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ROLE OF MFE IN PROTEIN THERMOSTABILITY IN THERMOPHILES

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ABSTRACT

Thermostability is the resistance to irreversibility of chemical or physical changes of a substance due to elevation in temperature. Protein thermostability is, therefore, the preservation of the unique structure and chemical properties of polypeptide chains under extreme temperatures. Thermophilic organisms provide a natural source of thermostable enzymes for industrial applications. Although thermostability-enhancing factors have been identified in other organisms, there was need to determine them in species of the *Methylobacterium* genus. This study aimed to determine factors that enhance thermostability in species of the *Methylobacterium* family. The protein thermostability was measured by optimum growth temperature, protein melting temperature (Tm), minimum folding energy (MFE) of RNA secondary structures. The increase in MFE values in *Methylobacterium extorquens PA1 and AM1* calculated for predicted mRNA secondary structures correlated with the rate of nucleotide substitutions (r = 0.37). These correlations were statistically significant as confirmed by a t-test. Thus, it can be concluded that significant changes in MFE and possible higher thermostability of mRNA molecules and encoded proteins are under positive evolutionary selection in these microorganisms.

KEYWORDS: Thermophilic organisms provide a natural source of thermostable enzymes for industrial applications.

INTRODUCTION

A hot water spring, also known as a thermal spring, is a natural discharge of hot water from the earth. Such springs normally occur in areas where underground water passes through hot igneous rock. The warm water of the springs allows an abundant growth of algae and bacteria which are called as thermophilic microorganisms. These organisms prefer to survive in high

temperatures, which are not normally found in nature. The thermophiles may among the first living things on the earth, developing and evolving during the primordial days of earth when surface temperatures were quite hot, and thus been called the "Universal Ancestor". The geological survey of India has identified 350 hot springs in India having temperature range from 60-120 ˚ C.

The hot spring under the present study is located at Vajreshwari on banks of river Tansa - lies in Bhiwandi city, Thane district, Maharashtra, India. It is at the foot of Mandakini Mountain, which was formed out of a volcanic eruption. It is this proximity that accounts for the number of hot springs in this region.

Hot-springs exhibit diverse bacteria and it serves as potential reservoirs for bacteria. These bacteria are unique in their metabolic activities. The temperature of this hot water varies between 37°C and 70°C. The bacteria thriving in this hot springs contain enzymes that can function at high temperatures. And such enzymes can find its use in biotechnology industries, food industries and for bioremediation.

Another most important characteristic of thermophilic organisms is their ability produce thermostable enzymes for industrial applications. Thermo stable enzymes are either extracted from cultured thermophilic organisms or are genetically engineered based on previously elucidated properties. Furthermore, organisms can be directly inoculated in chemical processes to achieve byproducts as a result of biochemical processes such as respiration, oxidation and degradation.

Examples of application of thermostable proteins, particularly those produced by Thermus genus, include bioremediation for eradication of heavy metal pollution, waste and contaminated water treatment. These enzymes, if modified, can be used in food and biotech industries. Sustaining life at high temperatures suggests an evolved metabolic network system and use of thermo stable proteins and enzymes which facilitate cellular biochemical processes necessary for survival. Such enzymes are both thermo stable and resistant to chemical reagents, salt concentrations, high pressure and acidic and alkaline conditions making them even more suitable for biotechnological applications.

The thermophiles of Vajreshwari hot springs are not been studied thoroughly so far. The studies conducted in this hot springs showed the presence of bacteria having anti-infective

potential (Pallavi Pednekar, 2011). The data available regarding the thermophiles of Vajreshwari hot springs is very limited, due to the inability to culture some taxa and also because of the limited knowledge for identification. The main problems in applying morphological criteria in bacterial classification arise from morphological features that vary with environmental conditions. Sometimes microscopy and enrichment cultures have limited role, since distinct species of bacteria can share similar simple morphological and cultivation limitations.

Significance of the study

As microbial ecology enters a new era, in which molecular techniques permit improved detection of specific populations, it seems useful that microbial ecologists should consider whether patterns of occurrence of microbial species are governed by principles similar to those that explain the evolution and ecology of larger, more complex species.

The studies will involve the use of 16S rRNA sequences (or the genes encoding them) as a means of avoiding the need to cultivate a microorganism to recognize its presence and measure its distribution in a community. Terrestrial hot spring microbial communities are among the first to be surveyed with this technology and thus are among the first in which the impressive diversity of uncultivated microbial populations in nature was revealed. Of course, this has been a typical finding in 16S rRNA gene surveys of microbial diversity in numerous habitats. 16S rRNA studies of hyperthermal hot spring habitats have led to the discovery of novel uncultivated bacteria (e.g., *Methylobacterium, Aquificales* and *Thermotogales* relatives and archaea e.g., *Korarchaeota* and others that are particularly interesting because they branch near the root of 16S rRNA-derived phylogenetic trees. Since such microorganisms may help us determine characteristics of the most ancestral cells, it is also quite exciting that some of them have recently been brought into culture.

MATERIALS AND METHODS

1. Isolation and characterization of thermophiles

Medium 77 as described in Bacterial Media Manual were used for enrichment and cultivation. All the media were digested and then autoclaved at 15 lb/in^2 pressure at 121^{o}C for 20 minutes. The autoclaved media was allowed to cool and set at room temperature. The media was incubated overnight to check for any contamination.

1.1. Preparation and composition of media

Medium 77
K_2HPO_4– 0.5 g
NH_4Cl – 1.0 g
$CaCl_2$. $2H_2O$ – 0.1 g
$MgSO_4$. $7H_2O$ – 0.1 g
Sodium lactate – 5.0 g
Yeast extract – 1.0 g
$FeSO_4$. $7H_2O$ – 5.0 g
Sodium thioglycolate– 1.0 g
Ascorbic acid – 1.0 g
Distilled water – 1000 ml
pH–7.0

1.2. Enrichment of culture

The enrichment was done in three steps. The first enrichment was carried out using sterile broths of above media, followed by second and third enrichment at $45^oC \pm 2^oC$ for 7 days in a shaker incubator. After the third enrichment, loopful of the enriched broth was plated out on respective sterile agar plates which were incubated at $45^oC \pm 2^oC$ for 48 hours. The isolated colonies were selected for further work.

2. Preservation of the cultures

The organisms were preserved in Eppendorf containing sterile Nutrient broth with 30% glycerol. These Eppendorf were maintained at freezing conditions (4 ± 2^oC) for 6 months. Regular viability was checked by plating the organisms on sterile Nutrient agar plates. For regular use, the organisms were cultured and maintained on sterile Nutrient agar slants and stored in refrigerator (Harley and Prescott, 2002).

3. Identification of selected strain by 16s rRNA partial sequencing and evolutionary relationship

The isolated colonies were sequenced for its conserved sequences and analyzed for partial 16s rRNA by geneOmbio, Pune, Maharashtra. The predicted 16S rRNA sequences from this study were compared with 16S rRNA sequences in a BLAS Table database constructed from sequences downloaded from the Ribosomal Database Project (release 8.1; http://rdp8.cme.msu.edu). Comparisons were made using the program BLAST (ftp://ftp.ncbi.nih.gov/BLAST/executables/LATEST/) and a FASTA-formatted file containing the predicted 16S rRNA sequences.

4. Detection of heavy metal reduction capacity by isolates

Sterile Nutrient broth containing 500 ppm of Cd, Cr, Cu, Fe, Zn were prepared and were inoculated with 24 hour old culture suspensions of all the five bacterial isolates. The inoculated flasks were kept at 45^{o}C±2^{o}C for 24 hrs. After that, the broths were centrifuged at 6000 rpm for 20 minutes (Sinha and Khare, 2012). The supernatant was analyzed for residual heavy metal by ICP-OES (At SAIF, IIT, Pawai, Mumbai, M. S.).

5. Calculation of minimum folding energy of mRNA secondary structures

The work aimed at determining factors that enhance protein thermostability in *Methylobacterium* species. A UNAFold algorithm was applied to DNA sequences to calculate MFE (kcal/mol) of predicted mRNA secondary structures. The folding energy of all coding sequences in a given genome in GenBank format was computed for all sequences at one run.

Thermostability was analyzed in SZP 4 (*Methylobacterium extorquens AM1),* SZP 8 (*Methylobacterium podarium* strain: DSM 15083), SZP 12 (*Methylobacterium nodulans*), SZP 16 (*Methylobacterium populi* isolate N8), SZP 18 (*Methylobacterium extorquens PA1*).All these organisms belong to the Methylobacteriaceae family and the GC-content of their genomes is in the range from 66.5% to 67.8%. Their genome sequences are available in the NCBI data- base under the given accession numbers. The analysis was narrowed and focused on coding sequences of *Methylobacterium extorquens AM1* and *Methylobacterium extorquens PA1,* in order to be generalized to *Methylobacterium* species. Although there are over fifty known *Methylobacterium* species, only three strains were sequenced at the commencement of this work: *Methylobacterium nodulans, Methylobacterium extorquens AM1*, *Methylobacterium extorquens PA1*.

6. Identification and analysis of orthologous sequences

Thermostability enhancing factors were analyzed between pairs of orthologous sequences of *Methylobacterium extorquens AM1* and *Methylobacterium extorquens PA1*. Orthologous sequences with largest negative difference in MFE as computed by UNAFold algorithm were analyzed. Orthologous sequences were identified BLASTp and MUSCLE alignments.

RESULT AND CONCLUSION

Characterization and Identification of the isolates

Enrichment and cultivation of the sample showed the presence of 5 isolates in Medium 77 Colony characteristics of all the isolates were studied.

Identification of selected thermophiles by 16s rRNA

The selected five strains were sequenced for 16s rRNA (Table 1). After comparing the sequence using BLAST database, genus and species were confirmed.

All the isolated the rmophilic strains were screened for their bioremediation capacity against five heavy metals namely (Cd, Cu, Cr, Fe and Zn). The five isolates from the twenty eight strains showed high tolerance for the selected five isolates. 16s rRNA analysis of these isolates have revealed the presence of SZP 4 (*Methylobacterium extorquens AM1)*, SZP 8 (*Methylobacterium podarium strain: DSM 15083*), SZP 12 (*Methylobacterium nodulans*), SZP 16 (*Methylobacterium populi* isolate N8), SZP 18 (*Methylobacterium extorquens PA1*). The isolates obtained have showed high bioremediation capacities in respect to cadmium, chromium, copper, iron and zinc.

Table 1: 16s rRNA sequence analysis of selected isolates.

>NC_012808.1 *Methylobacterium extorquens* AM1, complete genome
GTGCCCGTGCGCCGCGCGAATCGTCGTGCACGGCCATAGCTGGTTATGGCTGCGCGG
TGGAATTCCCCCT
CCCCCGCACGGTGCCGCCGCCATCCGTGCTCAGAAGCTCTCCGATTGCCCATCGTCTT
CACGCTAGGGAT
GGCCGCGCAGCGAAGGGGCGGTGAGAAACGCTCAGCCAAGCGCGCCTCGCCGCGCT
CGGGATCAGGCGCC
GCGCTGGGATTCGATCGGTCGCGGTTGTGCACCGACCGGTGATGCTTCTCCACAGTT
CGCGCCAGGTTAT
CCCGAATCGACGCTTGCACCTGCCTGTTCAGGGGCCCGTGAAGGTTTCGTTAACCAT
GCGGAAAGTGCTT
TCCATTGCGTGGTGCCACGCCGGCCGTGTAGCTTAACAAAACGTTGCCGATTGAGCG
GCTCGACAAGTTC
AGCACCGGCACCCCCAGAGTCGTCCCCAAGTTGCGGGCAGGCCTTCGACTCTTCGGT
CCCGGCCTGACGA
CTCTGTGGGCGCGAAGATGTTTTCCCCGATCGTCCCGCTGTTAGGAGAACGATAGAG
GATTGTTGATACA
CAGCCATCTTTAGCGATGGGCCGCGACGCCGCTCTTGACAGCGCCGCGGCCACTCTC
GGCGCCGCCCCGA
TCGGGCGGCGCCTCCTCTCGTGTGGCGTATGCGTACGCGTCCCCTCCGGTCTGCCGGC
TGAGGATGCGAG
GAGACACGGTGATGCACCTCGACGGCAGCATGTCGGACGGCGATGGCGGAAACGGC
AGAGGAAGCGTCGA
TCTTCCCGCCGCCTGGACGCGGGTGAAGCGGCGGCTGCGGGCGGAGCTTGGCGAGG

ACGTCTTCGCAAGC TGGTTCGCCCGCCTCGAACTGGAGACTGTGGACGGGGGGGTCGCCCGCCTCACCGTT CCGACCCGGTTTC TCAAGAGCTGGATCGAATCGCATTACATCGACCGGGTGCTGACGACCTTCCGCGCCG AGGCCGATGGCGT CAGCCGTATCGAGGTCGGCGTGCGCGGCCCCTCCGGCCCGGCCCGGCCGACTGCCGG CGTGCCGGCGAAG CCGAACGCGACGAGCGGGCCGCTGAACCGTCTCCACGCGATCGCGACGCCCGCGGC CCTCCAGGGGCCCG GCCCGATGATCGAGACCGAGATCGCCTCGCCCCGCGGCGATTCGGCCTCGGTCGATC TCAACGGCGCGCC GCTCGATGCGCGGCTCTCCTTCGCGAACTTCGTG
>AB302930.1 *Methylobacterium podarium* gene for 16S rRNA, partial sequence, strain: DSM 15083 CGAACGCTGGCGGCAGGCTTAACACATGCAAGTCGAACGGGCTTCTTCGGAAGTCAG TGGCAGACGGGTG AGTAACACGTGGGAACGTGCCCTTCGGTTCGGAATAACTCAGGGAAACTTGAGCTAA TACCGGATACGCC CTTANGGGGAAAGGTTGACTGCCGAAGGATCGGCCCGCGTCTGATTAGCTTGTTGGT GGGGTAACGGCCT ACCAAGGCGACGATCAGTAGCTGGTCTGAGAGGATGATCAGCCACACTGGGACTGA GACACGGCCCAGAC TCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGCAAGCCTGATCCAGC CATGCCGCGTGAGT GATGAAGGCCTTAGGGTTGTAAAGCTCTTTTGTCCGGGACGATAATGACGGTACCGG AAGAATAAGCCCC GGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGGGCTAGCGTTGCTCGGAAT CACTGGGCGTAAA GGGCGCGTAGGCGGCCGATTAAGTCGGGGGTGAAAGCCTGTGGCTCAACCACAGAA TTGCCTTCGATACT GGTTGGCTTGAGACCGGAAGAGGACAGCGGAACTGCGAGTGTAGAGGTGAAATTCG TAGATATTCGCAAG AACACCAGTGGCGAAGGCGGCTGTCTGGTCCGGTTCTGACGCTGAGGCGCGAAAGC GTGGGGAGCAAACA GGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAATGCCAGCCGTTGGTCTGC TTGCAGGTCAGTG GCGCCGCTAACGCATTAAGCATTCCGCCTGGGGAGTACGGTCGCAAGATTAAAACTC AAAGGAATTGACG GGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGCAGAACCT TACCATCCCTTGAC ATGGCATGTTACCCTGGGAGACCGGGGATCCTCTTCGGAGGCGTGCACACAGGTGCT GCATGGCTGTCGT CAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCACGTCCTT AGTTGCCATCATT NAGTTGGGCACTCTAGGGAG
>NC_011894.1 *Methylobacterium nodulans* ORS 2060, complete genome ATGCGTGTGGATGGCAGCTTGGCGGAAGGGTTCGATCCGGGCGGCGGCGGCAGTAG CGGCAGCGGCGGTG GCGGCGACGTCGCGGCCGCGTGGCAGCGGGTGAAGCGGCGCCTGCGGGCCGAGCTC GGCGAGGACGTGTT CGCGAGCTGGTTCGCGCGGCTCGAACTGCAGGAGGTCTCCGGGGGGACCGCCCGTCT CACCGTGCCGACC

CGTTTCCTCAAGAGCTGGATCGAGTCCCACTACCTCGACCGGGTGCTCGCCACCTTCC GCAGCGAGGCCG ACGGCGTCGAGGGGATCGAGGTCGGCGTGCGCGGGCCGATGGCGCCCGCCCGCGCG GCTCCGGTCGGCAT GGCGCCCGCCGCTCCGAAGGCGGCCACGCCCGTGCGGCTGGCCGCCTCCGCCCCGGC CGCCGCCGAGACG GCGGAGGCGGACCGGGCCGGCCGCTCCGAGGCCGCCGATCTCAGCGGCGCACCCCT GGATCCGCGCCTCA CCTTCCAGAGCTTCGTGGTCGGCCGCTCGAACGCGCTCGCGCATGCGGCGGCCGAGC GGGTCGCGGCCCA TGACGGCGGCGGGCCGGTCTACAACCCGCTCTACTTCCATGCGGGGGTGGGTCTGGG GAAGACGCATCTC CTGCACGCGATCGGGCATGCGGCCAAGGAGGTTGGGCGGCGGGTGATCTACCTCAC GGCCGACCGCTTCA TGTACGGCTTCGTCAACGCCCTGAAGACGCAGAACGCGCTTGCCTTCAAGGAGCGTC TGCGCGCCATCGA CGTGCTGATCCTCGACGACGTTCAGTTCATCCAGGGCCGCTCGATCCAGGCCGAGTT CGGCCATACGCTG AACGCCCTGATCGATGCCGGCCGGCAGGTCGTCGCCGCCGCCGACCGGCCGCCGACC GAGCTGGAGAGCC TCGACGAGCGCGTGCGCTCGCGCCTTGCGGG
>LK020736.1 *Methylobacterium populi* partial 16S rRNA gene, isolate N8 TAGAGTTTTGATCCTGGCTCAGAGCGAACGCTGGCGGCAGGCTTAACACATGCAAGT CGAACGGGCTTCT TCGGAAGTCAGTGGCAGACGGGTGAGTAACACGTGGGAACGTGCCCTTCGGTTCGG AATAACTCAGGGAA ACTTGAGCTAATACCGGATACGCCCTTACGGGGAAAGGTTTACTGCCGAAGGATCGG CCCGCGTCTGATT AGCTTGTTGGTGGGGTAACGGCCTACCAAGGCGACGATCAGTAGCTGGTCTGAGAGG ATGATCAGCCACA CTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGA CAATGGGCGCAAGC CTGATCCAGCCATGCCGCGTGAGTGATGAAGGCCTTAGGGTTGTAAAGCTCTTTTGT CCGGGACGATAAT GACGGTACCGGAAGAATAAGCCCCGGCTAACTTCGTGCCAGCAGCCGCGGTAATAC GAAGGGGGCTAGCG TTGCTCGGAATCACTGGGCGTAAAGGGCGCGTAGGCGGCCGATTAAGTCGGGGGTG AAAGCCTGTGGCTC AACCACAGAATTGCCTTCGATACTGGTTGGCTTGAGACCGGAAGAGGACAGCGGAA CTGCGAGTGTAGAG GTGAAATTCGTAGATATTCGCAAGAACACCAGTGGCGAAGGGG
>NC_010172.1 *Methylobacterium extorquens* 16S rRNA gene, isolate PA1, GTCCCCTCCGGTCCGCCGGCTGAGGATGCGAGGAGACACGGTGATGCACCTCGACGG CAGCATGTCGGAC GGCGATGGCGGAAACGGCAGAGGAAGCGTCGATCTTCCCGCCGCCTGGACGCGGGT GAAGCGGCGGCTGC GGGCGGAGCTTGGCGAGGACGTCTTCGCAAGCTGGTTCGCCCGCCTCGAACTGGAGA CTGTGGACGGGGG GGTCGCCCGCCTCACCGTTCCGACCCGGTTTCTCAAGAGCTGGATCGAATCGCATTA CATCGACCGGGTG CTGACGACCTTCCGCGCCGAGGCCGACGGCGTCAGCCGCATCGAGGTCGGTGTGCGC GGCCCCTCCGGCC

CGGCCCGGCCGAGTGCCGGCGTGCCGGCGAAGCCGAACGCGACGAGCGGGCCGCTG
AACCGTCTCCACGC
GATCGCGACGCCCGCGGCTCTCCAGGGGCCCGGCCCGATGATCGAGACCGAGATCG
CCTCGCCCCGCGGC
GATTCGGCCTCGGTCGATCTCAACGGCGCGCCGCTCGATGCGCGGCTCTCCTTCGCG
AACTTCGTGGTGG
GCCGCTCCAATGCGCTGGCCCATGCCGCCGCCGAGCGAATCGCCCGCAGCGACAGCG
ACGGCGCGCTTTA
TAACCCGCTCTACGTCCATGCCGGCGTGGGACTCGGCAAGACGCACCTGCTTCACGC
GGCCGGCCACGCC
GCCCGCGAGGCCGGCCGCCGGGTAATCTATCTCACCGCCGACCGCTTCATGTACGGC
TTCGTCAACGCCC
TGAAGACGCAGAACGCGCTGGCCTTCAAGGAGCGCCTGCGGGCGATCGACCTGCTC
ATCCTCGACGACGT
GCAGTTCATCCAGGGCAAGTCGATCCAGACCGAGTTCGGTCACACCCTCAACGCGTT
GATTGATTCGGGG
CGTCAGGTGGTGGTCGCCTCCGACCGGCCGCCGACGGAGCTGGAAGCGCTGGACGA
GCGCGTGCGCTCGC
GCCTCGCCGGTGGTCTGGTCGTCGAGATCGGCGGGCTCGACGAGGGGCTTCGTGCCT
CGATTCTCTCCGC
CCGGCTCGACGCCGTGCGCCAGAGCCACCCGAATTTCGAGGTCTCCCCGGCCGTGTC
GGCCTATGTCGCC
CGGGCGATCACGGCCAATGGCCGCGACCTCGAAGGGGCGGTGAACCGGCTCCTGGC
CCACGCAACCCTGA
CCGGCGCGCCGGTCACGGTCGAGACGGCCGAGACCGCGATCCGCGACCTCGTGAAG
AACCGCGAGCCCAA
GCGGGTGAAAATCGAGGACATCCAGAAGCTGGTGGCTTCGCGCTACAACGTCTCGC

Detection of metal reduction capacity by isolates

The bioremediation studies were carried out using the 5 isolates viz., SZP 4, SZP 8, SZP 12, SZP 16, SZP 18; because they were able to tolerate high amount of heavy metal salts. The bioremediation of heavy metals were determined by ICP-OES, at SAIF, IIT, Pawai, Mumbai, M. S. (Table 2).

Table 2: Metal reduction capacity by isolates.

Metal	Metal removal (%) after 24 hrs.				
	SZP 4	SZP 8	SZP 12	SZP 16	SZP 18
Cd	A66.00c±1.8	A64.00e±2.8	A54.00e±2.6	A59.30e±2.8	A44.00e±1.2
Cr	A45.80d±3.0	A78.20e±1.8	A41.30e±2.3	A78.60e±1.3	A61.60e±2.9
Cu	C57.40e±1.5	D51.00c±2.8	B64.20e±1.4	A51.00d±2.6	A52.20e±2.2
Fe	A50.20c±1.2	C29.00b±2.8	B60.80d±2.4	A58.80c±1.2	B53.60c±2.0
Zn	A64.00a±2.4	A44.60a±1.4	B32.00a±2.2	C58.60a±1.8	A37.40a±1.2

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	424.6	4	106.2	F (4, 20) = 0.6637	P = 0.6245
Residual (within columns)	3199	20	160.0	P value statistically significant (P < 0.05) No	
Total	3624	24			

Choice of MFE for prediction of thermostability in *Methylobacterium* proteins

Optimum growth temperature of an organism and protein melting temperature were considered to be impractical measures of thermostability. The ratio determines levels of thermostability based on amino acid composition in protein sequences. Particularly, the increased number of charged residues (Glu+Lys) against the decreased number of polar residues (Gln+His) to create a ratio (Glu+Lys)/ (Gln+His) which is used to identify thermostable proteins. In addition, analysis showed that the preferred amino acid usage also did not comply with the prescribed distribution based on which the ratio was developed. Bacteria of the genus *Methylobacterium* therefore, exploited alternative mechanisms of increasing thermostability.

MFE distribution curves and protein thermostability

The UNAFold algorithm was applied to determine thermostability of individual protein sequences. It was expected that in extreme thermophiles, these values would be higher than in thermotolerant Methylobacteriaceae. Calculated MFE values were normalized by the length of mRNA sequences to avoid bias. Figure 1 shows the distribution of MFE values calculated for all predicted genes in five Methylobacteriaceae genomes. Genes were ordered by MFE values and ranked into groups of approximately 0.5% of the total number of the genes in each genome for better presentation.

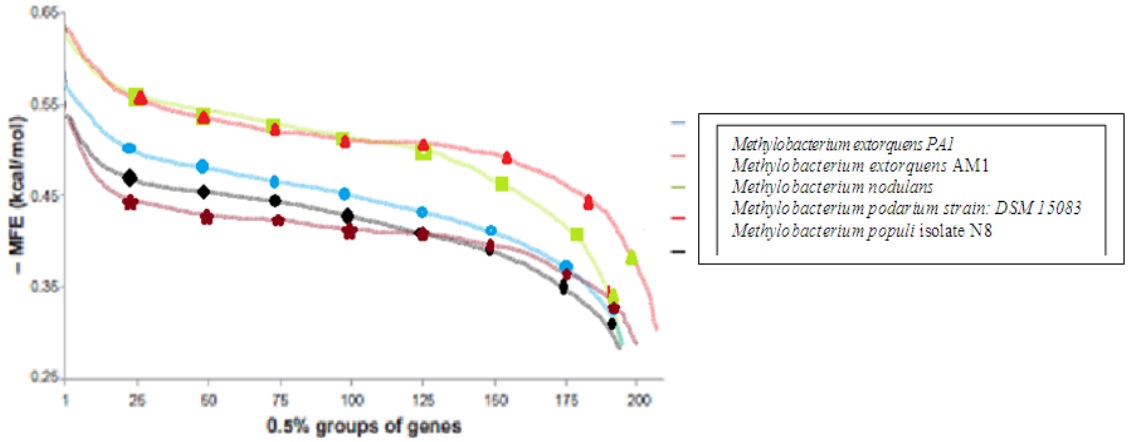

Figure 1: Distribution of MFE calculated for genes of five bacterial genomes. Negative MFE values are ordered along the axis Y from bigger to smaller.

MFE distribution curves calculated for all organisms were similar, but shifted to lower MFE in both thermophilic Methylobacterium strains indicating higher thermostability for all their mRNAs. These were followed by Methylobacterium extorquens PA1, Methylobacterium

populi N8, and Methylobacterium podarium DSM 15083, respectively. This observation was consistent with phylogenetic classification of micro-organisms based on 16s rRNA sequences and their optimum environmental growth temperature. Interestingly, the top 100 thermostable mRNAs in Methylobacterium extorquens PA1 were not the same as the top 100 ones in Methylobacterium nodulans.

These groups overlapped only by 35% and 30% *for Methylobacterium extorquens PA1* and *Methylobacterium nodulans*, respectively. Thus, some mRNAs acquired more thermostability than others. In an attempt to identify the mechanisms of enhancing of thermostability in Methylobacterium, correlations between differences in MFE values was calculated for orthologous proteins *Methylobacterium extorquens PA1 and AM1*, and *Methylobacterium nodulans*, and the rates of nucleotide and amino acid substitutions and deletions. The increase in MFE values in *Methylobacterium extorquens PA1 and AM1* calculated for predicted mRNA secondary structures correlated with the rate of nucleotide substitutions (r = 0.37). These correlations were statistically significant as confirmed by a t-test. It was assumed that the correlation between changes in MFE and frequencies of nucleotide and amino acid substitutions between orthologous protein coding genes implies a parallel adaptation of mRNA and proteins to higher temperatures. A statistically reliable correlation of 0.17 was also found between changes in MFE and dN/dS non-synonymous/synonymous nucleotide substitution rate ratios. An increased frequency of dN substitutions over dS indicate a positive selection. Thus, it can be concluded that significant changes in MFE and possible higher thermostability of mRNA molecules and encoded proteins are under positive evolutionary selection in these microorganisms.

CONCLUSION

The above study focuses broadly on the diversity of the thermophilic bacteria isolated from the natural thermal habitat, Vajreshwari and Ganeshpuri hot springs, Maharashtra. Further, the seasonal variation on the physico-chemical parameters of water samples and its effect on the bacterial diversity.

Physico-chemical analysis of water sample collected in three different seasons did not show any statistical difference. Also no specific trend was observed in the distribution of thermophilic isolates during particular seasons. The biochemical analysis together with the automated system revealed the presence of 28 different types of culturable thermophilic isolates with distinguishable morphological characteristics. Most of the heavy metals present

in the water samples were within the permissible limit. Considering this fact, we have screened all the isolated thermophiles for Bioremediation of heavy metals.

The seven isolates selected after secondary screening, were exposed to different concentration ranging from 50 ppm to 4500 ppm of five heavy metals i.e Cd, Cr, Cu, Fe, and Zn during third screening. The five isolates which showed maximum tolerance capacity to these heavy metals were selected for further study.

The UNAFold algorithm was extended in this work to efficiently handle large data sets to compute MFE for coding sequences in entire genomes. MFE values were higher in thermophiles *Methylobacterium,* which correlated with the general increase in the genomic GC-content. The prediction approach proved to be a useful for the identification of thermostable proteins in closely related organisms. Although this approach yielded consistent results when applied on *Escherichia coli* K-12 and *B. subtilis*, further testing of approaches on distantly related organisms with diverse GC-content is necessary. MFE values calculated for AT-rich sequences may vary, as they utilize different mechanisms of mRNA stabilization.

Optimum growth temperature and protein melting temperature were considered unsuitable to achieve the objectives of this work. The calculated ratio for *Methylobacterium extorquens PA1* was found to be below the prescribed value for thermophilic bacteria. This observation indicated that bacteria of different taxa exploit different evolutionary adaptation mechanisms to bio-stresses. The ratio is not ideal for computing thermostability of proteins from closely related organisms.

Comparison of orthologous protein sequences showed existence of dominant trends in amino acid substitutions and their properties consistent with the difference in thermostability between orthologous sequences. *Methylobacterium extorquens AM1 proteins* had an increased occurrence of non-polar, small, tiny, and charged amino acids. An abundance of alanine in thermophilic sequences *of Methylobacterium extorquens AM1* was substituted by serine and threonine *Methylobacterium extorquens PA1*. An abundant occurrence of arginine was observed in *nodulans* substituted by glutamine and lysine.

Thus, the present study will help in screening the industrially important thermophiles and the main factors that enhances the thermo stability of proteins in *Methylobacterium spp*.

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