CHARACTERIZATION AND 16S rDNA IDENTIFICATION OF THERMO-TOLERANT BACTERIA ISOLATED FROM HOT SPRINGS

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ABSTRACT: High water temperature exerts selection pressure on microbial species leading to specific flora that survives and tolerates heat stress. A total of 229 bacterial cultures were isolated from ten different hot springs at Siwa, Matrouh, Egypt. The number and percentage of heat tolerant bacteria were assessed. Only 13 (5.6%) of bacterial isolates were able to tolerate and survive 65°C. These bacterial isolates were genetically diverse according to RAPD and BOX-PCR analyses using different primers. RAPD, BOX-PCR and 16S rRNA sequence analysis confirmed the abundance of bacterial genotypes and that they were closely related to Bacillus licheniformis and Bacillus pumilus, based on 100% similarity in their 16S rDNA gene sequences. Bacillus licheniformis responded to one hour of thermal stress at elevated temperature from 30°C to 65°C by synthesizing different heat shock proteins (HSPs) with molecular weights ranging between 30 – 120 kDa.

KEY WORDS: Bacteria, Hot spring; Thermotolerance, PCR, Heat Shock Protein

INTRODUCTION

Temperature is one of the most important factors that govern species abundance and distribution. High temperatures in soil and/or water exert pressure on microbial species leading to the selection of specific flora capable of tolerating and surviving heat stress[10]. Some species can survive at the elevated temperatures of hot springs, or in various other adverse environments. The defense mechanism cells utilize when confronted with high temperatures in their local environment is known as the heat shock response. This response has been described extensively in both eukaryotes and prokaryotes[9]. When thermal stress is applied, the most prominent physiological reactions are the production of a set of novel proteins or an increase in the quantity of certain types of existing proteins. These proteins are known as heat shock proteins (HSPs)[11]. HSPs have been shown to play important roles in the protection of organisms under heat stress[12, 10]. Heat shock proteins can be classified into three categories according to their molecular size: 1) high molecular size, with molecular size between 39 and 68 kDa, 2) medium-molecular between 39 and 68 kDa and 3) low-molecular size, with molecular weight below 38 kDa[6].

Over the past decade, studies of the microbiology of high temperature terrestrial hot springs by both molecular-ecological and culture-based approaches have revealed phylogenetic and physiological diversity[23,13,14]. Bacterial communities are difficult to study because of their immense complexity and because of potential problems in culturability of many of the members. However, DNA-based analyses can contribute significantly in characterization of bacteria that have been successfully isolated from these environments. Genomic DNA fingerprinting using random amplification of polymorphic DNA (RAPD-PCR) and BOX-PCR have been found to be useful in differentiating between very closely related bacteria. The RAPD and BOX-PCR techniques are a polymerase chain reaction (PCR)-based assay that was developed to detect polymorphisms in genomic DNA [25,26]. Besides being simpler and cheaper, these methods are as effective as the more labor intensive RFLP for establishing genetic relationships and identifying bacterial genomes[21].

In this study, thermotolerant bacterial isolates from different hot springs were characterized using genomic patterns obtained through RAPD and BOX-PCR with the objective of identifying the dominant thermotolerant bacteria and to find out whether these thermophilic bacteria also responded to abrupt temperature changes with a heat shock-like response.

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MATERIALS AND METHODS

Sampling and Isolation of Bacteria: Water samples for viable bacterial counts were taken in sterile 250-ml screwcap bottles from ten different hot springs that are located at Siwa, Matrouh, Egypt. Temperature was measured in situ with a mercury bulb thermometer. The bottles were filled completely and then closed tightly to prevent the loss of dissolved gases. The water samples were brought back to the laboratory and analyzed within 24 h. Bacteria were isolated and enumerated using the standard plate method. 0.1 ml of the proper dilution was used to surface inoculate R2A media\textsuperscript{(17)}. From each dilution, five plates were inoculated and incubated at 30°C. Colonies were counted every 2 days until maximal plate counts were obtained. Numbers were expressed as colony forming units (c.f.u.) ml\textsuperscript{-1}. Colonies obtained on R2A were isolated and further purified on R2A medium. The isolates were stored at-80°C in R2A broth medium containing 50% glycerol.

Heat Tolerance: All isolates were examined for their ability to tolerate heat stress. The number and percentage of heat tolerant bacteria were assessed by transferring each colony onto R2A agar medium. The plates were incubated at different temperatures; 30, 37, 45, 50, 55, 60 and 65°C. Results of the inoculated plates were read in time intervals of 2 days for at least 4 days of incubation. Numbers and the percentage of bacteria grown at different temperatures were calculated.

Heat Shock Treatment: The highest thermotolerant bacterial isolate was grown overnight in 250-ml Erlenmeyer flasks containing 100ml of sterilized R2A broth on a shaker at 150 rpm. at 30°C until late log phase. Subsamples (5ml) of bacterial suspensions were transferred to test tubes. The bacterial cultures in each tube were then placed in water baths set at 37°C, 45°C, 55°C, 60°C and 65°C for 15 min as pre-treatment of heat shock\textsuperscript{(4)}. The cultures were maintained at the different temperatures for 60 minutes and then, after the heat shock treatment, bacterial cells were then harvested by centrifugation (14,000 xg, 20°C, 15 min) and the pellets were washed twice with sterile distilled water.

Electrophoresis Analysis of Proteins: Cell pellets was washed three times with 10mM ice-cold tris-HCl buffer, pH 7.6. Cells were suspended in 0.5ml 2x treatment buffer and disrupted by repeated rapid freezing at -80°C followed by thawing in a 45°C water bath. Cell preparations were centrifuged (10,000 xg) to obtain a clear supernatant containing the soluble proteins. The protein profile of bacterial strains was assayed by (Sodium Dodecyl Sulfate-Polyacrylamide Gel electrophoresis) SDS-PAGE Samples containing 10 mg protein/ml were prepared and electrophoresis was carried out according to the methods described in\textsuperscript{(20)}. The stacking gel was 5% (W/V) acrylamide and resolving SDS-containing gel was 10% (W/V) acrylamide.

DNA Extraction, RAPD and BOX-PCR Analysis: Bacterial DNA of thirteen thermotolerant bacterial isolates was extracted from 10-ml bacterial cultures grown overnight according to the method described by\textsuperscript{(9)}. DNA amplification reactions were conducted in a Perkin-Elmer 9600 thermocycler. RAPD-PCR amplification was performed as described by\textsuperscript{(21,22)} using the primers: A01, '5-CAGGCCCTTC-3'; A02, '5-TGGCCGAGCTG-3'; and A03 '5-AGTCAGCGAC-3'. The PCR protocol was a 35-cycle PCR (initial denaturation, 95°C for 5 min; subsequent denaturation, 95°C for 30 sec; annealing temperature, 45°C for 2 min; extension temperature, 72°C for 1 min and final extension, 72°C for 10 min). The oligonucleotide primers BOXA1R (5'-CTACGGCAAGCGACGCTGAC-3') used to amplify bacterial DNA\textsuperscript{(23)}. The PCR was initiated by incubating the reaction mixture at 95°C for 10 min, followed by 30 cycles of 30 sec at 94°C; 1 min at 52°C; and 6.5 min at 65°C. The reaction was terminated with an extension step consisting of 16 min incubation at 65°C. The PCR products were analyzed on 1.5% agarose gel and visualized by ultraviolet illumination after staining with 0.5µg ml\textsuperscript{-1} ethidium bromide. The fingerprint patterns resulting from RAPD and BOX-PCR analyses were scored for each template DNA by recording the presence or absence of bands to construct a rectangular binary matrix. The matrix was used to derive simple matching coefficients used in clustering analysis and the construction of dendrograms to illustrate the genetic relationships using NTSYS-PC 2.0\textsuperscript{(10)}.

16S Ribosomal RNA (rRNA) Sequencing: Oligonucleotide primers with specificity for eu-bacterial 16S rRNA genes, primers M16Sa and M16Sb were used to amplify the 16S rRNA gene fragments with template DNA originating from bacterial isolates and using PCR protocols described by\textsuperscript{(18)}. Subsamples (10µl) of the reaction mixtures were analyzed by 1% horizontal agarose gel electrophoresis to confirm the presence of products\textsuperscript{(23)}. PCR products were purified using QIAquick Spin columns (Qiagen Inc., Chatsworth, CA). Applied Biosystem 3100 Genetic analyzer in combination with a Dye Deoxy Terminator
Cycle Sequencing Kit (Perkin Elmer, Foster City, CA) were used for sequencing the purified PCR products as described previously[24]. A search of GenBank with BLAST[1] was used to identify named bacterial species with 16S rRNA gene sequences similar to those of the isolates.

RESULTS AND DISCUSSIONS

Bacterial Count and Response to Temperature: Total bacterial population size in the ten hot springs ranged from 2 to $4 \times 10^2$ CFU ml$^{-1}$ water. This relatively low bacterial count can be attributed to the relatively high temperatures ranging from 50 to 55°C as shown in Table 1. Elevated levels of temperatures affect the qualitative as well as the quantitative structure of microbial communities. Several studies found that temperature influence microorganisms by adversely affecting their growth, morphology and biochemical activities, resulting in decrease biomass and diversity[24,4,7,13]. A total of 229 morphologically distinct isolates were randomly selected from the ten different hot springs. All isolates were screened for their ability to grow at different temperatures. The cumulative percentages of bacterial strains resistance to various temperatures are shown in Table 2. The results shows that only 8 (20%), 4 (16.7%) and 1 (5.6%) of bacterial cultures isolated from Aen Zahra, Bar El-Dakour and Bar Quash, respectively, were able to grow at 65°C. A total of thirteen bacterial isolates with the highest rates of heat tolerance were selected for RAPD and BOX-PCR analysis.

The development and increased availability of techniques in molecular biology have made it possible to obtain information regarding the diversity of bacterial cultures isolated from different habitats.[2,9]. One such technique, a polymerase chain reaction (PCR)-based assay to fingerprint genomes using random amplification of polymorphic DNA (RAPD) and BOX are useful for differentiating between bacterial isolates.[27,8].

RAPD Fingerprinting: RAPD analysis of the thirteen isolates led to a minimum of 4 and a maximum of 7 discrete visible bands ranging in size from 0.1 to 3.5 kb (Figure 1). RAPD-PCR with primer A02 was the most informative since the highest numbers of polymorphisms were observed among the isolates. Primers A01, A03 and A04 were uninformative because no polymorphisms could be detected among the isolates (data not shown). The dendrogram derived from the RAPD dataset showed that bacterial isolates 2, 3, 4 and 5 from Bar El-Dakour hot springs shared 100% similarity and 8, 9, 10, 11 and 12 isolated from Aen Zahra hot springs also shared 100% similarity. The isolate numbers 7 and 13 shared 80% similarity with isolates 8, 9, 10, 11 and 12. From the RAPD patterns of the two remaining isolates it was concluded that these were more diverse, with similarities below 80% (Figure 2).

BOX Fingerprinting: Genetic variation within thirteen thermotolerant bacterial isolates was also assessed using BOX-PCR and was shown to a useful method for differentiating closely related bacteria[26,8]. BOX-PCR analysis led to a minimum 9 and a maximum 15 characteristic bands ranging in size from 5.1 to 0.2 kb as shown in figure 3. The dendrogram derived from the BOX-PCR dataset also showed that bacterial strains 2, 3, 4 and 5 shared 100% similarity and bacterial cultures 7, 8, 9, 12 and 13 shared 100% similarity and isolates number 7 and 13 shared 86% similarity with isolates 7, 8, 9, 12 and 13 (Figure 4). Isolate 6 shared 82% similarity with isolates 7, 8, 9, 12 and 13. Isolate number 1 shared 86% similarity with bacterial isolates 2, 3, 4 and 5.

The electrophoretic (RAPD and BOX-PCR) patterns of bacterial isolates showed the bacterial isolates 12 and 3 were predominant and represented 38.5% and 30.7%, respectively, of the 13 thermotolerant bacterial isolates.
Fig. 1: RAPD-PCR products from thirteen thermotolerant bacterial isolates (1, 6, 7, 8, 9, 10, 11 and 12 were isolated from Aen Zahra; 2, 3, 4 and 5 were isolated from Bar El-Dakour and 13 was isolated from Bar Qurash hot springs) generated by random primers A02. Lane M1, molecular size marker of Lambda DNA EcoRI digested with HindIII and M2, 100 bp DNA ladder.

Fig. 2: Dendrogram derived from the analysis of RAPD fingerprint patterns generated using A02 primer showing the diversity among thermotolerant bacterial isolates.

Fig. 3: Box polymerase chain reaction (PCR) fingerprint patterns of thermotolerant bacterial isolates. M1, molecular size marker of Lambda DNA EcoRI digested with HindIII and M2, molecular size marker of pBR322 digested with MspI.
16S rRNA Sequence Analysis: More than 1400 bp of the 16S rRNA genes of strains 12 and 3 were sequenced. Analysis of the 16S rRNA sequences confirmed the strain 12 and 3 were closely related to *Bacillus licheniformis* and *Bacillus pumilus*, respectively, based on 100% similarity in their 16S rDNA gene sequences.

Effects of elevated temperature on proteins synthesis: A short exposure of cells to elevated temperatures reduces the synthesis of normal cellular proteins and at the same time induces a transient overproduction of a specific group of proteins, the so-called heat shock proteins (HSPs)\(^\text{[5]}\). The optimum temperature for the production of HSPs varies from organism to organism. The heat shock temperature range for *E. coli* is 43-47°C, for the yeast 36°C and for the sickle fungus *Fusarium oxysporum* 40°C or 43°C. In general, a rise of 5°C above the normal physiological temperature will induce the synthesis of HSPs\(^\text{[11]}\).

Thermophilic microorganisms normally grow at high temperatures and therefore should have a higher thermotolerance. Thus it was of interest to find out whether these thermophilic bacteria (*B. licheniformis*) as a dominant bacterial isolates in hot spring responded to abrupt temperature changes with a heat shock-like response. For this, a comparison was made of the proteins which were synthesized in response to different temperature. The response of *B. licheniformis* to one hour of thermal stress at 65°C by synthesizing 11 heat shock proteins (HSPs) with molecular weights ranging between = 30 – 120 kDa as shown in Figure 5.
Conclusions: In the present study, a high proportion of heat tolerant bacteria were observed to be present in water samples that were collected from Aen Zahra, Bar El-Dakrour and Bar Qurash hot springs compared with other hot springs. A selected thirteen bacterial isolates could tolerate high temperature up to 65°C. The genetic methods used in this study differentiated these isolates. Similarity analysis of RAPD and BOX-PCR screening revealed 6 unique patterns among 13 bacterial isolates. The most abundant bacterial isolates were related to B. licheniformis (38%) and B. pumilus (31%). B. licheniformis responded to heat stress by synthesized different heat shock proteins. This bacterial strain can be used as a candidate for many industrial purposes like antibiotic and enzymes productions.

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REFERENCES


