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# Identification and characterization of thermophilic *Synechococcus* spp. isolates from Asian geothermal springs

Hongmei Jing, Hongbin Liu, and Stephen B. Pointing

**Abstract:** Two thermophilic cyanobacterial strains, Ts and Bs, collected from Asian geothermal springs were identified morphologically and phylogenetically as *Synechococcus* in the order *Chroococcales* and were isolated into axenic cultures. In addition to the high similarities between their full 16S rRNA gene sequences, both strains also shared similar pigment profiles and fatty acid compositions but with varied ratios. Strain Ts had elevated levels of photoprotective pigments such as carotenoid and scytonemin even after prolonged culture under identical laboratory conditions, whereas strain Bs produced more chlorophyll *a* per unit cell volume, perhaps resulting from UV adaptation in the natural habitats. In addition, strain Ts had more content than strain Bs in terms of the total fatty acids and the proportion of unsaturated fatty acids. Neither isolate was able to fix nitrogen, and they had zero susceptibility to ampicillin and streptomycin.

**Key words:** thermophilic *Synechococcus*, phylogeny, pigment, fatty acid, nitrogen fixation.

**Résumé :** Deux souches de cyanobactéries thermophiles, Ts et Bs, recueillies de sources géothermales asiatiques ont été identifiées morphologiquement et phylogéniquement comme appartenant à *Synechococcus*, de l'ordre des *Chroococcales* et ont été isolées en cultures axéniques. En plus de la grande similarité des séquences du gène codant l'ARNr 16S pleine longueur, les deux souches partageaient aussi des profils de pigments et de composition en acides gras similaires, mais selon des rapports différents. La souche Ts avait de fortes quantités de pigments photoprotecteurs comme les caroténoïdes et la scytonémine, même après une culture prolongée sous des conditions de laboratoire identiques, alors que la souche Bs produisait d'avantage de chlorophylle *a* par unité de volume cellulaire, ce qui pourrait résulter d'une adaptation aux UV dans les habitats naturels. De plus, la souche Ts avait un contenu supérieur à celui de Bs en termes d'acides gras totaux ainsi que dans la proportion d'acides gras insaturés. Qui plus est, aucun des isolats n'était capable de fixer l'azote et n'était sensible à l'ampicilline et à la streptomycine.

**Mots-clés :** *Synechococcus* thermophile, phylogénie, pigment, acide gras, fixation d'azote.

[Traduit par la Rédaction]

## Introduction

Thermophilic cyanobacteria are the primary producers in microbial communities in geothermal springs worldwide at temperatures up to 75 °C (Castenholz 2000), which is the maximum temperature limit for performing oxygenic photosynthesis (Ferris and Ward 1997). Previous studies have revealed great phenotypic plasticity in some cyanobacteria (Nadeau et al. 2001; de la Torre et al. 2003) and phylogenetic classification based on gene sequences would to some extent give a more accurate classification and evolutionary

placement (Wilmotte 1994), despite discrepancies between morphological and molecular (16S rRNA gene) taxonomy (Rippka et al. 1979; Rippka and Herdman 1992; Turner et al. 2001; Litvaitis 2002).

Fatty acids have been used as biological markers because different organisms are characterized by particular features of fatty acid composition (Ocarra and Oheocha 1976). Fatty acid composition of thermophilic cyanobacteria has been rarely studied. A recent study of fatty acid compositions of several thermophilic cyanobacteria revealed few polyenoic acids in filamentous thermophilic *Phormidium laminosum* and none in unicellular thermophilic *Synechococcus* spp. (Maslova et al. 2004). The authors observed that the ratio of saturated to unsaturated fatty acids decreased twofold with decreasing temperature, and hexadecenoic acids were suggested to play a major role in cyanobacterial adaptation to high temperatures. In addition, fatty acid chain length and degree of saturation in hot spring cyanobacterial mats vary with ambient temperature (Ward and Castenholz 2000).

Different photosynthetic organisms also contain a different suite of cellular pigments, but the contents and ratios of different pigments may be affected by environmental conditions such as nutrient status and the light regime. For instance, thermal *Synechococcus* populations shift between yellow and green colors, depending on the shading of light

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(Brock and Brock 1969). Some thermophilic microorganisms are capable of adjusting their physiology when exposed to high irradiance, including production of photoprotective compounds such as mycosporine-like amino acids and scytonemin (Sinha et al. 1998, 1999, 2001). Scytonemin is commonly produced by most (but not all) sheathed cyanobacteria from thermal springs and other natural habitats with high solar irradiance (Scherer et al. 1988; Garcia-Pichel and Castenholz 1991; Dillon and Castenholz 2003), and so is frequently found in terrestrial cyanobacterial crusts or dried mats and is very important to cyanobacteria inhabiting harsh habitats (Sinha et al. 1999). In addition to those UV-protective pigments, phycobiliproteins are disassembled and denatured at higher temperatures (Inoue et al. 2000) and are absent from all blue-green algae growing at temperatures above 60 °C (Brown and Richardson 1968).

Generally, cultivation of thermophilic cyanobacteria has been proved relatively difficult, with only very few species being characterized from axenic cultures. Successful cultivation of thermophilic cyanobacteria is the first step in bioprospecting for useful thermophilic enzymes. Physiological characterization of thermophilic microorganisms will help to determine their physicochemical growth limits and is also useful for understanding the basic mechanisms of thermal evolution, adaptation, and acclimation. In this paper we report the phylogenetic, biochemical, and physiological characteristics of two thermophilic cyanobacterial strains collected from Asian geothermal springs. The differences between their pigment and fatty acid compositions may reflect the adaptation to their natural habitats.

## Materials and methods

### Sample recovery

Microbial mat samples from an intertidal hot spring (56 °C) in Pasacao Bicol, the Philippines (13°29.416'N, 123°04.634'E) and a terrestrial hot spring (60 °C) in Daggyai Tso in Tibet, China (29°35.413'N, 85°44.486'E) were used for enrichment culture to isolate cyanobacteria of interest. Physicochemical conditions of the geothermal springs in Bicol were as follows: temperature 37–54 °C, pH 6.5–8.0, salinity 15‰–30‰, nitrate 0.02–0.2 mg·L<sup>-1</sup>, total phosphate 8.0–12.5 mg·L<sup>-1</sup>, and hydrogen sulphide 0–1.76 mg·L<sup>-1</sup>. Mats occurred at ≈65 °C in Daggyai Tso geothermal field, which was located in remote southern central Tibet, China, at an altitude of 5050 m.

### Isolation and purification

Enrichment culture with highly selective culture conditions that included serial dilution before inoculation, antibiotic treatment, and high temperature incubation were applied to selectively isolate desired cyanobacteria from natural samples and counter-select undesired microorganisms. Purification was made by plating and streaking; selected colonies were subcultured into liquid Castenholz D (CD) medium (Castenholz 1988) or onto solid plates, which incorporated Gelrite (Merck, Rahway, New Jersey) with high melting point (≈70.6 °C) as a substitute for normal agar (1.5% *m/v*). All isolates were grown with CD medium and were incubated under white fluorescent light of 124 μmol·m<sup>-2</sup>·s<sup>-1</sup> at 45 °C with a diel light:dark cycle of 14 h : 10 h.

Antibiotic treatments were performed to remove other potential thermophilic contaminations by adding filter-sterilized (Whatman, 0.2 μm) antibiotics (ampicillin and streptomycin sulphate, both at 100 μg·mL<sup>-1</sup>) into the media and (or) agar plates.

### Morphological characterization

Purity and morphological characters of two isolates were examined and recorded using an Olympus BX50™ light microscope (Olympus, Center Valley, Pennsylvania) and scanning electron microscopy. For scanning electron microscopy, cells were first collected by centrifugation at 13 400g for 10 min and fixed on a glass slide in 2.5% glutaraldehyde for 1 h at room temperature, rinsed by nano-distilled water for several times, and air-dried overnight. Each specimen was mounted onto an aluminum stub, gold-coated using a BAL-TEC SCD 005™ Sputter Coater (BAL-TEC, Balzers, Switzerland) and examined using an Stereoscan 440™ scanning electron microscope (Leica, Germany). Identification of morphotypes was made with reference to standard taxonomic keys in *Bergey's Manual of Systematic Bacteriology* (Castenholz and Waterbury 1989) and the one listed in Sompong et al. (2005).

### Phylogenetic placement

Genomic DNAs of two thermophilic cyanobacterial isolates were extracted using hot phenol methods. Full 16S rRNA gene sequences were amplified by a universal bacterial primer set: 27F (5'AGAGTTTGATCCTGGCTCAG3') and 1492R (5'GGTTACCTTGTACGACTT3') (de la Torre et al. 2003). PCR products were subsequently cloned into a pDrive vector (Qiagen, Valencia, California). Sequences of ten transformants were screened to establish homogeneity. Each colony with successful insertion was picked and sequenced using an ABI Prism 377™ sequencer (Applied Biosystems, Foster City, California) by using vector primer sets of QM13F (5'GTAAAACGACGGCCAGT3') and QM13R (5'AACAGCTATGACCATG3').

Phylogenetic placement was determined by using maximum likelihood analysis with reference to appropriate sister taxa. The full 16S rRNA gene sequences derived from this study were submitted to GenBank under the accession Nos. DQ131173–DQ131175.

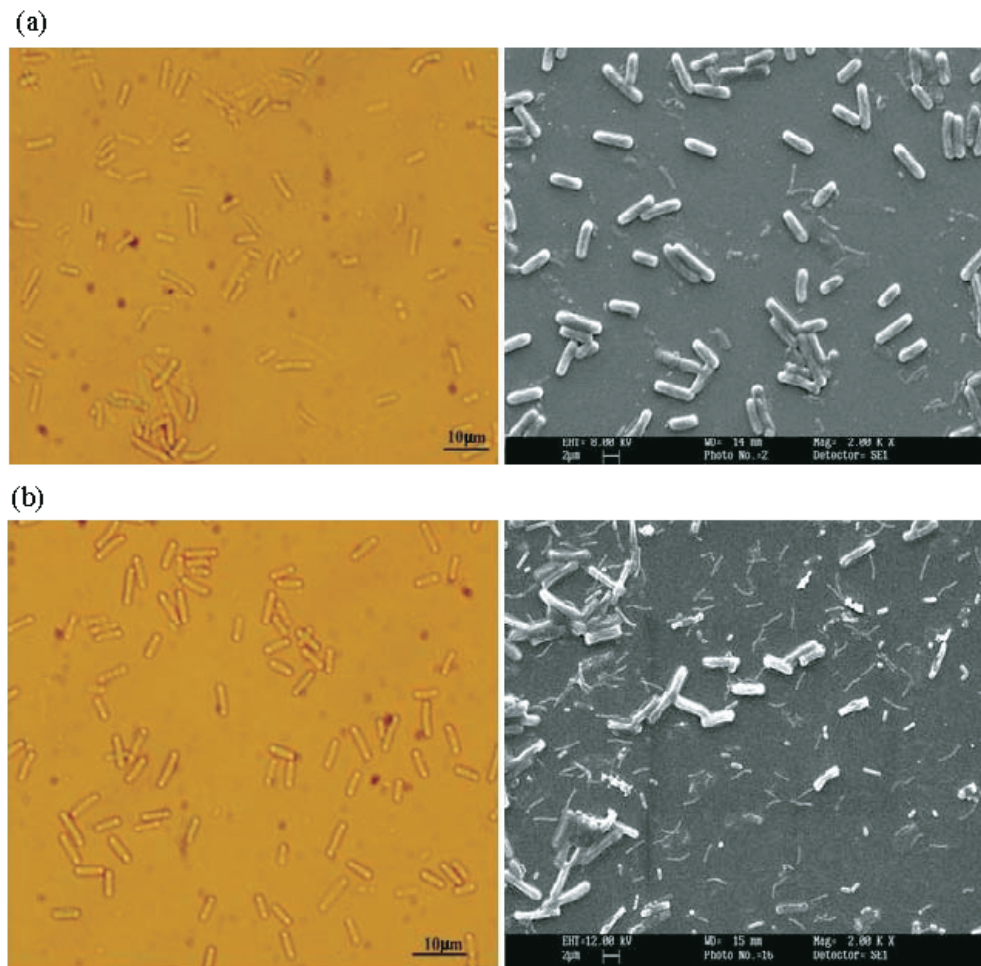
### Photosynthetic and photoprotective pigments

Biomass of cultures in stationary phase were collected by centrifugation at 13 400g for 20 min, the pellets were rinsed twice using distilled water, homogenized with 3 mL of 100% acetone, and were stored at 4 °C in darkness overnight (Garcia-Pichel and Castenholz 1991). Supernatants were obtained by centrifugation and quantified by spectrophotometry (Smartspect™plus; Bio-Rad, Hercules, California) at recorded spectra: chlorophyll *a* at 663 nm, carotenoid at 490 nm, and sytonemin at 384 nm. The absorbancy was converted to pigment concentration according to the following equation: pigment concn. (mg·mL<sup>-1</sup>) = ( $D \times V \times f \times 10$ )/2500, where *D* is absorbancy, *V* is the volume of the sample, *f* is the dilution factor, and 2500 is the extinction coefficient (Subramanian et al. 1992).

Similar procedures as mentioned above were used for the extraction of water-soluble phycobilins: c-phycocyanin at



**Fig. 1.** Micrographs of two thermophilic *Synechococcus* isolates. (a) Strain Ts from thermal springs in Tibet, China. (b) Strain Bs from thermal springs in Bicol, the Philippines. Light micrographs (left panels), scanning electron microimages (right panels).



615 nm, c-phycoerythrin at 562 nm, and allophycocyanin at 652 nm, except that phosphate buffer and a freeze-thaw step were used for extraction. Content ( $\text{mg}\cdot\text{mL}^{-1}$ ) of each pigment was calculated according to the following equations:

$$\text{c-phycoerythrin} = [A_{615} - 0.474(A_{652})]/5.34$$

$$\text{allophycocyanin} = [A_{652} - 0.208(A_{615})]/5.09$$

$$\text{c-phycoerythrin} = [A_{562} - 2.41(\text{c-phycoerythrin}) - 0.849(\text{allophycocyanin})]/9.62$$

Extra care was taken during the pigment extraction and testing, since all pigments were light sensitive.

#### Fatty acid profiles

Fatty acids were analyzed following the method described by Vazhappilly and Chen (1998). Biomass of isolates were harvested and lyophilized beforehand. Fatty acid methyl esters were prepared by trans-methylation with methanol – acetyl chloride and analyzed using a HP6890<sup>TM</sup> capillary gas chromatograph (Hewlett-Packard, Palo Alto, California)

equipped with a flame-ionization detector and an Innowax capillary column (30 m  $\times$  0.25 mm; J&W Scientific, Folsom, California). Nitrogen was used as carrier gas. Initial column temperature was set at 170 °C, which was subsequently raised to 230 °C at 1 °C $\cdot$ min<sup>-1</sup>. The injector was kept at 250 °C with an injection volume of 3  $\mu\text{L}$  under splitless mode. The flame ionization detector temperature was set at 270 °C. Fatty acid methyl esters were identified by chromatographic comparison with authentic standards (Sigma-Aldrich, St. Louis, Missouri). The quantities of individual fatty acid methyl esters were estimated from the peak areas on the chromatogram using nonadecanoic acid (C19:0) as the internal standard.

#### Growth in the absence of supplied nitrogen source

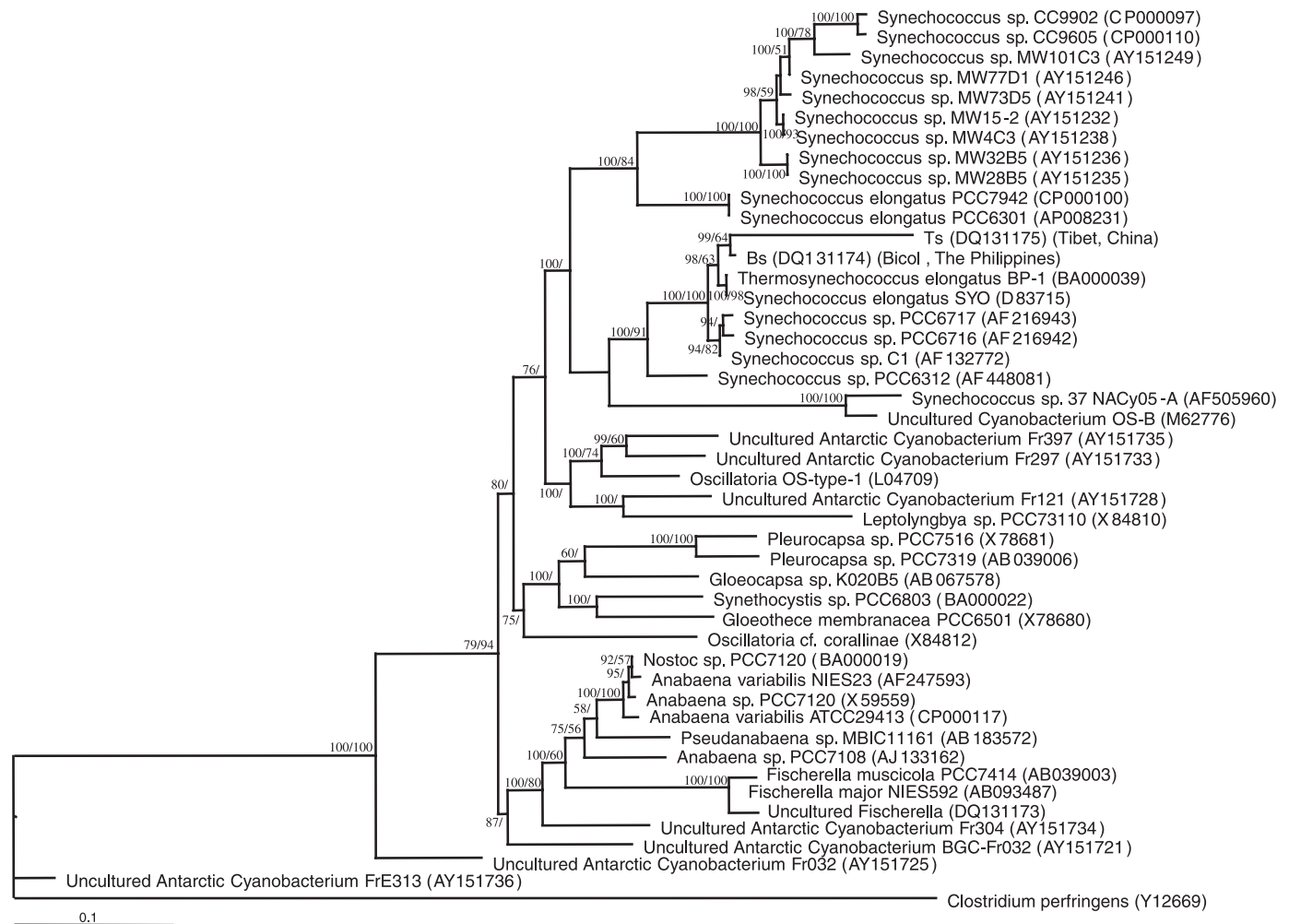
Both strains were cultured in CD medium with and without any supply of inorganic or organic nitrogen. Other culture conditions were the same as described above. Light microscopy was used to monitor cell viability.

## Results

#### Morphological characteristics

Two antibiotics added had no obvious effects on the survival of both cyanobacterial isolates, but they could effec-

**Fig. 2.** Phylogenetic positions of two thermophilic cyanobacterial isolates based upon maximum likelihood analysis of full 16S rRNA gene sequence data (*Escherichia coli* positions 27–1492). Tree topology is supported by Bayesian posterior probabilities (first number) and bootstrap values for 1000 replications (second number), shown for branches supported by more than 50% of the tree. Scale bar represents 0.1 nucleotide changes per position. Strain Bs, *Synechococcus* sp. from geothermal springs in Bicol, the Philippines; strain Ts, *Synechococcus* sp. from geothermal springs in Tibet, China.



tively reduce or remove some coexisting bacteria, which showed different morphologies in liquid cultures or formed different types of colonies on Gelrite plates.

Cells of strain Bs exhibited a short-rod morphology (Fig. 1), with average longitudinal diameter of  $3.89 \pm 0.415 \mu\text{m}$  and average transverse diameter of  $1.084 \pm 0.13 \mu\text{m}$  ( $n = 5$ ). Strain Ts was also rod shaped with an average longitudinal diameter of  $4.04 \pm 0.604 \mu\text{m}$  and average transverse diameter of  $1.21 \pm 0.173 \mu\text{m}$  ( $n = 5$ ). Both divided by binary fission in a single plane and lacked structured sheaths. Cell aggregates were only observed for strain Bs, and both cell aggregates and individual cells released from aggregates were visible. Morphological characters of both strains were in agreement with those described for the genus *Synechococcus* in order of *Chroococcales* in *Bergey's Manual of Systematic Bacteriology* (Castenholz and Waterbury 1989).

### 16S rRNA gene sequence determination and phylogenetic placement

The full length 16S rRNA genes of two thermophilic cyano-

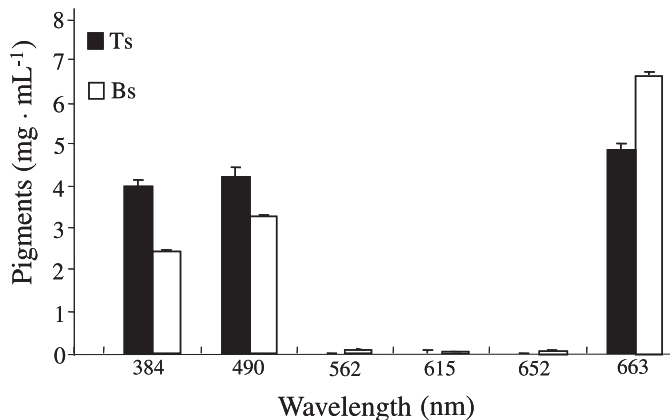
bacterial isolates were sequenced and submitted to the NCBI GenBank. Strain Ts (1474 bp, DQ131174) and strain Bs (1450 bp, DQ131175) shared 92% similarity in their full 16S rRNA gene sequences and had, respectively, 96% and 99% sequence identities to thermophilic *Synechococcus elongatus* BP-1 (BA000039) derived from a Japanese hot spring.

Phylogenetic tree based on maximum likelihood was constructed, including representative species of different cyanobacterial genera (Fig. 2). The two *Synechococcus* spp. were grouped together and formed a novel subclade within the Japanese thermophilic *Synechococcus* lineage C1 supported with high Bayesian posterior probabilities.

### Photosynthetic and photoprotective pigments

The two isolates shared identical pigment profiles but with different ratios of each component. Strain Ts contained more of the photoprotective pigments carotenoid and scytonemin than did strain Bs, which was relatively rich in chlorophyll *a* (Fig. 3). Both strains contained relatively little phycobilins (c-phycocyanin, allophycocyanin, and c-phycocerythrin).

**Fig. 3.** Pigment composition and contents of two thermophilic *Synechococcus* isolates in stationary phase. Cell abundances of the two cultures were adjusted to the same for comparison. Strain Bs, *Synechococcus* sp. from geothermal springs in Bicol, the Philippines; strain Ts, *Synechococcus* sp. from geothermal springs in Tibet, China.



### Fatty acid analysis

When growing under identical illumination conditions, the two thermophilic *Synechococcus* isolates possessed similar fatty acid composition with a difference in the concentration of each component (Fig. 4). The major features of the fatty acid profiles were high proportions of saturated straight-chain fatty acids, which accounted for 52.3% and 47.43% of the total fatty acid contents in strain Bs and Ts, respectively (Table 1). The total fatty acid content in strain Ts (6.02%) was far more than that of strain Bs (3.91%), although the latter contained more saturated straight-chain fatty acids.

In both strains, C16:0, C16:1, and C18:1n-9c fatty acids were the principal components of the total fatty acids. C16:0 was the most predominant saturated fatty acid, accounting for 43.57% and 40.58% of the total fatty acids in strain Bs and strain Ts, respectively. The content of each saturated fatty acid (except C17:0) was higher in strain Bs than in strain Ts, while in terms of unsaturated fatty acid (except C16:1, C18:3n-3, C18:3n-6), it was the opposite. The concentrations of each mono- and poly-unsaturated fatty acid were quantitatively different in these two isolates with an apparent pattern: strain Ts had more monounsaturated fatty acids, whereas strain Bs contained more polyunsaturated fatty acids. Each isolate had one polyunsaturated acid present at trace level (0.1% or less) and minor acids, C17:0, C17:1, and C14:0, accounting for less than 1% of the total. Furthermore, there were two unknown fatty acid components with minor peaks close to that of C17:1 and C16:1, and the former accounted for 6.14% and 8.72% of the total fatty acids of strain Bs and strain Ts, respectively.

### Nitrogen fixation

Both thermophilic *Synechococcus* isolates lacked the capability of fixing nitrogen and did not grow in CD medium without nitrogen source being added. Cells gradually became senescent and died within 5 days.

**Table 1.** Fatty acid compositions of two thermophilic *Synechococcus* strains.

Fatty acid	% fatty acid	
	Bs	Ts
13:0	1.07±0.13	0.54±0.06
14:0	0.48±0.17	0.46±0.16
14:1	2.24±0.21	2.56±0.66
15:0	3.32±0.52	1.83±0.06
16:0	43.57±0.51	40.58±0.73
Unknown 1	6.14±0.28	8.72±0.33
16:1	15.86±0.33	14.59±0.52
17:0	0.41±0.01	0.88±0.04
17:1	0.62±0.02	0.75±0.08
Unknown 2	0.92±0.06	1.57±0.23
18:0	3.45±0.13	3.15±0.52
18:1n-9c	17.95±0.23	23.04±0.17
18:2n-6	0.50±0.04	Trace <sup>a</sup>
18:3n-3	2.18±0.09	0.97±0.30
18:3n-6	1.18±0.01	0.00±0.00
18:4 <sup>b</sup>	Trace <sup>a</sup>	0.27±0.03
TFA <sup>c</sup>	3.91±0.02	6.02±0.07

**Note:** Data are expressed as the mean ± standard deviation of triplicates. Strain Bs, from geothermal springs in Bicol; strain Ts, from geothermal springs in Tibet.

<sup>a</sup>Trace means the percentage of fatty acid is equal to or less than 0.1% of the total fatty acids.

<sup>b</sup>18:4 was tentatively identified.

<sup>c</sup>Total fatty acid (TFA) content (%) = (TFA/cell dry mass) × 100%.

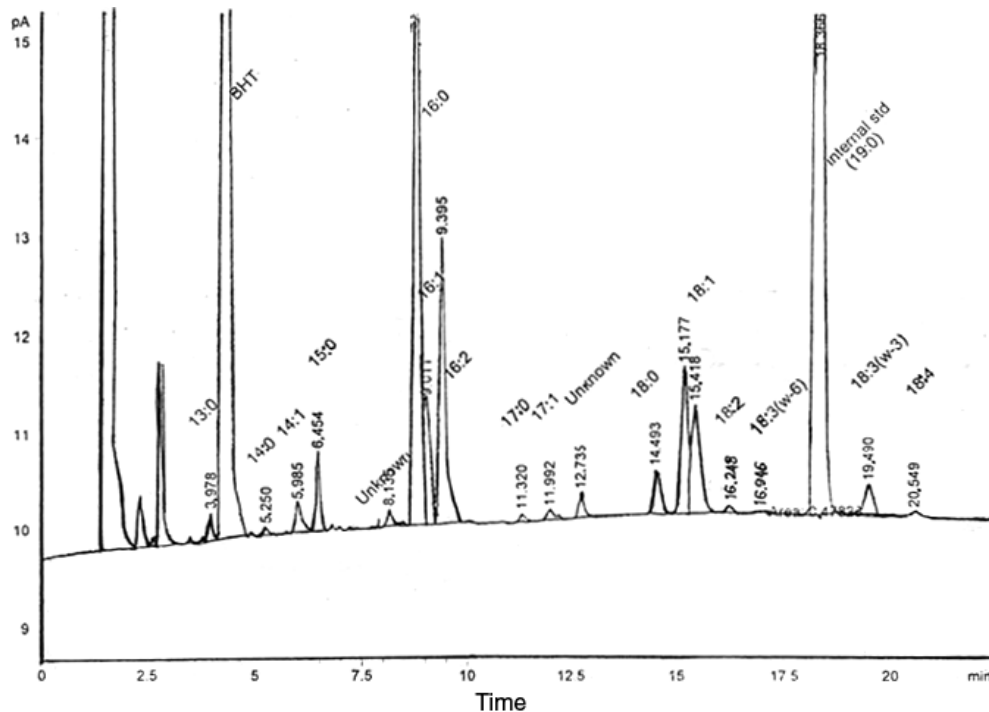
### Discussion

Enrichment culture techniques have been used in microbiological studies since the early 1900s and are still in application for selecting cultures of naturally occurring microorganisms that possess a desired metabolic trait. In this study, CD medium plus antibiotics was found to be quite effective during enrichment for obtaining axenic cultures of thermophilic *Synechococcus* strains. The purity of each culture was reflected by the colonies and sequencing results, since sequences of 10 randomly picked colonies for each isolate were all identical. 16S rRNA gene sequences of the two isolates were novel because they were not detected from molecular community diversity studies of the same sites (Jing et al. 2005, 2006). Therefore, they may represent novel species with very low abundance within the microbial community. Since the enrichment culture does not selectively recover the most abundant species but species that are physiologically adapted to the laboratory culture conditions, it may not reflect the real diversity of the cyanobacterial community. Therefore, molecular techniques are needed for studying microbial community diversity. Furthermore, different selecting culture conditions in enrichment cultures should be utilized in future to isolate other thermophilic cyanobacterial species from the natural samples.

Full 16S rRNA gene sequences allowed a higher resolution of phylogenetic analysis for the two newly derived thermophilic *Synechococcus* phylotypes. Both strains were phylogenetically closely affiliated despite being recovered



**Fig. 4.** Representative fatty acid profile of thermophilic *Synechococcus* strains.



from two distantly separated geothermal springs. Their close affinity with thermophilic *Synechococcus* phylotypes obtained from Japanese and Philippines geothermal springs is not unexpected, since those thermal locations are all geographically proximate compared with other geothermal springs being investigated.

Despite the close phylogenetic affiliation, the two strains differ in pigment composition. Strain Bs produces significant more photosynthetic chlorophyll *a* than strain Ts, while the later produces more ultraviolet (UV) photoprotective pigments. It has been reported that in thermophilic *Synechococcus* phycoerythrin is always absent, while phycocyanin is the major light-harvesting pigment and scytonemin content varies with genetic and environmental differences. For example, elevated temperature, salinity, and oxidative stress would enhance the production of scytonemin (Dillon et al. 2002), while high UV exposure could cause destruction of pigments including chlorophyll *a* (Ehling-Schulz and Scherer 1999). Therefore variation in pigment ratios in two strains may be the result of their distinctive natural habitats. The most obvious physicochemical differences between the two geothermal sites examined in this study were the water salinity and UV exposure. High UV irradiance, with hourly mean CIE-weighted biologically effective UV dose rate as high as 390.5 mW·m<sup>-2</sup>, was recorded at Lhasa (3648 m above sea level) on the Tibetan Plateau (Ren et al. 1997). Strong UV irradiation at the Tibetan geothermal location might have potentially induced the synthesis of more photoprotective pigment in strain Ts.

The reason that biomasses of stationary-phase cultures were used for fatty acid analysis was because the composition and content of fatty acids of the same species vary among different growth phases, and generally the proportions of polyunsaturated fatty acids are higher at stationary phase than at other growth phases. Hexadecanoic acids and

octadecanoic acids have long been known as the major fatty acids present in thermophilic unicellular cyanobacteria of the genus *Synechococcus* (Glazer 1977) and generally more C16 than C18 fatty acids are produced (Kenyon and Gray 1974; Fork et al. 1979; Knudsen et al. 1982; Miller et al. 1988). Compared with the nonthermophilic *Synechococcus* from the Pasteur Institute Culture Collection, which lack C17:0 and C17:1 fatty acids, the two thermophilic *Synechococcus* strains isolated in this study contained more fatty acids of C16:0, C18:0, and C18:1, although C16:2 fatty acid was absent. Lower light intensity in laboratory cultures than in nature may affect the relative amounts of complex polar lipids of photosynthetic and cytoplasmic membranes (Harwood and Russel 1984), and temperature is also known to affect both chain length and degree of saturation of fatty acid components in *Synechococcus* spp. (Fork et al. 1979; Miller et al. 1988). In addition, a correlation between fatty acid compositions and morphological and adaptational characteristics has been demonstrated (Wood 1974). For example, a high proportion of C18:3 fatty acids in the cytoplasmic membrane has been suggested as an important factor of *Nostoc flagelliforme* in its pronounced drought-tolerant ability. Similarly, in this study, the increased content of C18:3 fatty acids in strain Bs may contribute to the enhanced tolerance to desiccation stress. Also, the structure and identities of the two unknown fatty acids still need further investigation, since they may represent new or specific fatty acids in thermophilic cyanobacteria.

The finding that both thermophilic *Synechococcus* isolates could not fix nitrogen from air for their growth is not surprising, since no thermophilic species of *Synechococcus* have been reported as diazotrophic, and this also reflects that there is no nitrogen deficiencies in their natural thermal habitats.

Although most studies on thermophilic prefer culture independent molecular techniques, it is still desirable to isolate

and characterize axenic cultures to study their genetics and physiology in detail. Overall, this study used a range of tools to isolate and characterize two thermophilic cyanobacterial *Synechococcus* strains. The discrepancy between their pigment and fatty acid compositions may reflect adaptation to their natural habitats. Furthermore, the two *Synechococcus* strains obtained in this study would have application in future screening for enzymes of biotechnology interest.

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