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Potential new genera of cyanobacterial strains isolated from thermal springs of western Sichuan, China

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ABSTRACT

Cyanobacteria have shown great potential for energy and environmental applications. Cyanobacterial resources, however, are still largely unexplored. Here, forty-nine out of one hundred thirty-two cyanobacterial isolates recovered from thermal springs of western Sichuan, China, were characterized. The phylogenetic analysis of 16S rRNA gene sequences categorized the isolates into three genera, *Thermosynechococcus* (63.3%), *Leptolyngbya* (34.7%) and *Stanieria* (2.0%). Based on sequence similarity, five phylotypes were identified as either putative new species of genus *Leptolyngbya* or possibly completely new genera. Temperature test showed that all isolates were thermotolerant and twenty-five isolates were capable of growth at temperature of 60 °C, suggesting that these isolates may have strong biotechnological potential. Additionally, three isolates exhibited $NaHCO_3$ tolerance as high as 1 M, indicating that the isolates are promising candidates for bicarbonate-based cyanobacteria production system. Overall, this research laid solid basis for taxonomy and future exploration of extremophilic cyanobacteria for biotechnological and environmental applications.

1. Introduction

Microorganisms inhabiting extreme thermal environments play a crucial role in energy metabolism and matter cycling [1]. Cyanobacteria are a diverse group of microorganisms that are extensively distributed in various ecological niches including hot springs [2]. These autotrophic prokaryotes play an important part in sustaining the productivity of ecosystems through photosynthesis, nitrogen fixation, and accumulation of phosphorus as cytoplasmic polyphosphate granules [3]. Thermophilic strains from geothermal fields have been widely studied in numerous parts around the world, such as North Africa [4], Balkan Peninsula [5], Indian subcontinent [6], Kamchatka [7], and Yellowstone National Park [8].

The isolation and characterization of cyanobacteria from high-temperature environments is also of practical importance because these isolates may have biotechnological potential with regard to the production of bioactive molecules and useful temperature-tolerant enzymes, such as laccases, phosphatases and kinases [9, 10]. Besides, the thermophilic cyanobacteria can provide a robust photosynthetic platform for industrial CO_2 utilization.

Hot springs in western Sichuan of China are mostly located at Ganzi Prefecture and occur due to active tectonic movement. The hot springs in Ganzi span a wide range of temperature from 30 to 98 °C. Numerous prior studies verified that thermophilic cyanobacterial strains are often inhabitants of terrestrial hot springs like those in Ganzi prefecture [4, 6]. Therefore, the geothermal areas of western Sichuan might be a potential reservoir of genetic diversity of cyanobacterial community. To date, hot springs in Ganzi, however, are still untapped for cyanobacterial study.

The aim of this work was to isolate and characterize culturable cyanobacteria from the thermal spring ecosystems with a view to screen thermotolerant and thermophilic types. Cyanobacterial isolates were recovered from hot springs samples by plating and enrichment cultures. Characterization was achieved by analysis of morphology, 16S rRNA gene sequence and temperature tolerance.

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2. Materials and methods

2.1. Sample collection

Samples were collected from five sites of thermal springs in Ganzi Prefecture of Sichuan Province, China (30°05′14″ N, 101°56′55″ E (isolates A, one spring; temperature 40.8 °C; pH 6.32); 30°15′57″ N, 101°52′24″ E (isolates B, four springs, temperature range 53.1–85.0 °C; pH range 6.35–8.50); 30°36′39″ N, 101°41′9″ E (isolates C, one spring; temperature 64.6 °C; pH 6.56); 30°36′9″ N, 101°43′46 E″ (isolates D, one spring, temperature 95.0 °C; pH 6.80); 29°25′4″ N, 101°18′1″ E (isolates E seven springs, temperature range 41.1–68.5 °C; pH range 7.95–8.84)). Algal mats, sinter, and sediment samples were collected from multiple points of each site by scrapping with sterile forceps and spatula, and then samples were mixed together to ensure uniformity and directly transferred into sterile 50 mL Falcon tubes containing in situ hot spring water.

2.2. Isolation of cyanobacteria, morphological identification and tests of temperature/$NaHCO_3$ tolerance

All the samples were brought to the laboratory within 24 h for establishment of enrichment cultures and further isolation. A small portion (200 μL) of mixed samples were transferred to the Petri dish sealed tightly with Parafilm containing 20 mL of BG-11 liquid medium [11], and then cultured stationary in an incubator at 45 °C under a photoperiod of 16-h light (average 7.1 μmol $m^{-2} s^{-1}$) and 8-h dark for one week with gentle shaking once a day to establish enrichment culture. Cyanobacteria were isolated and established as pure culture by a combination of capillary pipette washing and plate streaking methods. The pure stock culture was regularly sub-cultured at three weeks interval. The isolated cultured cyanobacteria were identified by light microscopic observation by a microscope Olympus BX53 at 400–1000× magnification using morphological traits such as the dimensions of cells, the arrangement of cells in colonies or filaments, the terminal cell shape in a filament, and the presence of specialized structures, such as aerotopes (also known as gas vesicles used for regulating buoyancy), heterocysts (specialized cells for nitrogen fixation), and akinetes (specialized resting cells that allow organisms to survive harsh conditions and germinate when environmental conditions allow). Several sources were used to identify cyanobacteria based on morphology [12–14].

2.3. Preliminary tests of temperature/$NaHCO_3$ tolerance

Temperature tolerance experiment was performed by culturing 2 mL of cyanobacterial isolate culture in 20 mL capped test tubes containing 8 mL BG-11 liquid medium. The isolates were maintained at three levels of temperature (45, 50 and 60 °C) in a controlled incubator under a photoperiod of 16-h light (average 7.1 μmol $m^{-2} s^{-1}$) and 8-h dark for 3 weeks to provide initial screening of the strains. All experiments were conducted in triplicate. Cellular growth was determined by the visual observation of the appearance of the cultures against control samples maintained at 45 °C in BG11 medium. Experiments testing the effects of different concentrations of $NaHCO_3$ (0.1, 0.3, 0.5, 1.0 M) were performed as modifications of BG11 growth media in an analogous manner. Unique strains were stored in Peking University Algae Collection (PKUAC) at 45 °C with limited lighting conditions and subcultured routinely every three weeks.

2.4. DNA extraction, amplification and sequencing

Genomic DNA was isolated using modified Xanthogenate method essentially as described by Avijeet et al. [15]. The DNA quality and quantity were assessed using agarose gel electrophoresis and Nanophotometer Classic (Impeln, Germany), respectively. The 16S rRNA genes were amplified from bacterial genomic DNA using bacterial universal primers (8F: 5′-AGAGTTTGATCCTGGCTCAG-3′, 1510R: 5′-GGCTACCTTGTTACGA-3′) [16]. DNA amplifications were performed using a C1000 Touch ™ thermal cycler (Bio-Rad Laboratories, USA) with 20 μL reaction mixtures containing 1 μL DNA (~50 ng/μL), 0.5 μL each primer (10 μM), 10 μL High-Fidelity PCR Master mix (GENERAY, China) and 8 μL sterile deionized water. PCR cycling conditions consisted of denaturation at 95 °C for 3 min, 30 cycles of 95 °C for 30 s, 52 °C for 30 s, and 72 °C for 2 min, and final extension at 72 °C for 10 min.

Positive PCR products were separated by 1% agarose gel electrophoresis and purified using a Gel extraction kit (Generay, China). Then, PCR products were verified by 1% agarose gel electrophoresis. For 16S rRNA gene sequencing, purified PCR products were ligated into vector pMD19-T (Takara Inc., Japan), and transformed into *E. coli* DH5α. Clones were screened by PCR for inserts of the correct size (ca. 1500 bases) using pMD19-T-specific primers (M13F: 5′-CGCCAGGGTTTTCC CAGTCACGAC-3′, M13R: 5′-AGCGGATAACAATTTCACACAGGA-3′). Inserts were commercially sequenced (BGI Shenzhen, China) to provide full-length 16S rRNA gene sequences. Nucleotide sequences generated in this study were all deposited in GenBank under the accession numbers MF405378-MF405397, MF405399, MF405401-MF405407, and MF405409-MF405429.

2.5. Phylogenetic analysis

In addition to sequences representing the strains isolated in this study, reference sequences of 16S rRNA gene of cyanobacteria were retrieved from GenBank and included in phylogenetic analysis. Sequences were aligned, edited and trimmed in Mega7 [17]. Maximum likelihood phylogenetic analyses were carried out using PhyML version 3.0 [18]. Parameters used in PhyML were set as described in Tang et al. [19], and 1000 nonparametric bootstrap replications were used to assess support. A phylogenetic tree was reconstructed using Mega7 software.

3. Results

3.1. Morphology summary

A total of one hundred thirty-two isolates were recovered from various samples of thermal springs. Forty-nine of these isolates were selected on the basis of differences in colony morphology, growth and temperature tolerance for further characterization, given our research interests. The morphology of the forty-nine isolates was summarized in Table 1. The dominant cell morphology was unicellular rod (63.3%), followed by filamentous (34.7%) and unicellular round (2.0%). Two types of color were observed for the isolates, namely blue green (87.8%) and brown (12.2%).

3.2. Phylogeny

The phylogenetic tree reconstructed from nearly full length 16S rRNA gene sequences of the forty-nine cyanobacterial isolates recovered from hot springs is shown in Fig. 1. Analysis of 16S rRNA gene sequences divided the cyanobacterial isolates into the three families, Dermocarpellaceae (order *Pleurocapsales*), Leptolyngbyaceae and Synechococcaceae (order *Synechococcales*). The result of phylogenetic clustering was in accord with the morphology data (Table 1). The Synechococcaceae accounted for the majority of the isolates, (63.3%) whereas the Leptolyngbyaceae and Dermocarpellaceae accounted for 34.7% and 2.0% of the isolates, respectively.

Among cyanobacteria assigned to the family Synechococcaceae, a cluster of thirty-one isolates was closely related to *Thermosynechococcus elongatus* BP1 (98.7–99.5% similarity). The thirty-one isolates showed > 99.5% sequence similarity to each other, but many of these