacteria and Archaea), since it is more relevant to the study and the information on eukaryotic microorganisms is relatively scarce.

Ward et al. (1994) evaluated the diversity of microbes in Octopus spring mat found in Yellowstone National Park and 21 different species were identified as being involved in the formation of Octopus spring mat. Their study was based on direct DNA extraction from spring mat and amplification of 16S rRNA gene using universal primers. Their most striking finding was that none of the rRNAs of previously cultivated species were recovered (Ward 1989).

Barns et al. (1994) reported remarkable archaeal diversity in a hot spring, Yellowstone National Park employing molecular techniques. They established that the Crenarchaeota was much more diverse than expected. The immense phylogenetic diversity among archaeal rDNA clones was unexpected during those initial stages of studies on thermal habitats. They found sequences affiliating to *Desulfurococcus*, *Pyrodictium*, *Pyrobaculum*, *Thermophilum* and *Archaeoglobus*.

Hugenholtz et al. (1998) carried out a survey on bacterial diversity in Obsidian pool, a hot spring in Yellowstone National Park. They used the small subunit rRNA gene as a candidate gene for diversity studies and found a remarkable diversity of bacteria present in the sediments. Surprisingly no archaeal sequences were encountered from clone libraries constructed using universal primers. A commonly held notion that *Archaea* dominates hydrothermal environments was thus disproved.

A study on bacterial diversity in hot springs of Bakreshwar, India was conducted using culture-independent approach. It revealed that the sediments were associated with gamma-Proteobacteria, Cyanobacteria, Green nonsulfur and low-GC Gram-positive bacteria. The presence of *Shewanella*-like thermophilic bacteria in the sediments were also reported (Ghosh et al. 2003). Meyer-Dombard et al. (2005) conducted a study on bacterial diversity as a function of pH and conductivity in thermal environments of Yellowstone National Park. They selected three sites Obsidian Pool, Sylvan Spring, and Bison Pool, all of which have similar temperature (79.9-82.6 °C), but have a variable chemistry in pH (5.5-8.1) and electrical conductivity (650-5050 µS/cm). 16S rDNA analysis revealed the presence of Aquificales in all the three pools but there was variation at the family level among the three springs which was attributed to differences in energy level of the springs, due to changes in pH and electrical conductivity.

Derekova et al. (2008) isolated 67 carbohydrate degrading bacilli from Bulgarian hot springs. Characterization of isolates employing 16S rDNA sequence analysis revealed the presence of eight species in four *Bacillus* and *Bacillus* derived genera. The genera are

Anoxybacillus, Geobacillus, Brevibacillus and *Bacillus*. Investigation of carbohydrate degraders revealed that isolates were able to degrade different carbohydrates like starch, arabinan, xylan, xanthan, glucan, cellulose, polygalacturonic acid and arabinoxylan, most of which are hemicellulose like substrates. These hot springs exhibit a preponderance of bacteria that degrade hemicelluloses. Diversity studies based on DGGE analysis of 16S rRNA on hydrolytic bacteria in hot springs of Kamchatka, Russia revealed the presence of genera *Caldicellulosiruptor* and *Dictyoglomus* and the order Thermotogales. Five isolates were obtained from *in situ* enrichments which represented bacteria *Dictyoglomus* and *Caldanaerobacter* as well as archaea of the phylum Crenarchaeota (Kublanov et al. 2009). Adiguzel et al. (2009) were able to isolate *Geobacillus pallidus*, *B. licheniformis*, and *Aeribacillus pallidus* while studying the culturable bacterial diversity from Pasinler, a hot spring in Turkey.

Bacterial diversity studies on Icelandic geothermal waters in UK were conducted by Tobler and Benning (2011). They chose six geothermal waters and in one of the sites they could isolate neither bacterial nor archaeal DNA, which they attributed to fast sinter growth rates. In one of the geothermal waters designated as KF (temperature 80 °C) phylotypes detected were closely related to some of the well known mesophiles (*Pseudomonas*, *Caulobacter* and *Acinetobacter*). Each of the remaining four study sites was characterised by a distinct bacterial community structure, dominated by one phylogenetic class. Temperature was found to affect bacterial diversity as indicated by Shanon-Weaver index; highest for the site HV (1.92) and lowest for the site GY1 (0.26). The temperature of GY1 was 20 °C higher than that of HV.

Diversity of bacteria and archaea was investigated in ten hot springs of Tibet using 16S rRNA gene phylogenetic analysis. Phylogenetic analysis revealed bacteria were more diverse than archaea. Bacteria were mainly affiliated to Firmicutes, Proteobacteria, Cyanobacteria and Chloroflexi. Archaea from these hot springs were affiliated to Thaumarchaeota, a newly proposed archaeal phylum. However, there was no correlation between diversity indices and temperature (Huang et al. 2011).

Bacterial and archaeal diversity were investigated in two hot spring microbial mats from the geothermal region of China (Pagaling et al. 2012). Phylogenetic analysis revealed the bacterial affiliation to various phyla like Cyanobacteria, Chloroflexi, Chlorobia, Nitrospira, Deinococcus, Proteobacteria, Firmicutes, Bacteroidetes and Actinobacteria. Archaeal population was dominated by Euryarchaeota, Crenarchaetoa. Coman Let al. (2013) observed a very low number of bacterial and archaeal operational taxonomic units (OTUs) in the hot spring microbial mats of geothermal region of Romania. At the phylum/class-level, the microbial communities investigated are dominated apparently by

Cyanobacteria, together with Chloroflexi. They observed metabolic features in the studied mats based on high percentage identity between the observed OTUs and bacterial taxa described in literature. The presence of Cyanobacteria and Choloroflexi in microbial mats suggests that the predominant metabolic pathway operating are oxygenic and anoxygenic photosynthesis.

Mackenzie et al. (2013) studied the seasonal shifts in bacterial diversity in three hot springs of Patagonia. Small seasonal variation in temperature and hence bacterial communities was observed in only one of the three hot springs studied for unknown reasons. They also found effects of rock substrate differences and geographical attributes on the bacterial communities. Three major phyla were detected in all the springs; phyla Cyanobacteria, Bacteroidetes and order Thermales, within the phyla Deinococcus-Thermus phylum.

Studies of Sahm et al. (2013) on two hydrothermal springs in Azores, Germany revealed the presence of heterotrophic bacterial populations. This study puts a question on common notion that H_2 based chemolithoautotrophic processes play a dominant role in hot-temperature ecosystems, based on the predominant presence of order *Aquificales*. The study used a combination of methods based on 16S rRNA, such as fluorescent *in situ* hybridization technique, DGGE and 16S rRNA pyrosequencing. The study emphasizes how different methods of bacterial community analyses can complement each other in understanding the complex ecosystems.

2.6 Mechanism of thermophily and thermotolerance

Microbes can maintain a steep pH and salt gradient so the principle of evasion can operate when microbes tolerate high salt concentration or high hydrogen ion concentration in the environment. But the microorganisms cannot insulate themselves from hot environments. Therefore, thermophile/thermotolerant microorganisms have to be adapted at all levels of cellular development. Each and every biomolecule has to be stabilized intrinsically and extrinsically to be active at high temperatures (Daniel and Cowan 2000). The various mechanisms operating at biochemical and molecular level for thermotolerance are reviewed here.

2.6.1 Biochemical and Physiological mechanisms

Accumulation of compatible solutes likes mannosyl glycerate, Dimyoinositol phosphate and others have been proposed to confer thermal protection in bacteria (Empadinhas and da Costa 2006). Borges et al. (2002) made a comparative study of thermostability properties of different compatible solutes on model enzymes. They reported high efficiency of mannosyl glycerate as compared to other compatible solutes like glucosyl glycerate and di-myo-inositol phosphate. Lamosa et al. (2006) reported a new compatible solute 1-glyceryl-1-myo-inositol phosphate in hyperthermophiles of the order Aquificales. They also found that at supraoptimal growth temperature, phosphate derivatives, di-myo-inositol phosphate and 1-glyceryl-1-myo-inositol phosphate become dominant, accounting for 88% of total solute pool. Nunes et al. (1995) reported the accumulation of mannosyl glycerate in *Rhodothermus marinus* and *Thermus thermophilus* when grown at 70° C and 65° C respectively. They suggested the role of mannosyl glycerate in thermophily of these organisms. Lamosa et al. (2000) studied the effect of diglycerol phosphate (DGP) on thermostability of lactate dehydrogenase, alcohol dehydrogenase and glutamate dehydrogenase and found that this DGP acts as a protein stabilizer. Since this solute accumulates at high level in *Archaebolus fulgidus*, it was suggested that this DGP could protect the protein *in vivo*.

Polycationic polyamines, which increase the melting temperature of DNA and protect *Sulfolobus solfataricus* ribosomes from thermal inactivation in vitro (Friedman 1986), have also been observed in hyperthermophiles (Friedman and Oshima 1989). Concentrations of putrescine, spermidine, norspermidine, thermospermine and spermine of up to 0.4 % (cell biomass) were detected in various *Sulfolobus* strains. A comprehensive analysis of the polyamines in 75 bacterial and archaeal isolates from mesophilic to hyperthermophilic sources has been carried out (Kneifel et al. 1986). The results showed that some polyamines (norspermine and norspermidine) occurred only in the hyperthermophilic archaea, but that there was no significant correlation between total intracellular polyamine concentration and the growth temperature of the source organism. However, the hyperthermophilic archaea were found typically to contain a greater diversity of polyamines than other organisms.

Thermal adaptation is associated with amino-acid substitutions modifying the balance of stabilization forces in proteins. A hypothesis has been formulated that thermoadaptation is associated with protein dynamics in the sense that the higher thermal stability of the thermophilic proteins would arise from increased rigidity and lower flexibility (Jaenicke 2000). England et al. (2003) compared the frequency of folding in mesophilic and thermophilic eubacteria and suggested that the thermophilic adaptation of bacteria is partly due to more designable folds in its proteins. Farias and Bonato (2003) carried out a comparative proteome analysis using whole genome sequence of 18 mesophiles, 4 thermophiles and 6 hyperthermophiles. Their study revealed an interesting fact that the ratio of sum of glutamate and lysine to sum of histidine and glutamine was highest for hyperthermophiles and lowest for mesophiles indicating the role of glutamate and lysine in thermotolerance.

The role of heat shock proteins in thermotolerance has been studied by various groups (Li and Werb 1982; Laszlo 1988; Guagliardi et al. 1994; Nakamoto et al. 2001; Singh et al. 2007) since its first report of in *Drosophila* (Ritossa 1962). Hsps can be classified into five major families; 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). A small heat shock protein containing 63 amino acids was shown to be involved in thermotolerance by regulating the operon that codes for HSP 60 (Nakamoto et al. 2001). HSP 60 has earlier shown to provide thermotolerance in various organisms (Tanaka et al. 1996; Martin 1998). Protein homologous to HSP 60 was found to be produced in response to heat stress in *Pyrodictium* and *Sulfolobus* (Holden et al. 2000). Glover and Lindquist (1998) found that HSP 70 in the presence of HSP 40 can rescue proteins aggragated due to thermal stress. The study reiterates the chaperonic activity of HSP 70. It was also found to play a role in multiple stress tolerance in *Staphylococcus aureus* (Singh et al. 2007).

2.6.2 Molecular mechanisms

Ever since the experimental demonstration that thermal denaturation of double stranded DNA molecules is affected by their nucleotide composition, Biologists thought the same principle can be applied in nature. The expectation is that the genomes of organisms at higher temperature would have high proportion of G+C than A+T. But there is no obvious correlation between G+C content of genome and optimal environmental growth temperature of organism (Galtier and Lobry 1997; Forsdyke and Bell 2004). Galtier and Lobry (1997) demonstrated that there is a significant correlation between G+C content of structural RNAs and growth temperature and that the high G+C content was concentrated in double stranded stem regions of the molecule. Singer and Hickey (2003) confirmed that the coding sequences of thermophiles are relatively rich in purines and found that increase is due to increased frequency of adenine. Farias and Bonato (2003) analysed the codon usage of hyperthermophiles and thermophiles and illustrated that the hyperthermophiles use more of AGR (R stands for G or A) codons for arginine than CGN (N stands for A, G, C or T) codons which are more common in mesophiles.

2.7 Genes conferring temperature tolerance

There is a logical progression from biodiversity to advances in biotechnology. Whether searching for new pharmaceuticals, compounds to protect agricultural crops against disease, unusual degradative pathways for remediation of recalcitrant pollutants or products of use in the food industry, availability of a broad diversity of microorganisms will improve the chances of success. Perhaps the greatest value of microbial diversity is the opportunity for a harvest of truly novel ideas (Combie and Runnion 1996). One such opportunity is prospecting genes for temperature tolerance. A number of genes have been proposed for thermotolerance which are reviewed here.

The gene *dnaK* which codes for HSP 70 was shown to play a role in thermoregulation in *B. subtilis*, as detected by the increased level of its mRNA after heat induction (Wetzstein et al. 1992). Rockabrand et al. (1998) showed that in *E. coli*, *dnaK* played both an independent and an RpoS-dependent role in starvation-induced thermotolerance. Deletion mutation studies in the *dnaK* region of *Staphylococcus aureus* showed inability of the organism to grow well at 37 °C or above indicating a role of *dnaK* in thermotolerance (Singh et al. 2007). In bacteria, it was found that HSP 40 coded by *dnaJ* and GrpE coded by g*rpE* modulate the activities of *dnaK* proteins by acting as co-chaperones (Georgopoulos et al. 1994; Liberek et al. 1991). In vitro studies conducted by Mendoza et al. (1996) revealed that the protein product of *groEL* protects a number of dehydrogenases from thermal inactivation, suggesting its role in thermotolerance. Transcriptional analyses of *Streptomyces coelicolor* A3 (2) revealed that the genes *gro*ES (coding for HSP 10) and *gro*EL (coding for HSP 60) are induced by heat stress, as indicated by higher cellular transcript levels corresponding to these genes (Duchene et al. 1994). *cod*A (which codes for choline oxidase) from *Arthrobacter globiformis* when transformed to *Brassica* provides the plant thermotolerance and salinity tolerance (Wang et al. 2010).

Heterologous expression of *dnaK* from a halotolerant cyanobacterium, *Aphanothece halophytica* enhanced thermotolerance in tobacco up to 55 °C at seedling stage (Ono et al. 2001). Transgenic rice expressing *dnaK* gene from *A. halophyitca* showed higher activity of ascorbate peroxidase and catalase. Rice transgenics exhibited enhanced activities of Calvin cycle enzymes and greater tolerance to high temperature of up to 37 °C for 16 h (Uchida et al. 2008). The same gene when over expressed in poplar, enhanced tolerance to drought and freezing (Takabe et al. 2008).

dnaK gene from *Trichoderma harzianum* when transferred to a model plant *Arabidopsis thaliana* imparted thermotolerance in the plant (Montero-Barrientos et al. 2010). A small heat shock protein gene (*hsp22.4*) was cloned from *Chaetomium globosum* using rapid amplification of cDNA ends (RACE). The hsp22.4 gene was amplified using specific primers in the 5’ and 3’ untranslated regions of the *hsp22.4* cDNA. The *hsp22.4* transgenic yeast displayed significantly greater resistance to heat (Liu et al. 2007). A small heat shock protein gene *hsp 23* from *T. virens* confered thermotolerance in *T. harzianum* (Montero-Barrientos et al. 2007).

Materials and Methods

3. MATERIALS AND METHODS

3.1 Sample sites and Sampling

Five different hot springs from diverse ecologies were selected for the present study. The sample sites and their details are presented in Table 3.1. The temperature and pH of the hot springs were recorded at the time of collection.

Water samples collected from each of the hot springs was brought to the lab in thermos flasks within 12-16 h of collection and processed immediately for isolation of culturable bacteria.

3.2 Enumeration and isolation of culturable bacteria

Water samples were plated on five different media using standard spread plate technique and incubated at 37 °C for 48-72 h. Five different media used were Nutrient Agar (Peptone 0.5 %, Beef extract 0.3 %, NaCl 0.5 % and Agar 1.8 %), Thermus Agar (Peptone 0.5 %, Yeast extract 0.2 %, Beef extract 0.4 %, NaCl 0.5 % and Agar 1.8 %), R2A medium (Proteose Peptone 0.05 %, Casamino acid 0.05 %, Yeast extract 0.05 %, Dextrose 0.05 %, Soluble starch 0.05 %, Dipotassium hydrogen phosphate 0.03 %, Sodium Pyruvate 0.03 %, Magnesium sulphate heptahydrate 0.005 %), King's B medium (Protease Peptone 2 %, Dipotassium hydrogen phosphate 0.15 %, Magnesium sulphate 0.15 % and Agar 1.8 %) and Thermus Peptone Meat extract Yeast extract medium (TPMY : Peptone 0.35 %, Meat extract 0.5 % Yeast extract 0.2 % NaCl 0.15 % and Agar 1.8 %). After incubation, plates were observed for colony morphology and the total viable counts were recorded for each sample in the different media employed. Based on differences in colony morphologies, the various morphotypes from the five hot springs were axenized, numbered and preserved for further studies.

3.3 Screening of isolates for temperature tolerance

Isolates obtained from five hot springs were screened for temperature tolerance by incubating the culture spot inoculated plates at different temperatures (40, 45, 50, 55 and 60 °C) for 72 h. The isolates tolerant to 60 °C were screened further by inoculating in broth and incubating in water bath shaker at 150 rpm for 72 h at 70 °C. The optical density of the broth was measured at 600 nm and compared with the growth at 37 °C. All the isolates from the five hot springs that showed growth at 45 °C and above were selected for further characterization.

3.4 Molecular characterization of thermotolerant isolates

3.4.1 Isolation of genomic DNA

All the isolates that were able to grow at/above 45 °C were inoculated in their specific broth and allowed to grow to achieve a cell density of $OD_{600nm} > 1.0$. Five ml culture from each of the isolate was pelleted down and washed thrice with TE buffer (10 mM Tris and 1mM EDTA, pH 8.0). Pellets were then resuspended in 750 µl of TE buffer. Using Zymo Research

Table 3.1 Sample sites and their details

Sample sites	Ordinates	Temperature range	pH range
Manikaran	32°02’ N 77°34’ E	89° C to 95° C	7.8-8.2
Bakreshwar	23°88’ N 87°37’ E	55° C	9.2
Balrampur	27°43’ N 82°18’ E	90° C	7.8
Chumathang	33°36’ N 78°34’E	72° C	8.4
Vashisht	32°26’ N 77°18’ E	47° C	7.2

Fungal/Bacterial DNA MicroPrep™, genomic DNA was isolated from the suspended pellet by following the standard protocol prescribed by the manufacturer.

3.4.2 Amplification of 16S rRNA gene employing PCR

Primers used for amplification of 16S rRNA gene from the genomic DNA of isolates are pA (5′AGA GTT TGA TCC TGG CTC AG3′) and pH (5′-AAG GAG GTG ATC CAG CCG CA-3′) (Edwards et al. 1989). In a reaction mixture containing 50-100 ng of template DNA, primers pA and pH (100ng each), dATP, dCTP, dTTP and dGTP (200µM each), Taq Polymerase reaction buffer (10X) 10 µl and 1.0 U Taq Polymerase, amplification of 16S rRNA gene was carried from all the isolates under study. The final volume of reaction mixture was adjusted to 100 µl in all the amplification reactions. Conditions used for amplification were as follows: initial denaturation for 5 min at 95 °C, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 52 °C for 40 s and extension at 72 °C for 1 min and a final extension at 72 °C for 10 min. PCR products were resolved by electrophoresis at 60 V for 1 hour in 1.2 % agarose gel in 1X TAE buffer. Gels were then stained with ethidium bromide (10 mg/ml) and visualized on a gel documentation system (Alpha Imager).

3.4.3 Amplified rDNA restriction analysis (ARDRA)

Purified PCR products were digested separately with three restriction endonucleases - *Alu*I, *Msp*I and *Hae*III in a 30 µl reaction volume, using recommended buffers at 37 °C. Restricted PCR products were resolved by electrophoresis at 45 V for 1.5 to 2 h in 2.5 % agarose gels in 1X TAE buffer. Gels were then stained with ethidium bromide and visualized. Strong and clear bands were scored as binary data (presence and absence of bands). Numerical taxonomy analysis program (NTYSIS) package, Exerter software – 2.02e package, USA was used to score similarity and clustering analysis using the binary data. Jaccard's coefficient was used to calculate the similarity among the isolates and dendrogram was constructed using the UPGMA method (Nei and Li 1979).

3.4.4 16S rRNA gene sequencing and phylogenetic analysis

PCR amplified 16S rRNA genes were purified and sequenced using both pA and pH for forward and reverse reactions respectively. Sequencing was done employing a dideoxy cycle with fluorescent terminators and run in a 3130xl Applied Biosystems ABI prism automated DNA sequencer. The partial 16S rRNA gene sequences were compared with sequences available in NCBI database. Identification of isolates up to species level was done on the basis of 16S rRNA gene sequence similarity of ≥97 % with the sequences in Genbank. Sequence alignment and comparison was performed using the multiple sequence alignment tool CLUSTAL W2 (Thompson et al. 1994) with default parameters. The phylogenetic tree was constructed on aligned data sets using Neighbour joining (NJ) method (Saitou and Nei

1987) using the program MEGA 4.0.2 (Tamura et al. 2007). Bootstrap analysis was performed on 1000 random samples taken from multiple alignments (Felsentein 1981).

3.5 Biolog analysis of selected isolates

For the set of isolates tolerant to temperature of 70 °C, biochemical profiling was carried out using GEN III MicroPlate™. The profiles were based on 92 biochemical tests (70 Carbon sources and 22 chemical sensitivity tests). Numerical taxonomy analysis program (NTYSIS) package and Exerter software – 2.02e package, USA was used to score similarity and clustering analysis using the binary data generated through Biolog. Jaccard's coefficient was used to calculate the similarity among the isolates along with their reference strains and dendrogram was constructed using the UPGMA method (Nei and Li 1979). Microbial Identification System software of Biolog was used to identify the bacterium from its phenotypic pattern in the GEN III MicroPlate.

3.6 Protein profiling of the selected isolate

3.6.1 Bacterial strain and cultural condition

A single colony of the selected isolate was grown in nutrient broth (5 ml) at 37 °C for 16 h and was used for further inoculation of 100 ml nutrient broth in 250 ml Erlenmeyer flasks. One set was grown at 37 °C (control) and other set at 60 °C (stressed). Flasks were incubated up to mid exponential growth phase (OD_{600nm} 0.6).

3.6.2 Cell lysis and protein estimation

5 ml of culture from both the control and stressed ones were pelleted down by centrifugation (7000 rpm for 10 min) and washed thrice with Tris-Hcl buffer (50 mM, pH 7.2). Cells pellets were then resuspended in 2 ml of lysis buffer containing Tris-Hcl 50 mM, NaCl 100 mM, DTT 5 mM, Glycerol 5%, PMSF 5 mM, sonicated for 5 min and centrifuged (7000 rpm for 10 min) to pellet down cell debris. Supernatants were transferred to a fresh 1.5 ml autoclaved eppendorf tubes. The total protein concentration was determined by Bradford method using BioRad reagents and using bovine serum albumin as the standard. Aliquots of 400 µg of protein were stored at -80 °C.

3.6.3 Two dimensional gel electrophoresis (2DE)

Aliquots of 400 µg of protein from both the control and stressed samples of the culture were cleaned using a BioRad 2DE clean up kit by following the protocol prescribed by the manufacturer. The total protein concentration was determined again after clean up employing Bradford method using BioRad reagents. An aliquot containing 100 µg of protein was made up to a volume of 125 µl using rehydration buffer (Urea 8M, CHAPS 2 %, DTT 50 mM, 100X Biolyte Ampholyte 0.2 % and bromophenol blue 0.0002 %). The suspension containing 100 µg of protein was loaded on to immobilized pH gradients strips (pH 4.0-7.0, 7 cm, BioRad) in a rehydration tray overlaid with mineral oil. Gels were passively rehydrated for 16

h. Isoelectric focussing was carried out on a BioRad IEF assembly at 60000 V/h and focussing strips were stored at -20 °C until separation in second dimension was performed. Immobilized pH gradient strips were then equilibrated in a series of equilibration buffer I (Urea 6 M, Tris-HCl 0.375 M, SDS 2 %, Glycerol 20 % and DTT 2 %) and equilibration buffer II (Urea 6 M, Tris-HCl 0.375 M, SDS 2 %, Glycerol 20 % and Iodoacetamide 2.5%). The second dimension was performed by SDS- PAGE on gels containing 12.5 % polyacrylamide and carried out in a Genei PAGE assembly. Proteins were resolved at 80 V for 3 hours.

3.6.4 Silver staining of 2DE gels and analysis of gels

The 2DE gels of both control and stressed samples were stained following a silver staining protocol. Gels were incubated in a fixer solution (40 % ethanol, 10 % acetic acid, 50 % water) for 1 h. Gels were washed in deionized water for 30 min. Gels were then immersed in deionized water overnight to remove the traces of acetic acid. After overnight washing the gels were submerged in 0.2 % sodium thiosulphate and washed thrice with deionized water. Gels were then incubated for 20 min in 4 °C cold 0.1 % silver nitrate solution. After incubation, gels were washed thrice with deionized water. Gels were then developed with 3 % sodium carbonate and 0.05 % formaldehyde and once the spots became clearly visible the development was terminated using 5 % acetic acid for 5 min. The gels were scanned using a BioRad gel documentation unit and analysed using PDQuest 2D analysis software. Analyses were done to locate spots/proteins that are expressed only in stressed sample.

3.6.5 Protein identification using MALDI-TOF mass spectrometry

Individual spots were excised from the gels and subjected to MALDI-TOF mass spectrometry that was carried out by 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).

3.7 Identification of allelic variations

3.7.1 Amplification of partial region of *dna*K

Genus specific primers were selected for amplification of *dnaK* locus from different isolates under study. The primers used for amplification of dnaK from all the *Bacillus* isolates under study are BadnaKF (5'CCG GCG ACA ACC GTC TGG GTG GGG A3') and BadnaKR (5'GGG CGC TCA CCT TGA AGA ACA TGG A3') (Tokunaga et al. 1998). Similar primers were designed for partial amplification of *dnaK* loci from other genera like *Exiguobacterium, Rhodococcus* and *Pseudomonas*. For designing primers, a number of sequences were downloaded from NCBI GeneBank and aligned using ClustalW to look for the conserved region within each genus for that locus. Primers were designed for each genus and the details of the primers are tabulated in Table 3.2. *Primer*3, an online tool was used for primer designing.

3.7.2 PCR amplification of full length *dnaK* region

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 3.3. 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 hour 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).

3.7.3 Restriction analysis of *dnaK* gene

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 1X TAE buffer. Gels were then stained with ethidium bromide to visualize the profiles. Strong and clear bands were scored as binary data.

3.7.4 Sequencing and analysis of *dnaK* gene

DNA sequencing was performed by Scigenome Labs Pvt. Ltd. (Cochin, India) employing primer walking technique. The sequence was then analysed using ORF finder available in the National Centre for Biotechnological Information (NCBI) tools. The deduced amino acid sequence were analysed with the programs BLAST-X from NCBI.

3.8 Cloning and expression of *dnaK* of *Bacillus pumilus* (B3)

3.8.1 Competent cell preparation and cloning

Competent cells of *E.coli* cells 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 *B. pumilus dnaK* deduced was amplified using the primers BKEF (5’CGCGGATCCATGAGTAAAA TCATTGGTATTGAC3’) and BKER (5’CGAGCTCGTTATTTTTCTCTTGATCGTCG3’). 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 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. pET29-BpdnaK plasmid was then transformed to expression host *E.coli* BL21 (DE3).

3.8.2 Expression of B3 (*B. pumilus*) *dnaK* in *E.coli*

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

Table 3.2 Primers used for amplification of partial region of *dnaK*

Genera	**Primer sequence**
Bacillus	F 5’CCG GCG ACA ACC GTC TGG GTG GGG A3’ R 5’GGG CGC TCA CCT TGA AGA ACA TGG A 3’
Exiguobacterium	F 5’ TGT GTC GCA GTA ATG GAA GG 3’ R 5’ CGA TTC CGT TTG CAT CGA TGT CG 3’
Pseudomonas	F 5’ATC GGT ATC GAC CTG GG GAC3’ R5’CGT CAC CCT GGT TAC GGG CA3’
Rhodococcus	F 5’ATG GCG ACT GCT GTA GGC ATC GAC 3’ R 5’GCC GTT GGC GTC GAT ATC GAA 3’

Table 3.3 Primers used to amplify full length gene of *dnaK*

Strains	Probable species	Primer sequence
M4	*Bacillus licheniformis*	5’GCAGGTTGAGGATGAAGCGT3’ 5’ CTCATAGTAATCACGCTTAC3’
M5	*Bacillus pumilus*	5’CGATCCAAACCTTCATCAAGCC3’ 5’CTCATCTCTTCACACTCCCG3’
M7	*Bacillus pumilus*	-do-
B3	*Bacillus pumilus*	-do-
B5	*Bacillus pumilus*	-do-
B12	*Bacillus pumilus*	-do-
B34	*Bacillus pumilus*	-do-
M8	*Bacillus* sp.	-do-
M6	*Bacillus subtilis*	5’ GTCGAAGCCATCGAAGCTGT3’ 5’ ATAGTAATCACGCTTACTCA3’
M55	*Bacillus megaterium*	5’ TGTTGAAGCAATTGAATCAG3’ 5’ ATCTCGCTTACTCATCAATA3’
M46	*Pseudomonas psychrophila*	5’ CGCCTTCGATTGACGAGAAG3’ 5’ TTGGTTCATGCGGTATTTGC3’
M47	*Exiguobacterium acetylicum*	5’CGTCCAAGTATGGTCAAAGTCGC3’ 5’GACATGGAAAGAGCCAAAGC3’

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 $O.D_{600nm}$ reached upto 0.6. Recombinant protein expression was induced with IPTG to a final concentration of 0.2 mM and cultivated further. One ml 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.

3.8.3 SDS – PAGE of cell lysates

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.

3.8.4 MALDI-TOF analysis of expressed protein

The band corresponding to 70 kDa from the SDS-PAGE gel excised from the gel and subjected to MALDI-TOF mass spectrometry that was carried out by 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).

3.8.5 Heat stress experiments

In order to check for 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_{600nm}. Growth was monitored by measuring the optical density at 600 nm at regular intervals.

Research paper I

4. RESEARCH PAPER I

Deciphering the diversity of culturable thermotolerant bacteria from Manikaran hot springs

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Abstract

The aim of this study was to analyze and characterize the diversity of culturable thermotolerant bacteria in Manikaran hot springs. A total of 235 isolates were obtained employing different media, and screened for temperature tolerance (40 °C-70 °C). A set of 85 isolates tolerant to 45 °C or above were placed in 42 phylogenetic clusters after Amplified ribosomal DNA restriction analysis (ARDRA). Sequencing of 16S rRNA gene of 42 representative isolates followed by BLAST search revealed that the majority of isolates belonged to Firmicutes, followed by equal representation of Actinobacteria and Proteobacteria. Biolog based identification of thirteen isolates exhibiting temperature tolerance upto 70 °C, using carbon utilization patterns and sensitivity to chemicals revealed a high degree of correlation with identification based on 16S rRNA gene sequencing for all the isolates, except for one isolate (M48). These promising isolates showing a range of useful metabolic attributes need to be explored for their industrial and agricultural applications.

Keywords: 16S rRNA gene; ARDRA; Biolog analysis; Culturable bacteria; Sequencing

Introduction

Exotic niches, like thermal springs harbour populations of microorganisms that are a source of several commercially important products like enzymes, sugars, compatible solutes and antibiotics (Satyanarayana et al. 2005). Thermal springs are a manifestation of geological activity and represent aquatic microcosms that are produced by the emergence of geothermally heated groundwater from earth’s crust. Prokaryotes are the major component of most ecosystems, being ubiquitous in nature because of their small size, easy dispersal, metabolic versatility, ability to utilize a broad range of nutrients and tolerance to unfavourable extreme conditions. Thermal springs are, therefore, no exception to colonization by these prokaryotes. Diversity analysis of such extreme environments has grown in significance because of its diverse and unusual chemistry and the opportunity it provides to identify rare compounds and genes (Kuddus and Ramtekke 2012).

In the past, phylogenetic studies combined with cultivation studies and in situ microbial physiological and ecological studies have revealed the abundant diversity of thermophilic microorganisms inhabiting hot springs around the world like Japan, New Zealand, Iceland and Yellowstone National Park, USA (Tobler and Benning 2011). Physiologically diverse microorganisms are likely to exhibit diverse chemistry, hence increasing the chance of finding novel compounds. Several hot springs in different regions of India are known to geologists for years now (Ghosh et al. 2003). Although few attempts have been made to study and utilize microorganisms from hot springs in India (Nawani and Kaur 2000; Ghosh et al. 2003), most of them are fragmented in nature. Manikaran hot springs are located in the Beas and Parvati valley geothermal system, at an elevation of 1760 metres (Cinti et al. 2009). These hot springs are the hottest in the country, with a temperature range of 89-95 °C (Dwivedi et al. 2012). Despite intensive studies on terrestrial thermal springs, very little is known about bacterial diversity of thermal springs at high elevation (Huang et al. 2011). Hence, a comprehensive approach is needed to analyze the bacterial diversity of such niche, in terms of their taxonomic and biochemical attributes.

Studies on the diversity of bacterial communities from various environments have been undertaken mainly using traditional methods of isolating and culturing the microorganisms. Such methods involve isolation of microbes on standard cultivation media and characterization of microbes (Ranjard et al. 2000). Culture based methods of analyzing microbes have been the mainstay of microbiology since their origin in pioneering works of Robert Koch and Louis Pasteur. However, culture based techniques have many limitations in analysing the bacterial diversity of a particular environmental niche. Aerobic and anaerobic organisms cannot be cultured together; fastidious organisms will often not grow, because essential nutrients for growth or optimal environmental conditions such as temperature, pH, essential mixtures of gases may not be present (Piterina and Pembroke 2010). Despite all these limitations, there is immense scope for culture based diversity studies because of the advantages it offers in the generation of valuable germplasm. Working with strains isolated from hot springs offer a major advantage of preserving those strains for future studies and exploring them in due course for potential biotechnological applications (Akanbi et al. 2010; Acharya and Chaudhary 2012).

In the 1980s, a new tool for identifying bacteria was developed in the laboratories of Woese and other researchers for evaluating the phylogenetic relationships of bacteria. It was found that all life forms could be identified by comparing a stable part of the genetic code. Candidate genes used for these evolutionary studies included as many as 20; including 5S rRNA, 16S rRNA, 23S rRNA and 16-23S rRNA internal transcribed spacer (ITS) region. Among all these genes, 16S rRNA gene is considered the best evolutionary chronometer because of its universal presence in all bacteria, relative stability over evolutionary time and appropriate size (1,500 bp), which is large enough for bioinformatic analyses (Patel 2001). Amplified ribosomal DNA restriction analysis (ARDRA) represents a further improvement in the analysis of 16S rRNA gene, which involves the comparison of restriction patterns (Yadav et al. 2010) and analyses using bioinformatic tools. ARDRA is generally considered valuable for strain typing and screening clone libraries to identify phylogenetic clusters within a microbial community (Sklarz et al. 2009).

In the present study, an attempt was made to decipher the bacterial diversity of culturable thermotolerant bacteria from Manikaran thermal springs employing different media, followed by screening of isolates for temperature tolerance. ARDRA analysis was done for phylogenetic clustering of the thermotolerant isolates. Sequencing of 16S rRNA gene of representative thermotolerant strains was undertaken for identification. Biolog and analyses of other important biochemical attributes helped in further screening and selection of promising isolates, as a prelude to their applications in agriculture and industry.

Materials and methods

Sampling and isolation of culturable bacteria

Water samples were collected from four different sites of Manikaran hot springs. Manikaran is located at 32° 02' N latitude and 77 ° 34' E longitude. The temperature and pH of Manikaran hot springs ranges from 89 °C to 95 °C and 7.8 to 8.2 respectively. Water samples were collected in thermos flasks and brought to lab within 12 h of collection and processed for isolation of culturable bacteria.

For enumeration and isolation from water, samples were plated on five different media using standard spread plate technique and incubated at 37° C in an incubator for 48-72 h. The different media used were, Nutrient Agar (Peptone 0.5 %, Beef extract 0.3 %, NaCl 0.5 % and Agar 1.8 %), Thermus Agar (Peptone 0.5 %, Yeast extract 0.2 %, Beef extract 0.4 %, NaCl 0.5 % and Agar 1.8 %), R2A medium (Proteose Peptone 0.05 %, Casamino acid 0.05 %, Yeast extract 0.05 %, Dextrose 0.05 %, Soluble starch 0.05 %, Dipotassium hydrogen phosphate 0.03 %, Sodium Pyruvate 0.03 %, Magnesium sulphate heptahydrate 0.005 %), King's B medium (Protease Peptone 2 %, Dipotassium hydrogen phosphate 0.15 %, Magnesium sulphate 0.15 % and Agar 1.8 %) and Thermus Peptone Meat extract Yeast extract medium (TPMY : Peptone 0.35 %, Meat extract 0.5 % Yeast extract 0.2 % NaCl 0.15 % and Agar 1.8 %). After incubation, plates were observed for colony morphology and the total viable count was recorded for each sample in the different media employed. Based on differences in colony morphologies, 235 morphotypes were picked up from different plates and axenized.

Screening of isolates for temperature tolerance

All the 235 isolates were screened for temperature tolerance by incubating the culture spot inoculated plates at different temperatures (40, 45, 50, 55 and 60 °C) for 72 h. The cultures tolerant to 60 °C were screened further by inoculating in broth and incubating in shaker at 150 rpm for 72 h at 70 °C. The optical density of the broth was measured at 600 nm and compared with the growth at 37 °C.

Genomic DNA extraction

Isolates were grown in specific broth, until they reached an $OD_{600\ nm} > 1.0$. The cells were pelleted from 5 ml of culture, washed thrice with TE buffer (10 mM Tris and 1mM EDTA, pH 8.0) and the pellet was resuspended in 750 µl of TE buffer. Genomic DNA was isolated from the suspended pellet using Zymo Research Fungal/Bacterial DNA MicroPrep^{TM} following the standard protocol prescribed by the manufacturer.

PCR amplification of 16S rRNA gene

The primers pA (5′AGAGTTTGATCCTGGCTCAG3′) and pH (5′AAGGAGGTGATCCAG CCGCA3′) were used for amplification of 16S rRNA gene from the genomic DNA of isolates (Edwards et al. 1989). Amplification was carried out in a 100 µl reaction volume containing 50-100 ng of template DNA, primers pA and pH (100ng each), dATP, dCTP, dTTP and dGTP (200µM each), *Taq* Polymerase reaction buffer (10X) 10 µl and 1.0 U *Taq* Polymerase. Conditions used for amplification were as follows: initial denaturation for 5 min at 95 °C, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 52 °C for 40 s and extension at 72 °C for 1 min and a final extension at 72 °C for 10 min. PCR products were resolved by electrophoresis at 60 V for 1 hour in 1.2 % agarose gel in 1X TAE buffer. Gels were then stained with ethidium bromide (10 mg/ml) and visualized on a gel documentation system (Alpha Imager).

Amplified rDNA restriction analysis (ARDRA)

Purified PCR products were digested separately with three restriction endonucleases - *Alu*I, *Msp*I and *Hae*III in a 30 µl reaction volume, using recommended buffers at 37 °C. Restricted PCR products were resolved by electrophoresis at 45 V for 1.5 to 2 h in 2.5 % agarose gels in 1X TAE buffer. Gels were then stained with ethidium bromide and visualized. Strong and clear bands were scored as binary data (presence and absence of bands). Numerical taxonomy analysis program (NTYSIS) package, Exerter software – 2.02e package, USA was used to score similarity and clustering analysis using the binary data. Jaccard’s coefficient was used to calculate the similarity among the isolates and dendrogram was constructed using the UPGMA method (Nei and Li 1979).

16S rRNA gene sequencing and Phylogenetic analysis

PCR amplified 16S rRNA genes were purified and sequenced using both pA and pH for forward and reverse reactions respectively. Sequencing was done employing a dideoxy cycle with fluorescent terminators and run in a 3130xl Applied Biosystems ABI prism automated DNA sequencer. The partial 16S rRNA gene sequences were compared with sequences available in NCBI database. Identification of isolates upto species level was done on the basis of 16S rRNA gene sequence similarity of ≥97 % with the sequences in Genbank. Sequence alignment and comparison was performed using the multiple sequence alignment tool CLUSTAL W2 (Thompson et al. 1994) with default parameters. The phylogenetic tree was constructed on aligned data sets using Neighbour joining (NJ) method (Saitou and Nei 1987) using the program MEGA 4.0.2 (Tamura et al. 2007). Bootstrap analysis was performed on 1000 random samples taken from multiple alignments (Felsentein 1981).

Biolog analyses of selected isolates

The set of selected isolates tolerant to 70 °C were characterized using the GEN III MicroPlate™, to generate a profile based on 92 biochemical tests (70 Carbon sources and 22 Chemical sensitivity tests). Numerical taxonomy analysis program (NTYSIS) package, Exerter software – 2.02e package, USA was used to score similarity and clustering analysis using the binary data generated through Biolog. Jaccard's coefficient was used to calculate the similarity among the isolates along with their reference strains and dendrogram was constructed using the UPGMA method (Nei and Li 1979). Microbial Identification System software of Biolog was used to identify the bacterium from its phenotypic pattern in the GEN III MicroPlate.

Results and Discussion

Thermal springs represent extreme niches whose pristine quality is maintained over a period of time. Many terrestrial hot springs exist on earth (Tobler and Benning 2011) which represent hot spots for unusual forms of life, genes and metabolites. In the last two decades, a number of researchers have investigated various facets of the bacterial diversity in hot springs of different parts of world (Kanokratna et al. 2004; Meyer-Dombard et al. 2005; Pagaling et al. 2012). The diversity of bacterial communities in various ecologies has been investigated mostly using culture - dependent approaches, although it is estimated that this may represent 0.1 to a maximum of 10 % of the total population (Ranjard et al. 2000). 16S rRNA gene sequencing, in combination with ARDRA analyses was effectively used for diversity studies in the past (Yadav et al. 2010). Our investigation therefore focussed towards the isolation of microbes using different media and growth at high temperature; so as to generate diverse nutritional types, undertake ARDRA analyses, evaluate the biochemical characterization of representative isolates and identify the thermotolerant isolates by Biolog analyses and 16S rRNA gene sequencing.

Manikaran hot springs are located at Manikaran village in Kullu district of Himachal Pradesh, India, which represents a high altitude environment. The thermal discharges of Manikaran hot springs are classified as Na-HCO_3-Cl type. Chemical analysis of thermal waters from these hot springs indicated the presence of calcium, magnesium, sodium, potassium, sulphate, chloride and carbonate at concentrations 38-70 ppm, 4.6-10.4 ppm, 75-106 ppm, 15-20 ppm, 55-70 ppm, 87-117 ppm and 120-200 ppm respectively (Chandrasekharam et al. 2005).

Enumeration and Isolation of culturable bacteria

In order to obtain a diverse range of nutritional types and bacteria with various metabolic requirements, five types of media were employed. These included a nutrient rich medium (Nutrient Agar) for isolating a larger proportion of the available diversity, a medium suitable for oligotrophs (R2A medium), specific media designed for thermophiles (Thermus Agar, Thermus Peptone Meat extract Yeast extract medium) and a medium mostly used for *Pseudomonas* and related genera (King's B medium). The population of aerobic heterotrophic bacteria in the water samples of Manikaran thermal springs ranged from 72 to 241 × 10^4 cfu/ml. Among the media used, highest population of 241× 10^4 cfu/ml was recorded on Nutrient agar medium. A total of 235 isolates were selected based on colony morphologies among which, 137 isolates were from Nutrient Agar medium, 47 isolates were from Thermus Agar medium, 13 Isolates were from King's B medium, and 19 each from TPMY and R2A media (Table 4.1).

Screening for temperature tolerance

All the 235 isolates were screened for temperature tolerance of which 85 were found to tolerate 45 °C, whereas 13 isolates could tolerate 70 °C. Isolate M7 was found to be most tolerant and exhibited more than 70 % growth when compared to its growth at 37 °C. Evaluation of temperature tolerance of the isolates revealed that only 13 isolates were thermotolerant up to 70 °C. Huang et al. (2011), while studying the archaeal and bacterial diversity in twelve hot springs of Tibetan Plateau, China reported that one of the springs GL81 with the highest temperature among the 12 studied was dominated by mesophilic microbial community. Lau et al. (2006) and Mitchell (2009) also reported that there is no monotonic relationship between microbial diversity and thermal stress. Based on the temperature tolerance, 85 isolates were selected for molecular characterization.

Amplified rDNA restriction analysis (ARDRA)

A set of 85 isolates that were tolerant to temperatures 45 °C or above were analysed using ARDRA, which is known to be an important technique for distinguishing among closely related taxonomic groups, especially for screening endospore forming bacilli (Ettoumi et al. 2009). PCR amplification of 16S rRNA gene followed by ARDRA with three restriction enzymes was carried out to analyze the variation among the 85 isolates. PCR amplification of 16S rRNA gene yielded a single amplicon of 1.5 Kbp from all the isolates. When the amplicons were digested with restriction enzymes, the different patterns comprising of 3 to 6 fragments ranging in size from 100 to 800 bp, were characterized. ARDRA revealed that restriction digestion with *Alu*I (31 groups) was more discriminative, as compared to *Msp*I and *Hae*III. A combined dendrogram (based on patterns generated using three enzymes individually) was constructed to determine the percent similarity among the isolates (Fig.

Table 4.1 Media based quantification of bacterial morphotypes from Manikaran hot spring samples

Media	**Number of morphotypes**	**Total Viable count (10^4 CFU/ml)**			
		MI	**MII**	**MIII**	**MIV**
Nutrient Agar	137	214	241	198	182
Thermus Agar	47	226	207	234	221
TPMY	19	116	148	121	128
King’s B	13	88	104	72	85
R2A	19	133	106	144	115

TPMY: Thermus peptone meat extract yeast extract; MI-MIV: Sites of samples

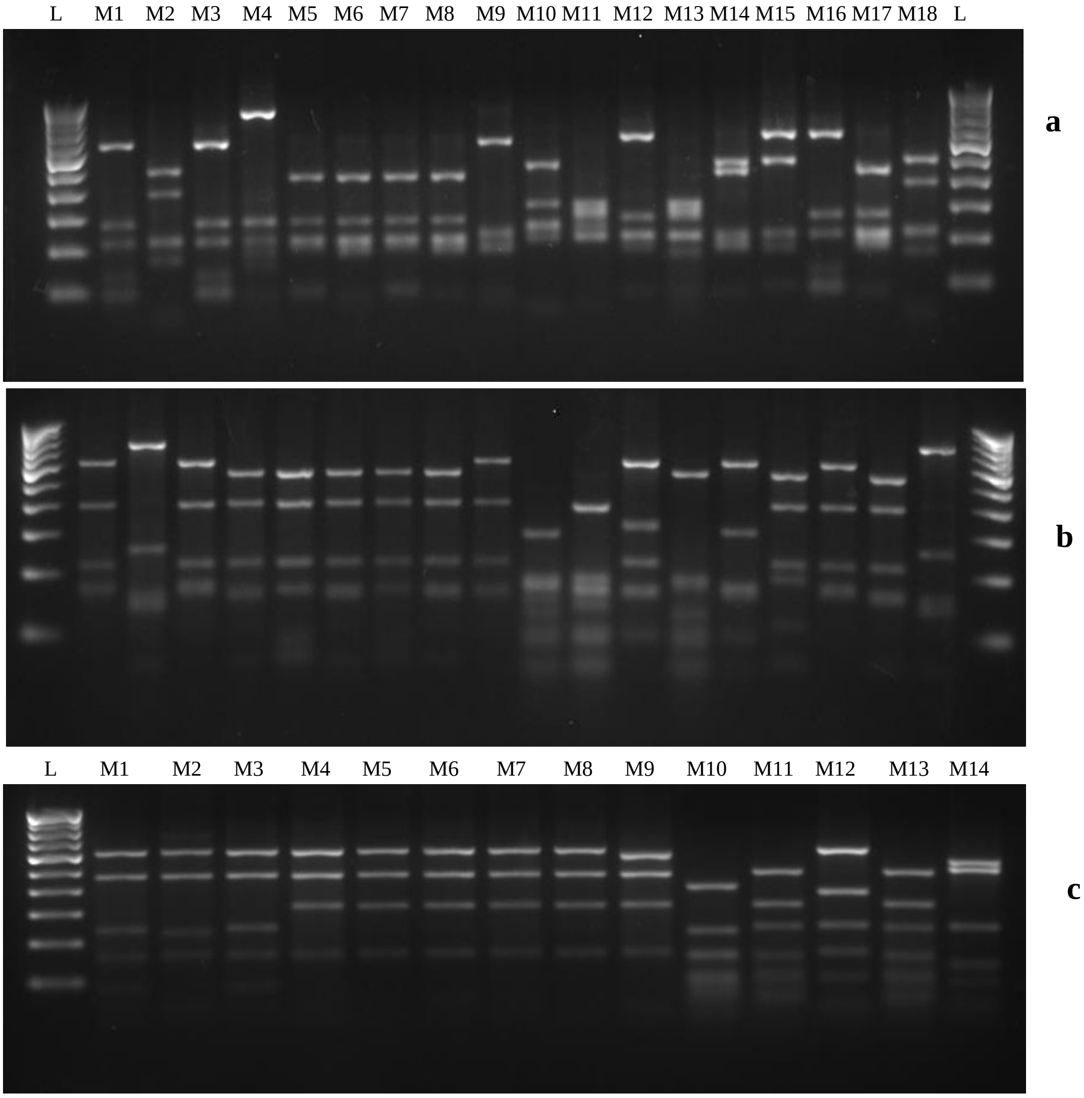

Figure 4.1 Representative restriction patterns of amplified 16S rRNA gene of isolates generated using the restriction enzymes a) *Alu*I b) *Msp*I c) *Hae*III.
L - 100 bp - 1000 bp ladder.
M1 - M18 - Restriction patterns of different isolates.

4.1). The isolates were grouped into 42 clusters at 95 % similarity level, based on the combined dendrogram (Table 4.2).

16S rRNA gene sequencing and phylogenetic analysis

Sequencing of 16S rRNA gene was carried out for the 42 representative isolates and the sequence data were analysed by BLAST. The nearest match from the GenBank database for each of the 42 representative isolates has been reported (Table 4.2). A phylogenetic tree was constructed using these 42 isolates along with the closest sequences in the NCBI Genebank (Fig. 4.2 a; Fig. 4.2 b). From 42 sequences obtained in this study, 11 sequences were 100 % identical to those already reported in GenBank (NCBI) database, and the remaining isolates had 98-99 % identity to reported sequences in database (as consulted in September 2012). Among the 42 isolates 72 % (30 isolates) belonged to Firmicutes which is in accordance with Sayeh et al. (2010). BLAST analysis on the sequences of 42 representative isolates revealed a majority of isolates showed closeness to the genus *Bacillus*. Apart from this, many *Bacillus* derived genera like *Brevibacillus*, *Aneurinibacillus*, and *Lysinibacillus* were also recorded.

Gram positive prokaryotes are known to be comparatively stress resistant, besides being long range migrants, especially the Firmicutes and Actinobacteria (Cerritos et al. 2011). The genus *Bacillus* and its related genera have been explored extensively for their applications in industry and agriculture, especially as a source of hydrolytic enzymes, because of their temperature tolerance, which is an inherent trait also attributed to this genus (Archana and Satyanarayana 2003). The members of this genus are spore formers and also produce a number of biocidal metabolites/enzymes, which makes them a common inhabitant of diverse extreme habitats (Nicholson et al. 2000). Enough evidence from recent studies for the reclassification of thermophilic bacteria in the genus *Bacillus* into *Brevibacillus, Aneurinibacillus*, *Amphibacillus*, *Virgibacillus, Alicyclobacillus, Paenibacillus, Halobacillus*, *Geobacillus* (Bae et al. 2005) has accumulated, based on the data of 16S rRNA gene sequence analysis. This supports the presence of genera *Brevibacillus* and *Aneurinibacillus* in the hot springs in our study. The presence of genera like *Micrococcus*, *Microbacterium*, *Staphylococcus*, *Kocuria* and *Exiguobacterium*, in our investigation is supported by the work of Cerritos et al. (2011), as thermoresistant aquatic bacteria. These apart, representatives from *Planococccus*, *Rhodococcus*, *Pseudomonas*, and *Chelatococcus* were also present. A novel species of *Chelatococcus* – *C. sambhunathii* sp. nov was isolated and characterized from hot spring sediments by Panday and Das (2010). Phylogenetic analyses of bacterial communities in hot springs from low and high elevations of the Tibetan peninsula revealed no significant influence of elevation on diversity (Huang et al. 2011). This illustrates the predominance of *Bacillus* as an aggressive coloniser of diverse types of extreme habitats of the world.

Table 4.2 Phylogenetic clusters based on ARDRA and sequenced isolates along with available closest sequences in NCBI Genebank

RFLP Pattern	**Representative Isolate**	**Accession number**	**Temperature tolerance**	**Nearest phylogenetic neighbour**	**16S Similitude**	**Division**
1	M1	JX312613	45°C	*Bacillus aryabhattai* (JQ904723)	100	Firmicutes
2	M2	JX312614	45°C	*Brevibacillus* sp. (AJ313027)	99	Firmicutes
3	M4	JX312615	70°C	*Bacillus licheniformis* (GQ280087)	99	Firmicutes
4	M5	JX312616	70°C	*Bacillus pumilus* (JQ435673)	99	Firmicutes
5	M6	KC315773	70°C	*Bacillus subtilis* (EF442670)	100	Firmicutes
6	M7	KC315774	70°C	*Bacillus pumilus* (HF536558)	100	Firmicutes
7	M8	KC315775	70°C	*Bacillus* sp. (KC121051)	100	Firmicutes
8	M9	KC315776	45°C	*Bacillus cereus* (GU011948)	98	Firmicutes
9	M10	JX312617	45°C	*Rhodococcus* sp. (DQ285075)	99	Actinobacteria
10	M11	JX312618	45°C	*Kocuria* sp. (DQ448711)	99	Actinobacteria
11	M12	JX312619	45°C	*Planococcus* sp. (JX312584)	100	Firmicutes
12	M13	JX312620	45°C	*Micrococcus* sp. (JN866765)	98	Actinobacteria
13	M21	JX312623	45°C	*Staphylococcus haemolyticus* (JQ624771)	99	Firmicutes
14	M25	KC315762	45°C	*Bacillus arbutinivorans* (FJ380988)	99	Firmicutes
15	M26	KC315763	45°C	*Bacillus* sp. (FN397517)	99	Firmicutes
16	M27	KC315764	45°C	*Bacillus niacini* (AB680904)	99	Firmicutes
17	M30	JX312624	45°C	*Bacillus vireti* (HQ397585)	99	Firmicutes
18	M35	JX312625	45°C	*Aneurinibacillus danicus* (NR_028657)	99	Firmicutes
19	M36	JX312626	45°C	*Brevibacillus* sp. (FJ529026)	99	Firmicutes
20	M40	JX312627	45°C	*Staphylococcus succinus* (HQ423378)	99	Firmicutes
21	M70	KC315777	70°C	*Staphylococcus succinus* (HQ423378)	99	Firmicutes
22	M41	JX312628	45°C	*Bacillus* sp. (JF901703)	99	Firmicutes
23	M43	KC315765	70°C	Uncultured *Klebsiella* sp.(GQ416648)	99	Proteobacteria

RFLP Pattern	**Representative Isolate**	**Accession number**	**Temperature tolerance**	**Nearest phylogenetic neighbour**	**16S Similitude**	**Division**
24	M44	JX312629	50°C	*Pseudomonas* sp. (EU680995)	99	Proteobacteria
25	M45	JX312630	55°C	*Microbacterium oxydans* (EU714340)	99	Actinobacteria
26	M46	KC315766	70°C	*Pseudomonas psychrophila* (JQ782895)	100	Proteobacteria
27	M49	KC315769	70°C	*Pseudomonas psychrophila* (JQ782895)	100	Proteobacteria
28	M47	KC315767	70°C	*Exiguobacterium acetylicum* (JX307688)	99	Firmicutes
29	M48	KC315768	70°C	*Rhodococcus baikunurensis* (JX683682)	100	Actinobacteria
30	M50	KC315770	70°C	*Pseudomonas fluorescens* (JX127246)	99	Proteobacteria
31	M52	JX312631	50°C	*Lysinibacillus xylaniticus* (JQ739716)	99	Firmicutes
32	M53	JX312632	55°C	*Staphylococcus hominis* (JN644561)	99	Firmicutes
33	M55	JX312633	70°C	*Bacillus megaterium* (EU931553)	99	Firmicutes
34	M59	JX312635	45°C	*Chelatococcus daeguensis* (NR_044297)	99	Proteobacteria
35	M61	JX312636	45°C	*Bacillus flexus* (JQ936679)	100	Firmicutes
36	M63	KC315771	45°C	*Bacillus* sp. (HE821233)	99	Firmicutes
37	M65	KC315772	45°C	*Bacillus subtilis* (JX845578)	99	Firmicutes
38	M68	JX312637	55°C	*Lysinibacillus* sp. (FN397524)	99	Firmicutes
39	M73	JX312638	45°C	*Brevibacillus agri* (HQ222834)	100	Firmicutes
40	M74	JX312639	45°C	*Bacillus beijingensis* (JQ799102)	100	Firmicutes
41	M75	JX312640	45°C	*Bhargavaea cecembensis* (JQ071510)	99	Firmicutes
42	M80	KC315778	45°C	*Microbacterium* sp.(DQ339613)	98	Actinobacteria

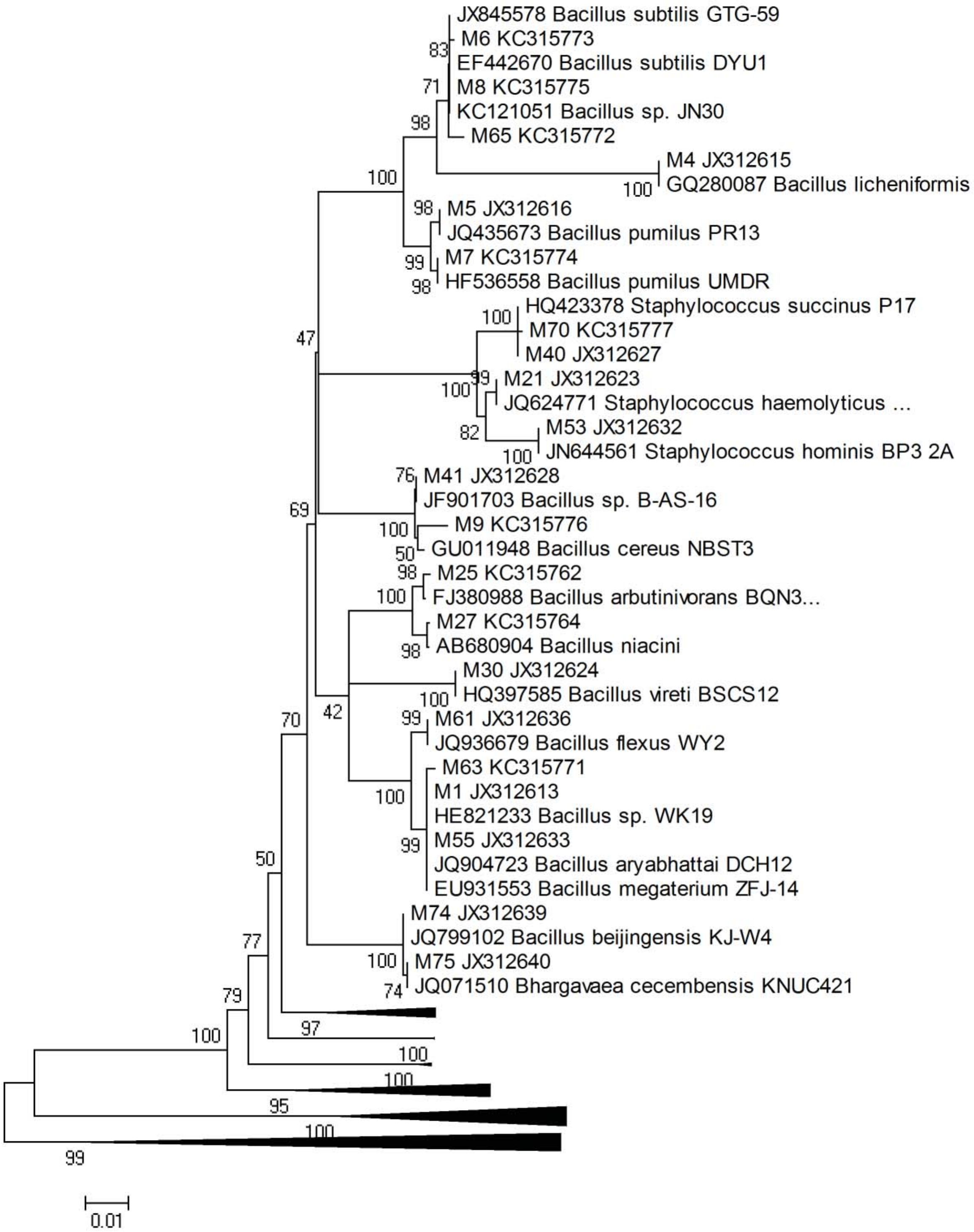

Figure 4.2 a) Unrooted phylogenetic tree based on comparison of 16S rDNA sequences of isolates with their closest phylogenetic relatives.

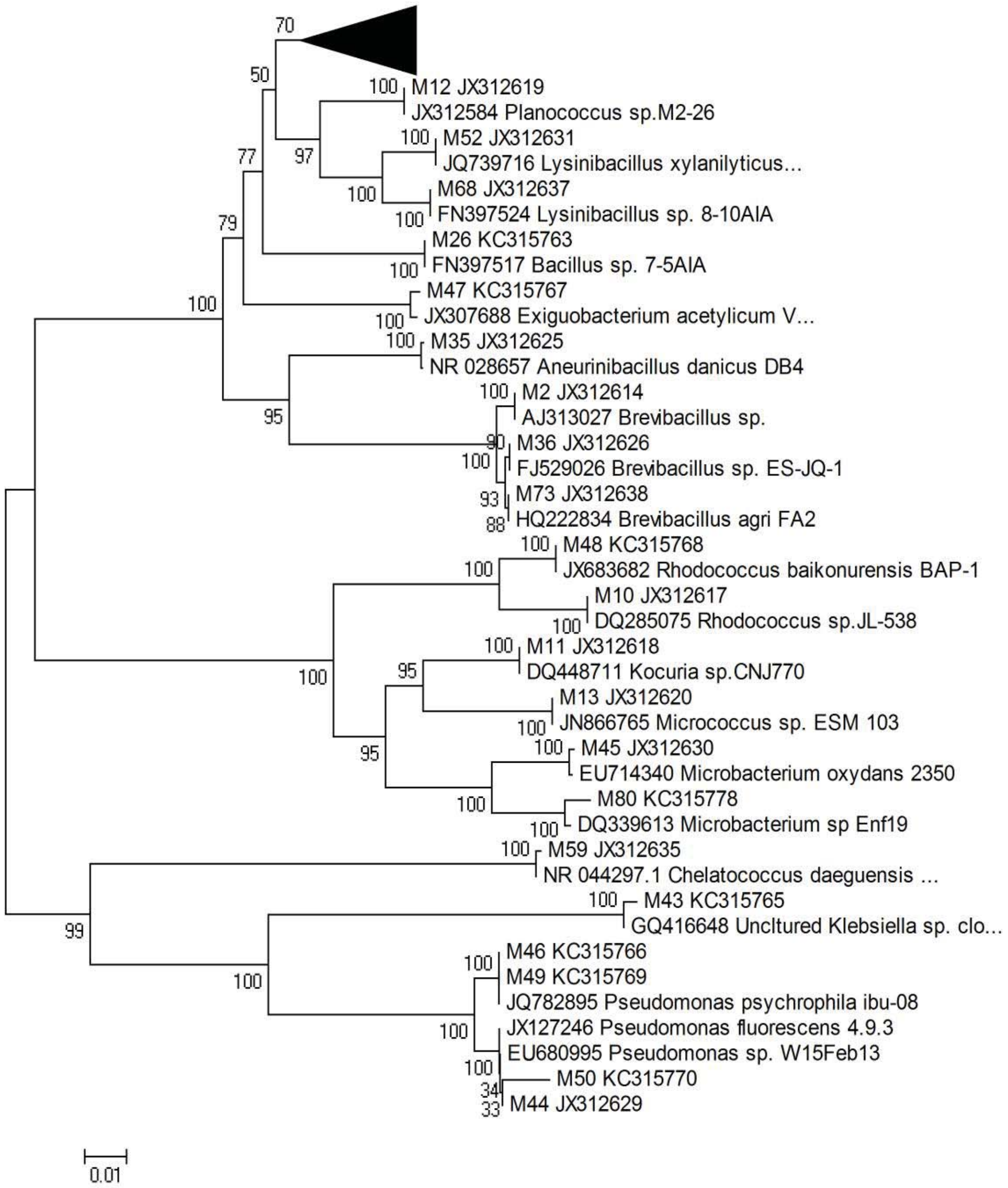

Figure 4.2 b) Unrooted phylogenetic tree based on comparison of 16S rDNA sequences of isolates with their closest phylogenetic relatives.

Biochemical tests and substrate utilization profiles for selected isolates using Biolog

Biolog based phenotyping was done for the set of 13 highly thermotolerant isolates, for deriving unique fingerprints of the isolates, besides revealing their ability to utilize routine and unusual substrates. This assumes immense importance in the characterization of microbial isolates from extreme environment and their potential utilization in agriculture and industry. The data on substrate utilization patterns and sensitivity to chemicals for the 13 temperature tolerant isolates were tabulated (Supplementary Table 4.1). Significant variation was observed among the isolates with regard to the utilization of sugars, sugar derivatives, metabolic intermediates and amino acids and peptides. A dendrogram was generated based on the data obtained through Biolog for the thirteen isolates and reference strains available in the database (Fig. 4.3). Among the 20 sugars tested, D-fructose was utilized by all isolates, except M55. Similarly α-D-glucose was utilized by all isolates, except M48, while 3-methyl glucose was utilized only by M43. Among the 13 isolates tested for utilization of sugars, isolate M43 was able to utilize all but one sugar (Raffinose) and M8 could utilize 17 sugars out of 20 tested. Isolate M55 was able to utilize only two sugars (α D-glucose and sucrose). Isolates M43 and M48 were able to utilize maximum number of sugar derivatives, 18 and 17 respectively. Among all the sugar derivatives, glucoronamide was utilized by 12 isolates followed by L-galactonic acid and D-gluconic acid (11 isolates). Isolate M55 was able to utilize only one sugar derivative, glucoronamide.

A total of 19 metabolic intermediates were tested for growth of isolates. Isolate M48 was able to utilize the maximum number of metabolic intermediates (15) followed by isolates M8 and M70, which were able to utilize 12 metabolic intermediates each. Isolate M55 utilized the least number of metabolic intermediates (2). The metabolic intermediate, D-fructose-6-PO_4 was utilized by all the isolates tested and L-malic acid was utilized by 11 isolates. P-hydroxy-phenylacetic acid, α-hydroxy-butyric acid and α-keto-butyric acid were the least utilized, and supported the growth of only two isolates. The results on the utilization of amino acid and peptides revealed that isolate M43 was able to use all the 12 substrates. Both M8 and M48 utilized 10 substrates each. The isolate M55 could not utilize all the substrates tested and only two substrates were utilized by isolate M50. Amino acids, L-alanine and L-glutamic acid were utilized by 9 isolates while L-arginine and L-serine were utilized by 7 isolates. Among the isolates, based on carbon utilization patterns, M55 (*Bacillus* sp.) was able to utilize only 7 %, while M43 *Klebsiella* sp. was able to utilize 85.7 % of the C substrates.

Sensitivity and Resistance to antimicrobials

Isolate M50 was found to be resistant to 15 antimicrobials out of 17 tested, closely followed by isolate M49 which was resistant to 13 antimicrobials. Isolate M6 showed resistance to least number of antimicrobial compounds (4) (Supplementary Table 4.2).

Supplementary Table 4.1 Substrate utilization patterns of the thermotolerant isolates

Substrates	**M4**	**M5**	**M6**	**M7**	**M8**	**M43**	**M46**	**M47**	**M48**	**M49**	**M50**	**M55**	**M70**
Saccharides													
α-D-Glucose	+	+	+	+	+	+	+	+	-	+	+	+	+
D – Mannose	+	-	+	-	+	+	+	+	-	+	+	-	+
D – Fructose	+	+	+	+	+	+	+	+	+	+	+	-	+
D – Galactose	-	-	-	-	+	+	+	+	-	+	+	-	+
3 – Methyl Glucose	-	-	-	-	-	+	-	-	-	-	-	-	-
D – Fucose	-	-	-	-	-	+	+	-	-	+	+	-	-
L – Fucose	-	-	-	-	+	+	+	-	+	+	+	-	+
L – Rhamnose	-	-	-	-	+	+	-	-	-	-	+	-	-
D – Maltose	+	-	-	-	+	+	-	+	-	-	-	-	+
D – Trehalose	+	-	+	-	+	+	-	+	-	+	-	-	+
D –Cellobiose	+	+	+	+	+	+	-	+	-	-	-	-	+
Gentiobiose	+	-	-	-	+	+	-	+	-	-	-	-	+
Sucrose	+	+	+	+	+	+	-	+	+	-	-	+	+
D – Turanose	+	-	-	-	+	+	-	+	-	-	-	-	+
α - D - Lactose	-	-	-	-	-	+	-	-	-	-	-	-	+
D – Melibiose	-	-	-	-	+	+	-	+	-	-	-	-	+
D-Raffinose	-	-	-	-	+	-	-	+	-	-	-	-	+
Stachyose	-	-	-	+	+	+	-	-	-	-	-	-	-
Dextrin	+	-	-	-	+	+	-	+	+	-	-	-	+
Pectin	-	+	-	+	+	+	-	+	-	-	-	-	+
Saccharide derivatives													
D – Sorbitol	+	-	+	-	+	+	-	-	+	-	-	-	+
D - Mannitol	+	-	+	-	+	+	-	+	+	-	-	-	+
D – Arabitol	-	-	-	-	-	+	-	-	+	-	-	-	+
myo - Inositol	+	-	+	-	+	+	-	+	+	-	-	-	+
Glycerol	+	-	+	-	+	+	+	+	+	+	-	-	+
β– Methyl – D - Glucoside	+	-	+	-	+	+	-	+	+	-	-	-	+
D – Salicin	+	+	+	+	+	+	-	+	+	-	-	-	+
D -Galacturonic acid	+	-	+	-	+	+	+	-	+	-	+	-	+

Substrates	**M4**	**M5**	**M6**	**M7**	**M8**	**M43**	**M46**	**M47**	**M48**	**M49**	**M50**	**M55**	**M70**
L – Galactonic acid	+	+	+	+	+	+	+	-	+	+	+	-	+
D – Gluconic acid	+	+	+	+	+	+	+	+	+	+	-	-	+
D – Glucuronic acid	+	-	-	-	+	+	+	-	+	+	+	-	+
Glucuronamide	+	+	+	+	+	+	+	+	-	+	+	+	+
Mucic acid	+	-	+	-	+	+	-	-	+	-	+	-	-
Quinic acid	-	+	-	+	-	+	+	+	+	+	+	-	-
D – Saccharic acid	+	-	+	-	-	+	-	+	+	-	-	-	-
N- Acetyl-D - Glucosamine	+	-	-	-	+	+	-	+	+	-	-	-	+
N – Acetyl – β - D - Mannosamine	-	-	-	-	+	-	-	+	+	-	-	-	+
N – Acetyl – D - Galactosamine	-	-	-	-	-	+	-	+	-	-	-	-	-
N – Acetyl Neuraminic acid	-	-	-	-	-	+	-	+	+	-	-	-	+
Metabolic intermediates													
D -Glucose - 6 -PO4	-	-	-	-	+	+	-	+	+	-	-	-	-
D -Fructose - 6 – PO4	+	+	+	+	+	+	+	+	+	+	+	+	+
p - Hydroxy -Phenylacetic acid	-	-	-	-	-	-	-	+	-	-	+	-	-
Methyl Pyruvate	-	-	-	-	-	+	+	-	-	-	-	-	+
D – Lactic acid Methyl Ester	-	-	-	-	+	+	-	+	+	-	-	-	+
L – Lactic acid	+	-	-	-	+	+	+	+	+	+	-	-	+
Citric acid	-	+	+	+	+	+	+	-	+	+	+	-	+
α - keto- Glutaric acid	-	+	-	+	+	-	+	-	+	+	-	-	+
D -Malic acid	-	+	-	+	-	-	+	-	-	+	-	-	-
L - Malic acid	+	+	+	+	+	+	+	+	+	+	-	-	+
α -Hydroxy -Butyric acid	-	-	-	-	-	-	-	-	+	-	-	-	-
β-Hydroxy - Butyric acid	-	-	-	-	-	+	-	+	+	-	-	-	-
α - keto- Butyric acid	-	-	-	-	-	-	-	-	+	+	-	-	-
Acetoacetic acid	-	-	-	-	+	-	-	+	+	+	+	-	+
Propionic acid	-	-	-	-	+	-	-	-	+	-	-	-	-
Acetic acid	-	-	-	-	+	+	-	+	+	-	+	-	+
Formic acid	-	-	-	-	+	-	-	-	-	-	-	+	+
γ – Amino Butyric acid	-	+	-	+	-	-	+	-	+	+	+	-	+

Substrates	**M4**	**M5**	**M6**	**M7**	**M8**	**M43**	**M46**	**M47**	**M48**	**M49**	**M50**	**M55**	**M70**
Bromosuccinic acid	-	+	-	+	+	+	-	-	+	-	-	-	+
Amino acids and peptides													
D -Aspartic acid	-	+	-	+	+	+	-	-	+	-	-	-	-
D - Serine	-	-	-	-	-	+	-	-	+	-	+	-	-
L-Alanine	+	+	+	+	+	+	-	+	+	-	-	-	+
L - Arginine	-	+	-	+	+	+	-	+	+	-	-	-	+
L -Aspartic acid	-	+	-	+	+	+	-	-	+	-	-	-	+
L – Glutamic acid	-	+	-	+	+	+	+	+	+	+	-	-	+
L - Histidine	-	-	-	-	-	+	-	-	+	-	-	-	+
L – Pyroglutamic acid	-	-	-	-	+	+	-	-	+	-	-	-	-
L - Serine	-	+	-	+	+	+	-	+	-	-	+	-	+
Inosine	-	-	-	-	+	+	-	+	-	-	-	-	-
Glycyl -L - Proline	-	-	-	-	+	+	-	+	+	-	-	-	+
Gelatin	-	+	-	+	+	+	-	+	+	-	-	-	-

Supplementary Table 4.2 Resistance against chemical agents and stress conditions

Antimicrobial compounds/Growth conditions	**M4**	**M5**	**M6**	**M7**	**M8**	**M43**	**M46**	**M47**	**M48**	**M49**	**M50**	**M55**	**M70**
Antimicrobial compounds													
Troleandomycin	-	-	-	-	-	+	+	-	-	+	+	-	-
Rifamycin	+	-	-	-	-	+	+	-	-	+	+	-	-
Minocycline	-	-	-	-	-	-	-	-	-	-	+	-	-
Fusidic acid	-	-	-	-	-	+	+	-	-	+	+	-	-
Lincomycin	+	-	-	-	-	+	+	-	-	+	+	-	-
Vancomycin	-	-	-	-	-	+	+	-	-	+	+	-	-
Nalidixic acid	-	-	-	-	-	-	+	+	+	+	+	-	+
Aztreonam	+	+	+	+	+	+	+	+	+	+	+	-	+
Guanidine HCL	+	+	-	+	+	+	+	+	+	+	+	+	+
Niaproofu	-	-	-	-	-	+	+	-	-	+	+	-	-
Potassium Tellurite	+	+	+	+	+	+	-	+	+	-	+	+	+
Sodium Butyrate	+	+	-	+	+	+	-	+	-	-	-	+	+
Sodium Bromate	-	-	-	-	-	-	-	+	-	-	-	-	-
1% Sodium Lactate	+	+	+	+	+	+	+	+	+	+	+	+	+
Lithium Chloride	+	-	+	-	+	+	+	+	-	+	+	+	+
Tetrazolium Violet	-	-	-	-	-	+	+	-	-	+	+	-	-
Tetrazolium Blue	-	-	-	-	-	+	+	-	-	+	+	-	-
Growth conditions													
pH6	+	+	+	+	+	+	+	+	+	+	+	+	+
pH5	+	+	+	+	+	+	+	-	-	+	+	-	+
1% NaCl	+	+	+	+	+	+	+	+	+	+	+	+	+
4% NaCl	+	+	+	+	+	+	+	+	-	+	+	+	+
8% NaCl	+	+	+	+	+	-	-	+	-	+	-	+	+

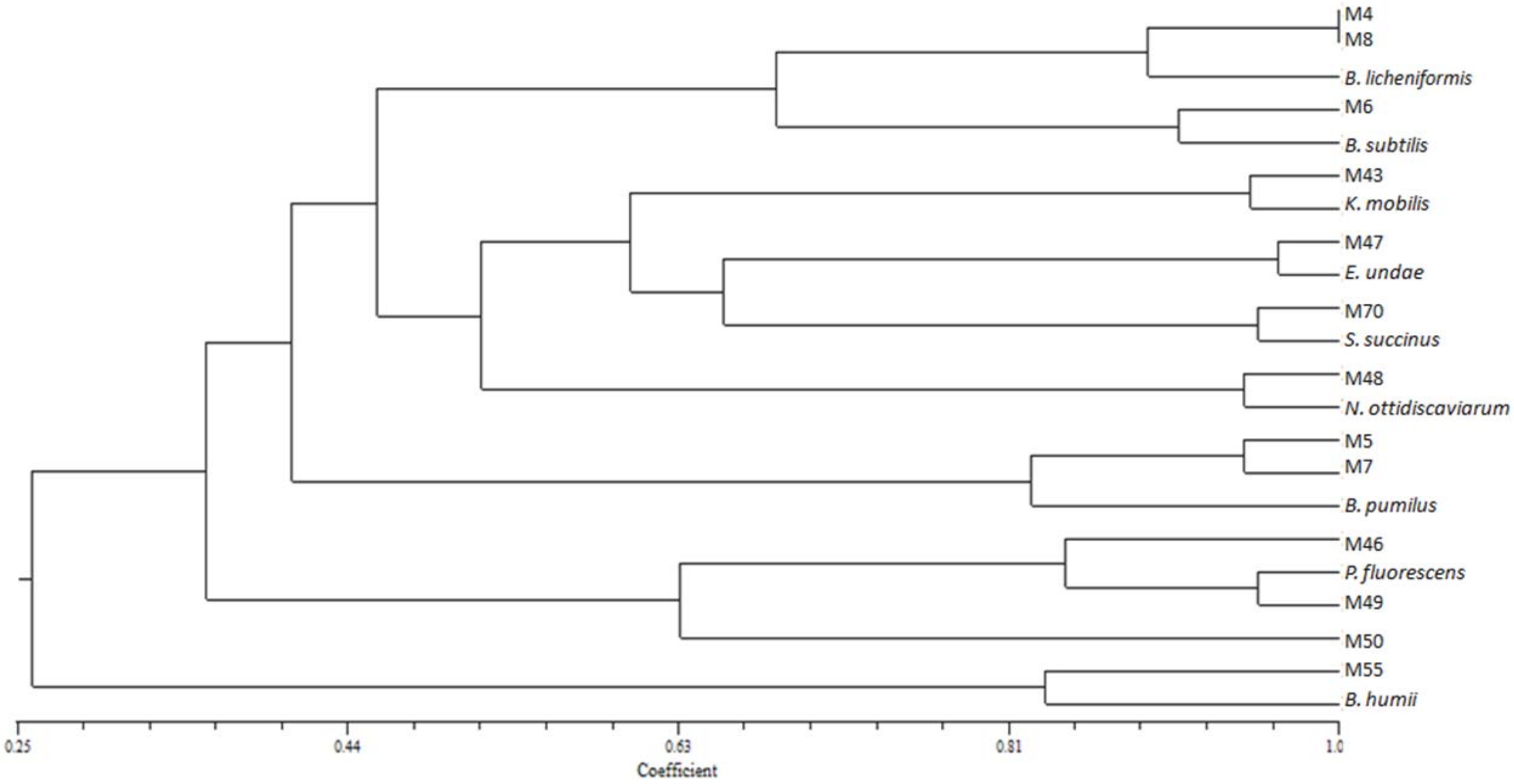

Figure 4.3 Dendrogram showing the clustering of thermotolerant isolates along with reference strains based on substrate utilization pattern generated by Biolog analyses.

In terms of resistance to chemical agents, sodium lactate which is known for its bactericidal properties and acts as a preservative, acidity regulator, and bulking agent, did not inhibit any of the isolates tested. This reveals the unusual nature of our isolates. Also, minocycline inhibited the growth of all the isolates, except M50 (*Psuedomonas fluorescens*), which was found to be most resistant to many of the chemical agents tested. The isolate M47 (*Exiguobacterium acetylicum*) was the only isolate capable of growth on sodium bromate.

Response of selected isolates to abiotic stress

The ability of isolates to show growth at low pH and in the presence of salt was tested and the results are tabulated (Supplementary Table 4.2). Seven isolates (M4, M5, M6, M7 M8, M49 and M70) were able to show growth at pH up to 5 and 8 % NaCl. Isolate M47 and M55 were able to grow up to 8 % NaCl concentration and a pH of 6. Isolate M48 was not able to grow either at low pH (5) or at high salt concentration (4/8 % NaCl). Growth at different pH (5 and 6) revealed that all the 13 isolates (originally isolated from a pH of 7.8-8.2) could grow at 6, but three isolates (M47, 48 and 50) were unable to show growth at 5. Salinity upto 1 % NaCl was tolerated by all the isolates; however M48 did not grow at higher salt concentrations (4/8 % NaCl). A total of 92 % isolates and 69 % of the thermotolerant isolates were able to grow at 4 and 8 % NaCl concentrations, revealing their promise for use in saline environments.

Biolog analysis in this study revealed interesting results, especially providing valuable information on the temperature tolerant isolates showing positive results in terms of growth in both low pH (pH up to 5) and high salt concentrations (NaCl concentration up to 8 %). Such isolates could be explored for their potential applications in agriculture and industry.

Identification based on Biolog analysis

Using the Biolog Microlog 3 software, the 13 temperature tolerant isolates were identified based on the profiles generated using GEN III MicroPlate™. The results of the identification of temperature tolerant isolates are given in Table 4.3, among which 6 belonged to the genus *Bacillus*; three to *Pseudomonas* and one each were placed in the genera, *Nocardia, Staphylococcus, Klebsiella* and *Exiguobacterium.* Biolog based identification of the temperature tolerant isolates in our study matched with 16S rRNA gene sequence based identification up to genera level, for all but one isolate (M48). This isolate M48 had a 16S rRNA gene sequence similitude with *Rhodococcus baikunurensis,* however, matched with *Nocardia ottidiscaviarum* based on Biolog based analyses. There are reports that C-utilization patterns and identification through Biolog have certain limitations, as it is difficult to distinguish closely related organisms (Singh et al. 2010). Also, limited availability exists on members belonging to Mycobacteria and Corynebacteria in the database.

Rhodococcus is a genus of aerobic, non-sporulating, non- motile Gram-positive bacterium, which is closely related to Mycobacteria and Corynebacteria and thrives in a wide range of environments. Its importance in biotechnological applications comes from

Table 4.3 Biolog based identification of the thermotolerant isolates

Isolates	**Biolog identity**	**16S rRNA gene sequence based identity**
M4	*Bacillus licheniformis*	*Bacillus licheniformis* (GQ280087)
M5	*Bacillus pumilus*	*Bacillus pumilus* (JQ435673)
M6	*Bacillus subtilis*	*Bacillus subtilis* (EF442670)
M7	*Bacillus pumilus*	*Bacillus pumilus* (HF536558)
M8	*Bacillus licheniformis*	*Bacillus sp.* (KC121051)
M43	*Klebsiella mobilis*	Uncultured *Klebsiella* sp. (GQ416648)
M46	*Pseudomonas fluorescens*	*Pseudomonas psychrophila* (JQ782895)
M47	*Exiguobacterium undae*	*Exiguobacterium acetylicum* (JX307688)
M48	*Nocardia ottidiscaviarum*	*Rhodococcus baikunurensis* (JX683682)
M49	*Pseudomonas fluorescens*	*Pseudomonas psychrophila* (JQ782895)
M50	*Pseudomonas fluorescens*	*Pseudomonas fluorescens* (JX127246)
M55	*Bacillus humii*	*Bacillus megaterium* (EU931553)
M70	*Staphylococcus succinus*	*Staphylococcus succinus* (HQ423378)

its ability to catabolize a wide range of compounds (including harmful pollutants), besides production of thermostable enzymes (Kuddus and Ramtekke 2012). In our study, the novelty of this strain was emphasized, as it was not capable of utilizing D-glucose or glucuronimide, but able to utilize α hydroxyl-butyric acid. This isolate exhibits unusual properties, as to date published literature reveals that the majority of hydroxyl-butyrate degrading bacteria belong to Gram negative genera such as *Acidovorax* /*Variovorax* sp. (Mergaert et al. 1993), and *Rhodococcus* is not reported in this context.

The bacterial community composition of Manikaran hot springs exhibited a phylogenetically distinct character, resembling in part, those previously reported from other geothermal hot springs (Pagaling et al. 2012; Sayeh et al. 2010), but comprising isolates with novel substrate utilization patterns, besides tolerance to biocidal agents and abiotic stress such as 8 % NaCl.

Research paper II

5. RESEARCH PAPER II

Evaluating the diversity of culturable thermotolerant bacteria from four hot springs of India

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Abstract

The present investigation was focussed towards determining the patterns of biogeographic differentiation of bacterial divisions/taxa/genera from four hot springs belonging to different parts of north and central India. Niche-specific genera were also identified and the number of morphotypes obtained employing different media included 60 from Bakreshwar, 48 from Balrampur, 46 from Chumathang and 52 from Vashisht hot springs, which were screened for their temperature tolerance (40 °C – 70 °C). A set of 112 isolates, based on their ability to grow at or above 45 °C were utilized for Amplified ribosomal DNA restriction analysis (ARDRA). Sequencing of 16S rRNA gene of the representative isolates followed by BLAST search, revealed that 86 %, 93 %, 44 % and 44 % of the sequenced isolates respectively from Bakreshwar, Balrampur, Chumathang and Vashisht hot springs belonged to Firmicutes. Actinobacterial members were present in all the four hot springs with Chumathang hot springs (18 % of the sequenced isolates) recording highest values. Members of Proteobacteria were present only in Chumathang and Vashisht hot springs, while members of Bacteroidetes were found only in Bakreshwar hot springs. To our knowledge, this is the first report of the presence of *Aurantimonas* and *Brevundimonas* isolates in hot springs. Identification using Biolog, based on carbon utilization patterns and sensitivity to chemicals was undertaken for three isolates (*Bacillus pumilu*s, *B. licheniformis*/*B.subtilis* and *Kocuria*/*Turicella* sp.), growing at or above 60 °C. Observations revealed the unique ability of these isolates, in terms of utilization of substrates and unusual resistance patterns, which can be useful for use as inoculants in high temperature habitats.

Keywords: ARDRA; Biolog; Culturable bacteria; Sequencing

Introduction

Among the many extreme environments on this planet, thermal springs are of considerable interest to researchers worldwide, since they are analogs of primitive earth. Microbial communities present in such habitats therefore constitute valuable sources for various biotechnological products (Satyanarayana et al. 2005; Sayeh et al. 2010). India is the home to several hot springs, most of which have been less explored in terms of biotic components (Ghosh et al. 2003), and limited published literature is available on the microbial diversity and their utility (Jha 1992; Ghosh et al. 2003).

Worldwide, studies on microbial communities in hot springs have mainly concentrated on habitats at low elevations like Yellowstone National Park (Mitchell 2009), Kamchatka in Russia, Iceland (Reigstad et al. 2009), Indonesia (Aditiawati et al. 2009) and Tunisia (Sayeh et al. 2010). Although intensive studies were carried out in terrestrial hot springs, very little is known about the bacterial diversity in thermal springs from highly elevated regions. The Himalayan Geothermal Belts (HGB) contains nearly 150 thermal springs. Chumathang, well known a site of high temperature geothermal manifestation is located in the HGB (Chandrasekharam 2000). Vashisht, in Himachal Pradesh is another hot spring located in proximity to Kullu (Pathania et al. 2012), which also comes under the HGB. Bakreshwar is a well known hot spring located in West Bengal, India. These hot springs show temperature in the range of 35 °C to 66.5 °C (Mukhopadhyay and Sarolkar 2012). Balrampur, located in Chattisgarh is the home to a low altitude hot spring, which is less investigated. In the present investigation, an attempt was made to compare the diversity of culturable bacteria in these four hot springs from northern and central regions of India and identify niche-specific bacterial genera/groups and promising thermotolerant isolates.

In the recent years there has been an increasing emphasis on metagenomic approaches to analyse diversity, especially of microorganisms (Sayeh et al. 2010; Huang et al. 2011; Pagaling et al. 2012). However, the methods involved are expensive, require high-throughput sequencing and in depth expertise on bioinformatic tools. Therefore, in the current scenario, most of the diversity studies rely on traditional methods of isolating the microorganisms, culturing, bacterial communities from various environments, complemented with PCR based approaches. Such methods of isolating the microbes on standard media and culturing, followed by their characterization have been the mainstay of microbiology, since their origins in the pioneering works of Robert Koch and Louis Pasteur. Traditional methods of isolation and culturing have several limitations and can be considered inefficient in a way that they cannot recover symbiotic, slow growing organisms and viable but non-culturable fractions that are believed to make up the bulk of environmental samples (Piterina and Pembroke 2010). However, the value of such less competitive microbes or with low counts is questionable. Therefore, despite limitations, the advantage of culture based diversity studies outweighs metagenomic approaches in the opportunity it offers to generate a large and valuable germplasm. Also, the main advantage in working with isolated strains is that they can be preserved for further studies and can be explored for biotechnological applications as and when the need arises (Akanbi et al. 2010; Acharya and Chaudhary 2012).

Advances in molecular biology techniques such as fatty acid methyl ester analysis, rep-PCR profiling and sequencing a stable part of genetic code have provided an excellent opportunity for complementing the identification and characterization of bacteria at species and subspecies levels (Adiguzel et al. 2009). Candidate genes used for these phylogenetic studies included as many as 20 including 5S rRNA, 16S rRNA, 23S rRNA and 16-23S rRNA internal transcribed spacer (ITS) region. Among all these genes, 16S rRNA is considered the best evolutionary chronometer because (i) it is universally present in all bacteria; (ii) its function over time has not changed, suggesting that random sequence changes are a more accurate measure of time (i.e. evolution); and (iii) the 16S rRNA gene (1,500 bp) is large enough for bioinformatic analyses (Patel 2001). Amplified ribosomal DNA restriction analysis (ARDRA) represents a further improvement in the analysis of 16S rRNA genes and involves amplification of 16S rRNA gene followed by digestion of amplified product using selected restriction enzymes and generation of restriction patterns. Restriction patterns can then be compared (Yadav et al. 2010) and analysed using bioinformatic tools. The utility of ARDRA in genotypic characterization of bacteria (Divya et al. 2010) and screening clone libraries (Sklarz et al. 2009) for grouping them into phylogenetic clusters is undisputed. ARDRA has been utilized in the past for identification of bacteria up to species level also (Krizova et al. 2006).

In the present investigation on diversity of thermotolerant bacteria in four hot springs of India (Bakreshwar, Balrampur, Chumathang and Vashisht), the culture based method of isolating bacteria on different media, followed by screening of isolates for temperature tolerance was followed. ARDRA analysis was done for phylogenetic clustering of the thermotolerant isolates, and sequencing of 16S rRNA gene of representative thermotolerant strains was undertaken for identification. Niche specific taxonomic groups and genera of bacteria were also defined. A set of selected highly thermo tolerant strains were further characterized using Biolog and other useful biochemical attributes.

Materials and Methods

Sampling and Isolation of bacteria

Four thermal springs of India - Bakreshwar, Balrampur, Chumathang and Vashisht were selected as sites for collecting water samples, which were brought to the laboratory in thermos flasks within 12 hours. These samples were then processed for the isolation of culturable bacteria. The physico-chemical characteristics of the water samples were tested in terms of pH and temperature. In order to capture the majority of culturable bacteria present in these samples, five different media were used for enumeration and isolation, using standard spread plate technique and incubated at 37 °C for 48-72 h. Details on different media employed were given in our earlier study (Kumar et al. 2013). The total viable count was recorded in the different media. Based on colony morphology, a set of 60, 48, 46, and 52 morphotypes were selected from Bakreshwar, Balrampur, Chumathang and Vashisht respectively. These isolates were axenized and maintained for further characterization.

Screening for temperature tolerance

All the isolates from each of the four springs were screened for temperature tolerance by spotting the cultures on specific media at different temperatures *viz* 40, 45, 50, 55 and 60 for 72 h. Isolates growing at 60 °C on solid media were tested further at temperatures beyond 60 °C in liquid media by incubating them in water bath-shaker.

General molecular techniques and Bioinformatic analyses

Methods of DNA extraction, PCR amplification of 16S rDNA, ARDRA, 16S rRNA gene sequencing and phylogenetic analysis were as followed in our earlier studies (Kumar et al. 2013).

Biolog analyses of selected isolates

A set of isolates capable of growing upto a temperature of 60 °C and above were characterized using the GEN III MicroPlate™, to generate a profile based on 92 biochemical tests (70 Carbon sources and 22 Chemical sensitivity tests). Biolog's Microbial Identification System software was used to identify the bacterium from its phenotypic pattern in the GEN III MicroPlate.

Results and Discussion

Hot springs are manifestations of geological activity and represent extreme environments which have been mainly explored in terms of their physico-chemical characteristics. The major focus of such studies has been mainly towards their potential as sources for electricity generation, radon monitoring, health hazards associated and their utility in prediction of earthquakes (Cinti et al. 2009). Among the different extreme environments, thermal springs are analogous to primitive earth and hydrothermal processes on Mars. Hence, the microbial communities thriving in geothermal hot springs have been the subject of extensive research (Tobbler and Benning, 2011). Ever since Thomas Brock discovered the presence of *Thermus aquaticus* in the thermal vents of Yellowstone National Park, a number of research groups started exploring similar environments all around the world (Kumar et al. 2004; Aditiawadi et al. 2009; Mitchell 2009; Reigstad et al. 2009; Sayeh et al. 2010). These studies brought out the unusual and novel aspects of diversity of microbial communities present in these environments and also revealed that the major factors influencing abundance and diversity of microbes are pH, temperature, dissolved hydrogen sulphide levels and biogeography (Huang et al. 2011). Despite intensive studies on geothermal springs, scanty information is available on diversity of bacteria in moderate altitudes or highly elevated regions.

The Indian subcontinent is a host to innumerable number of geothermal hot springs, especially the Himalayan Geothermal Belt which contains close to 150 thermal springs.

Chumathang which is at an elevation of 4023 m above mean sea level and Vashisht, which is at an elevation of 1982 m come under HGB. Bakreshwar hot springs present in Eastern part of India, is at an elevation of 84 m above mean sea level and Balrampur hot springs present in central India, is located at an elevation of 623 m above mean sea level. Our investigation focussed on the diversity of culturable thermotolerant bacteria from hot springs present in both the high altitude region and plains. Chumathang, Vashisht and Balrampur hot springs represent high altitude regions in the descending order of altitude and Bakreshwar represents the geothermal springs of plains (Fig. 5.1). Among the different samples, the temperature was highest in the Balrampur hot springs and lowest in Vashisht hot springs.

Enumeration and isolation of culturable bacteria

Five different types of media were employed to capture the maximum possible diversity. These included a nutrient rich medium (Nutrient Agar) suitable for most nutritional types, a medium suitable for oligotrophs (R2A), specific media designed for thermophiles (Thermus Agar and Thermus Peptone Meat extract Yeast extract medium) and a medium mostly used for *Pseudomonas* and related genera (King's B medium). Enumeration of bacterial population revealed that aerobic heterotrophic bacteria in Bakreshwar thermal springs ranged from 119 to 183 × 10^4 CFU/mL and a similar trend was recorded for Vashisht thermal springs, which was in the range of 158 to 229 × 10^4 CFU/mL. Among the different media used, R2A yielded highest population of 229 × 10^4 CFU/mL from Vashisht samples and for Bakreshwar samples, while TPMY yielded the highest population of 183 × 10^4 CFU/mL. In the samples from Balrampur and Chumathang, the population of aerobic heterotrophs were in the range from 89 to 193 × 10^3 CFU/mL and 69 to 112 × 10^4 CFU/mL respectively (Supplementary Table 5.1). Thermus agar yielded highest values from both Balrampur and Chumathang samples. A total of 60, 48, 46 and 52 morphotypes were selected from Bakreshwar, Balrampur, Chummathang and Vashisht hot spring samples respectively, for further studies.

Screening for temperature tolerance

From the selected morphotypes, only 27, 38, 18, and 29 isolates from Bakreshwar, Balrampur, Chumathang and Vashisht were able to grow at 45 °C. Seven isolates were able to grow at 50 °C, while five were able to grow at 55 °C and three were able to grow at 60 °C. Only one isolate was able to grow at 70° C. Interestingly, only the isolates from Bakreshwar were able to grow at temperatures beyond 45 °C, although Bakreshwar hot springs did not exhibit the highest temperature among the four hot springs. This is in consonance with reports regarding the absence of a monotonic relation between temperature stress and microbial community present (Mitchell 2009; Lau et al. 2006). The microbial community obtained from these hot springs encompasses mesophiles also, which reveals the possibility of syntrophic partnerships of such high temperature adapted mesophiles existing with more thermotolerant

Supplementary Table 5.1 Media based quantification of bacteria from the four investgated hot springs

Media	**Total Viable count (10^4 CFU/ml)**			
	Bakreshwar	**Balrampur**	**Chumathang**	**Vashisht**
Nutrient Agar	156	18.1	112	223
Thermus Agar	174	19.3	123	207
TPMY	183	11.3	69	215
King’s B	119	12.6	83	158
R2A	136	8.9	95	229

TPMY-Thermus peptone meat extract yeast extract

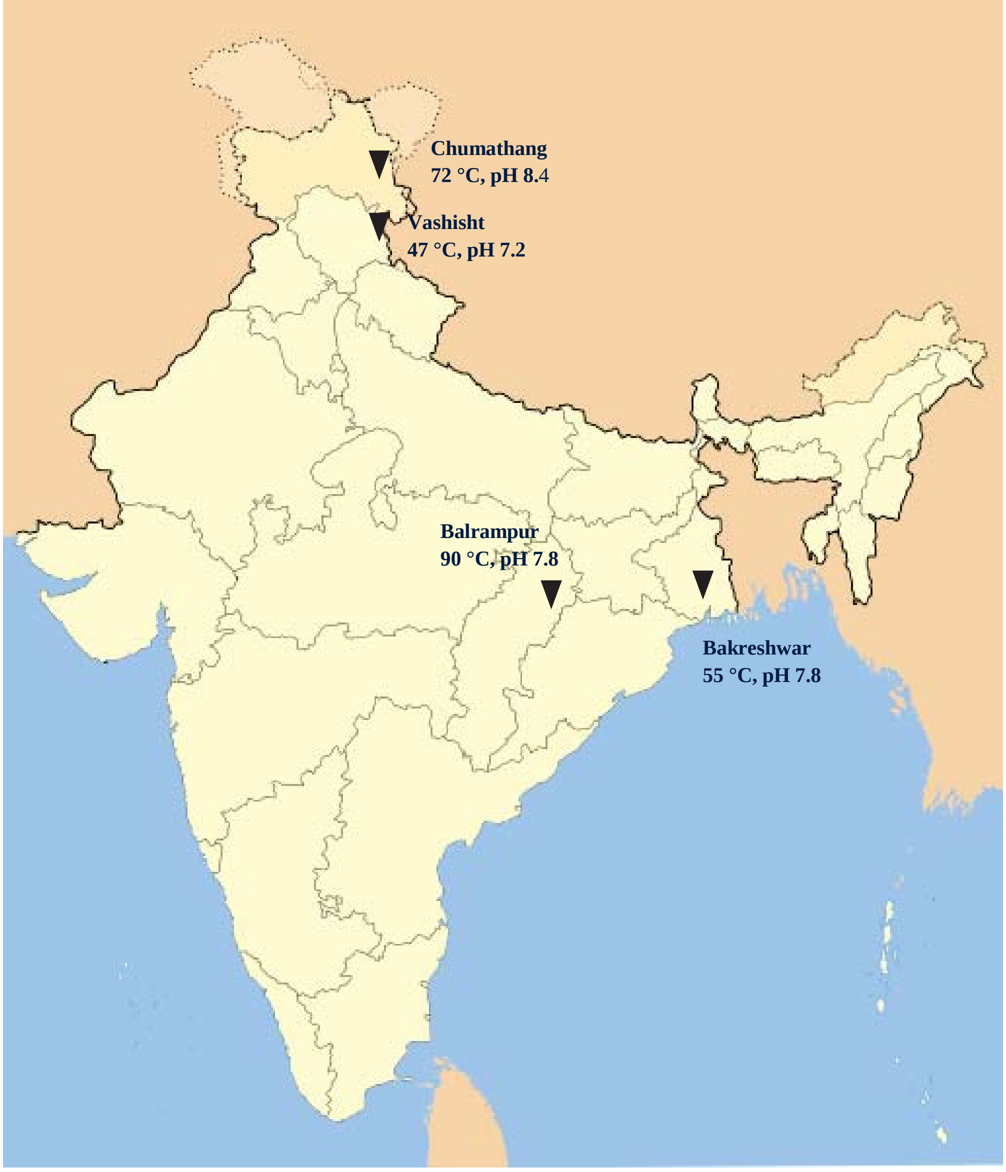

Figure 5.1 Map showing the location of the four hot springs and their physico-chemical characteristics.

non culturable bacteria, as has been recorded in several extreme habitats (Mitchell 2009). Metagenomic analyses can help further thro light on our observations. Isolates which were able to grow at 45 °C and above were selected for molecular characterization.

Amplified rDNA restriction analysis (ARDRA)

PCR amplification of 16S rRNA gene yielded a single amplicon of 1.5 Kbp from all the isolates. The amplicons were digested with three restriction enzymes, the different patterns comprising of 3 to 6 fragments ranging in size from 100 to 800 bp, were characterized. ARDRA revealed that restriction digestion with *Alu*I was more discriminative, as compared to *Msp*I and *Hae*III. A combined dendrogram (based on patterns generated using three enzymes individually) was constructed to determine the percent similarity among the isolates from each of the three thermal springs. At 90 % similarity level, thermotolerant isolates from Bakreshwar, Balrampur, Chumathang and Vashisht thermal springs grouped into 14, 14, 11 and 9 clusters respectively. ARDRA has been effectively utilized in the past for both culture dependent and culture independent diversity studies as a tool to group the isolates and clones into phylotypes (Sklarz et al. 2009; Divya et al. 2010; Yadav et al. 2010).

16S rDNA sequencing and phylogenetic analysis

Representative isolates from each cluster from each of the four thermal springs were sequenced and the data was analysed by BLAST and the nearest match from the GenBank database is illustrated (Table 5.1). Among the 14 representative isolates sequenced from Bakreshwar hot springs, 10 belonged to the genera *Bacillus*. Sequence results from Chumathang hot springs revealed that 4 out of 11 isolates were *Bacillus*. Two isolates of the 9 representative isolates of Vashisht also belonged to *Bacillus*. In Balrampur samples, out of 14 representative isolates, 12 belonged to the genus *Bacillus*. A taxonomic grouping of the isolates based on the divisions of Eubacteria revealed that Firmicutes was the dominant division in both Bakreshwar and Balrampur, but Chumathang and Vashisht hot water springs exhibited a greater diversity with both containing members from Firmicutes, Proteobacteria and Actinobacteria. Actinobacterial members were present in all the four hot springs studied and Chumathang hot springs recorded highest number i.e. 18% of the sequenced isolates. Members of Proteobacteria were present only in Chumathang and Vashisht hot springs and members of Bacteroidetes were found only in Bakreshwar hot springs (Fig. 5.2). Phylogenetic trees were constructed from the sequences of the representative isolates from each of the four thermal springs along with closest sequences in the NCBI Genebank (Fig. 5.3, 5.4, 5.5 and 5.6). The use of 16S rDNA sequencing in combination with ARDRA for grouping into phylogenetic clusters and identification of bacteria is well documented (Krizova et al. 2006; Sklarz et al. 2009; Divya et al. 2010). Although the number of phylogenetic clusters is less in Vashisht, the average bacterial population was highest in Vashisht and the lowest in

Table 5.1 Phylogenetic clusters and sequenced representative isolates of thermal springs

RFLP Pattern	Representative isolates	Temperature tolerance	GeneBank accession number	Nearest phylogenetic neighbour	16S rDNA Similitude (%)	Division
Bakreshwar						
1	B1	50°C	KC492101	*Bacillus cereus* (GQ375259)	99	Firmicutes
2	B3	60°C	KC492102	*Bacillus pumilus* (JX841107)	99	Firmicutes
3	B4	45°C	JX312592	*Bacillus licheniformis* (JN366726)	99	Firmicutes
4	B6	45°C	JX312593	*Pontibacter niistensis* (FJ897494)	98	Bacteroidetes
5	B9	45°C	JX312594	*Bacillus* sp. (DQ448753)	99	Firmicutes
6	B10	45°C	JX312595	*Lysinibacillus fusiformis*(EU430993)	99	Firmicutes
7	B16	45°C	JX312596	*Bacillus flexus* (DQ837543)	99	Firmicutes
8	B19	45°C	KC492103	*Bacillus* sp. (JX402419)	99	Firmicutes
9	B21	50°C	JX312597	*Bacillus megaterium* (EU931553)	99	Firmicutes
10	B25	55°C	JX312598	*Exiguobacterium* sp. (AY745848)	99	Firmicutes
11	B27	55°C	JX312599	*Bacillus* sp.(EU584536)	99	Firmicutes
12	B31	60°C	KC492104	*Bacillus licheniformis* (AB301007)	99	Firmicutes
13	B36	45°C	KC492106	*Bacillus* sp. (AB681430)	99	Firmicutes
14	B38	70°C	KC492107	*Kocuria* sp. (JF834545)	100	Actinobacteria
Balrampur						
1	BC1	45°C	JX312575	*Bacillus cereus* (JQ739719)	100	Firmicutes
2	BC2	45°C	JX312576	*Brevibacillus* sp. (AJ313027)	99	Firmicutes
3	BC5	45°C	JX312578	*Bacillus pumilus* (JQ435673)	99	Firmicutes
4	BC6	45°C	JX312579	*Bacillus aryabhattai* (HQ242769)	100	Firmicutes
5	BC7	45°C	JX312580	*Bacillus firmus* (JQ249909)	100	Firmicutes
6	BC9	45°C	JX312581	*Bacillus megaterium* (EU931553)	100	Firmicutes
7	BC10	45°C	JX312582	*Bacillus* sp. (HM567109)	100	Firmicutes
8	BC11	45°C	JX312583	*Bacillus flexus* (HQ875778)	99	Firmicutes

RFLP Pattern	Representative isolates	Temperature tolerance	GeneBank accession number	Nearest phylogenetic neighbour	16 Similitude (%)	Division
9	BC12	45°C	JX312584	*Planococcus* sp. (EF471920)	100	Firmicutes
10	BC16	45°C	JX312587	*Bacillus licheniformis* (GU377281)	99	Firmicutes
11	BC17	45°C	JX312588	*Lysinibacillus* sp. (FN397524)	100	Firmicutes
12	BC21	45°C	JX312589	*Staphylococcus haemolyticus* (JQ624771)	99	Firmicutes
13	BC27	45°C	JX312590	*Rhodococcus* sp.(DQ285075)	99	Actinobacteria
14	BC31	45°C	JX312591	*Exiguobacterium acetylicum* (HM047519)	100	Firmicutes
Chumathang						
1	LC4	45°C	JX312600	*Paracoccus* sp. (AY864654)	99	Proteobacteria
2	LC5	45°C	JX312601	*Cellulosimicrobium cellulans* (EU931556)	99	Actinobacteria
3	LC7	45°C	JX312602	*Aurantimonas altamirensis* (AB682666)	99	Proteobacteria
4	LC8	45°C	JX312603	*Bacillus pumilus* (JF803782)	99	Firmicutes
5	LC10	45°C	JX312604	*Kocuria palustris* (HM218471)	100	Actinobacteria
6	LC11	45°C	JX312605	*Klebsiella* sp. (GU290319)	100	Proteobacteria
7	LC13	45°C	JX312607	*Bacillus* sp. (JF901711)	100	Firmicutes
8	LC15	45°C	KC492091	*Bacillus megaterium* (JX997394)	99	Firmicutes
9	LC17	45°C	JX312609	*Brevundimonas terrae* (NR_043726)	100	Proteobacteria
10	LC19	45°C	JX312610	*Staphylococcus arlettae* (JN315890)	100	Firmicutes
11	LC24	45°C	JX312612	*Bacillus firmus* (JQ249909)	100	Firmicutes
Vashisht						
1	V1	45°C	KC492092	*Bacillus pumilus* (HF536558)	100	Firmicutes
2	V6	45°C	KC492093	*Acinetobacter baumanii* (JQ337945)	99	Proteobacteria
3	V7	45°C	KC492094	*Acinetobacter junii* (JX307680)	100	Proteobacteria
4	V8	45°C	KC492095	*Enterobacter cloacae* (JF894166)	100	Proteobacteria
5	V13	45°C	KC492096	*Kocuria* sp. (JF778721)	99	Actinobacteria
6	V21	45°C	KC492097	*Exiguobacterium* sp. (GU397441)	99	Firmicutes

RFLP Pattern	Representative isolates	Temperature tolerance	GeneBank accession number	Nearest phylogenetic neighbour	16 Similitude (%)	Division
7	V26	45°C	KC492098	*Bacillus subtilis* (JX502843)	99	Firmicutes
8	V28	45°C	KC492099	*Gulbenkiania mobilis* (NR_042548)	99	Proteobacteria
9	V29	45°C	KC492100	*Brevibacillus* sp. (DQ116777)	100	Firmicutes

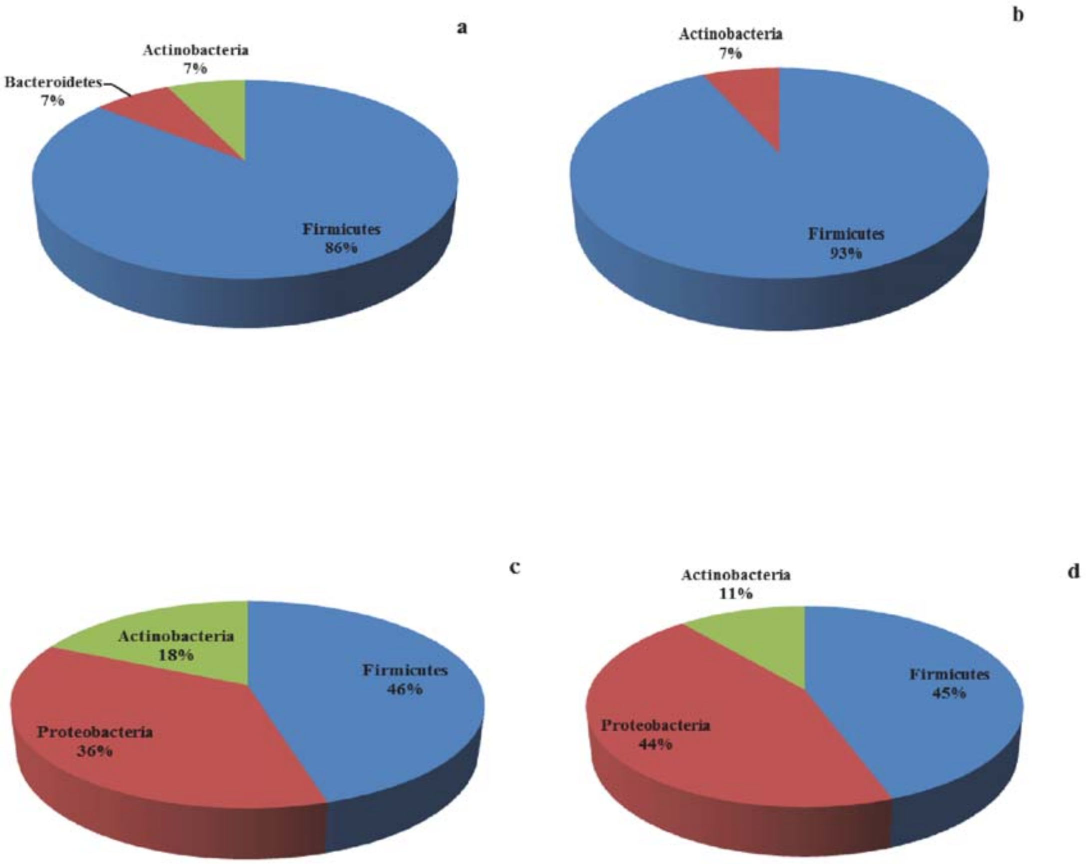

Figure 5.2 Distribution of different Divisions of Eubacteria in
a) Bakreshwar hot springs
b) Balrampur hot springs
c) Chumathang hot springs
d) Vashisht hot springs

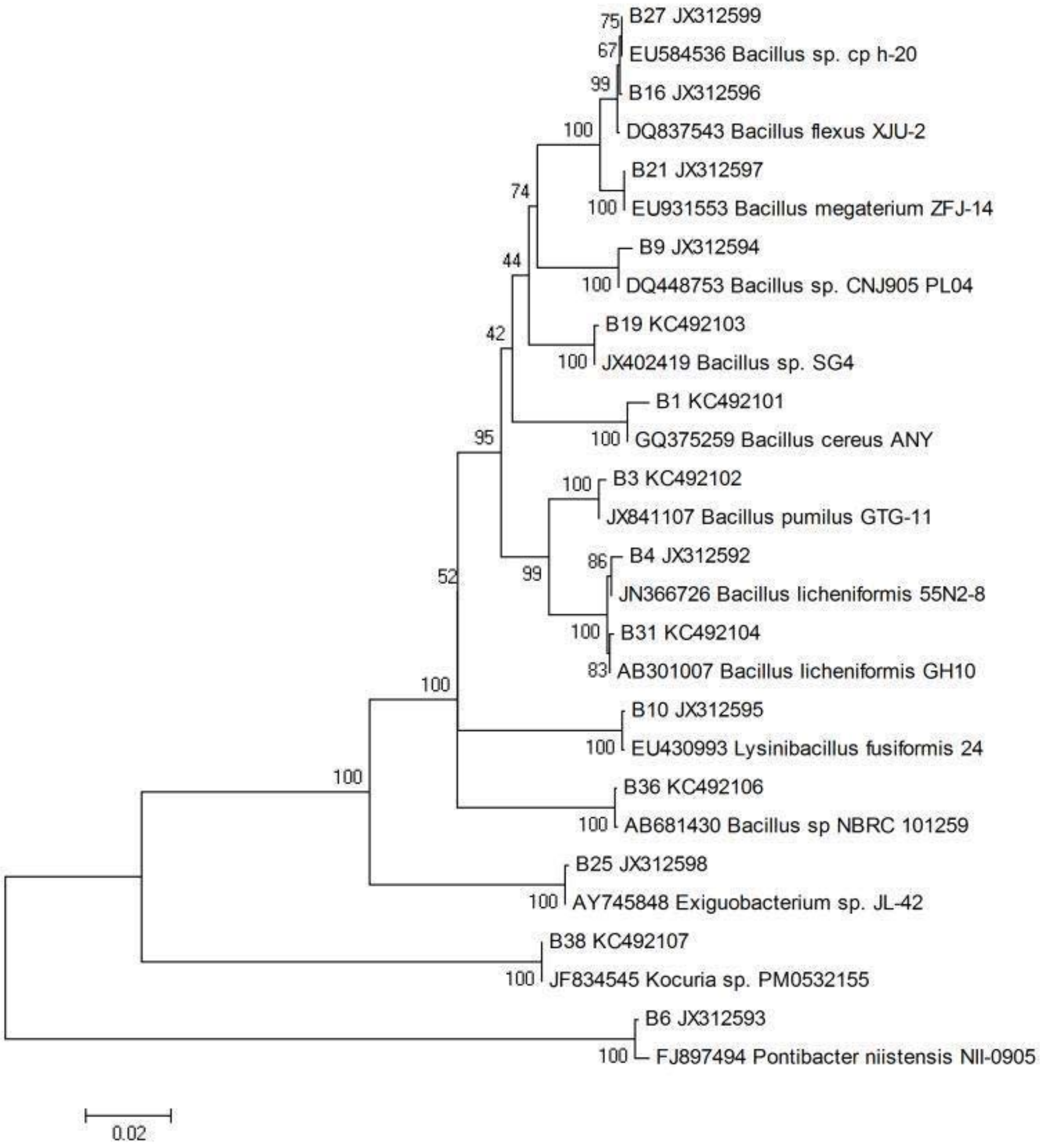

Figure 5.3 Unrooted phylogenetic tree based on comparison of 16S rDNA sequences of 14 isolates of Bakreshwar along with closest phylogenetic relatives.

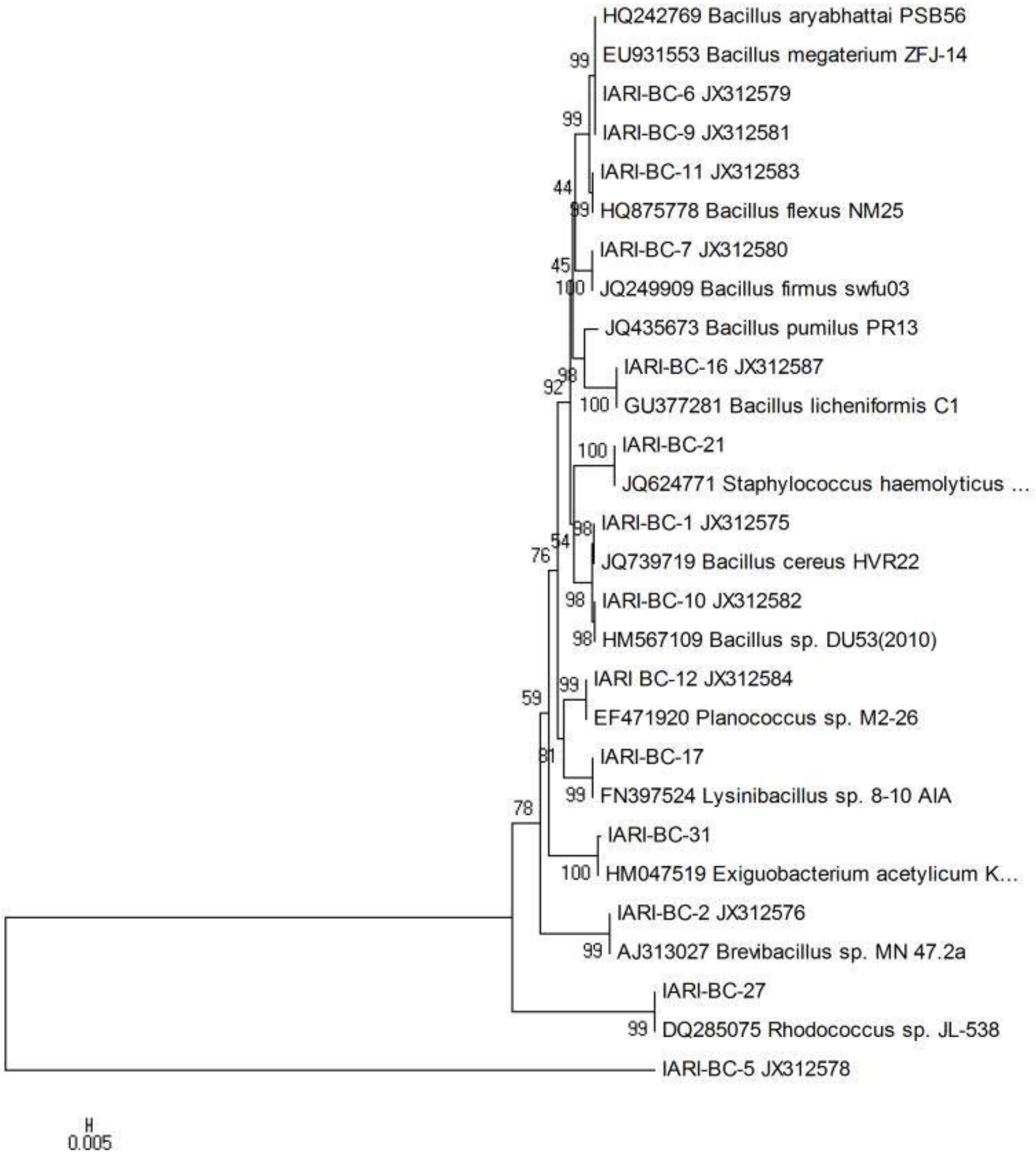

Figure 5.4 Unrooted phylogenetic tree based on comparison of 16S rDNA sequences of 14 isolates of Balrampur along with closest phylogenetic relatives.

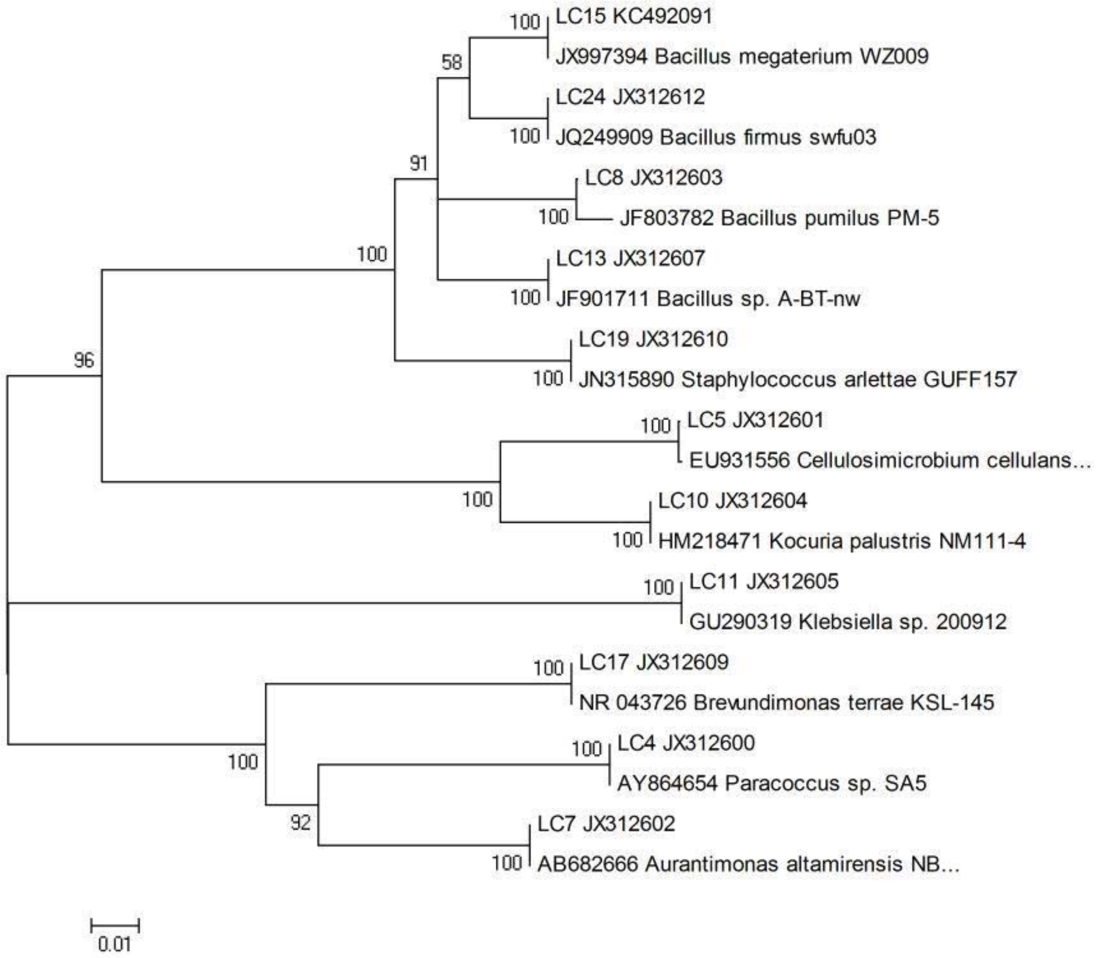

Figure 5.5 Unrooted phylogenetic tree based on comparison of 16S rDNA sequences of 11 isolates of Chumathang along with closest phylogenetic relatives.

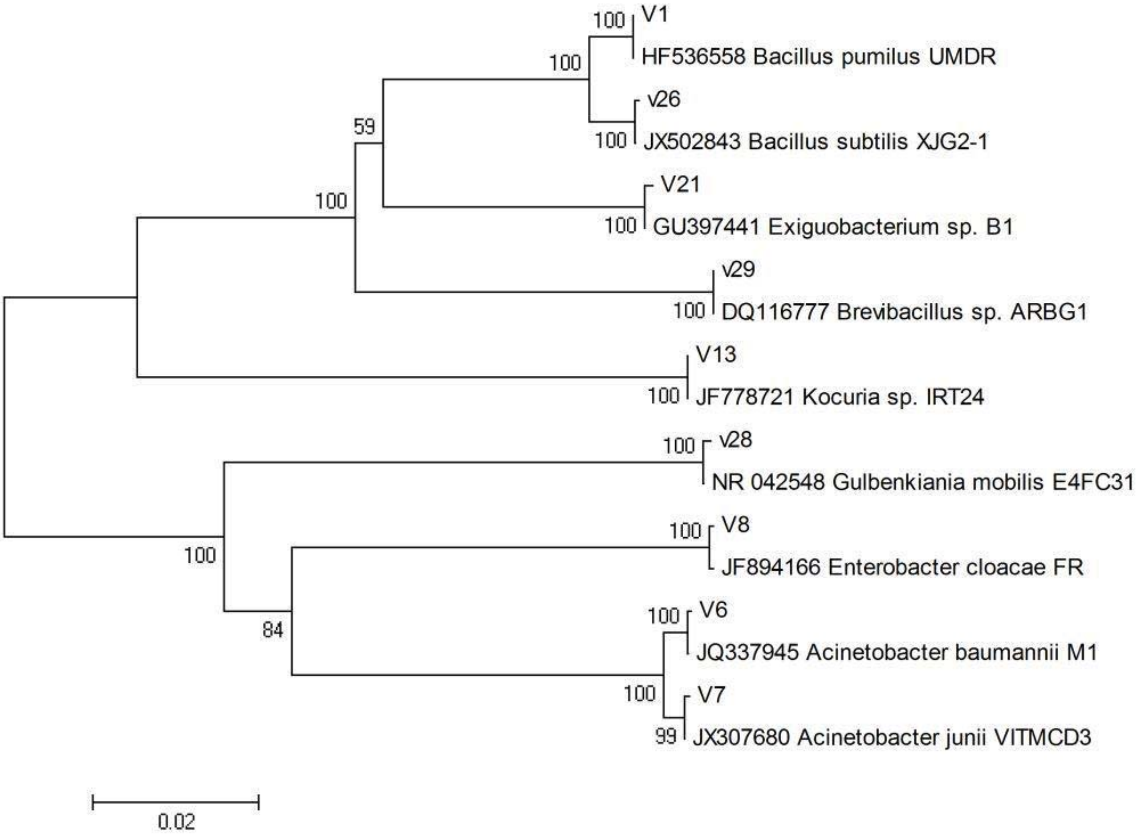

Figure 5.6 Unrooted phylogenetic tree based on comparison of 16S rDNA sequences of nine isolates of Vashist along with closest phylogenetic relatives.

Balrampur. This may be related to the variation in the temperature of the springs, which was least for Vashisht and highest for Balrampur. Temperature showed a significant negative correlation with CFU (r = -0.9950). It is interesting to note that the pH of Vashisht hot spring is also in the neutral range, suggesting the role of temperature and pH in bacterial abundance in geothermal waters, which is in accordance with earlier investigations (Purcell et al. 2007).

In the present study, 16S rRNA gene sequencing of representative isolates from each phylotype, followed by BLAST search revealed that a majority of sequences from both Balrampur and Bakreshwar (71.4 % and 85.7 % respectively) showed closeness to *Bacillus*, whereas only 36.4 % and 22.2 % of the sequenced isolates from Chumathang and Vashisht repectively showed closeness to *Bacillus*. Apart from *Bacillus*, representatives from *Bacillus* derived genera were present in both Bakreshwar (*Brevibacillus* and *Lysinibacillus*) and Vashisht (*Brevibacillus*) hot springs. The members of the genus *Bacillus* are spore formers and also produce a number of biocidal metabolites/enzymes which makes them a common inhabitant of diverse extreme habitats (Nicholson et al. 2000). Sufficient evidence exists for reclassification of thermophilic bacteria in the genus *Bacillus* into *Brevibacillus*, *Aneurinibacillus*, *Amphibacillus*, *Virgibacillus*, *Alicyclobacillus*, *Paenibacillus*, *Halobacillus* and *Geobacillus* based on the 16S rRNA gene sequence (Adiguzel et al. 2009).

A majority of sequenced isolates from all the four hot springs belonged to Firmicutes division. It was found that 86 %, 93 %, 44 % and 44 % of the sequenced isolates respectively from Bakreshwar, Balrampur, Chumathang and Vashisht hot springs belonged to Firmicutes (Fig. 5.2). The bacterial communities in many hot springs are predominated by Firmicutes (Huang et al. 2011, Tobler and Benning 2011; Pagaling et al. 2012). Another commonality in these four hot springs is the presence of isolates belonging to Actinobacteria division, which represent 18 % of the isolates. Gram positive prokaryotes are known to be comparatively stress resistant, besides being long range migrants, especially the Firmicutes and Actinobacteria (Cerritos et al. 2011). The bacterial communities in many hot springs studied are predominated by Firmicutes (Huang et al. 2011; Tobler and Benning 2011; Pagaling et al. 2012).

It is interesting to note that the hot springs in Himalayan Geothermal Belt (Chumathang and Vashisht) alone posses the isolates belonging to the division Proteobacteria. Bacterial communities in the hot springs of high elevated regions of Tibet are known to be predominated by Proteobacteria, alongside Firmicutes as recorded in an earlier study (Huang et al. 2011) which supports our observation. Bacteroidetes is present only in the Bakreshwar hot spring which is a representative of plain region hot springs. The genus in this division is *Pontibacter* which is normally a soil bacterium (Dastager et al. 2010) and has not been isolated from hot springs. The presence of *Pontibacter* suggests a migration of bacterial communities from the sediments to the spring water aided by the flow of springs through the sediments. The numbers of different genera obtained in this study are 8, 7, 7, and 5 from Chumathang, Balrampur, Vashisht and Bakreshwar respectively. It is interesting to note that the three hot springs exhibit high level of diversity of bacteria belong to high altitude region. Chumathang exhibited the highest number of different genera, while Bakreshwar had the least number. This observation supplemented with the presence Proteobacteria in the hot springs of Himalayan Geothermal Belt, reemphasizes the effect of geographic location on the type of bacterial communities in any particular environment (Whitaker et al. 2003), whether it is hot springs or salt lakes.

The isolates from Chumathang hot springs showed closeness to many bacteria apart from *Bacillus* and its derived genera like *Paracoccus, Kocuria, Klebsiella, Staphylococcus,* all of which were also recorded in our earlier study on diversity of thermotolerant bacteria in Manikaran hot springs (Kumar et al. 2013) which also falls under HGB. Besides these, representatives from *Cellulosimicrobium, Aurantimonas*, *Brevundimonas* were niche specific to Chumathang hot springs. *Cellulosimicrobium* is very closely related to *Cellulomonas.* Schuman et al. (2001) suggested that *Cellulomonas* should be reclassified as *Cellulosimicrobium*. *Cellulomonas* has previously been reported as a culturable thermoresistant aquatic bacterium from the oligotrophic pools in Mexico (Cerritos et al. 2011). *Brevundimonas* are lactose fermenting Gram negative bacilli, previously classified under the genus *Pseudomonas* and later placed in new genus on the basis of genotypic and phenotypic characteristics (Segers et al. 1994). *Aurantimonas* spp. has been reported from environmental sources (Jurado et al. 2006) and human clinical materials, but not from high temperature regions. To our knowledge, this is the first report on *Aurantimonas* and *Brevundimonas* in a hot spring.

Among the different genera obtained from Vashisht hot springs *Exiguobacterium, Kocuria* and *Brevibacillus* were reported in our earlier study on diversity of thermotolerant bacteria from Manikaran (Kumar et al. 2013) and also in the studies on the diversity of culturable thermoresistant aquatic bacteria in Mexico (Cerritos et al. 2011). Representatives from *Acinetobacter*, *Enterobacter* and *Gulbenkiania*, which are pathogens and associated with human intestine, may have been transmitted as contaminants from people taking shower in the spring, as this spring is known for its medicinal properties. The genera *Exiguobacterium*, *Staphylococcus* and *Brevibacillus* were also present in Balrampur hot springs. There are also representations from *Planococcus* and *Rhodococcus,* both being niche specific to Balrampur, but have also been reported in our early study on Manikaran hot springs. Bakreshwar, which showed least diversity with reference to number of genera obtained, has representatives from *Exiguobacterium*, *Kocuria*, *Lysinibacillus* and *Pontibacter*;

among which *Pontibacter* was niche specific to Bakreshwar hot springs and the others are also present in other hot springs under study.

Biochemical tests and substrate utilization profiles for selected isolates using Biolog

The data on substrate utilization patterns and sensitivity to chemicals for the 3 temperature tolerant isolates from Bakreshwar were tabulated. Significant variation was observed among the isolates in terms of utilization of sugars, sugar derivatives, metabolic intermediates and amino acids and peptides. Among the three isolates tested for a total of 20 sugars, isolate B31 was able to utilize all, except one sugar. α-D-glucose, sucrose and pectin were utilized by all the three isolates. Generally known as refractile substrates and not commonly utilized by bacteria, compounds such as D-turanose, D-glucuronic acid, glucuronamide, mucic acid, quinic acid, D-saccharic acid, formic acid and D-serine were not utilized by any of the three isolates. Isolate B31 was able to utilize maximum number of sugar derivatives (15), followed by B3 which was able to utilize 9 of the 19 sugar derivatives tested (Table 5.2). Out of 19 metabolic intermediates tested, 15 were found utilized by B38 and B31 utilized 13 sugar derivatives (Table 5.2). The results on amino acids and peptides utilization revealed that the isolate B31 was able to utilize a maximum of 11 amino acids followed by B3, which utilized 7 amino acids (Table 5.2).

Among the three isolates tested for 17 antimicrobial compounds, the isolate B38 was found to be resistant to nine antimicrobial compounds. All the three isolates exhibited resistance to aztreonam and potassium tellurite. The results also revealed that the isolates were sensitive to five antimicrobial compounds. All the three isolates were able to show growth at pH up to 6 and salt concentration up to 8%. B38 is the lone isolate which was not able to show growth at pH 5. Biolog based phenotyping of the three highly thermotolerant isolates generated useful unique fingerprints of the isolates (Table 5.3), besides providing valuable information on the utilization of routine and rare substrates and sensitivity/resistance to antimicrobial compounds. Among the three isolates, two belonged to the genus *Bacillus* and one belonged to the genus *Turicella*.

Biolog based identification of temperature tolerant isolates matched with 16S rDNA sequence based identification up to genera level only for two isolates. One isolate for which there was no match was B38, which according to Biolog analysis, identifies it as *Turicella otitidis,* but according to 16S rDNA sequence based analyses it was placed in the genus *Kocuria* sp. *T. otitidis* is known to be the closest phylogenetic neighbour of the genus *Corynebacterium* species and is mainly a clinical specimen (Funke et al. 1994). Members of the genus *Kocuria,* which are coccoid, gram-positive bacteria, placed under Actinobacteria, have been isolated from a wide variety of natural sources, including mammalian skin, soil, the rhizosphere, fermented foods, clinical specimens, fresh water and marine sediments. It

Table 5.2 Profiling of the promising isolates using Biolog

Isolates	**Substrates Utilized**				**Resistance to antimicrobials and stress conditions**	
	Saccharides (20)[a]	Saccharide derivatives (19)	Metabolic intermediates (19)	Amino acids and peptides (12)	Antimicrobial compounds (17)	Growth conditions (5)
B3	9	9	8	7	5	5
B31	19	15	13	11	4	5
B38	9	8	15	2	9	4

a; Numbers in parentheses indicates the number of substrates/antimicrobial compounds tested

Table 5.3 Unique traits of promising isolates in terms of substrate utilization and response to antimicrobial compounds

Isolates	Substrates	Antimicrobial Compounds	
		Sensitive to	Resistant to
B3	D-arabitol	Lithium chloride	Guanidine HCl and Sodium butyrate
B31	L-fucose, L-rhamnose, D-melibiose, Stachyose, N-acetyl-β-D-mannosamine, N- acetyl-D-galactosamine, N-acetyl neuraminic acid, L-histidine, L-pyroglutamic acid, Inosine and Glycyl-L-proline	-	-
B38	β-hydroxy-butyric acid	Sodium lactate	Minocycline, Fusidic acid and Sodium bromate

belongs to the family *Micrococcaceae,* in the suborder *Micrococcineae*, a divergent bacterial group, and limited amount of genomic information is currently available. Morphotyping of isolate B38 placed it as an actinobacterium, which corresponds to *Kocuria* and its unique resistance profile against 3 chemical agents- minocycline, fusidic acid and sodium bromated and utilization of ß-hydroxy butyric acid. Biolog analyses showed it to be uniquely resistant to 3 antimicrobials. The genome sequence of *K. rhizophila* ATCC 9341, formerly *Micrococcus luteus*, is designated as a quality-control strain in a number of applications, including susceptibility assays for a variety of antibiotics and is known to exhibit tolerance to a wide variety of organic solvents (Tang and Gillevet 2003). Its small genome size, supplemented with the ability to grow rapidly, and its robustness at various growth conditions has made it a model system for development of bacterial bioconversion system, especially under harsh conditions such as in organic solvents. Earlier studies using Biolog assays based on carbon utilization patterns have shown its utility in differentiation of *Bacillus* species (Chun et al. 2003). However Biolog analyses have certain limitations, as it is unable to distinguish closely related organisms (Singh et al. 2010). Also, limited availability exists on members belonging to Actinobacteria in the database; therefore in the study clear cut identification was difficult; however this strain can have tremendous biotechnological potential.

The identification of isolate B3 using both Biolog based and 16SrRNA based identification was congruent and placed it as *B. pumilus*. B3 was able to utilize only 35.71% of the substrates tested. Strains of *B. pumilus* generally show high resistance to environmental stresses, including UV light exposure, desiccation, and the presence of oxidizers such as hydrogen peroxide and produce compounds antagonist to fungal and bacterial pathogens (Yadav et al. 2010). In the present investigation, this strain was able to show growth on 45 % of the substrates and chemical agents tested, besides being uniquely able to use D-arabitol, and uniquely exhibiting resistance to guanidine and sodium butyrate (Table 5.3).

Isolate B31, identified as *B.licheniformis* based on Biolog, was found to show closest sequence based identity with *B. subtilis.* This isolate was able to uniquely utilize 11 substrates. Rey et al. (2004) determined the complete genome sequence of *B. licheniformis* ATCC 14580 and compared the information generated with other species of *Bacillus*, notably with *B. subtilis* 168. Although there exists unmistakable organizational similarities (84.6 % identical at the nucleotide level) between the *B. licheniformis* and *B. subtilis* genomes, notable differences in the numbers and locations of prophages, transposable elements and a number of extracellular enzymes and secondary metabolic pathway operons have aided in distinguishing these species. The isolate B31 corresponding to *B. licheniformis* was able to utilize 82.85 %

of the substrates tested while isolate. In-depth analyses of our isolate may provide more definitive information.

Our study clearly illustrated the significance of evaluating microbial diversity using a combination of traditional morphotyping, followed by molecular approaches and Biolog analyses of promising isolates. Niche specific taxa or genera were also identified. These promising isolates can be of immense significance, as their unique substrate utilization and antibiotic resistance profiles, can make them competitive in the rhizosphere and benefit the crop and soil as eco-friendly options.

Research paper III

6. RESEARCH PAPER III

Differential expression of stress related proteins in *Bacillus pumilus* B3 at high temperature

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Abstract

The Bakreshwar hot springs of India is located at an altitude of 84 metres above mean sea level with an average temperature of 55 °C. *Bacillus pumilus* B3 was isolated from this hot spring and found to tolerate high growth temperatures, hence used to identify the differentially expressed proteins. The strain showed good growth at normal temperature (37 °C) and high temperature (60 °C), recording an optical density (600 nm) of 1.85 and 1.26 at 37 °C and 60 °C respectively. Two-dimensional gel electrophoresis (2DE) of proteins extracted from cultures grown at different temperatures revealed that eight proteins were up regulated and four were down regulated at 60 °C as compared to 37 °C. Identification by MALDI-TOF mass spectrometry (MS) analysis revealed the up regulation of proteins involved in energy metabolism and transport. The study provides important information on thermal stress tolerance in *Bacillus*, and represents the first proteomic study on thermotolerance in *B. pumilus*.

Keywords: *Bacillus*; Differential expression; Proteins; MALDI-TOF-MS; Two-dimensional gel electrophoresis

Introduction

In order to survive in extreme environments, cells trigger programs of specific gene expression, which are manifested as an increase or decrease in the amount of a set of proteins synthesized in response to stress (Duche et al. 2002). Bacteria have evolved adaptive networks to face the challenges of changing environments and to survive under conditions of stress (Abee and Wouters 1999).

Physiological stresses like high or low temperatures, increased salt or acid concentration and oxidative stress induces the synthesis of class of proteins called heat shock proteins (Hsps). Classical HSPs are molecular chaperones or ATP dependant proteases and play a role in protein folding assembly repair and prevention of aggregation under stress conditions (Periago et al. 2002). Response to heat stress, the archetypal stress of different physiological stress (Csermely and Yahara, 2005) has been studied in different bacteria (Hecker et al. 1996; Singh et al. 2007; Rodrigues et al. 2008) and several proteins induced during heat stress have been reported. The molecular genetics of heat shock response has been investigated most extensively in *Escherichia coli* and *B. subtilis* (Hecker et al. 1996; Yura et al. 2000). Heat shock response in a food borne pathogen *B. cereus* ATCC 14579 was studied and it was found that in addition to stress related proteins, proteins involved in metabolism and transport were also regulated (Periago et al. 2002). However, the proteomic analyses as influenced by high temperature are not much investigated.

In our earlier studies on diversity of culturable thermotolerant bacteria from Indian hot springs, a number of thermotolerant bacteria capable of growth at incubation temperatures upto 70 °C were identified (Kumar et al. 2013). *B. pumilus* B3 isolated from Bakreshwar hot springs showed tolerance to high temperatures upto 60 °C and good growth, both under mesophilic (37 °C) and thermophilic (60 °C) conditions. The present study focussed on employing a proteomic approach to identify differentially expressed proteins at high temperature in *B. pumilus* B3.

Materials and methods

Bacterial strains and growth conditions

The thermotolerant strain *B. pumilus* B3 used in the study was isolated from Bakreshwar hot springs India. Cells were grown in Nutrient broth (Peptone 0.5 %, Beef extract 0.3 %, NaCl 0.5 %) with shaking at 180 rpm. One set of cultures were grown at 37 °C (control) and other at 60 °C (stress). Growth of culture was monitored by measuring the optical density at 600nm.

Cell lysis and protein estimation

Five ml of culture from both the control and stressed ones were pelleted down by centrifugation (7000 rpm for 10 min) and washed thrice with Tris-HCl buffer (50 mM, pH 7.2). Cell pellets were resuspended in 2 ml of lysis buffer containing Tris-HCl 50 mM, NaCl 100 mM, DTT 5 mM, Glycerol 5%, PMSF 5 mM, sonicated for 5 min and centrifuged (7000 rpm for 10 min) to pellet down cell debris. Supernatants were transferred to a fresh 1.5 ml autoclaved eppendorf tubes. The total protein concentration was determined by Bradford method using BioRad reagents and using bovine serum albumin as the standard. Aliquots of 400 µg of protein were stored at - 80 °C.

Two dimensional electrophoresis (2DE)

Aliquots of 400 µg of protein from both the control and stressed samples were cleaned using a BioRad 2DE clean up kit following the protocol prescribed by the manufacturer. The total protein concentration was determined again after clean up employing Bradford method using BioRad reagents. An aliquot containing 100 µg of protein was made up to a volume of 125 µl using rehydration buffer (Urea 8M, CHAPS 2 %, DTT 50 mM, 100X Biolyte Ampholyte 0.2 % and bromophenol blue 0.0002 %). The suspension containing 100 µg of protein was loaded on to immobilized pH gradients strips (pH 4.0-7.0, 7 cm, BioRad) in a rehydration tray overlaid with mineral oil. Gels were passively rehydrated for 16 h. Isoelectric focussing was carried out on a BioRad IEF assembly at 60000 V/h and focussing strips were stored at -20 °C until separation in second dimension was performed. Immobilized pH gradient strips were then equilibrated in a series of equilibration buffer I (Urea 6 M, Tris-HCl 0.375 M, SDS 2 %, Glycerol 20 % and DTT 2 %) and equilibration buffer II (Urea 6 M, Tris-HCl 0.375 M, SDS 2 %, Glycerol 20 % and Iodoacetamide 2.5%). The second dimension was performed by SDS-PAGE on gels containing 12.5 % polyacrylamide and carried out in a Genei PAGE assembly. Proteins were resolved at 80 V for 3 hours. The gels were silver stained as described by Chevallet et al. (2006). The gels were analysed using PD-Quest software (Bio-Rad). Analysis was done to locate spots/proteins that are expressed only in stressed sample.

Protein identification using peptide mass fingerprinting

Individual spots were excised from the gels and subjected to MALDI-TOF mass spectrometry analyses that were carried out by Sandor Proteomics Pvt. Ltd, 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).

Results and Discussion

In the present study, a strain of *B. pumilus* B3 isolated from Bakreshwar hot springs with a temperature of 55 °C was found to grow well upto a temperature of 60 °C. A Biolog based phenotyping was done to generate unique biochemical fingerprint of this strain, which showed the strain's ability to grow at pH 6, high NaCl concentration (8 %) and on 45 % of the substrates and chemical agents tested (data submitted elsewhere).

Growth of B. pumilus B3 under thermal stress

To study the effect of incubation temperature on growth of *B. pumilus* B3, time course study on growth was undertaken by measuring the optical density (600nm) after incubation under the two growth conditions, 37 °C representing the normal growth temperature and 60 °C representing the thermal stress conditions. The results revealed that the *B. pumilus* B3 strain attains maximum growth 16 h after inoculation at 37 °C, exhibiting an optical density (600 nm) of 1.84 and reaching stagnation thereafter. At 60 °C, the cells reached a maximum optical density of only 1.26 in 20 h after inoculation (Fig. 6.1). This clearly showed that the strain was thermotolerant upto 60 °C and able to show 68 % of the growth observed at 37 °C. The response of a bacterium to a particular stress is related to its ecological niche (Belfiore et al. 2013) and this strain was therefore able to grow at high temperature. *B. pumilus* is generally a soil dwelling bacterium and the species is also reported as second most predominant species of *Bacillus* in spacecrafts. This bacterium is highly resistant to extreme environmental conditions such as low or no nutrient availability, desiccation, irradiation, H_2O_2 and chemical disinfection (Gioia et al. 2007).

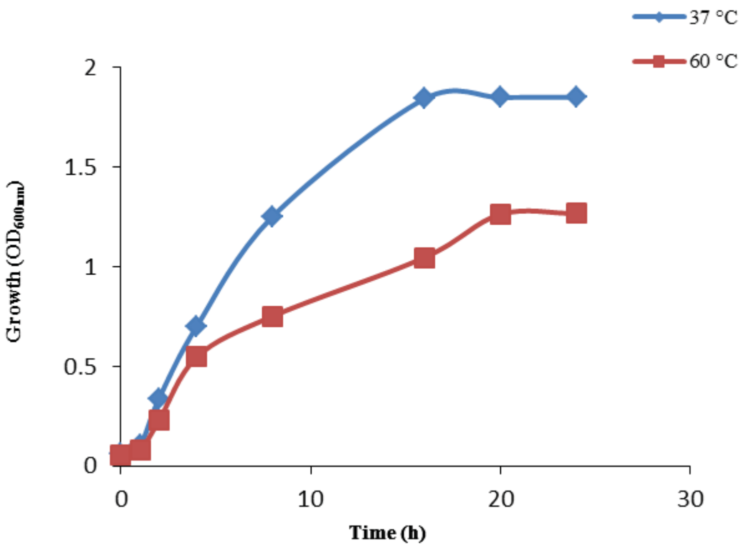

Figure 6.1 Growth curves of *Bacillus pumilus* B3 at different temperature. Cells were inoculated in Nutrient broth and incubated at 37 °C and 60 °C.

Proteomic analysis of B. pumilus B3

Thermal stress response of the strain was investigated by the proteomics approach. Cells were grown in nutrient broth incubated at 37 °C (control) and 60 °C (stressed), and harvested at mid log phase growth (OD_{600nm} ~ 0.6). These samples were subjected to two dimensional electrophoresis which led to the identification of proteins differentially expressed under high temperature stress (Fig. 6.2). Identification of differentially expressed proteins was based on PD-Quest software (Bio-Rad) and spot by spot comparison of gels containing samples extracted under differential conditions. Two dimensional protein patterns revealed a total of 12 spots which were differentially expressed. Among them, eight were present only in the stressed cells, indicating up regulation of these eight proteins at growth temperature of 60 °C, and four were present only under control conditions, indicating the down regulation of these four proteins. Among the eight up regulated proteins, five prominent proteins were selected and carried forward for MALDI-TOF/MS analysis and peptide mass finger printing. The results of the peptide mass fingerprinting of the five proteins followed by MASCOT search are given in the Table 6.1. *B. pumilus* B3 showed overexpression of two hypothetical proteins, ATP binding protein, ATP synthase subunit E and an aminotransferase.

Among the various anti stress strategies of bacteria, one of the most powerful is the high thermal stability of enzymes which can protect their metabolism at high temperatures. Aspartate aminotransferase (AST) is a key enzyme of Krebs cycle, involved in the formation of oxaloacetate. Synthesis of various other essential amino acids is also dependant on AST. AST catalyzes the α-amino group reversible transfer between four- and five-carbon dicarboxylic amino acids and the α-keto-acids by a mechanism named “ping-pong bi-bi”, which is pyridoxal phosphate dependent (Deu et al. 2002). Chen et al. (2013) have shown that AST forms complex with GroEL (HSP 60) to protect proteins in thermophilic microorganisms. Hence the aminotransferase, up regulated in the strain *B. pumilus* B3 at high temperature might play a role along with GroEL (HSP 60) in protecting the proteins at that temperature. Although the ATP binding protein and ATP synthase subunit E are not known to play any direct role in thermotolerance, their up regulation at high temperature is indicative of their indirect involvement in thermotolerance. All molecular chaperones which protect the protein from unfolding and denaturation at higher temperature require the presence of ATP and it has been shown that ATP enhances the chaperonic activity of molecular chaperones (Muchowski and Clark 1998). As cells might require more ATP to support the chaperonic activity, the ATP binding protein and ATP synthase subunit E might indirectly enhances the cells’ thermotolerance by supplying sufficient ATP for molecular chaperones to function effectively under stress.

Employing a proteomic approach to understand thermotolerance in a strain of *B. pumilus* isolated from hot water springs has provided important pointers for research on other

Table 6.1 MALDI-TOF/MS analysis of differentially expressed proteins

Spot	**Accession number**	**Name**	**Biological functions**
B1	gi|13471403	ATP Synthase subunit E	Respiratory chain complex 1
B2	gi|62390402	Hypothetical protein	Unknown
B3	gi|118713231	Hypothetical protein	Unknown
B4	gi|83589279	ATP Binding Protein	Sulfonate/nitrate transport
B5	gi|11662631	Aminotransferase	Amino acid transfer from aspartate to pyridoxal phosphate

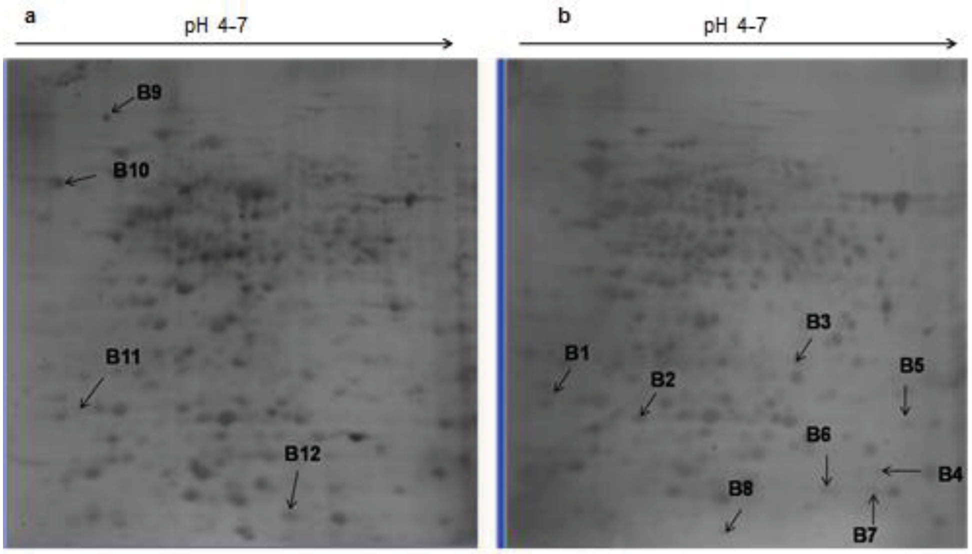

Figure 6.2 Image of 2-D gel electrophoresis analysis of whole-cell protein extracts from *Bacillus pumilus* B3 a) grown at 37 °C b) grown at 60 °C
B1-B8; upregulated proteins, B9-B12; downregulated proteins.

extremophiles. Future research needs to be directed towards using a combination of gene expression coupled with protein expression analyses for a more comprehensive understanding of the mechanisms involved in thermo tolerance.

Research paper IV

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

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Abstract

A set of thermotolerant bacilli were analysed in terms of the variation in the *dnaK* gene, which codes for DnaK/HSP 70 protein. A partial fragment of *dnaK* was amplified from seven different bacilli isolated from Manikaran hot water springs. Phylogenetic analyses of *dnaK* in the set of strains generated 4 clusters, three representing highly thermotolerant strains and one of a moderately thermotolerant strain. However, analyses of translated amino acid sequence 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 polymorphisms in the DnaK could be related to the difference in the temperature tolerance limit of the two strains.

Keywords: DnaK; Heat shock protein; Polymorphism; Sequencing

Introduction

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 and other proteins/chaperone proteins (Wang et al. 2004). Heat shock proteins (Hsps) are the class of proteins that are induced in cells experiencing abiotic stress, which were first discovered in *Drosophila* (Ritossa 1962). The archetypal stress response is a sudden rise in the outside temperature, called heat shock (Csermely and Yahara 2005). 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). Hsp 70 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). Hsp 70 consists of a NH_2-terminal ATPase domain, COOH-terminal substrate binding domain and an 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 HSP70 in the domain Bacteria is called *dnaK* (Ward-Reiney 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 have 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 drought and freezing stress tolerance in poplar (Takabe et al. 2008).

In our earlier studies on diversity of culturable thermotolerant bacteria from Indian hot springs, many *Bacillus* strains capable of tolerating growth temperatures upto 70 °C were identified (Kumar et al. 2013). Perhaps the greatest value of microbial diversity in extreme environments is its opportunity to mine novel genes and metabolites (Combie and Runnion 1996). Allele/gene mining for tolerance to extreme condition is one such novel approach which exploits the nucleotide sequence of one organism to isolate useful alleles from different organisms (Latha et al. 2004). The present study is designed with an aim to look for allelic variation in the gene *dnaK* of different bacilli, isolated from thermal springs and found to be thermotolerant. A moderately thermotolerant strain is also used in the study for comparison of *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 (Kumar et al. 2013). A list of strains used with their NCBI Accession number and temperature tolerance are given in Table 7.1. 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 hours.

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 1mM 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 MicroPrep^{TM} following the standard protocol prescribed by the manufacturer (Zymo Research, The Epigenetics company^{TM}).

PCR amplification of dnaK region

A partial fragment of dnaK gene (650bp) was amplified using a forward primer (5’CCGGCGACAACCGTCTGGTGGGGA3’) and reverse primer (5’GGGCGCTCACC TTGAAGAACATGGA3’) (Tokunaga et al. 1998). 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

Table 7.1 Strains used and temperature tolerance

S. No	**Strains**	**16S rRNA sequence NCBI Accession Number**	**Temperature tolerance**
1	M4 *Bacillus licheniformis*	JX312615	70 °C
2	M5 *Bacillus pumilus*	JX312616	70 °C
3	M6 *Bacillus subtilis*	KC315773	70 °C
4	M7 *Bacillus pumilus*	KC315774	70 °C
5	M8 *Bacillus* sp.	KC315775	70 °C
6	M36 *Brevibacillus* sp.	JX312626	45 °C
7	M55 *Bacillus megaterium*	JX312633	70 °C

U Advantage2 Polymerase. PCR products were resolved by electrophoresis at 60 V for 1 hour 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 ExPASy Translate tool from ExPASy Bioinformatics Resource Portal (http://web.expasy.org/translate/). Both nucleotide sequence and amino acid sequence were aligned using the multiple sequence alignment tool CLUSTAL W2 (Thompson et al. 1994) with default parameters. Dendrogram was constructed on aligned data sets using Neighbour joining (NJ) method (Saitou and Nei 1987) using the program MEGA 4.0.2 (Tamura et al. 2007). Bootstrap analysis was performed on 1000 random samples taken from multiple alignments (Felsentein 1981).

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 resultant reducing crop yields. Recognizing the importance of the gene *dnaK* in thermoregulation and its potential role in management of abiotic stress in crop plants (Ono et al. 2001; Uchida et al. 2007; Montero-Barrientos et al. 2010) and possibility of recombinant overexpression of particular protein of interest (Schlicker et al. 2002), an attempt was made to explore the polymorphisms in the gene *dnaK* of seven bacilli isolated from Manikaran hot water springs.

Among the seven strains, six were tolerant to incubation temperatures upto 70 °C and one strain (M36) was tolerant only upto 45 °C. Amplification with the primers yielded a PCR product of 650 bp from all the seven strains (Fig. 7.1). Sequencing of the purified PCR product followed by BLAST search showed that sequence had an overall similarity to well described *dnaK* homologues (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Liang et al. (2009) identified and characterized a heat shock protein 70 (Hsp 70/Dna K) in *B. licheniformis,* which was 1839 bp 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 in to 4 clusters; Cluster I (M5, M6 and M55) Cluster II (M7 and M8), Cluster III (M4) and Cluster IV (M36) (Fig. 7.2a). Alignment of 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. 7.2b). This suggests that the

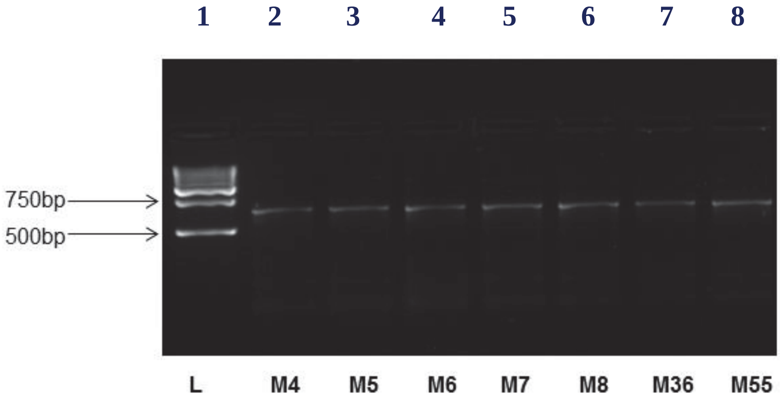

Figure 7.1 Amplification of *dnaK* from selected thermotolerant bacilli inhabiting Manikaran hot springs. Lane 1-Marker; Lane 2 to 8 Amplicons of *dnaK*.

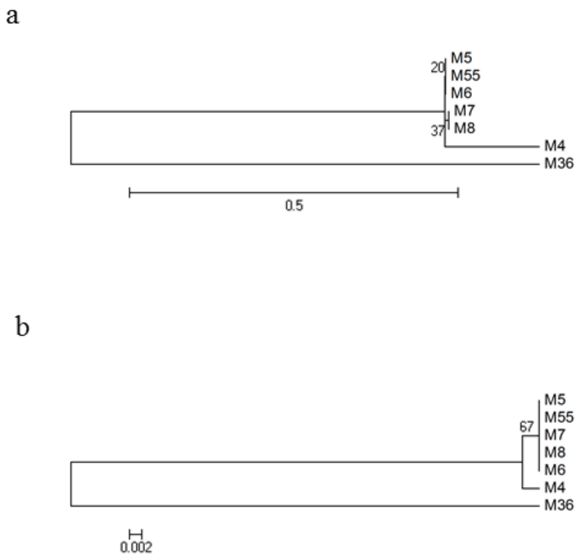

Figure 7.2 Unrooted dendrogram based on comparison of **a)** *dnaK* nucleotide sequences and **b)** DnaK amino acid sequence of seven thermotolerant bacilli isolated from Manikaran hot springs.

nucleotide sequence variations between Cluster I and Cluster II are sense variations and they both code for same amino acid sequence. 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 upto 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 and moderately thermotolerant strain, a pairwise alignment between DnaK of M5 and M36 was done for 185 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 between them (Fig. 7.3). Within these 27 amino acid differences, 26 were 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 DnaK (Liang et al. 2009). 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 (Flynn et al. 1989; Gragerov et al. 1994). Polymorphism between the DnaK of M5 and M36 in the ATPase domain may be the reason for difference in the thermotolerance limit between these two strains. Although in the present study, not much of polymorphism was observed in the DnaK protein analysed, among the highly thermotolerant strains, it may exist in the other regions. Future research needs to be pursued towards extensive screening with 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. Earlier report exists on the overexpression of *dnaK* from a halotolerant cyanobacterium being able to enhance the high temperature tolerance of tobacco seeds in the germination and early growth stages (Ono et al. 2011). It may be plausible to utilize the promising *dnaK* from our study for developing stress tolerant transgenics in future.

Figure 7.3 Pairwise alignment of DnaK proteins of M5 (highly thermotolerant strain) and M36 (moderately thermotolerant strain) showing variations in the amino acid sequence.

Research paper V

8. RESEARCH PAPER V

Cloning, sequencing and expression analysis of *dnaK* from *Bacillus pumilus* inhabiting hot water springs

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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/Hsp70 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 *B. 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 hours, 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.

Keywords: Heat shock protein, DnaK, Heterologous expression

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 such as soil moisture, high temperatures, soil salinity/alkalinity, low pH and metal toxicity (Grover et al. 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). Hsp 70 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). Hsp 70 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 HSP70 in the domain Bacteria is called *dnaK* (Ward-Reiney 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 have been reported in various organisms like *E. coli* (Liang et al. 2009), tobacco (Ono et al. 2001), Arabidopsis (Montero-Barrientos et al. 2010) and Rice (Uchida et al. 2008). *dnaK* gene from *Trichoderma harzianum* has been found to enhance drought and freezing stress in poplar (Takabe et al. 2008).

In our earlier studies on diversity of culturable thermotolerant bacteria from Indian hot springs members of the genus *Bacillus*, *Exiguobacterium*, and *Pseudomonas* were found to tolerate temperatures upto 70°C The present study is designed with an aim to isolate gene *dnaK* from these thermotolerant strains. In the domain Bacteria, *dnaK* gene is a 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* (http://www.ncbi.nlm.nih.gov/genome) allowed for the designing of primers in the region flanking *dnaK* gene. In the present study we demonstrated successful cloning and expression of *dnaK* from the native strain of *B. pumilus* B3 in the *E. coli* host leading to enhanced thermotolerance. *B. pumilus* strain B3 is a gram-positive spore forming bacterium isolated from Bakreshwar hot springs and was phylogenetically related to *B. pumilus* SAFR-032 for which the whole genome sequence is available in the NCBI database (Gioia et al. 2007).

Materials and methods

Bacterial strains, vectors and growth conditions

The bacterial strains used in the study were isolated from Manikaran and Bakreshwar hot springs (Kumar et al. 2013). A list of strains used with their NCBI Accession number and temperature tolerance are given in Table 8.1. *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.

Materials

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).

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 1mM 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.

PCR amplification of dnaK region

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 8.2. 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 hour 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).

Restriction analysis of dnaK gene

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

Table 8.1 Strains used in the study

Strain	**16S rRNA sequence NCBI Accession number**	**Hot water springs**	**Temperature tolerance**
M4 *Bacillus licheniformis*	GQ280087	Manikaran	70 °C
M5 *Bacillus pumilus*	JQ435673	Manikaran	70 °C
M6 *Bacillus subtilis*	EF442670	Manikaran	70 °C
M7 *Bacillus pumilus*	HF536558	Manikaran	70 °C
M8 *Bacillus* sp.	KC121051	Manikaran	70 °C
M46 *Pseudomonas psychrophila*	JQ782895	Manikaran	70 °C
M47 *Exiguobacterium acetylicum*	JX307688	Manikaran	70 °C
M55 *Bacillus megaterium*	EU931553	Manikaran	70 °C
B3 *Bacillus pumilus*	KC492102	Bakreshwar	60 °C
B5 *Bacillus pumilus*	KC596003	Bakreshwar	60 °C
B12 *Bacillus pumilus*	KC596004	Bakreshwar	60 °C
B34 *Bacillus pumilus*	KC492105	Bakreshwar	60 °C

Table 8.2 List of primers used to amplify *dnaK* from different strains

Strains	**Probable species**	**Primer sequence**
M4	*Bacillus licheniformis*	5’GCAGGTTGAGGATGAAGCGT3’ 5’ CTCATAGTAATCACGCTTAC3’
M5	*Bacillus pumilus*	5’CGATCCAAACCTTCATCAAGCC3’ 5’CTCATCTCTTCACACTCCCG3’
M7	*Bacillus pumilus*	-do-
B3	*Bacillus pumilus*	-do-
B5	*Bacillus pumilus*	-do-
B12	*Bacillus pumilus*	-do-
B34	*Bacillus pumilus*	-do-
M8	*Bacillus* sp.	-do-
M6	*Bacillus subtilis*	5’ GTCGAAGCCATCGAAGCTGT3’ 5’ ATAGTAATCACGCTTACTCA3’
M55	*Bacillus megaterium*	5’ TGTTGAAGCAATTGAATCAG3’ 5’ ATCTCGCTTACTCATCAATA3’
M46	*Pseudomonas psychrophila*	5’ CGCCTTCGATTGACGAGAAG3’ 5’ TTGGTTCATGCGGTATTTGC3’
M47	*Exiguobacterium acetylicum*	5’CGTCCAAGTATGGTCAAAGTCGC3’ 5’GACATGGAAAGAGCCAAAGC3’

to 2 h in 2.5% agarose gels in 1X 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).

Sequencing and analysis of dnaK gene

DNA sequencing was performed by Scigenome Labs Pvt. Ltd. (Cochin, India) employing primer walking technique. The sequence was then analysed using ORF finder available in the National Centre for Biotechnological Information (NCBI) tools. The deduced amino acid sequence was analysed with the program BLAST-P from NCBI. Alignment of amino acid sequence was done by T-Coffee alignment method (Notredame et al. 2000).

Competent cell preparation and cloning

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’CGCGGATCCATGAGTAAAATCATTGGTA TTGAC3’) and BKER (5’CGAGCTCGTTATTTTTCTCTTGATCGTCG 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 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 transformation and expression in the host *E.coli* BL21 (DE3).

Expression of dnaK of B. pumilus B3 in E.coli

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 $O.D_{600nm}$ reaches up to 0.6. Recombinant protein expression was induced with IPTG to a final concentration of 0.2 mM and cultivated further. One ml 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.

SDS – PAGE of cell lysates

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.

MALDI-TOF analysis of expressed protein

The band corresponding to 70 kDa from the SDS-PAGE gel excised from the gel and subjected to MALDI-TOF mass spectrometry that was carried out by 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).

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 volume equivalent to 0.6 OD_{600nm}. Growth was monitored by measuring the optical density at 600 nm at regular intervals.

Results and Discussion

PCR amplification and restriction analysis of dnaK region

Amplication 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. 8.1 a). 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 (Kumar et al. 2013). 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-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. 8.1 b) revealing that there are no variations in the *dnaK* among the five strains. This suggests that 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.

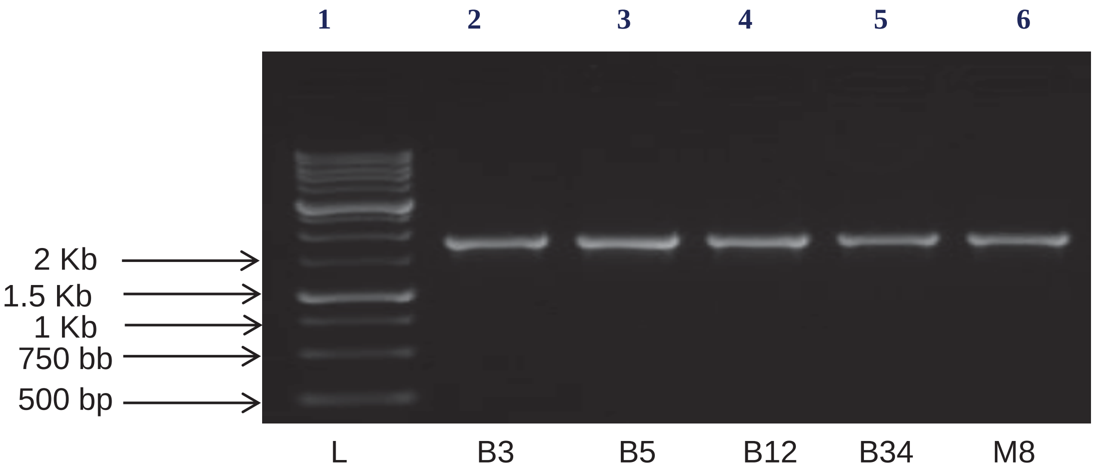

Figure 8.1 a) Amplification of *dnaK* in selected thermotolerant strains from extreme habitats. Lane 1-Ladder; Lane 2 to 6-Amplicons of *dnaK* with flanking region.

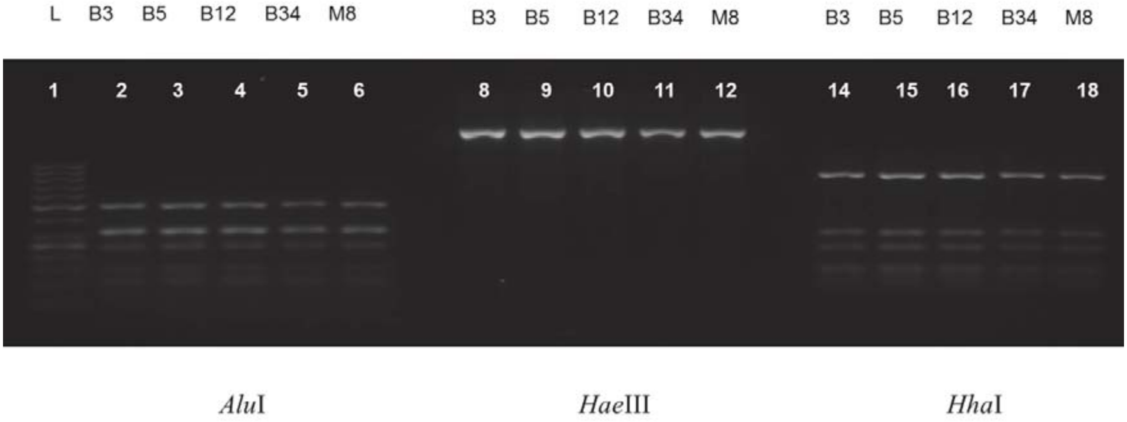

Figure 8.1 b) Restriction patterns of amplified *dnaK* region of thermotolerant isolates generated using the restriction enzymes *Alu*I, *Hae*III and *Hha*I.

Lane 1 - Ladder; 100 bp-1000 bp.

Lane 2 - 6 - Restriction patterns generated using the enzyme *Alu*I.

Lane 8 - 12 - Restriction patterns generated using the enzyme *Hae*III.

Lane 14 – 18 - Restriction patterns generated using the enzyme *Hha*I.

Sequencing and analysis of dnaK of B. pumilus B3

Sequencing of the purified PCR product through primer walking and analysis allowed for identification of an open reading frame of 1842 bp (Fig. 8.2) 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/TrEMBL) DnaK proteins contain an amino acid length ranging from 596 to 656 (Liang et al. 2009). Molecular weight and pI of the protein was 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/HSP70 of *B. pumilus* B3 share an overall similarity to the well described homologues. The DnaK/HSP70 of *B. pumilus* B3 shared 93 %, 58 % 56 % and 56 % amino acid sequence identity with DnaK of *B. subtilis*, *P. 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. 8.3). 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. 8.4). 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 Hsp70/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*.

Expression of dnaK of B. pumilus B3

For expression of *dnaK* of *B. pumilus* B3, the *E*.coli BL21 (DE3) harbouring pET29-BpdnaK was grown in liquid broth up to an OD_{600nm} of 0.6. Once the required growth was achieved, the expression of recombinant protein was induced with IPTG at a final concentration of 0.2mM. Cells were harvested one hour 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 with pET29-BpdnaK plasmid but without induction (Fig. 8.5). MADI-TOF analysis of the 70 kDa protein

ATCGTTGTAGAAGAATTGCAAAAGGGCTATAAACTCAAAGACCGAGTTATTCGTCCATCAATGGTAAAAGTAAATCAA
TAACTACATAATGGGAGGTCATCGAAT**ATGAGTAAAATCATTGGTATTGACTTAGGAACAACAAACTCATGCGTT**
GCAGTACTCGAAGGCGGCGAGCCAAAAGTTATTGCAAACGCTGAAGGAGCACGTACAACACCATCTGTTGTCGC
TTTTAAAAATGGAGAGCGCCAAGTAGGTGAAGTAGCGAAACGTCAATCAATTACAAACCCGAACACCATCATGT
CTGTTAAAAGACATATGGGTACAGATTATAAAGTAGAAGTTGAAGGCAAGAACTACACGCCGCAGGAAATCTCT
GCAATTATTCTTCAACACCTTAAATCTTATGCTGAAGCTTATCTTGGCGAAGAAGTAACAAAAGCGGTTATTACA
GTTCCTGCTTACTTCAACGATGCAGAGCGCCAAGCAACAAAAGATGCTGGTAAAATTGCTGGTCTTGAAGTAGA
ACGTATCATCAACGAACCAACAGCAGCTGCACTTGCTTATGGTTTAGATAAAACAGATGAAGATCAAACGATCCT
TGTATATGACCTTGGCGGCGGTACATTTGACGTATCAGTCCTTGAGCTTGGAGACGGCGTCTTTGAAGTACGCT
CAACTGCTGGGGACAACCGTCTAGGTGGAGACGATTTTGACCAAGTCATCATCGATCATCTAGTGGCTGAGTTC
AAAAAAGAAAACGGCATTGATCTTTCTAAAGATAAAATGGCGCTTCAGCGTTTAAAAGATGCTGCTGAAAAAGC
GAAAAAAGATCTTTCTGGTGTATCTTCTACACAAATCTCACTGCCATTTATCACAGCTGGAGATGCAGGTCCTCT
TCACCTAGAATTAACGCTAACGCGTGCTAAATTCGAAGAGCTTTCTGCAGACCTTGTAGAGCGTACAATGACAC
CTGTACGTCAATCACTGAAAGATGCTGGTCTATCTGCTAGCGAGATTGATAAAGTTATCCTTGTTGGTGGATCAA
CTCGTATTCCTGCAGTACAAGAAGCAATCAAAAAAGAAACAGGTAAAGAGCCTCATAAAGGCGTAAACCCTGAT
GAAGTGGTTGCACTTGGTGCGGCAATTCAAGGTGGAGTTATCACAGGAGATGTCAAAGACGTTGTTCTTCTTGA
CGTCACACCACTTTCTTTAGGAATTGAAACAATGGGCGGCGTATTCACAAAGCTGATTGAACGTAATACAACCAT
TCCAACAAGTAAATCTCAAGTATTCTCAACGGCTGCTGACAACCAAACTGCTGTAGACATTCACGTCTTACAAGG
TGAGCGTCCAATGGCAGCAGACAACAAAACATTAGGTCGTTTCCAATTGACTGATATCCCGCCAGCACCACGCG
GCGTACCACAAATCGAAGTATCTTTTGATATCGATAAAAACGGTATTGTCAACGTACGTGCGAAAGATATGGGT
ACTGGAAAAGAACAAAACATCACAATCAAATCTTCTTCAGGTCTTTCTGATGATGAGATCGAAAAAATGGTCAAA
GAAGCAGAAGAAAATGCTGAAGCAGATGCAAAGAAAAAAGAAGAAATCGAAGTGCGCAATGAAGCAGATCAAT
TAGTGTTTACAACAGAAAAAACATTAAAAGATCTTGAAGGCAAAATCGATGAAGAGCAAGTGAAAAAAGCGAAT
GATGCAAAAGATGCGCTAAAAGCTGCGATCGAAAAAGGCGAGCTTGAAGACATCAAAGCGAAAAAAGATGAGC
TACAAACAATCGTTCAAGAATTAACGACAAAGCTCTATGAAGAAGCTGCAAAACAAGCACAAGCTCAGCAAGAA
GGCGGCGCTGAAGGTGCTCAAAAAGCAGATGACAATGTAGTGGATGCAGAATACGAAGAAGTAAACGACGATC
AAGAGAAAAAATAATCACCTTTACTTTTGAAAAAGACTGCTGAAACGTACTCGTCAGAAAGTCAAAGTCAGGATCT
CTTGGCTTTGACTTTTTTCTTTTCAACGATGAAAAGGAATGAAAAAGATCAATTAAAAT

Figure 8.2 Nucleotide sequence showing amplified *dnaK* region of *Bacillus pumilus* B3. Open reading frame is marked in a different colour (red) and start and stop codons are marked in different colour (green).

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B3                1 MSKIIGIDLGTTNSCVAVLEGGEPKVIANAEGARTTPSVVAFK-NGERQVGEVAKRQSITNP-NTIMSV   67
Bacillus          1 MSKVIGIDLGTTNSCVAVLEGGEPKVIANAEGNRTTPSVVAFK-NGERQVGEVAKRQSITNP-NTIMSV   67
Escherichia       1 MGKIIGIDLGTTNSCVAIMDGTTPRVLENAEGDRTTPSIIAYTQDGETLVGQPAKRQAVTNPQNTLFAI   69
Pseudomonas       1 MGKIIGIDLGTTNSCVAILENGNVKVIENAEGARTTPSIIAYTNDGETLVGQSAKRQAVTNPHNTLYAV   69
Thermus           1 MAKAVGIDLGTTNSVIAVLEGGKPVVLENAEGERVTPSVVAFR-DGEILVGRMAKRQAVLNPEGTIFEI   68

cons              1 *.* :********* :*:::.    *: **** *.***::*:  :**  **. ****:: ** .*:  :   69


B3               68 KRHMGTDY------------------------KVEVEGKNYTPQEISAIILQHLKSYAEAYLGEEVTKA  112
Bacillus         68 KRHMGTDY------------------------KVEIEGKDYTPQEVSAIILQHLKSYAESYLGETVSKA  112
Escherichia      70 KRLIGRRFQDEEVQRDVSIMPFKIIAADNGDAWVEVKGQKMAPPQISAEVLKKMKKTAEDYLGEPVTEA  138
Pseudomonas      70 KRLIGRRFDEDVVQKDIKLVPYKIVKADNGDAWVEVKGQKMAPPQISAEVLKKMKKTAEDYLGEPVTEA  138
Thermus          69 KRFIGRRFE--EVQEEAKRVPYKVVPGPDGGVRVEVKGKLYTPEEISAMILRKLVEDASKKLGEKITKA  135

cons             70 ** :*  :                         **::*:  :* ::** :*::: . *.  *** :::*  138


B3              113 VITVPAYFNDAERQATKDAGKIAGLEVERIINEPTAAALAYGLDKTDEDQTILVYDLGGGTFDVSVLEL  181
Bacillus        113 VITVPAYFNDAERQATKDAGKIAGLEVERIINEPTAAALAYGLDKTDEDQTILVYDLGGGTFDVSILEL  181
Escherichia     139 VITVPAYFNDAQRQATKDAGRIAGLEVKRIINEPTAAALAYGLDKGTGNRTIAVYDLGGGTFDISIIEI  207
Pseudomonas     139 VITVPAYFNDSQRQATKDAGRIAGLDVKRIINEPTAAALAYGMDKAKGDRTVVVYDLGGGTFDVSVIEI  207
Thermus         136 VITVPAYFNNAQREATANAGRIAGLEVLRIINEPTAAALAYGLDKK-GNETVLVFDLGGGTFDVTILEI  203

cons            139 *********:::*:** :**:****:* **************:**   :.*: *:********::::*:  207


B3              182 ----GDGVFEVRSTAGDNRLGGDDFDQVIIDHLVAEFKKENGIDLSKDKMALQRLKDAAEKAKKDLSGV  246
Bacillus        182 ----GDGVFEVRSTAGDNRLGGDDFDQVIIDHLVSEFKKENGIDLSKDKMALQRLKDAAEKAKKDLSGV  246
Escherichia     208 DEVDGEKTFEVLATNGDTHLGGEDFDSRLINYLVEEFKKDQGIDLRNDPLAMQRLKEAAEKAKIELSSA  276
Pseudomonas     208 AEVDGEHQFEVLATNGDTFLGGEDFDLRLIDYLVDEFKKESGIDLKGDPLAMQRLKEAAEKAKIELSSS  276
Thermus         204 ----GEGVFEVKATSGDTHLGGSDMDHAIVNWLAEEFKREYGVDLKADRQALQRLIEAAEKAKIELSST  268

cons            208     *:  *** :* **. ***.*:*  ::: *. ***:: *:**  *  *:*** :****** :**.   276


B3              247 SSTQISLPFITAGDA--GPLHLELTLTRAKFEELSADLVERTMTPVRQSLKDAGLSASEIDKVILVGGS  313
Bacillus        247 SSTQISLPFITAGEA--GPLHLELTLTRAKFEELSSHLVERTMGPVRQALQDAGLSASEIDKVILVGGS  313
Escherichia     277 QQTDVNLPYITADAT--GPKHMNIKVTRAKLESLVEDLVNRSIEPLKVALQDAGLSVSDIDDVILVGGQ  343
Pseudomonas     277 QQTDVNLPYITADQT--GPKHLNVKISRAKLEALVEDLVARTLEPCRVALKDAGLDVGSINDVILVGGQ  343
Thermus         269 LETTISLPFIALDPASKTPLHLEKKLTRAKFEELIQPLLKRLRGPVEQALKDAGLTPAQIDEVILVGGA  337

cons            277  .* :.**:*: . :   * *:: .::***:* *   *: *   * . :*:****  ..*:.******   345
B3              314 TRIPAVQEAIKKETGKEPHKGVNPDEVVALGAAIQGGVITGDVKDVVLLDVTPLSLGIETMGGVFTKLI  382
Bacillus        314 TRIPAVQEAIKKETGKEAHKGVNPDEVVALGAAIQGGVITGDVKDVVLLDVTPLSLGIETMGGVFTKLI  382
Escherichia     344 TRMPMVQKKVAEFFGKEPRKDVNPDEAVAIGAAVQGGVLTGDVKDVLLLDVTPLSLGIETMGGVMTTLI  412
Pseudomonas     344 TRMPLVQQKVAEFFGREPRKDVNPDEAVAMGAAIQGAVLAGDVKDVLLLDVTPLTLGIETMGGVMTPLI  412
Thermus         338 TRVPAVQRVVKELLGKEPNRSVNPDEVVAMGAAIQAGVLMGEVQDVVLLDVTPLSLGVETKGGVMTVLI  406

cons            346 **:* **. : :  *:*..:.*****.**:***:*..*: *:*:**:*******:**:** ***:* **  414


B3              383 ERNTTIPTSKSQVFSTAADNQTAVDIHVLQGERPMAADNKTLGRFQLTDIPPAPRGVPQIEVSFDIDKN  451
Bacillus        383 DRNTTIPTSKSQVFSTAADNQTAVDIHVLQGERPMSADNKTLGRFQLTDIPPAPRGVPQIEVSFDIDKN  451
Escherichia     413 AKNTTIPTKHSQVFSTAEDNQSAVTIHVLQGERKRAADNKSLGQFNLDGINPAPRGMPQIEVTFDIDAD  481
Pseudomonas     413 EKNTTIPTKKSQVFSTAEDNQSAVTIHVLQGERKQAAQNKSLGKFDLTDIPPAPRGVPQIEVTFDIDAN  481
Thermus         407 PRNTTIPTRKCEIFTTAEHNQTAVEIHVLQGERPMAQDNKSLGRFRLEGIPPMPAGVPQIEVCFDIDAN  475

cons            415  :****** :.::*:** .**:** ********  : :**:**:* * .* * * *:***** **** :  483


B3              452 GIVNVRAKDMGTGKEQNITIKSSSGLSDDEIEKMVKEAEENAEADAKKKEEIEVRNEADQLVFTTEKTL  520
Bacillus        452 GIVNVRAKDLGTGKEQNITIKSSSGLSDDEIERMVKEAEENADADAKKKEEIEIRNEADQLVFQTEKTL  520
Escherichia     482 GILHVSAKDKNSGKEQKITIKASSGLNEDEIQKMVRDAEANAEADRKFEELVQTRNQGDHLLHSTRKQV  550
Pseudomonas     482 GILHVSAKDKATGKHQSIVIKANSGLSDEEIEKMIRDAEANAEEDRKFEELAAARNQGDALVHATRKML  550
Thermus         476 GILHVTAKELSTGREASITIQNTTTLSEEEIQRMIEEAQRHAEEDRRRREHAELKNALDSARIQAERVL  544

cons            484 **::* **:  :*:. .*.*: .: *.::**::*:.:*: :*: * : .*    :*  *     :.: :  552

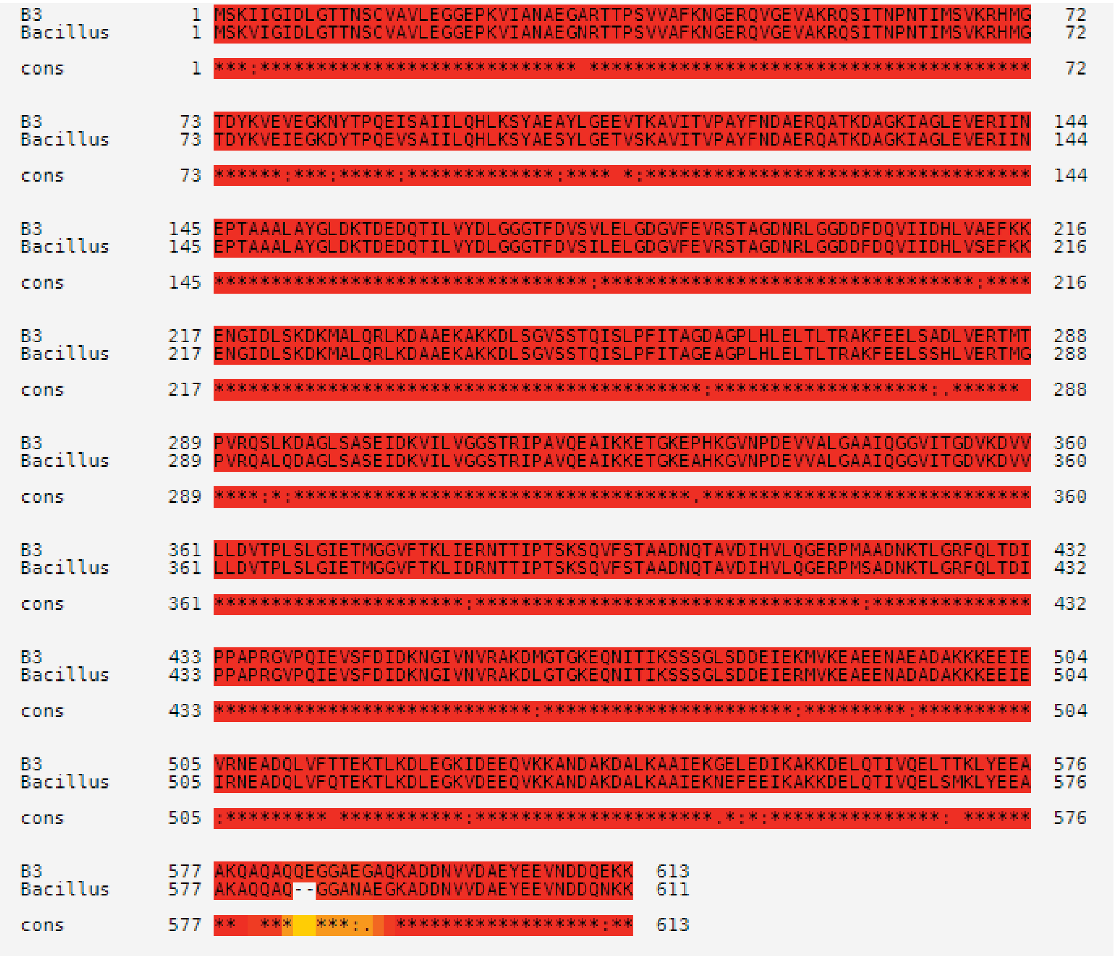

B3              521 KDLEGKIDEEQVKKANDAKDALKAAIEKG-ELEDIKAKKDELQTIVQELTTKLYEEAAK---QAQAQQE  585
Bacillus        521 KDLEGKVDEEQVKKANDAKDALKAAIEKN-EFEEIKAKKDELQTIVQELSMKLYEEAAK---AQQA--Q  583
Escherichia     551 EEAGDKLPADDKTAIESALTALETALKGE-DKAAIEAKMQELAQVSQKLMEIAQQQHAQQQHAQQQ--T  616
Pseudomonas     551 NEAGDKATADEKAAIEKALGELEAAVKGD-DKATIEAKMAALSEATTPLAQKMYAEQSQ---PGAA--A  613
Thermus         545 QEKQG---------APEAKARLEAAIQKAKELVERDAPDPELKAATEELLRA-VEEYEK----------  593

cons            553 ::  .           .*   *::*::   :    .*    *      *      :  :            621


B3              586 GGAEG-AQ-KADDNVVDAEYEEVNDDQEKK  613
Bacillus        584 GGANA-EG-KADDNVVDAEYEEVNDDQNKK  611
Escherichia     617 AGADASANNAKDDDVVDAEFEEVKD---KK  643
Pseudomonas     614 QGAEE-PK-GGADDVVDAEFEEVKD---NK  638
Thermus         594 -GAQAASS-KGPDDVIDADYKP------AE  615

cons            622  **:        *:*:**:::        :  651
```

Figure 8.3 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* (*Thermus*) are shown.



```
B3           1 MSKIIGIDLGTTNSCVAVLEGGEPKVIANAEGARTTPSVVAFKNGERQVGEVAKRQSITNPNTIMSVKRHMG  72
Bacillus     1 MSKVIGIDLGTTNSCVAVLEGGEPKVIANAEGNRTTPSVVAFKNGERQVGEVAKRQSITNPNTIMSVKRHMG  72

cons         1 ***:**************************** ***************************************  72


B3          73 TDYKVEVEGKNYTPQEISAIILQHLKSYAEAYLGEEVTKAVITVPAYFNDAERQATKDAGKIAGLEVERIIN 144
Bacillus    73 TDYKVEIEGKDYTPQEVSAIILQHLKSYAESYLGETVSKAVITVPAYFNDAERQATKDAGKIAGLEVERIIN 144

cons        73 ******:***:*****:*************:**** *:********************************** 144


B3         145 EPTAAALAYGLDKTDEDQTILVYDLGGGTFDVSVLELGDGVFEVRSTAGDNRLGGDDFDQVIIDHLVAEFKK 216
Bacillus   145 EPTAAALAYGLDKTDEDQTILVYDLGGGTFDVSILELGDGVFEVRSTAGDNRLGGDDFDQVIIDHLVSEFKK 216

cons       145 *********************************:*********************************:**** 216


B3         217 ENGIDLSKDKMALQRLKDAAEKAKKDLSGVSSTQISLPFITAGDAGPLHLELTLTRAKFEELSADLVERTMT 288
Bacillus   217 ENGIDLSKDKMALQRLKDAAEKAKKDLSGVSSTQISLPFITAGEAGPLHLELTLTRAKFEELSSHLVERTMG 288

cons       217 *******************************************:*******************:.******  288


B3         289 PVRQSLKDAGLSASEIDKVILVGGSTRIPAVQEAIKKETGKEPHKGVNPDEVVALGAAIQGGVITGDVKDVV 360
Bacillus   289 PVRQALQDAGLSASEIDKVILVGGSTRIPAVQEAIKKETGKEAHKGVNPDEVVALGAAIQGGVITGDVKDVV 360

cons       289 ****:*:***********************************.***************************** 360


B3         361 LLDVTPLSLGIETMGGVFTKLIERNTTIPTSKSQVFSTAADNQTAVDIHVLQGERPMAADNKTLGRFQLTDI 432
Bacillus   361 LLDVTPLSLGIETMGGVFTKLIDRNTTIPTSKSQVFSTAADNQTAVDIHVLQGERPMSADNKTLGRFQLTDI 432

cons       361 **********************:**********************************:************** 432


B3         433 PPAPRGVPQIEVSFDIDKNGIVNVRAKDMGTGKEQNITIKSSSGLSDDEIEKMVKEAEENAEADAKKKEEIE 504
Bacillus   433 PPAPRGVPQIEVSFDIDKNGIVNVRAKDLGTGKEQNITIKSSSGLSDDEIERMVKEAEENADADAKKKEEIE 504

cons       433 ****************************:**********************:*********:********** 504


B3         505 VRNEADQLVFTTEKTLKDLEGKIDEEQVKKANDAKDALKAAIEKGELEDIKAKKDELQTIVQELTTKLYEEA 576
Bacillus   505 IRNEADQLVFQTEKTLKDLEGKVDEEQVKKANDAKDALKAAIEKNEFEEIKAKKDELQTIVQELSMKLYEEA 576

cons       505 :********* ***********:*********************.*:*:***************: ****** 576


B3         577 AKQAQAQQEGGAEGAQKADDNVVDAEYEEVNDDQEKK   613
Bacillus   577 AKAQQAQ--GGANAEGKADDNVVDAEYEEVNDDQNKK   611

cons       577 **  ***  ***:.  ******************:**   613
```

Figure 8.4 Pairwise alignment of DnaK proteins of *Bacillus pumilus* B3 (B3) and *Bacillus subtilis* (*Bacillus*) showing substitutions and insertion of amino acids.

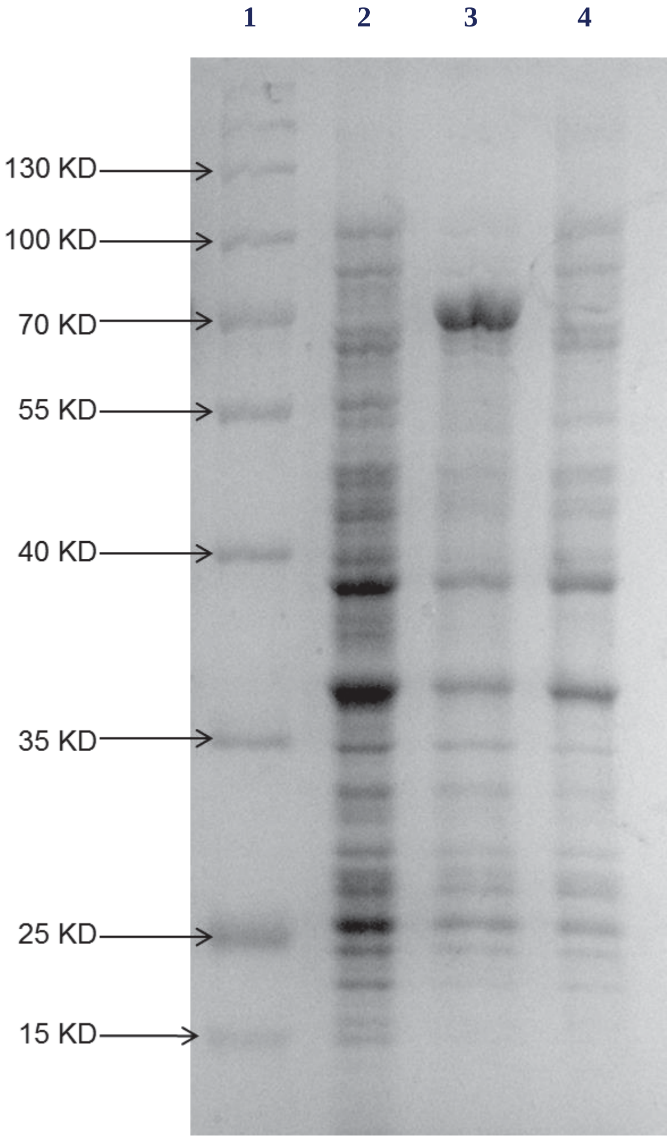


Figure 8.5 SDS-PAGE of crude cell extracts of transformants.
Lane 1-Protein molecular mass marker,
Lane 2-*E. coli* transformant (uninduced),
Lane 3- *E. coli* transformant induced (Cell lysates collected 1 hour after induction),
Lane 4- *E. coli* transformant induced (Cell lysates collected immediately after induction).

revealed matches with well described homologues of DnaK of bacteria. Hence *dnaK* of *B. pumilus* B3 was successfully expressed in *E.* coli BL21 (DE3).

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_{600nm} 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. 8.6 a and 8.6 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 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 up to 16 h for both the transformants, 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. 2007). 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 provide 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 machinery operating in the stressed cell (Wang et al. 2004). Although the specific mechanism of action of DnaK is not known from our results, previous studies (Ono et al. 2001; Uchida et al. 2007; Montero-Barriento et al. 2010) suggest 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 at 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.

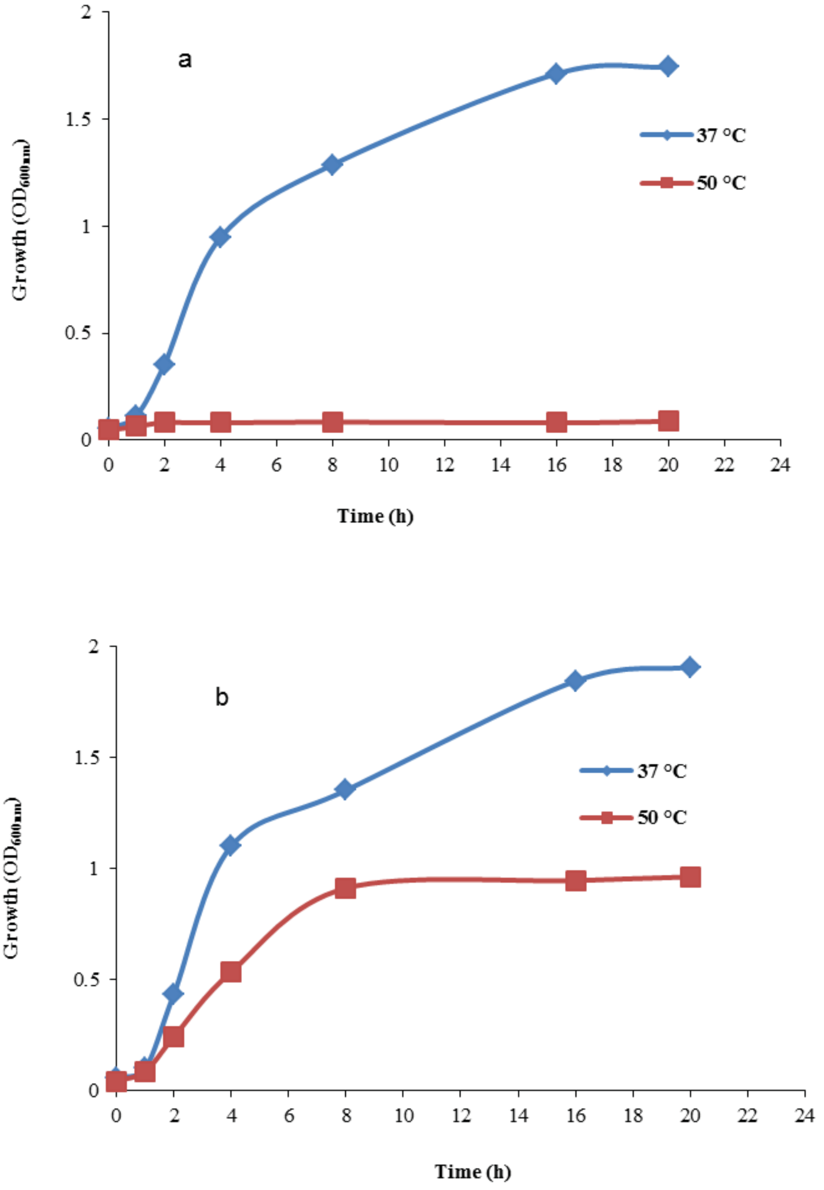


Figure 8.6 High temperature/Thermal stress tolerance of *E. coli* cells carrying *dnaK* of *Bacillus pumilus*. **a)** The *E. coli* cells with pET 29 (a) plasmid and **b)** with pET29-BpdnaK plasmid; cultured in LB medium at 37 °C and 50 °C. Cell growth was monitored by measuring the OD at 600nm.

Discussion

9. DISCUSSION

Extreme habitats such as hydrothermal vents, thermal springs, cold deserts, salt lakes, coal mines possess physiologically diverse groups of microorganisms, which are sources of several commercially important products like enzymes, sugars, compatible solutes and antibiotics (Satyanarayana et al. 2005). Diversity analyses of extreme environments have grown in significance, because the cellular chemistry exhibited by constituent microorganisms increases the base to mine novel metabolites/products of commercial interest. There is a logical progression from biodiversity to advances in biotechnology (Hunter-Cevera 1998); whether searching for new pharmaceuticals, compounds to protect agricultural crops against disease, unusual degradative pathways for remediation of recalcitrant pollutants or products of use in the food industry, availability of broad germplasm can definitely yield fruitful results. The possibility of generating new ideas during the analyses of microbial diversity from unique and extreme environments fascinates microbiologists and microbiologists (Kuddus and Ramtekke 2012). Identification of new biomolecules and the relevant genes can be a boon to agriculture as these genes could be utilized to develop transgenics tolerant to abiotic stress.

Thermal springs represent extreme niches whose pristine quality is maintained over a period of time. Many terrestrial hot springs exist on earth (Tobler and Benning 2011) which represent hot spots for unusual forms of life, genes and metabolites. Prokaryotes, because of their small size, easy dispersal, metabolic versatility, ability to utilize broad range of nutrients and tolerance to unfavourable extreme conditions, are ubiquitous in nature. Thermal springs, therefore are expected to be colonized by these prokaryotes. Phylogenetic studies using Small subunit ribosomal RNA sequence, combined with cultivation studies and in situ microbial physiological and ecological studies have shown that an abundant diversity of microorganisms inhabit thermal springs around the world like Iceland, Tunisia, Yellowstone National Park, USA and Kamchatka, Russia (Mitchell 2009; Reigstad et al. 2009; Sayeh et al. 2010).

India is the home to several hot springs and the Himalayan Geothermal Belts alone contain nearly 150 thermal springs. Hot springs in India are less explored in terms of biotic components and a few studies have been undertaken to study and utilize microorganisms from Indian hot springs (Jha 1992; Ghosh et al. 2003; Kumar et al. 2004). All these studies are fragmented in nature, concentrating on some hot springs or a particular group of microorganisms. A comprehensive approach was needed to bring microorganisms from hot springs into culture, analyse them for diversity and explore them for their possible applications. Hence the present study was designed to analyse the diversity of culturable thermotolerant bacteria from five Indian hot springs and study the mechanisms of thermotolerance employing proteomics and genomics based approaches.

Diversity of culturable thermotolerant bacteria from five Indian hot springs

Five different hot springs from India were selected for analyzing the diversity of culturable thermotolerant bacteria. Sites include Chumathang, Vashisht, Manikaran, Balrampur and Bakreshwar. Studies on microbial communities in terrestrial hot springs worldwide have mainly concentrated on habitats of low elevations like Yellowstone National Park, Kamchatka of Russia, Iceland, Indonesia and Tunisia. Despite intensive studies on terrestrial hot springs, not much of research has been carried out on bacterial diversity of hot springs of medium and high altitude regions (Huang et al. 2011). The sites selected for diversity analysis in the present study represent hot springs from different altitude regions. Chumathang (4023 m), Vashisht (1982 m) and Manikaran (1760 m) represent hot springs from high altitude regions, while Balrampur which is at elevation of 623 m above mean sea level and Bakreshwar at an elevation of 84 m above mean sea level represent hot springs from medium altitude and low altitude regions respectively. The limitations of culture based diversity studies are; aerobic and anaerobic microorganisms cannot be cultured together, fastidious organism will not often grow, and others (Piterina and Pembroke 2010). This method still offers major advantage in terms of generation of valuable germplasm. Working with the strains isolated from natural environments also offers a major advantage of generating core collections of strains future studies and exploring their potential biotechnological and industrial applications.

Hence in the present investigation a culture based approach was used to generate a germplasm of bacteria from these hot springs. In order to obtain a diverse range of nutritional types and bacteria with various metabolic requirements, five types of media were employed. These included a nutrient rich medium (Nutrient Agar) for isolating a larger proportion of the available diversity, a medium suitable for oligotrophs (R2A medium), specific media designed for thermophiles (Thermus Agar, Thermus Peptone Meat extract Yeast extract medium) and a medium mostly used for *Pseudomonas* and related genera (King's B medium). The population of aerobic heterotrophic bacteria in the water samples of Manikaran thermal springs ranged from 72 to 241 × 10^4 cfu/ml. Among the media used, highest population of 241× 10^4 cfu/ml was recorded on Nutrient agar medium. A total of 235 isolates were selected based on colony morphologies among which, 137 isolates were from Nutrient Agar medium, 47 isolates were from Thermus Agar medium, 13 Isolates were from King's B medium, and 19 each from TPMY and R2A media. Enumeration of bacterial population on different media from each samples revealed that aerobic heterotrophic bacteria in Bakreshwar thermal springs ranged from 119 to 183 × 10^4 cfu/mL and a similar trend was recorded for Vashist thermal springs, which was in the range from 158 to 229 × 10^4 cfu/mL. Among the different media used, R2A yielded maximum population of 229 × 10^4 cfu/mL from Vashisht samples and for Bakreshwar samples, while TPMY yielded maximum population of 183 × 10^4 cfu/mL. It is interesting to note that the pH of Vashist hot spring is in the neutral range, thus suggesting the role of temperature and pH in bacterial abundance in geothermal waters which is in accordance with earlier investigations (Purcell et al. 2007). In the samples from Balrampur and Chumathang, the population of aerobic heterotrophs were in the range from 89 to 193 × 10^3 cfu/mL and 69 to 112 × 10^4 cfu/mL, respectively. Thermus agar yielded maximum population from both Balrampur and Chumathang samples. A total of 235, 60, 48, 46 and 52 morphotypes were obtained from the hot springs, Manikaran, Bakreshwar, Balrampur, Chumathang and Vashist respectively. Screening of these morphotypes for thermotolerance revealed that a total of 85, 27, 38, 18 and 29 isolates were able to grow at 45 °C. Interestingly, bacteria from Manikaran and Bakreshwar alone were able to grow at temperatures above 45 °C. Although the temperature of other two hot springs Balrampur and Chumathang are high; 90 °C and 72 °C respectively, bacteria isolated from these hot springs had a tolerance limit of only 45 °C. There might be two reasons for not obtaining isolates tolerant to temperatures above 45 °C from these two hot springs; either absence of a monotonic relation between temperature stress and microbial community present (Mitchell 2009, Lau et al. 2006) or thermotolerant/thermophilic bacteria present in these hot springs might be more fastidious to nutrient status, hence not isolated with the media used. The inability to attain fastidious bacteria is an inherent limitation of culture based approach (Ranjard et al. 2000). Future research needs to be focused on development of special media to bring those bacteria, if any in culture and analyse their diversity. Metagenomic approach can also be used to throw light on the present observations. Bacterial isolates tolerant to temperature of 45 °C and above were selected for molecular characterization and diversity analyses.

Woese and other researchers have developed a new tool to identify bacteria and evaluate phylogenetic relationships among bacteria in the early 1980s. They found that all life forms could be identified by comparing a stable part of genetic code (Claridge 2004). There are more than 20 candidate genes available to carry out these evolutionary studies. Genes include 5S rRNA, 16S rRNA, 23S rRNA and 16-23S rRNA internal transcribed spacer (ITS) region. 16S rRNA is considered the best evolutionary chronometer among all these genes, the reasons being; its universal presence in all bacteria, relative stability over evolutionary time and appropriate size (1,500bp) which is large enough for variation analyses (Patel 2001). A further improvement of 16S rRNA gene analysis is Amplified ribosomal DNA restriction analysis (ARDRA), which involves restriction digestion of the amplified 16S rRNA gene product and comparison of restriction patterns and analysis using numerical taxonomy systems (Yadav et al. 2010). ARDRA is generally considered valuable for strain typing and screening clone libraries to identify phylogenetic clusters within a microbial community (Sklarz et al. 2009). Hence in the present study for molecular characterization and identification of thermotolerant isolates, initially ARDRA analyses were done for phylogenetic clustering of thermotolerant isolates from each hot springs. Sequencing of 16S rRNA gene of representative thermotolerant strain was undertaken for identification.

ARDRA analyses were done with three restriction enzymes viz., *Alu*I, *Hae*III, and *Msp*I. It was found that in all the five sites studied, *Alu*I was more discriminative as compared to *Msp*I and *Hae*III. A similar result of *Alu*I yielding a higher polymorphism among the 16S rRNA gene of plant growth promoting bacilli of moderately acidic soil was reported by Yadav et al. (2010). A combined dendrogram (based on patterns generated using three enzymes individually) revealed a total of 42, 14,14 11 and 9 phylogenetic clusters from thermotolerant isolates of Manikaran, Bakreshwar, Balrampur, Chumathang and Vashisht hot springs respectively. Utilization of ARDRA in the present study helped to reduce the cost and involved in sequencing by avoiding redundancy. ARDRA has been effectively utilized in the past for both culture dependent and culture independent studies, as a tool to group the isolates and clones in the phylotypes (Sklarz et al. 2009, Divya et al. 2010, Yadav et al. 2010).

Sequencing of 16S rRNA gene of representative isolates from all the five hot springs revealed that the majority of isolates from Balrampur (71.4 %), Bakreshwar (85.7 %), Manikaran (38 %) and Chumathang (36.4 %) belonged to *Bacillus* genus while *Bacillus* represented only 22.2 % of isolates from Vashisht. The members of the genus *Bacillus* are spore formers and a common inhabitant of diverse extreme habitats due to their ability to produce endospores (Nicholson et al. 2000). Apart from *Bacillus*, representatives from *Bacillus* derived genera like *Brevibacillus*, (Manikaran, Balrampur and Vashisht) *Lysinibacillus*, (Manikaran Bakreshwar and Balrampur) and *Aneurinibacillus* (Manikaran) were also present. Bae et al. (2005) has given sufficient evidence to reclassify thermophilic bacteria in the genus *Bacillus* into *Brevibacillus*, *Aneurinibacillus*, *Amphibacillus*, and *Geobacillus* based on the 16S rRNA gene sequence analysis. This supports the presence of genera *Brevibacillus* and *Aneurinibacillus* in the different hot springs studied. The presence of genera like *Micrococcus*, *Microbacterium*, *Staphylococcus*, *Kocuria* and *Exiguobacterium* in the hot springs studied is supported by the work of Cerritos et al. (2011), who referred to them as aquatic thermoresistant bacteria. *Chelatococcus daeguensis* a species of Proteobacteria was found in hot springs of Manikaran. Panday and Das (2010) have shown the presence of *Chelatococcus* spp. in hot spring sediments. *Cellulosimicrobium* isolated from Chumathang hot springs is closely related to *Cellulomonas*. Schuman et al. (2001) suggested that *Cellulomonas* should be reclassified as *Cellulosimicrobium*. *Cellulolomonas* has previously been reported as a culturable thermoresistant aquatic bacterium from the oligotrophic pools in Mexico (Cerritos et al. 2011). The present observations support the presence of *Cellulosimicrobium* in the hot springs.

A number of genera identified in the study from the hot springs were first reports of them being identified in the hot springs. *Aurantimonas*, *Brevundimonas*, *Planococccus*, *Rhodococcus*, *Pontibacter* and *Pseudomonas* isolated from different hot springs in the present study have not been reported in thermal springs earlier. *Pontibacter niistensis* isolated from Bakreshwar hot springs is normally a soil bacterium (Dastager et al. 2010) and its presence in the hot springs suggests a migration of bacterial communities from the sediments to the spring water aided by the flow of springs through the sediments. Representatives from *Acinetobacter*, *Enterobacter* and *Gulbenkiania* which are pathogens and associated with human intestine were isolated from Vashisht hot springs.This can be a result of contamination from people taking shower in the spring as this site is known for its medicinal properties. *Acinetobacter* represents group of bacteria commonly found in soil and water, they can also be found on the skin of healthy people, especially healthcare personnel. Although there are many types or “species” of *Acinetobacter* and all can cause human disease, *Acinetobacter baumannii* account for about 80% of reported infections. In a study to look for possible risks of exposure to harmful agents, hazardous pollutants at 57 natural hot springs from 11 provinces in northern, central, eastern and southern Thailand were analysed. Pathogenic, free-living amoebae of the genera *Naegleria* and *Acanthamoeba*, which can cause central nervous system infection, were found in 26.3% (15/57) and 15.8% (9/57), respectively. *Legionella pneumophila* (serogroups 1, 3, 5, 6, 7 10 and13) were found in samples from 71.9% (41/57) of studied sites (Sukthana, 2005). In India, opportunist human pathogens have been reported from the thermal springs of Rajgir, which are frequently used by tourists and local people for bathing and healing wounds (Singh et al 2013). Identification of Niche specific species of these hot springs have shown that 12 different species being niche specific to Manikaran hot springs, one to Bakreshwar, six to Chumathang and four to Vashisht hot springs (Table 9.1).

Phylogenetic grouping of strains from these hot springs have shown that members of Firmicutes and Actinobacteria inhabit all the five hot springs studied. Firmicutes was the predominant phylum in all the hot springs studied amounting for 72 %, 86 %, 93 %, 44 % and 44 % of the isolates in Manikaran, Bakreshwar, Balrampur, Chumathang and Vashisht respectively. The bacterial communities in many hot springs are dominated by Firmicutes (Huang et al. 2011; Tobler and Benning 2011; Pagaling et al. 2012). Gram positive prokaryotes like Firmicutes and Actinobacteria are known to be comparatively stress tolerant besides being long range migrants. It is interesting to note that hot springs in the highly elevated regions (Chumathang, Manikaran and Vashisht) alone possess members of phyla

Table 9.1 Niche specificity of species

Hot springs	Niche specific organisms
Manikaran	*Bacillus arbutinivorans*, *B. niacini*, *B. viretti*, *B. beijingensis*, *Aneurinibacillus danicus*, *Staphylococcus succinus*, *Microbacterium oxydans*, *Lysinibacillus xylaniticus*, *Rhodococcus baikunurensis*, *Cheltococcus daeguensis*, *Bhargavaea cecembensis* and *Planococcus*
Bakreshwar	*Pontibacter niistensis*
Balrampur	-
Chummathang	*Brevundimonas terrae*, *Kocuria palustris*, *Staphylococcus arlettae*, *Aurantimonas altamirensis*, *Paracoccus* sp. and *Cellulosimicrobium cellulans*
Vashist	*Acinetobacter baumanii*, *A. junii* and *Gulbenkiania mobilis*

Proteobacteria. Bacterial communities in the hot springs of high elevated regions of Tibet are known to be predominated by Proteobacteria alongside Firmicutes which was recorded in an earlier study (Huang et al. 2011) which supports our observation. This also reemphasizes the effect of geographical location on the type of bacterial communities in any particular environment (Whitaker et al. 2003), whether it is hot springs or soil environment.

Sixteen highly thermotolerant bacteria which were tolerant to growth temperatures 60 °C and above were further characterized for utilization of carbon substrates using Biolog. Based on the profiles generated, the identity of cultures matched with identification based on 16S rRNA gene sequencing only species level. However, for members from Actinobacteria, the 16S rRNA based identity did not match with Biolog based identity which may be attributed to insufficient information for Actinobacteria in the Biolog database. Biolog analysis in the present study revealed interesting results especially providing valuable information on the temperature tolerant isolates, which were positive results in terms of growth at low pH and high salt concentration. Such strains could be explored for their potential applications in agriculture and industry.

Proteomic analysis of thermotolerant strain

In order to survive in extreme environments, bacteria use adaptive networks elaborated during the process of evolution (Abee and Wouters 1999). Cells trigger programs of specific gene expression, to survive and grow in extreme conditions. Such variations in gene expression are manifested as an increase or decrease in the set of proteins (Duche et al. 2002). In the present study, among the 16 strains that were thermotolerant, one strain *B. pumilus* B3 isolated from Bakreshwar hot springs was selected. This strain showed tolerance to high temperatures upto 60 °C and growth, both under mesophilic (37 °C) and thermophilic (60 °C) conditions and hence used for proteomic analysis under different growth conditions. Studies on the heat shock response in *B. cereus* ATCC 14579 have shown that in addition to stress related proteins, proteins involved in metabolism and transport are also regulated (Periago et al. 2002). Therefore, the present study focussed on employing a proteomic approach to identify differentially expressed proteins at high temperature in *B. pumilus* B3. Proteomic analysis by 2DE followed by MALDI-TOF/MS peptide mass fingerprinting showed the expression of two hypothetical proteins, an ATP binding protein, ATP synthase subunit E and an aminotransferase.

Among the various anti stress strategies of bacteria, one of the most powerful is the high thermal stability of enzymes which can protect their metabolism at high temperatures.

Aspartate aminotransferase (AST) is a key enzyme of Krebs cycle, involved in the formation of oxaloacetate. Synthesis of various other essential amino acids is also dependant on AST. AST catalyzes the reversible transfer of α-amino group between four- and five-carbon dicarboxylic amino acids and the α-keto-acids by a mechanism named “ping-pong bi-bi”, which is pyridoxal phosphate dependent (Deu et al. 2002). Chen et al. (2013) have shown that AST forms complex with GroEL (HSP 60) to protect proteins in thermophilic microorganisms. Hence the aminotransferase, up regulated in the strain *B. pumilus* B3 at high temperature might play a role along with GroEL (HSP 60) in protecting the proteins at that temperature. Although the ATP binding protein and ATP synthase subunit E are not known to play any direct role in thermotolerance, their up regulation at high temperature is indicative of their indirect involvement in thermotolerance. All molecular chaperones which protect the protein from unfolding and denaturation at higher temperature require the presence of ATP and it has been shown that ATP enhances the chaperonic activity of molecular chaperones (Muchowski and Clark 1998). As cells might require more ATP to support the chaperonic activity, the ATP binding protein and ATP synthase subunit E might indirectly enhance the cells’ thermotolerance by supplying sufficient ATP for molecular chaperones to function effectively under stress. Employing a proteomic approach to understand thermotolerance in a strain of *B. pumilus* B3 isolated from hot water springs has provided important pointers for research on other extremophiles.

Allelic variation in *dnaK* of thermotolerant strains

Physiological stress such as sudden change in temperature, an increase in the salt or acid concentration and oxidative stress induce the synthesis of class of proteins called heat shock proteins (Hsps). The first report on a 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). Hsp 70 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). Hsp 70 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 HSP70 in the domain Bacteria is called *dnaK* (Ward-Reiney et al. 1997). The role of *dnak* in thermoregulation is well established by gene expression studies at mRNA level (Wetzstein et al. 1992) and use of deletion mutants (Singh et al. 2007). Enhancement of thermotolerance in heterologous systems have 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 drought and freezing stress in poplar (Takabe et al. 2008).

Gene or allele mining for abiotic stress tolerance is among the major goals of research worldwide in the light of climate change and resultant reducing crop yields. The greatest value of microbial diversity in extreme environments is its opportunity to mine novel genes and metabolites (Combie and Runnion 1996). Allele/gene mining for tolerance to extreme condition is one such novel approach which exploits the nucleotide sequence of one organism to isolate useful alleles from different organisms (Latha et al. 2004). Recognizing the importance of the gene *dnaK* in thermoregulation and its potential role in management of abiotic stress in crop plants (Ono et al. 2001; Uchida et al. 2007; Montero-Barrientos et al. 2010) and possibility of recombinant overexpression of particular protein of interest (Schlicker et al. 2002), an attempt was made to explore the polymorphisms in the gene *dnaK* of thermotolerant strains isolated from thermal springs. Extensive screening with different primers against different strains yielded amplification of partial gene of *dnaK* from seven bacilli isolated from Manikaran hot springs. Among those seven strains, six (M4, M5, M6, M7, M8 and M55) were highly thermotolerant (tolerate upto 70 °C) and one strain, M36 was moderately thermotolerant (tolerate upto 45 °C). Sequencing and analyses revealed polymorphism in the ATPase domain of DnaK protein in the highly thermotolerant and moderately thermotolerant strains. Nucleotide binding and ATP hydrolysis, activities of ATPase are important steps in the chaperonic activity of DnaK (Liang et al. 2009). 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 (Flynn et al. 1989; Gragerov et al. 1994). Polymorphism between the DnaK of thermotolerant strains in the ATPase domain may be the reason for difference in the thermotolerance limit of those strains. Although studies on partial gene sequence and analysis give us an idea of variability in the gene, for generating more information, cloning of full length gene is inevitable. Hence, in the present study a PCR based approach to clone full length *dnaK* from thermotolerant strains was employed. Representatives from genera *Bacillus*, *Exiguobacterium* and *Pseudomonas* found to tolerate growth temperatures upto 70 °C were selected to clone full length *dnaK* gene. In the domain Bacteria *dnaK* gene is a 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* (http://www.ncbi.nlm.nih.gov/genome) allowed for the designing of primers in the region flanking *dnaK* gene. Screening with different primers against different strains yielded amplification of expected size from five *Bacillus* strains. Among these, four (B3, B5, B12 and B34) had been earlier identified as *B. pumilus* and one strain (M8) was identified as *Bacillus* sp. based on 16S rRNA gene based identity. The failure to attain amplification of *dnaK* from other thermotolerant strains cannot be considered as non-availability of the gene in the organism as *dnaK* or its homologue, as it 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). Allelic variations, lack of annealing with primers used or amplifying conditions being not conducive for the specific organism can be a possible reason.

For identifying any possible variation in the gene *dnaK* of these five strains, Restriction fragment length polymorphism (RFLP) was employed. 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 RFLP with three restriction enzymes (*Alu*I, *Hha*I and *Hae*III) separately, yielded similar restriction patterns, suggesting no variations in the *dnaK* region of these five strains. Hence, *dnaK* from one representative strain *B. pumilus* B3 was selected for sequencing, cloning and characterization. This strain *B. pumilus* B3 was selected because of its ability to grow well under both mesophilic and thermophilic conditions. Sequencing and analysis of *dnaK* gene of *B. pumilus* B3 revealed that the protein DnaK of *B. pumilus* was different from the DnaK of a mesophilic counterpart *B. subtilis.* Alignment revealed 36 amino acids substitution in the DnaK of *B. pumilus* B3 as compared to DnaK of *B. subtilis*. Substitutions included 18 in the ATPase domain, 2 in the substrate binding domain and 16 in the α-helical domain. Considering the importance of ATPase domain in the chaperonic activity of DnaK (Flynn et al. 1989; Gregarov et al. 1994; Liang et al. 2009), the 18 amino acid substitutions in the ATPase domain of DnaK of *B. pumilus* B3 can be hypothesized to be responsible for its thermotolerance.

For obtaining a more straightforward proof for *dnaK* of *B. pumilus* B3 imparting thermotolerance, this gene was transformed into a heterologous host *E.coli* BL21 (DE3). Cloning and expression analysis showed that this gene imparted ability to the host to grow at 50 °C and survive upto 16 h at 60 °C. 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 machinery operating in the stressed cell (Wang et al., 2004). Although the specific mechanism of action of DnaK is not known from our results, previous studies (Ono et al., 2001; Uchida et al. 2007; Montero-Barriento et al. 2010) suggest 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 at 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.

Summary and Conclusion

10. SUMMARY AND CONCLUSION

Bacteria colonizing thermal springs and other extreme habitats are known to produce various commercially important products like enzymes, sugars and antibiotics. Investigations on bacterial diversity of extreme habitats like thermal springs have grown in significance because of the potential industrial applications of these microorganisms. The Indian subcontinent is a home to innumerable number of hot springs which are of ancient origin. Vast information on geochemical characteristics of several hot springs of India is available. Yet, only a countable number of them have been explored microbiologically. Almost in every instance, the investigation was fragmented and a comprehensive approach is therefore needed to culture bacteria from hot springs, collate the information on their diversity and explore them for their possible utility. Hence, an investigation was carried out with an aim to analyse diversity of culturable bacteria from five hot springs of India, understand the mechanism of tolerance and mine genes conferring high temperature tolerance.

The five hot springs selected for the study were Chumathang, Vashisht, Manikaran, Balrampur and Bakreshwar. Three of them Chumathang, Vashisht and Manikaran represent sites in the Himalayan geothermal belts, belonging to high altitude regions. Balrampur represents a site from medium altitude region and Bakreshwar is located in a low altitude region. Culturable bacteria were isolated from these hot springs employing five media having different nutrient composition. A total of 235, 60, 48, 46 and 52 morphotypes were obtained from Manikaran, Bakreshwar, Balrampur, Chumathang and Vashisht hot springs respectively. They were screened for thermotolerance which revealed a total of 85, 27, 38, 18 and 29 isolates from Manikaran, Bakreshwar, Balrampur, Chumathang and Vashisht hot springs respectively, were able to grow at 45 °C. Screening at higher temperatures revealed that only the isolates from hot springs of Manikaran and Bakreshwar were able to grow at temperatures upto 70°C. All the isolates tolerant to incubation temperature of 45 °C and above were selected for molecular characterization using 16S rDNA analysis. ARDRA with three restriction enzymes (*Alu*I, *Hae*III and *Msp*I) generated 42, 14, 14, 11 and 9 phylogenetic clusters from Manikaran, Bakreshwar, Balrampur, Chumathang and Vashisht hot springs respectively. Sequencing and analyses of 16S rDNA from one representative isolate from each phylogenetic cluster showed that a majority of isolates from Balrampur (71.4 %), Bakreshwar (85.7 %), Manikaran (38 %) and Chumathang (36.4 %) belonged to the genus *Bacillus*. However, only 22.2 % of isolates from Vashisht were identified as *Bacillus*. There were also representatives from *Bacillus* derived genera like *Brevibacillus* (Manikaran, Balrampur and Vashisht) *Lysinibacillus* (Manikaran Bakreshwar and Balrampur) and *Aneurinibacillus* (Manikaran). A number of genera such as *Aurantimonas*, *Brevundimonas*, *Planococccus, Rhodococcus* and *Pontibacter* were identified for the first time from hot springs.

Phylogenetic grouping of strains have shown that majority of the isolates (> 70%) belonged to Firmicutes, except at Chumathang and Vashist, where the combined population of Proteobacteria and Actinobacteria was in proportion equal to Firmicutes. It was also noted that members of phylum Proteobacteria were present only in hot springs of high altitude region which include Manikaran, Vashisht and Chumathang. Thermal springs specifically enrich certain species of bacteria and niche specific genera and species were identified from each thermal spring. There were 12 species niche specific to Manikaran, one to Bakreshwar, six to Chumathang and four to Vashisht hot springs. Biolog analysis of sixteen highly thermotolerant strains (13 from Manikaran and 3 from Bakreshwar) illustrated the tolerance of some isolates to low pH and high salt concentration. This highlighted the unusual nature of these isolates, in terms of their biochemical and physiological attributes.

Among the sixteen highly thermotolerant strains, *B. pumilus* B3 (isolated from Bakreshwar hot springs) was selected for proteomics study, based on the strain's ability to grow well under both mesophilic and thermophilic conditions. Proteomic analysis by two-dimensional electrophoresis (2DE) revealed up regulation of eight proteins and down regulation of four proteins at high temperature (60 °C). Identification by MALDI-TOF/MS peptide mass fingerprinting revealed that among the upregulated proteins, two were hypothetical proteins, while one each was an ATP binding protein, ATP synthase subunit E and aminotransferase respectively. Employing a proteomic approach therefore helped in understanding thermotolerance in the selected bacteria besides provide additionally important baseline information for research on other extremophiles.

Efforts were made to analyse the role of allelic variation in the gene *dnaK* on thermotolerance of bacteria, by exploring the polymorphisms. Screening with different set of primers against different thermotolerant strains yielded partial length amplification products of *dnaK* from seven thermotolerant bacilli isolated from Manikaran hot springs. Among those seven strains, six (M4, M5, M6, M7, M8 and M55) were highly thermotolerant (tolerated upto 70 °C) and one strain, M36 was moderately thermotolerant (tolerated upto 45 °C). The *dnaK* of these seven strains were grouped into four clusters based on their nucleotide sequence. However, in silico translation and analysis of amino acid sequence of DnaK grouped the strains into three clusters, two representing the highly thermotolerant strain and one representing the moderately thermotolerant strain. The DnaK of the highly thermotolerant strains showed variation for only one amino acid, but a comparison of DnaK of the highly thermotolerant strain and moderately thermotolerant strain revealed a polymorphism for 27 amino acids.

The full length gene of *dnaK* along with the flanking region was cloned from five thermotolerant strains (B3, B5, B12, B34 and M8) and RFLP analyses showed them to be identical. Hence, the *dnaK* from one strain (*B. pumilus* B3) was selected for sequencing and further analyses. The DnaK of *B. pumilus* B3 differed from its mesophilic counterpart *B. subtilis*, with variations in 18 amino acids in the ATPase domain, two in the substrate binding domain and 16 in the α-helical domain. Cloning, followed by expression analyses revealed that the gene *dnaK* of *B. pumilus* B3 conferred thermotolerance in the heterologous host *Escherichia coli* BL21 (DE3).

Future research needs to be focussed on in depth analyses of the *dnaK* gene with metabolic networking of the cell in imparting thermotolerance. This can prove useful in the development of transgenics tolerant to high temperature stress.

ABSTRACT

Thermal springs represent aquatic bodies emerging from earth's crust which are habitats of pristine quality. India is home to several hot springs whose biological attributes are not well characterized. Exotic niches like thermal springs are expected to be colonized by a diverse group of bacteria that possess unexplored biotechnological potential. Identification of new biomolecules and the relevant genes can be a boon to Indian agriculture as they can be utilized in the development of transgenics tolerant to abiotic stress. Hence, the present investigation focused towards generating a germplasm of culturable bacteria from five hot springs in India and analyses of their diversity. Attempts were made to understand the mechanisms for thermotolerance in the bacteria employing proteomics and genomics approaches.

The diversity of culturable thermotolerant bacteria in the five hot springs of India *viz*., Manikaran, Vashisht, Chumathang, Balrampur and Bakreshwar was analysed. A total of 235, 52, 46, 48 and 60 different morphotypes from Manikaran, Vashisht, Chumathang, Balrampur and Bakreshwar respectively were selected and screened for thermotolerance. Results revealed that 85, 29, 18, 38 and 27 isolates from these hot springs respectively were tolerant to incubation temperatures of 45 °C. Only the isolates from Manikaran and Bakreshwar were able to grow at temperatures above 45 °C. A total of sixteen isolates were found to be highly thermotolerant exhibiting growth at incubation temperatures 60 °C and above. All the isolates tolerant to incubation temperatures 45 °C and above were selected for diversity analyses based on amplified ribosomal DNA restriction analysis (ARDRA) and 16S rDNA sequencing. ARDRA followed by 16S rDNA sequencing showed that the majority of isolates belonged to *Bacillus* and *Bacillus* derived genera. Phylogenetic groupings of isolates revealed members of the phyla Firmicutes were predominant in these hot springs and members of phylum Proteobacteria were present only in hot springs of high altitude region.

Among the sixteen strains that were highly thermotolerant, *B. pumilus* B3 isolated from Bakreshwar hot springs was selected for proteomic analyses based on the strain's ability to grow well under both mesophilic and thermophilic conditions. Proteomic analysis followed by 2DE and MALDI-TOF mass spectrometry revealed the possible role of ATP binding protein, ATP synthase subunit E and aminotransferase in the thermotolerance of this strain. Two hypothetical proteins were also found to be implicated in thermotolerance.

Screening of different thermotolerant strains with multiple set of primers yielded partial length amplification products of *dnaK* (a gene coding for DnaK/Hsp70, involved in thermotolerance) from seven thermotolerant bacilli isolated from Manikaran hot springs. The seven strains varied in their thermotolerance limit (six were highly thermotolerant and one moderately thermotolerant). Allelic variation studies in the highly thermotolerant strain and moderately thermotolerant strain, revealed polymorphisms for 27 amino acids in the DnaK, which may be responsible for the difference in their limits to thermotolerance. The full length gene of *dnaK* from the strain *B. pumilus* B3 was cloned and expressed in the heterologous host *Escherichia coli* BL21 (DE3). It was found that the *dnaK* of *B. pumilus* B3 conferred thermotolerance to the heterologous host *E. coli* BL21 (DE3).

The study revealed the enormous diversity of bacteria colonizing the different hot springs, and allelic variation in *dnaK* gene, which is involved in thermotolerance. The study also identified a candidate gene *dnaK* in the strain *B. pumilus* B3 for thermotolerance, which can be explored for its possible role in developing transgenics.

सारांश

गर्म झरनें भूपटल पर पाए जाने वाले प्राचीन गुणवत्ता वाले निवासों व उभरते हुए जलीय निकायों का प्रतिनिधित्व करते हैं | भारत ऐसे अनेक गर्म झरनों का घर है जिनके जैविकीय गुणों की विशेषताओं का अच्छी प्रकार वर्णन नही किया गयाहै | असाधारण आले, जैसे गर्म झरने, जीवाणुओं के एक ऐसे विविध समूह द्वारा उपनिवेश होने की उम्मीद कर रहें हैं जो अनेकों जैव प्रोद्यौगिकी क्षमताएं रखते हैं तथा जिनका अभीतक कोई पता नही लगाया गया | नयेजैव अणुओं एवं प्रांसगिक जीनों की पहचान भारतीय कृषि के लिए एक वरदान साबित हो सकती है क्योंकि इन जीनों को अजैवकीय तनाव के प्रति पराजीवी सहनशीलता विकसित करने के लिए प्रयोग किया जा सकता है |अत: वर्तमान अन्वेषण में भारत में पाए जाने वाले पांच गर्म झरनों से कृषि उपयोगी जीवाणुओं का जननद्रव्य सृजन कर उनकी विविधता प्रथक्करण करने पर ध्यान केंद्रित किया गया है |नियुक्त जीवाणुओं के प्रोटिओमिक एवं जीनोमिक दृष्टिकोण को ध्यान में रख उष्म सहनशीलता करने की प्रक्रिया को समझने के प्रयास किये गए हैं |

भारत के पांच गर्म झरनों नामत: मनिकर्ण, वशिष्ठ, चुमाथंग, बलरामपुरएवं बकरेश्वर में पाए जाने वाले कृषियोग्य उष्मसहनशील जीवाणुओं की विविधता का प्रथक्करण किया गया | विभिन्न आकारिकी वाले कुल 235 मनिकर्ण से,52 वशिष्ठ से,46 चुमाथंग से,48बलरामपुर से एवं 60 जीवाणु बकरेश्वर से क्रमश: चयनित किये गए एवं उनकी उष्म सहनशीलता की जाँच की गयी |परिणाम से पता चला कि इन गर्म झरनों से क्रमश: 85, 29, 18, 38 एवं27नियोजन (आईसोलेटस)सहनशील पाए गए जो 45^{0}Cऊष्मायन तापमान को सहन कर लेते हैं | केवल मनिकर्ण एवं बकरेश्वर से नियोजित ही 45^{0}C और उससे अधिक तापमान में विकसित करने में सक्षम थे | कुल 16 नियोजन ऐसे पाए गए जिन्होंने उच्च उष्म सहनशीलता ऊष्मायन तापमान 60^{0}C एवं अधिक पर भी विकसित होने का प्रदर्शन किया | वे सभी नियोजन जिनमे ऊष्मायन तापमान 45^{0}C और उससे अधिक सहन करने कि क्षमता थी उनका चयन प्रवर्धित राईबोसोमल डीएनए विश्लेषण (एआरडीआरए) एवं16एसआरडीएनए अनुक्रमण के आधार पर विविधता विश्लेषण के लिए किया गया |एआरडीआरए के बाद 16एस आरडीएनए अनुक्रमण ने यह दर्शाया है कि ज्यादातर नियोजन*बेसिलस* एवं *बेसिलस* व्युत्पन्न पीढ़ी से थे | नियोजनों (आईसोलेटस) के वंशावली समूह से यह बात प्रकट हुई कि गर्म झरनों में फर्मीक्यूटस संघ के सदस्य प्रभावशाली थे एवं प्रोटियोबैक्टेरिया संघ केवल उच्च ऊँचाई वाले क्षेत्र में ही मौजूद थे |

सोलह नियोजन जो उच्च सहनशीलता रखते थे, उनमे से *बेसिलसप्युमिलस* बी 3 जो बकरेश्वर के गर्म झरने से प्रथक किया गया था उसे प्रोटिओमिक विश्लेषण के लिए चयनित किया गया क्योंकि यह उपभेद मध्यमतापरागी (मीसोफिलिक) एवं तापरागी (थेर्मोफिलिक) दोनों

ही दशाओं में विकसित होने की क्षमता रखता है |प्रोटिओमिक विश्लेषण के उपरांत 2डीई एवं एमएएलडीआई - टीओएफ मास स्पेक्ट्रोमेट्री करने से एटीपी बाध्यकारी प्रोटीन, एटीपी सिंथेस की उपइकाई ई एवं अमीनों ट्रांस्फेरेज की संभावित भूमिका का पता चला जो इस उष्म सहनशील प्रभेद में पाए गए | उष्म सहनशीलता प्रदान करने वाले दो काल्पनिक प्रोटीन भी पाए गए | मनिकर्ण के गर्म झरनों से नियोजित सात उष्म सहनशील बेसिली का विभिन्न उष्म सहनशील प्रभेदों की विभिन्न प्राईमरों के द्वारा जाँच करने से डीएनएके (*dnaK*जीन)का आंशिक लम्बाई प्रवर्धन उत्पाद प्राप्त हुआ |(DnaK/Hsp70 के लिए एक जीन जो उष्म सहनशीलता में शामिल होता है)| सातों प्रभेदों की उष्म सहनशीलता की सीमा में भी विभिन्नता पाई गई जिनमे से छ: प्रभेद उच्च उष्मसहनशील थे जबकि एक मामूली उष्मसहनशील था |

उच्च उष्मसहनशीलएवं मामूलीउष्मसहनशील प्रभेदों की युग्म विकल्पित भिन्नता का अध्ययन करने सेडीएनएके (DnaK) में 27 अमीनो अम्लों के लिये बहुरूपताओं का पता चला जो उनकी उष्म सहनशीलता की विभिन्न सीमा के लिए जिम्मेदार हो सकते हैं |*बेसिलसप्युमिलस* बी 3 प्रभेद से डीएनएके (*dnaK*) का पूरी लम्बाई के जीन का क्लोन किया गया और उसे विषम जातीय मेजबान *ई.कोलाई* बी एल 21 (डीई3) में व्यक्त किया गया | यह देखा गया कि *बेसिलसप्युमिलस* बी 3 प्रभेद, विषमजातीय मेजबान *ई.कोलाई* बी एल 21 (डीई3)को उष्म सहनशीलता अर्पित करता है|

इस अध्ययन से गर्म झरनों में जीवाणुओं कि विशाल बस्तियों (उपनिवेश) एवं डीएनएके (*dnak*) जीन में विषमजातीय भिन्नता का पता चला जो उष्म सहनशीलता प्रदानकरता है | इस अध्ययन से *बेसिलसप्युमिलस* बी 3 प्रभेद के डीएनएके (*dnak*) जीन की पहचान भी उष्म सहनशीलता के लिए एक उम्मीदवार जीन के रूप में हुई, जिससे ट्रांसजेनिक विकासशील करने में उनकी संभव भूमिका का पता लगाया जा सकता है |

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Deciphering the diversity of culturable thermotolerant bacteria from Manikaran hot springs

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ORIGINAL ARTICLE

Deciphering the diversity of culturable thermotolerant bacteria from Manikaran hot springs

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Abstract The aim of this study was to analyze and characterize the diversity of culturable thermotolerant bacteria in Manikaran hot springs. A total of 235 isolates were obtained employing different media, and screened for temperature tolerance (40 °C–70 °C). A set of 85 isolates tolerant to 45 °C or above were placed in 42 phylogenetic clusters after amplified ribosomal DNA restriction analysis (16S rRNA-ARDRA). Sequencing of the 16S rRNA gene of 42 representative isolates followed by BLAST search revealed that the majority of isolates belonged to Firmicutes, followed by equal representation of Actinobacteria and Proteobacteria. Screening of representative isolates (42 ARDRA phylotypes) for amylase activity revealed that 26 % of the isolates were positive, while 45 % exhibited protease activity, among which one amylase and six protease producers were tolerant up to 70 °C. BIOLOG-based identification of 13 isolates exhibiting temperature tolerance up to 70 °C, using carbon utilization patterns and sensitivity to chemicals, revealed a high degree of correlation with identification based on 16S rRNA gene sequencing for all isolates, except one (M48). These promising isolates showing a range of useful metabolic attributes demand to be explored further for industrial and agricultural applications.

Keywords Culturable bacteria · 16S rRNA gene · ARDRA · Sequencing · BIOLOG analysis · Enzymes

Introduction

Exotic niches, such as thermal springs, harbor populations of microorganisms that can be a source of commercially important products like enzymes, sugars, compatible solutes and antibiotics (Satyanarayana et al. 2005). Thermal springs are a manifestation of geological activity and represent aquatic microcosms that are produced by the emergence of geothermally heated groundwater from the Earth's crust. Prokaryotes are the major component of most ecosystems, being ubiquitous in nature because of their small size, easy dispersal, metabolic versatility, ability to utilize a broad range of nutrients, and tolerance to unfavorable and extreme conditions. Thermal springs are, therefore, no exception to colonization by prokaryotes. Diversity analysis of such extreme environments has grown in significance because of their diverse and unusual chemistry and the opportunity they provide to identify rare compounds and genes (Kuddus and Ramtekke 2012).

In the past, phylogenetic- combined with cultivation- and in situ microbial physiological- and ecological-studies have revealed the abundant diversity of thermophilic microorganisms inhabiting hot springs around the world in locations like Japan, New Zealand, Iceland and Yellowstone National Park in the US (Tobler and Benning 2011). Physiologically diverse microorganisms are likely to exhibit diverse chemistry, hence increasing the chance of finding novel compounds. Several hot springs in different regions of India have been known to geologists for years (Ghosh et al. 2003). Although a few attempts have been made to study and utilize microorganisms from hot springs in India (Nawani and Kaur 2000; Ghosh et al. 2003), most such studies are fragmented in nature.

Manikaran hot springs are located in the Beas and Parvati valley geothermal system, at an elevation of 1,760 metres (Cinti et al. 2009). These hot springs are the hottest in the country, with a temperature range of 89–95 ° C (Dwivedi et al. 2012). Despite intensive studies on terrestrial thermal springs, very little is known about the bacterial diversity of thermal

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springs at high elevation (Huang et al. 2011). Hence, a comprehensive approach is needed to analyze the bacterial diversity of such a niche, in terms of its taxonomic and biochemical attributes.

Studies on the diversity of bacterial communities from various environments have been undertaken mainly using traditional methods of isolating and culturing microorganisms. Such methods involve isolation of microbes on standard cultivation media and characterization of microbes (Ranjard et al. 2000). Culture-based methods of analyzing microbes have been the mainstay of microbiology since their origin in the pioneering works of Robert Koch and Louis Pasteur. However, culture-based techniques have many limitations when analyzing the bacterial diversity of a particular environmental niche. Aerobic and anaerobic organisms cannot be cultured together; fastidious organisms will often not grow, because essential nutrients for growth or optimal environmental conditions such as temperature, pH, essential mixtures of gases may not be present (Piterina and Pembroke 2010). Despite these limitations, there is immense scope for culture-based diversity studies because of the advantages they offer in the generation of valuable germplasm. Working with strains isolated from hot springs offers the major advantage of preserving those strains for future studies and exploring them in due course for potential biotechnological applications (Akanbi et al. 2010; Acharya and Chaudhary 2012).

In the 1980s, a new tool for identifying bacteria and evaluating their phylogenetic relationships was developed in the laboratories of Woese and other researchers. It was found that all life forms could be identified by comparing a stable part of their common genetic code. Candidate genes used for these evolutionary studies numbered as many as 20; including 5S rRNA, 16S rRNA, 23S rRNA and the 16-23S rRNA internal transcribed spacer (ITS) region. Among these latter, the 16S rRNA gene is considered the best evolutionary chronometer because of its universal presence in all bacteria, its relative stability over evolutionary time, and its appropriate size (1,500 bp), i.e., large enough for bioinformatic analyses (Patel 2001). Amplified ribosomal DNA restriction analysis (ARDRA), which involves the comparison of restriction patterns (Yadav et al. 2010) and analyses using bioinformatic tools, represents a further improvement in the analysis of the 16S rRNA gene. ARDRA is generally considered valuable for strain typing and screening clone libraries to identify phylogenetic clusters within a microbial community (Sklarz et al. 2009).

The present study attempted to decipher the bacterial diversity of culturable thermotolerant bacteria from Manikaran thermal springs employing different media, followed by screening of isolates for temperature tolerance. ARDRA analysis was done for phylogenetic clustering of the thermotolerant isolates. Sequencing the 16S rRNA gene of representative thermotolerant strains was undertaken for identification. BIOLOG and analyses of other important biochemical attributes helped in further screening and selection of promising isolates, as a prelude to their application in agriculture and industry.

Material and methods

Sampling and isolation of culturable bacteria

Water samples were collected from four different sites of Manikaran hot springs. Manikaran is located at 32 ° 02′ N latitude and 77 ° 34′ E longitude. The temperature and pH of Manikaran hot springs ranges from 89 °C to 95 °C and 7.8 to 8.2, respectively. Water samples were collected in thermos flasks and brought to laboratory within 12 h of collection and processed for isolation of culturable bacteria.

For enumeration and isolation from water, samples were plated on five different media using a standard spread plate technique and incubated at 37 °C in an incubator for 48–72 h. The different media used were, nutrient agar (peptone 0.5 %, beef extract 0.3 %, NaCl 0.5 % and agar 1.8 %), thermus agar (peptone 0.5 %, yeast extract 0.2 %, beef extract 0.4 %, NaCl 0.5 % and agar 1.8 %), R2A medium (proteose peptone 0.05 %, casamino acid 0.05 %, yeast extract 0.05 %, dextrose 0.05 %, soluble starch 0.05 %, dipotassium hydrogen phosphate 0.03 %, sodium pyruvate 0.03 %, magnesium sulfate heptahydrate 0.005 %), King's B medium (protease peptone 2 %, dipotassium hydrogen phosphate 0.15 %, magnesium sulfate 0.15 % and agar 1.8 %) and thermus peptone meat extract yeast extract medium (TPMY: peptone 0.35 %, meat extract 0.5 % yeast extract 0.2 % NaCl 0.15 % and agar 1.8 %). After incubation, plates were observed for colony morphology and the total viable count was recorded for each sample in the different media employed. Based on differences in colony morphologies, 235 morphotypes were picked up from different plates and axenized.

Screening of isolates for temperature tolerance

All 235 isolates were screened for temperature tolerance by incubating the culture spot inoculated plates at different temperatures (40, 45, 50, 55 and 60 °C) for 72 h. Cultures tolerant to 60 °C were screened further by inoculating in broth and incubating in a shaker at 150 rpm for 72 h at 70 °C. The optical density of the broth was measured at 600 nm and compared with growth at 37 °C.

Genomic DNA extraction

Isolates were grown in specific broth, until they reached an $OD_{600\ nm} > 1.0$. The cells were pelleted from 5 ml culture, washed thrice with TE buffer (10 mM Tris and 1 mM EDTA, pH 8.0) and the pellet was resuspended in 750 µl 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.

PCR amplification of 16S rRNA gene

The primers pA (5′-AGA GTT TGA TCC TGG CTC AG-3′) and pH (5′-AAG GAG GTG ATC CAG CCG CA-3′) were used for amplification of 16S rRNA gene from the genomic DNA of isolates (Edwards et al. 1989). Amplification was carried out in a 100 μl reaction volume containing 50–100 ng template DNA, primers pA and pH (100 ng each), dATP, dCTP, dTTP and dGTP (200 μM each), *Taq* polymerase reaction buffer (10X) 10 μl and 1.0 U *Taq* polymerase. Conditions used for amplification were as follows: initial denaturation for 5 min at 95 °C, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 52 °C for 40 s and extension at 72 °C for 1 min and a final extension at 72 °C for 10 min. 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 (10 mg/ml) and visualized on a gel documentation system (Alpha Imager, Alpha Innotech, Santa Clara, CA).

Amplified rDNA restriction analysis

Purified PCR products were digested separately with three restriction endonucleases - *Alu* I, *Msp* I and *Hae* III in a 30 μl reaction volume, using recommended buffers at 37 °C. Restricted PCR products were resolved by electrophoresis at 45 V for 1.5 to 2 h in 2.5 % agarose gels in 1X TAE buffer for amplified rDNA restriction analysis (ARDRA). Gels were then stained with ethidium bromide and visualized. Strong and clear bands were scored as binary data (presence and absence of bands). The numerical taxonomy analysis program (NTYSIS) package (version 2.02e, Exeter Software, Setauket, NY) was used to score similarity and clustering analysis using the binary data. Jaccard's coefficient was used to calculate the similarity among the isolates and dendrogram was constructed using the UPGMA method (Nei and Li 1979).

16S rRNA gene sequencing and phylogenetic analysis

PCR amplified 16S rRNA genes were purified and sequenced using both pA and pH primers for forward and reverse reactions, respectively. Sequencing employed a dideoxy cycle with fluorescent terminators and was run in a 3130xl Applied Biosystems ABI prism automated DNA sequencer (Applied Biosystems, Foster City, CA). The partial 16S rRNA gene sequences were compared with sequences available in the NCBI database. Isolates were identified to species level on the basis of 16S rRNA gene sequence similarity of ≥97 % with the sequences in GenBank. Sequence alignment and comparison used the multiple sequence alignment tool CLUSTAL W2 (Thompson et al. 1994) with default parameters. The phylogenetic tree was constructed on aligned data sets using the neighbour joining (NJ) method (Saitou and Nei 1987) and the program MEGA 4.0.2 (Tamura et al. 2007). Bootstrap analysis was performed on 1,000 random samples taken from multiple alignments (Felsentein 1981).

Amylase production

Amylase production was tested qualitatively on Starch Agar (peptone 0.5 %, yeast extract 0.2 %, soluble starch 2 %, NaCl 0.5 %). Isolates were streaked on starch agar plates and incubated at 45 °C for 24–36 h and after incubation period plates were flooded with Lugol's iodine solution (10 g potassium iodide, 5 g iodine crystals, 100 ml distilled water). Isolates showing a zone of clearance against a dark blue background were recorded as positive for amylase production.

Protease production

Protease production was tested qualitatively by placing isolated colonies of distinct cultures on 10 % skim milk agar plates and incubating at 45 °C for 24–36 h. A zone of clearance against an opaque background indicated a positive result for protease production.

BIOLOG analyses of selected isolates

The set of selected isolates tolerant to 70 °C was characterized using the GEN III MicroPlate™ (http://www.biolog.com), to generate a profile based on 92 biochemical tests (70 Carbon sources and 22 Chemical sensitivity tests). The NTYSIS package (Exeter software, version 2.02e) was used to score similarity and clustering analysis using the binary data generated through BIOLOG. Jaccard's coefficient was used to calculate the similarity among isolates along with their reference strains, and a dendrogram was constructed using the UPGMA method (Nei and Li 1979). Microbial Identification System software of BIOLOG was used to identify the bacterium from its phenotypic pattern in the GEN III MicroPlate.

Results and discussion

Thermal springs represent extreme niches whose pristine quality is maintained over a period of time. The terrestrial hot springs that exist on Earth (Tobler and Benning 2011) represent hot spots for unusual forms of life, genes and metabolites. In the last two decades, a number of researchers have investigated various facets of the bacterial diversity in hot springs of different parts of world (Kanokratna et al. 2004; Meyer-Dombard et al. 2005; Pagaling et al. 2012). The diversity of bacterial communities in various ecologies has been investigated mostly using culture-dependent approaches, although it is estimated that this may represent 0.1 to a maximum of 10 % of the total population (Ranjard et al. 2000). 16S rRNA gene sequencing, in combination with ARDRA analyses was used effectively for diversity studies in the past (Yadav et al. 2010). Our investigation therefore focused towards the isolation of microbes using different media and growth at high temperature so as to generate diverse nutritional types, undertake ARDRA analyses, evaluate the biochemical characterization of representative isolates and identify the thermotolerant isolates by BIOLOG analyses and 16S rRNA gene sequencing.

Manikaran hot springs are located at Manikaran village in Kullu district of Himachal Pradesh, India, which represents a high altitude environment. The thermal discharges of Manikaran hot springs are classified as the Na-HCO_3-Cl type. Chemical analysis of thermal waters from these hot springs indicated the presence of calcium, magnesium, sodium, potassium, sulphate, chloride and carbonate at concentrations 38–70 ppm, 4.6–10.4 ppm, 75–106 ppm, 15–20 ppm, 55–70 ppm, 87–117 ppm and 120–200 ppm, respectively (Chandrasekharam et al. 2005).

Enumeration and isolation of culturable bacteria

In order to obtain a diverse range of nutritional types and bacteria with various metabolic requirements, five types of media were employed. These included a nutrient-rich medium (nutrient agar) for isolating a larger proportion of the available diversity, a medium suitable for oligotrophs (R2A medium), specific media designed for thermophiles (thermus agar, TPMY medium) and a medium used mostly for *Pseudomonas* and related genera (King's B medium). The population of aerobic heterotrophic bacteria in the water samples of Manikaran thermal springs ranged from 72 to 241×10^4 cfu/ml. Among the media used, the highest population of 241×10^4 cfu/ml was recorded on nutrient agar medium. A total of 235 isolates was selected based on colony morphology, among which 137 isolates were from nutrient agar medium, 47 from thermus agar medium, 13 from King's B medium, and 19 each from TPMY and R2A media (Table 1).

Table 1 Media-based quantification of bacterial morphoytpes from Manikaran hot spring samples. *TPMY* Thermus peptone meat extract yeast extract

Medium	Number of morphotypes	Total viable count (10^4 CFU/ml)			
		MI	MII	MIII	MIV
Nutrient agar	137	214	241	198	182
Thermus agar	47	226	207	234	221
TPMY	19	116	148	121	128
King's B	13	88	104	72	85
R2A	19	133	106	144	115

Screening for temperature tolerance

All 235 isolates were screened for temperature tolerance of which 85 were found to tolerate 45 °C, and 13 isolates could tolerate 70 °C. Isolate M7 was found to be most tolerant, exhibiting more than 70 % growth when compared to its growth at 37 °C. Evaluation of temperature tolerance of the isolates revealed that only 13 isolates were thermotolerant up to 70 °C. While studying the archaeal and bacterial diversity in 12 hot springs of Tibetan Plateau of China, Huang et al. (2011), reported that one of the springs (GL81) with the highest temperature among the 12 studied was dominated by a mesophilic microbial community. Lau et al. (2006) and Mitchell (2009) also reported that there is no monotonic relationship between microbial diversity and thermal stress. Based on the temperature tolerance, 85 isolates were selected for molecular characterization.

Amplified rDNA restriction analysis

A set of 85 isolates that were tolerant to temperatures 45 °C or above were analyzed using ARDRA—an important technique for distinguishing closely related taxonomic groups, especially for screening endospore-forming bacilli (Ettoumi et al. 2009). PCR amplification of the 16S rRNA gene followed by ARDRA with three restriction enzymes was carried out to analyze the variation among the 85 isolates. PCR amplification of 16S rRNA gene yielded a single amplicon of 1.5 kb from all the isolates. When the amplicons were digested with restriction enzymes, the different patterns comprising three to six fragments ranging in size from 100 to 800 bp, were characterized. ARDRA revealed that restriction digestion with *Alu* I (31 groups) was more discriminative as compared to *Msp* I and *Hae* III. A combined dendrogram (based on patterns generated using three enzymes individually) was constructed to determine the percent similarity among the isolates (Fig. 1). The isolates were grouped into 42 clusters at 95 % similarity level, based on the combined dendrogram (Table 2).

16S rRNA gene sequencing and phylogenetic analysis

Sequence data from the 16S rRNA gene of 42 representative isolates were analyzed by BLAST. The nearest match from the GenBank database for each of the 42 representative isolates is reported (Table 2). A phylogenetic tree was constructed using these 42 isolates along with the closest sequences in the NCBI GenBank (Fig. 2a, b). Of the 42 sequences obtained in this study, 11 were 100 % identical to those already reported in the GenBank (NCBI) database, and the remaining isolates had 98–99 % identity to reported sequences in database

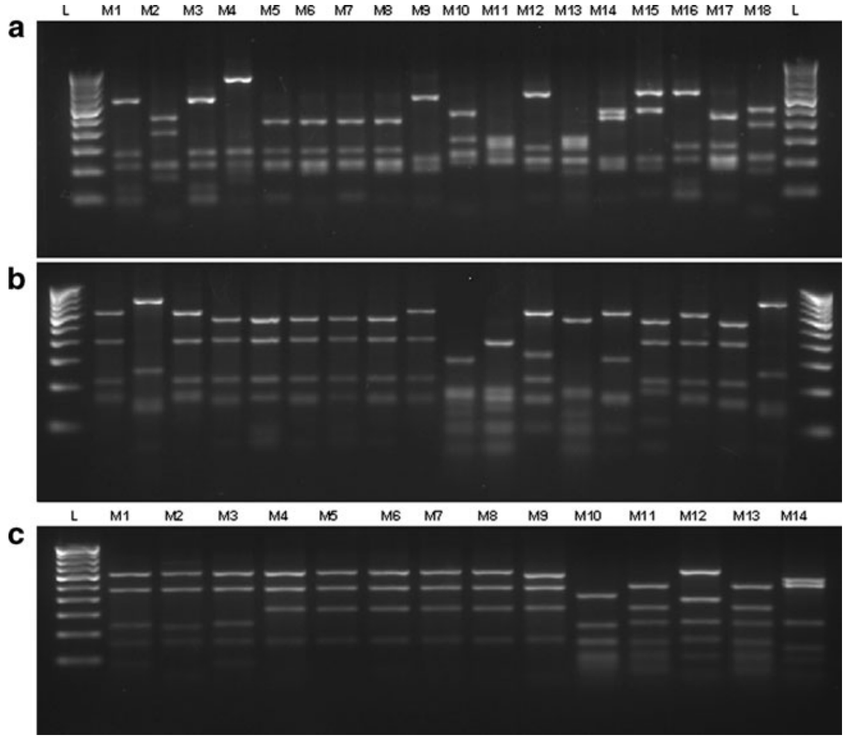

Fig. 1a–c Representative restriction patterns generated using three different restriction enzyme digests of amplified 16S rRNA gene of thermotolerant isolates. **a** *Alu*I **b** *Msp*I **c** *Hae*III

(as consulted in September 2012). Among the 42 isolates 72 % (30 isolates) belonged to Firmicutes, which is in accordance with Sayeh et al. (2010). BLAST analysis on the sequences of 42 representative isolates revealed the majority of isolates showing relatedness to the genus *Bacillus*. Apart from this, many *Bacillus* derived genera like *Brevibacillus*, *Aneurinibacillus*, and *Lysinibacillus* were also recorded.

Gram positive prokaryotes, especially the Firmicutes and Actinobacteria, are known to be comparatively stress resistant, besides being long range migrants (Cerritos et al. 2011). Because of their temperature tolerance, which is an inherent trait also attributed to this genus (Archana and Satyanarayana 2003), the genus *Bacillus* and its related genera have been explored extensively for their applications in industry and agriculture, especially as a source of hydrolytic enzymes. The members of this genus are spore formers and also produce a number of biocidal metabolites/enzymes, which makes them a common inhabitant of diverse extreme habitats (Nicholson et al. 2000). Enough evidence from recent studies has accumulated, based on the data of 16S rRNA gene sequence analysis, for the reclassification of thermophilic bacteria in the genus *Bacillus* into *Brevibacillus*, *Aneurinibacillus*, *Amphibacillus*, *Virgibacillus*, *Alicyclobacillus*, *Paenibacillus*, *Halobacillus*, and *Geobacillus* (Bae et al. 2005). This supports the presence of genera *Brevibacillus* and *Aneurinibacillus* in the hot springs in our study. The presence of genera like *Micrococcus*, *Microbacterium*, *Staphylococcus*, *Kocuria* and *Exiguobacterium* as thermoresistant aquatic bacteria in our investigation is supported by the work of Cerritos et al. (2011). These apart, representatives from *Planococccus*, *Rhodococcus*, *Pseudomonas*, and *Chelatococcus* were also present. A novel species of *Chelatococcus*—*Chelatococcus sambhunathii* sp. nov—was isolated and characterized from hot spring sediments by Panday and Das (2010). Phylogenetic analyses of bacterial communities in hot springs from low and high elevations of the Tibetan peninsula revealed no significant influence of elevation on diversity (Huang et al. 2011). This illustrates the predominance of *Bacillus* as an aggressive colonizer of diverse types of extreme habitats of the world.

Amylase and protease production

All 42 representative isolates were screened for production of the extracellular enzymes amylase and protease. Plate assays revealed that 26 % of the isolates were amylase producers and 45 % were protease producers (Table 2). Amylases hydrolyze starch molecules into dextrins and progressively into small polymers containing glucose units. Amylases are available from different sources and have extensive commercial applications in industrial processes involving starch conversions (Gupta et al. 2003), as well as in the detergent (Olsen and Falholt 1998), fuel ethanol production, food, textile (Ahlawat et al. 2009) and paper (van der Maarel et al. 2002) industries. Unique properties are required for each application; hence, screening of microorganisms for amylase production allows us to discover novel amylases required for specific industrial applications (Abdel-Fattah et al. 2012). Among the many properties of an effective amylase, thermostability is the most

Table 2 Restriction fragment length polymorphism (RFLP) clusters and sequenced isolates along with the closest available sequences in NCBI GenBank and their hydrolytic ability

RFLP pattern	Representative isolate	GenBank accession number	Temperature tolerance	Nearest phylogenetic neighbor	16S similarity (%)	Division	Amylolytic ability	Proteolytic ability
1	M1	JX312613	45 °C	*Bacillus aryabhattai* (JQ904723)	100	Firmicutes	+	+
2	M2	JX312614	45 °C	*Brevibacillus* sp. (AJ313027)	99	Firmicutes	-	-
3	M4	JX312615	70 °C	*Bacillus licheniformis* (GQ280087)	99	Firmicutes	+	+
4	M5	JX312616	70 °C	*Bacillus pumilus* (JQ435673)	99	Firmicutes	-	+
5	M6	KC315773	70 °C	*Bacillus subtilis* (EF442670)	100	Firmicutes	-	+
6	M7	KC315774	70 °C	*Bacillus pumilus* (HF536558)	100	Firmicutes	-	+
7	M8	KC315775	70 °C	*Bacillus* sp. (KC121051)	100	Firmicutes	-	+
8	M9	KC315776	45 °C	*Bacillus cereus* (GU011948)	98	Firmicutes	-	-
9	M10	JX312617	45 °C	*Rhodococcus* sp. (DQ285075)	99	Actinobacteria	-	-
10	M11	JX312618	45 °C	*Kocuria* sp. (DQ448711)	99	Actinobacteria	+	-
11	M12	JX312619	45 °C	*Planococcus* sp. (JX312584)	100	Firmicutes	-	-
12	M13	JX312620	45 °C	*Micrococcus* sp. (JN866765)	98	Actinobacteria	-	-
13	M21	JX312623	45 °C	*Staphylococcus haemolyticus* (JQ624771)	99	Firmicutes	-	-
14	M25	KC315762	45 °C	*Bacillus arbutinivorans* (FJ380988)	99	Firmicutes	-	-
15	M26	KC315763	45 °C	*Bacillus* sp. (FN397517)	99	Firmicutes	-	-
16	M27	KC315764	45 °C	*Bacillus niacini* (AB680904)	99	Firmicutes	-	-
17	M30	JX312624	45 °C	*Bacillus vireti* (HQ397585)	99	Firmicutes	-	-
18	M35	JX312625	45 °C	*Aneurinibacillus danicus* (NR_028657)	99	Firmicutes	-	-
19	M36	JX312626	45 °C	*Brevibacillus* sp. (FJ529026)	99	Firmicutes	-	+
20	M40	JX312627	45 °C	*Staphylococcus succinus* (HQ423378)	99	Firmicutes	-	-
21	M70	KC315777	70 °C	*Staphylococcus succinus* (HQ423378)	99	Firmicutes	-	-
22	M41	JX312628	45 °C	*Bacillus* sp. (JF901703)	99	Firmicutes	+	+
23	M43	KC315765	70 °C	Uncultured *Klebsiella* sp.(GQ416648)	99	Proteobacteria	+	-
24	M44	JX312629	50 °C	*Pseudomonas* sp. (EU680995)	99	Proteobacteria	-	+
25	M45	JX312630	55 °C	*Microbacterium oxydans* (EU714340)	99	Actinobacteria	-	-
26	M46	KC315766	70 °C	*Pseudomonas psychrophila* (JQ782895)	100	Proteobacteria	-	-
27	M49	KC315769	70 °C	*Pseudomonas psychrophila* (JQ782895)	100	Proteobacteria	-	-
28	M47	KC315767	70 °C	*Exiguobacterium acetylicum* (JX307688)	99	Firmicutes	-	+
29	M48	KC315768	70 °C	*Rhodococcus baikunurensis* (JX683682)	100	Actinobacteria	-	-
30	M50	KC315770	70 °C	*Pseudomonas fluorescens* (JX127246)	99	Proteobacteria	-	+
31	M52	JX312631	50 °C	*Lysinibacillus xylaniticus* (JQ739716)	99	Firmicutes	+	-
32	M53	JX312632	55 °C	*Staphylococcus hominis* (JN644561)	99	Firmicutes	+	+
33	M55	JX312633	70 °C	*Bacillus megaterium* (EU931553)	99	Firmicutes	-	+
34	M59	JX312635	45 °C	*Chelatococcus daeguensis* (NR_044297)	99	Proteobacteria	-	-
35	M61	JX312636	45 °C	*Bacillus flexus* (JQ936679)	100	Firmicutes	+	+
36	M63	KC315771	45 °C	*Bacillus* sp. (HE821233)	99	Firmicutes	-	+
37	M65	KC315772	45 °C	*Bacillus subtilis* (JX845578)	99	Firmicutes	+	+
38	M68	JX312637	55 °C	*Lysinibacillus* sp. (FN397524)	99	Firmicutes	-	-
39	M73	JX312638	45 °C	*Brevibacillus agri* (HQ222834)	100	Firmicutes	-	+
40	M74	JX312639	45 °C	*Bacillus beijingensis* (JQ799102)	100	Firmicutes	+	+
41	M75	JX312640	45 °C	*Bhargavaea cecembensis* (JQ071510)	99	Firmicutes	-	+
42	M80	KC315778	45 °C	*Microbacterium* sp.(DQ339613)	98	Actinobacteria	+	-

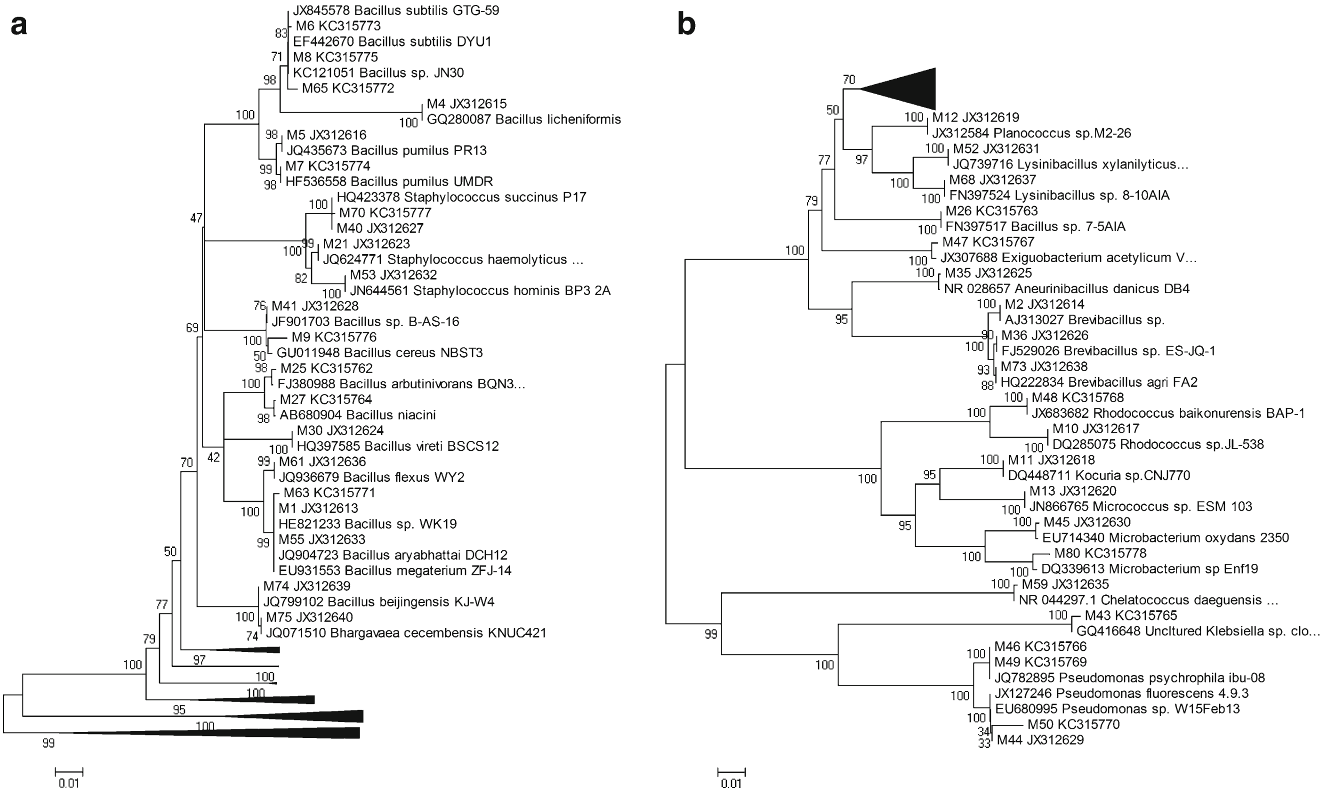

Fig. 2 Unrooted phylogenetic trees based on comparison of 16S rDNA sequences of 42 isolates along with their closest phylogenetic relatives. The phylogenetic tree was constructed based on aligned datasets using the neighbor joining (NJ) method in the program MEGA 4.0.2. Numbers on the tree indicate percentage of bootstrap sampling derived from 1,000 random samples

important, as liquefaction and saccharification of starch are performed at high temperatures. In our present study, out of 42 isolates from Manikaran hot springs, 11 were positive for amylase production. Among the amylase positive isolates, six were identified as belonging to *Bacillus* based on their 16S rRNA gene sequence similarity and one as *Lysinibacillus*, which is again a *Bacillus* derived genus. There was one representative each from the genera *Kocuria*, *Staphylococcus*, *Klebsiella* and *Microbacterium*. Sahay et al. (2011) reported similar results, in which many *Bacillus* sp. tested positive for amylase production. Interestingly, the isolate M4 (*Bacillus licheniformis*), which tested positive for amylase production, also possessed thermotolerance. Considering the requirement for thermostability of amylases in industrial applications, there is the possibility of exploring this isolate further.

Proteases are hydrolytic enzymes that catalyze total hydrolysis of proteins (Rao et al. 1998). Proteases find commercial applications in the food processing, tannery, detergent, and pharmaceutical industries (Chakrabarti et al. 2000; Kim et al. 2005); microbial proteases dominate commercial applications (Kuddus and Ramtekke 2012). As with amylases, thermal stability is the major requirement for any proteases to find commercial importance (Kristjansson and Kinsella 1990). In our study, among the 42 isolates tested for extracellular protease production, 19 tested positive and a similar trend as for amylase was recorded , as a majority (12) of them were *Bacillus* based genera based on their 16S rDNA sequence similarity. There were also representatives from *Brevibacillus* (2) and *Bhargavaea* (1), both being *Bacillus*-derived genera. Of the remaining four, there were two isolates of *Pseudomonas* and one each of *Exiguobacterium* and *Staphylococcus*. Isolates M4 (*Bacillus licheniformis*), M5 (*Bacillus pumilus*), M6 (*Bacillus subtilis*), M7 (*Bacillus pumilus*), M8 (*Bacillus* sp.) and M47 (*Exiguobacterium acetylicum*), which tested positive for protease production, were also thermotolerant. Hence, these isolates could be explored for thermostable proteases, as there is a need for such enzymes in industrial processes operating at high temperatures (Kristjansson and Kinsella 1990).

Biochemical tests and substrate utilization profiles for selected isolates using BIOLOG

BIOLOG-based phenotyping of the set of 13 highly thermotolerant isolates generated unique fingerprints of the isolates, besides revealing their ability to utilize routine and unusual substrates. This assumes immense importance in the characterization of microbial isolates from extreme environments and their potential utilization in agriculture and industry. The data on substrate utilization patterns and

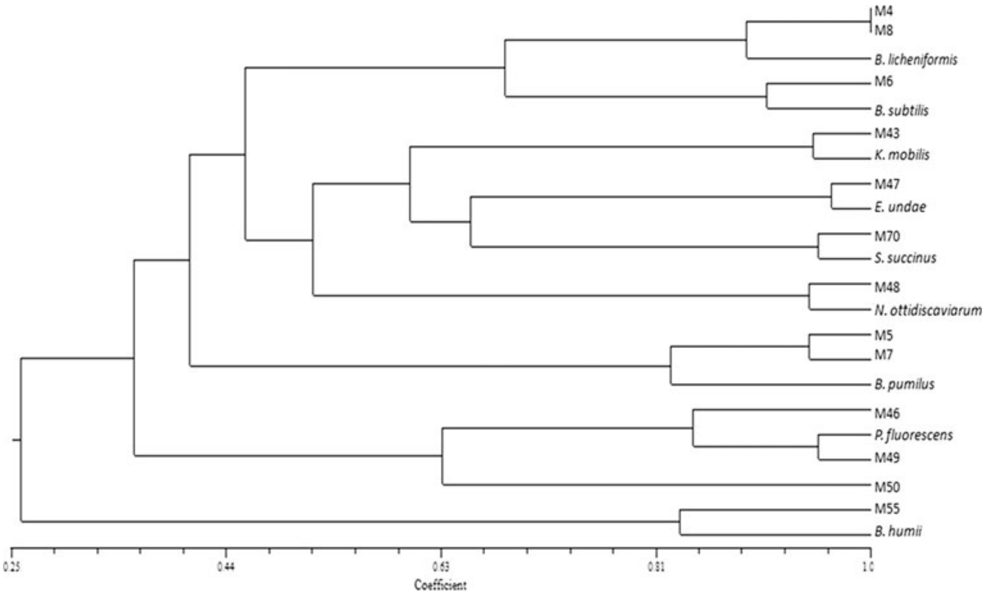

Fig. 3 Dendrogram showing the clustering of thermotolerant isolates along with reference strains based on substrate utilization patterns generated by BIOLOG analyses. The dendrogram was constructed using the UPGMA algorithm and the Jaccard's co-efficient. Distances were calculated based on the utilization of substrates and resistance to chemical agents

sensitivity to chemicals for the 13 temperature tolerant isolates were tabulated (Supplementary Table 1). Significant variation was observed among the isolates with regard to the utilization of sugars, sugar derivatives, metabolic intermediates and amino acids and peptides. A dendrogram was generated based on the data obtained through BIOLOG for the 13 isolates and reference strains available in the database (Fig. 3). Among the 20 sugars tested, D-fructose was utilized by all isolates except M55. Similarly α-D-glucose was utilized by all isolates except M48, while 3-methyl glucose was utilized only by M43. Among the 13 isolates tested for utilization of sugars, isolate M43 was able to utilize all but one sugar (raffinose) and M8 could utilize 17 sugars out of 20 tested. Isolate M55 was able to utilize only two sugars (α-D-glucose and sucrose). Isolates M43 and M48 were able to utilize the highest number of sugar derivatives, 18 and 17, respectively. Among all the sugar derivatives, glucoronamide was utilized by 12 isolates followed by L-galactonic acid and D-gluconic acid (11 isolates). Isolate M55 was able to utilize only one sugar derivative (glucoronamide).

A total of 19 metabolic intermediates was tested for growth of isolates. Isolate M48 was able to utilize the maximum number of metabolic intermediates (15) followed by isolates M8 and M70, which were able to utilize 12 metabolic intermediates each. Isolate M55 utilized the fewest metabolic intermediates (2). The metabolic intermediate, D-fructose-6-PO_4 was utilized by all isolates tested, and L-malic acid was utilized by 11 isolates. p-hydroxy-phenylacetic acid, α-hydroxy-butyric acid and α-keto-butyric acid were the least utilized, and supported the growth of only two isolates. The results on the utilization of amino acids and peptides revealed that isolate M43 was able to use all 12 substrates. Both M8 and M48 utilized ten substrates each. Isolate M55 could not utilize all substrates tested and only two substrates were utilized by isolate M50. Amino acids, L-alanine and L-glutamic acid were utilized by nine isolates while L-arginine and L-serine were utilized by seven isolates. Among the isolates, based on carbon utilization patterns, M55 (*Bacillus* sp.) was able to utilize only 7 %, while M43 *Klebsiella* sp. was able to utilize 85.7 % of the C substrates.

Table 3 BIOLOG-based identification of the thermotolerant isolates

Isolates	BIOLOG identity	16S rRNA gene sequence based identity
M4	*Bacillus licheniformis*	*Bacillus licheniformis* (GQ280087)
M5	*Bacillus pumilus*	*Bacillus pumilus* (JQ435673)
M6	*Bacillus subtilis*	*Bacillus subtilis* (EF442670)
M7	*Bacillus pumilus*	*Bacillus pumilus* (HF536558)
M8	*Bacillus licheniformis*	*Bacillus* sp. (KC121051)
M43	*Klebsiella mobilis*	Uncultured *Klebsiella* sp. (GQ416648)
M46	*Pseudomonas fluorescens*	*Pseudomonas psychrophila* (JQ782895)
M47	*Exiguobacterium undae*	*Exiguobacterium acetylicum* (JX307688)
M48	*Nocardia ottidiscaviarum*	*Rhodococcus baikunurensis* (JX683682)
M49	*Pseudomonas fluorescens*	*Pseudomonas psychrophila* (JQ782895)
M50	*Pseudomonas fluorescens*	*Pseudomonas fluorescens* (JX127246)
M55	*Bacillus humii*	*Bacillus megaterium* (EU931553)
M70	*Staphylococcus succinus*	*Staphylococcus succinus* (HQ423378)

Sensitivity and resistance to antimicrobials

Isolate M50 was found to be resistant to 15 antimicrobials out of 17 tested, closely followed by isolate M49, which was resistant to 13 antimicrobials. Isolate M6 showed resistance to least number of antimicrobial compounds (4) (Supplementary Table 2).

In terms of resistance to chemical agents, sodium lactate, which is known for its bactericidal properties and acts as a preservative,acidity regulator, and bulking agent, did not inhibit any of the isolates tested. This reveals the unusual nature of our isolates. Also, minocycline inhibited the growth of all the isolates, with the exception of M50 (*Psuedomonas fluorescens*), which was found to be most resistant to many of the chemical agents tested. Isolate M47 (*Exiguobacterium acetylicum*) was the only isolate capable of growth on sodium bromate.

Response of selected isolates to abiotic stress

The ability of isolates to show growth at low pH and in the presence of salt was tested and the results are tabulated in Supplementary Table 2. Seven isolates (M4, M5, M6, M7 M8, M49 and M70) were able to show growth at pH values up to 5 and 8 % NaCl. Isolate M47 and M55 were able to grow up to 8 % NaCl concentration and a pH of 6. Isolate M48 was not able to grow either at low pH (5) or at high salt concentration (4/8 % NaCl). Growth at different pH (5 and 6) revealed that all the 13 isolates (originally isolated from a pH of 7.8–8.2) could grow at 6, but three isolates (M47, 48 and 50) were unable to show growth at 5. Salinity up to 1 % NaCl was tolerated by all isolates; however, M48 did not grow at higher salt concentrations (4/8 % NaCl). A total of 92 % isolates and 69 % of the thermotolerant isolates were able to grow at 4 % and 8 % NaCl concentrations, revealing their promise for use in saline environments.

BIOLOG analysis in this study revealed interesting results, especially providing valuable information on the temperature tolerant isolates showing positive results in terms of growth in both low pH (pH up to 5) and high salt concentrations (NaCl concentration up to 8 %). Such isolates could be explored for their potential applications in agriculture and industry.

Identification based on BIOLOG analysis

Using the BIOLOG Microlog 3 software, the 13 temperature tolerant isolates were identified based on the profiles generated using GEN III MicroPlate™. The results of the identification of temperature tolerant isolates are given in Table 3, among which six belonged to the genus *Bacillus*; three to *Pseudomonas* and one each were placed in the genera *Nocardia*, *Staphylococcus*, *Klebsiella* and *Exiguobacterium*. BIOLOG-based identification of the temperature tolerant isolates in our study matched with 16S rRNA gene sequence based identification up to genera level, for all but one isolate (M48). This isolate M48 had a 16S rRNA gene sequence similar to that of *Rhodococcus baikunurensis*; however, this isolate matched with *Nocardia ottidiscaviarum* based on BIOLOG-based analyses. There are reports that C-utilization patterns and identification through BIOLOG have certain limitations, as it is difficult to distinguish closely related organisms (Singh et al. 2010). Also, information on members belonging to Mycobacteria and Corynebacteria in the database is limited.

Rhodococcus is a genus of aerobic, non-sporulating, non-motile Gram-positive bacterium, which is closely related to Mycobacteria and Corynebacteria and thrives in a wide range of environments. Its importance in biotechnological applications comes from its ability to catabolize a wide range of compounds (including harmful pollutants), besides production of thermostable enzymes (Kuddus and Ramtekke 2012). Our study emphasized the novelty of this strain, as it was not capable of utilizing D-glucose or glucuronimide, but was able to utilize α hydroxy-butyric acid. This isolate exhibits unusual properties as, in the published literature to date, the majority of hydroxy-butyrate degrading bacteria belong to Gram negative genera such as *Acidovorax* /*Variovorax* sp. (Mergaert et al. 1993), and *Rhodococcus* is not reported in this context.

The bacterial community composition of Manikaran hot springs exhibited a phylogenetically distinct character, resembling in part that previously reported from other geothermal hot springs (Pagaling et al. 2012; Sayeh et al. 2010), but comprising isolates with novel properties of substrate utilization patterns, tolerance to biocidal agents and abiotic stress such as 8 % NaCl.

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