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IDENTIFICATION AND CHARACTERIZATION OF EXTREMOPHILE MICROORGANISMS WITH SIGNIFICANCE TO ASTROBIOLOGY

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Introduction

It is now well recognized that microorganisms thrive in extreme ecological conditions such as geothermal vents, polar region, acid and alkaline lakes, and the cold pressurized depth of the ocean floor of this planet. Morphological, physiological, biochemical and genetic adaptations to extreme environments by these extremophile microorganisms have generated immense interest amongst astrobiologists who increasingly believe in the existence of extraterrestrial life. The evidence collected by NASA's space probe Galileo suggested the presence of liquid water and volcanic activity on Mars and Jupiter's satellite Europa. Volcanic activity provides some of the heat necessary to keep the water on Europa from freezing that could provide important dissolved chemicals needed by living organisms. The possibility of the existence of hypersaline alkaline lakes and evaporites confined within closed volcanic basins and impact craters on Mars, and a layer of liquid water under the ice on Europa provide sufficient "raison d'être" to study microorganisms in similar extreme environments on Earth, which could provide us with a model that would help establish the existence of extraterrestrial life on other planetary bodies [4].

The objectives of the summer research project were as follows: (1) application of molecular approaches to help establish new species of extremophile microorganisms isolated from a hypersaline alkaline lake; and (2) identification of a major cold-shock gene (*cspA*) homolog from a psychrotolerant microorganism, PmagG1.

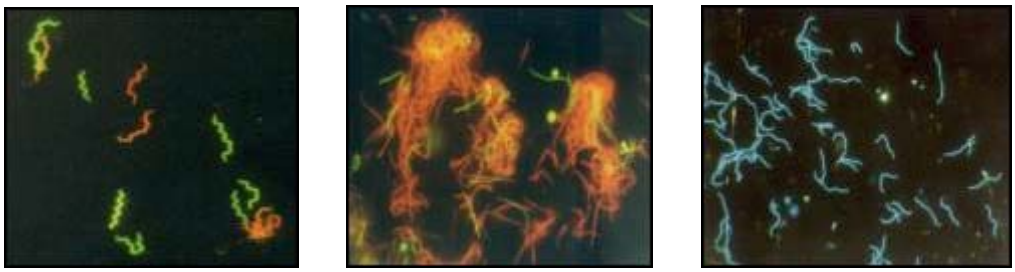
Objective 1: Application of molecular approaches to help establish new species of extremophile microorganisms isolated from a hypersaline alkaline lake.

Characterization of Spirochaeta americana str. ASpG1^T from soda Mono Lake. Some of the *Spirochaeta* are extremophiles and may serve as a model for the existence extraterrestrial life. *S. amaricana* ASpG1^T was isolated from black mud samples with strong odor of sulfur collected from Mono Lake in California by Richard Hoover.

Methods: Exponentially grown and stationary phase cultures of *S. amaricana* ASpG1^T were subjected to BacLight™ Live vs. Dead fluorescent stain (Molecular Probes, Inc.) and nucleic acid DAPI stain. The DAPI staining was was adopted from a previously described and examined under a *Leitz* Diaplan epifluoroscent microscope at a 1000x magnification.

Figure 1a. Soda Mono Lake in California with Tufa columns.

Results: The morphology of an exponentially grown culture of *S. americana* ASpG1^T exhibited typical spiral cells of 0.20-0.25 μm in diameter and 8-15 μm in length with rotating undulatory movement (Figure 2). The DAPI stained cells exhibited typical blue fluorescent color of the nucleic acids in the cells. Uniform blue color throughout the cell body suggests that unlike in other Gram-negative microorganisms the chromosomal DNA in *S. amaricana* ASpG1^T does not form a nucleoid structure (Figure 2). The stationary phase culture exhibited round irregular spheroplast cells possibly a mechanism to remain in "dormant" state (Figure 2).



a. **b.** **c.**

Figure 2. BaLight™ and DAPI fluorescent stained *S. americana* ASpG1^T cells from (a) an exponentially grown culture with typical spiral morphologies and (b) a stationary phase culture with spheroplasts and aggregated cells. Green="live" cells; Red= "dead"cells; (c) DAPI fluorescent stained *S. americana* ASpG1^T cells with uniform blue color of the cell bodies.

Molecular Analyses: Phylogenetic analysis of *S. americana* ASpG1^T based on the 16S rDNA sequence exhibited 99.2% sequence homology with another haloalkaliphilic miroorganism, *Spirochaeta alkalica* Z-7491^T. However, the morphological, biochemical and total mol %GC content suggested that these two spirochetes are separate species. Therefore, it was necessary to conduct further experiments on the genome of these two microorganisms to firmly establish that *S. americana* ASpG1^T is a new species.

Methods: (i) Melting temperature (Tm): Melting temperatures (*Tm*) of total genomic DNA from *S. alkalica* Z-7491^T and *S. americana* ASpG1^T were determined by following the procedures described elsewhere [2,3]. Purified genomic DNA from *S. alkalica* Z-7491^T and *S. americana* ASpG1^T were subjected to sonication to generate DNA fragments between 400-800 bp. The sonicated DNA was treated with boiled RNase I and S1 nuclease [1]. The concentration and the purity of the DNA were determined using a spectrophotometer (Shimadzu UV-160). The denaturation process of the sonicated genomic DNA in 2x SSC was carried out in an uvonic cuvette placed in a spectrophotometer by increasing the temperature of the sample from 26 ºC to 100 ºC, and absorbance was recorded at 260 nm wavelengths. The melting temperature (*Tm*) was determined by calculating the temperature at which the hyperchromicity has reached half of the value obtained after completed melting.

(ii) DNA-DNA Hybridization: To determine the percent homology of genomic DNA between *S. alkalica* Z-7491^T and *S. americana* ASpG1^T, DNA-DNA hybridization was performed followed by DNA reassociation kinetics as described by DeLey *et al.* [2]. Sonicated genomic DNA (80 μg) from each of these microorganisms was added to 2x SSC and 25% formamide and then denatured by raising the temperature to 100°C and cooled to melting temperatures in the spectrophotometer. The samples were kept at melting temperature for 3 minutes and the optical density at 260 nm wavelengths was recorded at 5 seconds interval for a total of 20 minutes. The initial reassociation kinetics was determined by linear regression analysis. The percent homology of the DNA from these two microorganisms was calculated using the equation described by DeLay *et al.* [2]. All statistical analysis was performed using the Microsoft™ Excel software.

(iii) Genome size: The genome size of *S. americana* ASpG1^T was determined using the DNA-reassociation kinetics, following the Equation described by Gillis *et al.* [3], and by using the reported molecular weight of the genome of *S. alkalica* Z-7491^T.

Results: The *Tm* value of total fragmented genomic DNA of *S. americana* ASpG1^T was 68±2 ºC whereas it was 74±2 ºC for *S. alkalica* Z-7491^T. The regression analysis during the first 1.5 min of DNA reassociation kinetics for *S. americana* ASpG1^T exhibited a decrease of 0.03386±0.00074 (r^2=0.9937) OD units per min, whereas, *S. alkalica* Z-7491^T had a decrease of 0.03510±0.00049 (r^2=0.9974) OD units per min. The DNA from *S. americana* ASpG1^T and *S. alkalica* Z-7491^T strains in a mixture exhibited a decrease of 0.02563±0.00052 (r^2=0.9946) OD units per min. DNA-DNA hybridization established 48.7% homology between the genomes of *S. americana* ASpG1^T and *S. alkalica* Z-7491^T based on the reassociation kinetics and the equation described by Gillis *et al.* [3]. The genome size for *S. americana* ASpG1^T was 2.98x10^9 daltons. From this study, manuscript entitled "*Spirochaeta americana* sp. nov., a new halo-alkaliphilic, obligatory anaerobic spirochete isolated from soda Mono Lake in California" has been submitted to a peer reviewed journal for consideration for publication.

B. Characterization of Tindallia californiensis APO^T *from soda Mono Lake.*

This strictly anaerobic hyperalkaliphilic microorganism was isolated from black mud samples collected from soda Mono Lake in California by Richard Hoover and Elena Pikuta. Phylogenetic analysis based on the16S rDNA sequence exhibited 99.1% homology with *Tindallia magadii* Z-7934^T. Therefore, it was necessary to conduct additional genetic analysis to establish that in fact *T. californiensis* APO^T is a new species. The *Tm* values, DNA-DNA hybridization and genome size for these two species were conducted as described above. The *Tm* values for *T. californiensis* APO^T was 70°C±2°C and for *T. magadii* Z-7934^T was 63°C±2°C. DNA-DNA hybridization revealed that the homology between genomic DNA of these two species was 55%. Genome size for *T. californiensis* APO^T was 1.02x10^9 daltons and for *T. magadii* Z-7934^T was 1.15x10^9 daltons. Therefore, based on the genetic analysis along with the morphological and biochemical characteristics, it is determined that *Tindallia californiensis* APO^T is a new species. From this study, a manuscript entitled "*Tindallia californiensis* sp. nov., a new halo- alkaliphilic primary anaerobe, isolated from meromictic soda Mono Lake in California, and the correction of diagnosis for genus *Tindallia"* is currently being prepared.

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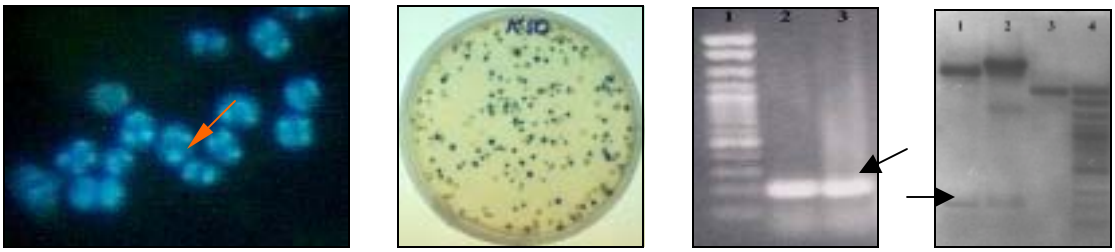
Objective 2: Identification of a major cold-shock gene (cspA) homolog from a psychrotolerant microorganism, PmagG1.

The PmagG1 was isolated from guano of the Magellanic Penguine in Patagonia, South America by Richard Hoover. PmagG1 is a Gram-positive, anaerobic and phylogenetically closely related to *Lactosphera pasteurii* as determined based on the 16S rDNA sequence homologies (98%). DAPI fluorescent stain exhibited round cell morphologies in the form of clusters with a distinct nucleoid in each cell body (Figure 3). PmagG1 is a psychrotolerant bacterium with a growth temperature between 27°C and –5.5°C. To determine the genetic mechanism of cold tolerance, genomic DNA of PmagG1 was subjected to PCR amplification of the *cspA* gene that codes for the major cold shock protein in *E. coli*. PCR amplification resulted into a 210 bp amplicon (Figure 3). The *cspA* amplicon has been cloned onto a TOPO TA™ cloning vector (Invitrogen, Inc.) and nucleotide sequence is being performed. Further analysis of the *cspA* and its regulation at cold temperature may help understand underlying genetic and mechanisms of their tolerance and survival at cold temperatures for PmagG1.



a. **b.** **c.** **d.**

Figure 3(a). DAPI-stained PmagG1 cells showing distinct chromosomes in nucleoid structures; (b) *E. coli* colonies with *cspA* clones (white); (c) Agarose gel electrophoresis showing the PCR amplified 210 bp *cspA* gene fragment from PmagG1 (Lane 2) and *E. coli* (Lane 3); lane 1, DNA size marker. (d). Agarose gel electrophoresis showing the *Eco*RI restriction endonuclease digested 210 bp fragment of the *cspA* gene fragments (arrows) from PmagG1 (Lane 1) and from *E. coli* (Lane 2); lane 3, undigested plasmid and lane 4, DNA size marker.

Discussion

The study of extremophile microorganisms is of particular interest to Astrobiology as extremophiles may provide models for extraterrestrial life. The haloalkaliphilic microbes in hypersaline soda alkaline lakes may represent possible analogs for microbial life that could have inhabited hypersaline alkaline lakes and evaporites confined within closed volcanic basins and impact craters of ancient Mars. Also, study of psychrotolerant and psychrophilic microorganisms in Antarctic ice and cryoconites provide model for the type of extraterrestrial life that may exist under the icy crust of Europa, polar ice caps in Mars and other frozen worlds of the Universe.

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