Extremophiles (2009) 13:533–540
DOI 10.1007/s00792-009-0240-8

ORIGINAL PAPER

Vertical partitioning and expression of primary metabolic genes in a thermophilic microbial mat

Maggie C. Y. Lau · Stephen B. Pointing

Received: 19 December 2007 / Accepted: 16 March 2009 / Published online: 4 April 2009

Abstract A thermophilic microbial mat with a relatively simple morphological composition was used to study the expression of key metabolic genes between mat layers. Mats comprised *Roseiflexus castenholzii*, *Synechococcus* sp., a *Sphingomonas*-like proteobacterial taxon and an unidentified member of the Thermotogae as determined by 16S rRNA phylotypes. The diversity of expressed loci for key genes involved in oxygenic photosynthesis (*cbb*L), anoxygenic photosynthesis (*puf*M) and nitrogen fixation (*nif*H) was assessed. The cyanobacterial surface layer supported two *cbb*L transcripts, with closest phylogenetic affinity to those from the cyanobacterium *Synechococcus* sp. and a proteobacterium *Nitrobacter* sp. This indicates that both photoautotrophic and chemolithoautotrophic carbon dioxide fixation may occur in this mat layer. Lower layers did not support *cbb*L transcripts. Anoxygenic photosynthesis was indicated by a single *puf*M transcript with closest affinity to that of *R. castenholzii.* Expression occurred in all layers beneath the cyanobacterial surface layer. Expression of a single *nif*H transcript with closest affinity to a proteobacterial nitrogenase occurred in samples throughout all mat layers.

Communicated by K. Horikoshi.

Electronic supplementary material The online version of this article (doi:10.1007/s00792-009-0240-8) contains supplementary material, which is available to authorized users.

M. C. Y. Lau · S. B. Pointing (✉)
School of Biological Sciences,
The University of Hong Kong, Pokfulam Road,
Hong Kong SAR, People's Republic of China
e-mail: pointing@hku.hk

Keywords Anoxygenic photosynthesis · Nitrogenase · RUBISCO · Thermophiles

Introduction

Thermophilic microbial mats in hot springs have received significant recent research attention due to the development of culture-independent molecular approaches for diversity assessment. Such mats are often characterized by the cyanobacterial component, and indeed this phylum has been the focus of most research to date. An extensive inventory of biodiversity at the 16S rRNA gene-defined level now exists for thermophilic cyanobacteria and to a lesser extent for other bacterial taxa from these mats. These include well-studied sites such as Yellowstone National Park (YNP) and the Great Basin hot springs in the USA (Ward and Castenholz 2000) and also some relatively less well-studied locations in China, Greenland, Japan, New Zealand and Thailand (Papke et al. 2003; Jing et al. 2005; Sompong et al. 2005; Jing et al. 2006; Lacap et al. 2007; Lau et al. 2006; Roesslers et al. 2007). Among lithic thermophilic mats it has emerged that the cyanobacterial taxon *Synechococcus* is a near-ubiquitous component of mat surface layers, while green non-sulphur bacteria and other heterotrophic and autotrophic taxa occur below this layer (Lau et al. 2006).

It has been envisaged that oxygenic photoautotrophy by the cyanobacteria represents a first trophic level in oligotrophic thermal waters, and photoexcretion supports successive layers of heterotrophic activity below (Bateson and Ward 1988). The anoxygenic photosynthetic green non-sulphur bacteria form a major portion of overall mat biomass and are thought to act photoheterotrophically and/or photoautotrophically, with levels of oxygenation determining the occurrence of anaerobic phototrophs such as the

Chlorobi (Lau et al. 2006). Fermentation of photosynthetically derived carbohydrates by cyanobacteria has been demonstrated to occur under dark conditions (Nold and Ward 1996) and this is a likely source of substrates such as acetate and propionate (Anderson et al. 1987) for other heterotrophs. Productivity estimates suggest that while cyanobacterial mats may be active across a broad temperature range (Tison et al. 1981), levels are nonetheless relatively low in thermophilic mats (Nold and Ward 1996). Evidence for diurnal flux in productivity suggests that mats are most active in carbon fixation during the day with a gradual decline in the afternoon (Miller 1998).

Another key metabolic process for nutrient input to thermophilic mat systems is nitrogen fixation, since thermal waters supporting *Synechococcus* mats are generally nitrogen-poor environments. Little is known about nitrogen fixation in such thermophilic mats, although several diazotrophic cyanobacterial mats dominated by the genera *Anabaena*, *Calothrix* and *Fischerella* occur at lower moderately thermophilic temperatures (Jing et al. 2005). The genome sequences of *Synechococcus* from YNP indicate that this taxon also has nitrogenase gene clusters, and both transcription and nitrogenase activity were detected in mats under anoxic conditions during night-time (Steunou et al. 2006). It is not clear that to what extent other mat taxa are involved in nitrogen fixation within thermophilic mats, but given the high diversity of phylotypes recovered from phyla with known diazotrophic representatives (Lau et al. 2006) it is likely that others are also important in nitrogen input to the mat assemblage.

Our current knowledge of thermophilic mat function is therefore limited by a general lack of data on the metabolic pathways that are active in situ within mats throughout different layers and the identity of taxa that are actually responsible. Here we describe experiments using a '*Synechococcus* mat' from the Daggyai Tso geothermal field in Tibet that supported relatively low 16S rRNA gene-defined diversity. We identify diversity among transcripts for three key metabolic genes involved in oxygenic photosynthesis, anoxygenic photosynthesis and nitrogen fixation from environmental samples throughout the vertical profile of layers in the thermophilic mat. This provides a valuable insight into the partitioning of metabolic function within thermophilic mats and likely interactions among taxa within biofilm assemblages.

Materials and methods

Sample recovery

Vertical sections of a lithic thermophilic mat were sampled from the Daggyai Tso Geyser field (29°36.083′N 85°44.856′E, 5,070 m altitude) at solar noon on 14 October 2005 as previously described (Lau et al. 2006). Three independent samples were obtained and for each sample the mat was sectioned according to visually identified bands of coloured biomass under aseptic conditions using a scalpel, taking care to avoid marginal areas between layers. For each mat sample a total of five layers were identified, and each of these was analysed separately in subsequent molecular studies. All samples were preserved in RNAlater solution (Ambion Inc, Austin, TX, USA) until processed after approximately 2 weeks (also applied to samples for DNA extraction since no freezing facilities were available in the field). Physico-chemical parameters known to have the greatest influence on thermophilic diversity (temperature, pH, hydrogen sulphide) were tested in the field. Temperature was 62.5°C (±2.5°C), pH 7 (±0.5) (combined temperature/pH electrode, Orion, Boston, MA, USA) and hydrogen sulphide was below the detection limit of 0.1 mg/l (as determined by methylene blue titration, HS-WR, Hach, Loveland, CA, USA). Morphological features of mat samples were observed using a SZH stereomicroscope and BX50 compound microscope (Olympus, Tokyo, Japan).

Recovery of DNA, mRNA and cDNA synthesis

Nucleic acid extraction was performed for each sample to yield environmental DNA and mRNA. DNA was recovered as previously described (Lau et al. 2006), mRNA was recovered using the TRI reagent (Molecular Research Center Inc, Ohio, USA) according to manufacturer's instructions. cDNA synthesis from mRNA was carried out using the ImProm-II Reverse Transcription System (Promega, WI, USA) according to manufacturer's instructions.

PCR amplification and DGGE

PCR of DNA and cDNA was carried out as previously described (Lau et al. 2006) with the following primer pairs (all numbers refer to equivalent *E. coli* nucleotide positions): universal bacterial primers 341F-907R encoding the 16S rRNA gene (Muyzer et al. 1993); oxygenic photosynthesis [Ribulose bisphosphate carboxylase (RUBISCO) large subunit] *cbb*LF-*cbb*LR (Elsaied and Naganuma 2001), anoxygenic photosynthesis (Reaction centre subunit M) *puf*MF–*puf*MR (Achenbach et al. 2001) and nitrogen fixation (dinitrogenase reductase H) *nif*HF–*nif*HR (Poly et al. 2001). Phylotypes for each locus within the environmental samples were resolved using DGGE (CBS Scientific, Del Mar, CA, USA) in a urea–formamide denaturant gradient as previously described (Lau et al. 2006). Electrophoresis profiles for each gene were

optimized and final electrophoresis conditions were as follows: 16S rDNA, 20–80% gradient × 12 h; *cbb*L, 10–85% gradient × 16 h; *puf*M, 10–65% gradient × 2.5 h; *nif*H, 10–65% gradient × 1.5 h. All bands were excised from gels and soaked overnight in deionized water at 4°C prior to re-amplification. Amplicons were then purified (GFX, Amersham, Bucks, UK) prior to automated sequencing using the BigDye terminator reaction (ABI PRISM, BigDye Terminator v3.1, Applied Biosystems, CA, USA) with an ABI PRISM 3700 DNA Analyzer (Applied Biosystems, CA, USA). Sequencing of all band migration classes from separate replicates (minimum 3 per treatment) yielded identical phylotypes for each band migration class. Sequence data for cDNA phylotypes have been submitted to the NCBI GenBank databases under accession numbers EF208567–EF208570.

Sequence analysis

Sequence data were checked against the ABI trace file for erroneously encoded nucleotides and counter-checked through pair-wise alignments using the freeware BioEdit v5.0.0 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Manual adjustment was made where necessary. Approximate phylogenetic affiliations were determined by BLAST search of the NCBI GenBank database (http://www.ncbi.nlm.nih.gov). All sequences were checked for the existence of chimeric artefacts using the freeware Chimera-Check v2.7 (http://rdp8.cme.msu.edu/cgis/chimera). For construction of phylogenetic relationships, all sequences obtained in this study together with appropriate sister and outgroup sequences from the NCBI GenBank database were aligned using the freeware ClustalX v1.83 (http://bips.ustrasb.fr/fr/documentation/ClustalX). Trees for each locus were constructed using a Maximum Likelihood model in the software package PAUP* v4.0b10 (Sinauer Associates Inc., MA, USA). Branch length correction was achieved by testing each alignment against 56 prescript models of evolution using the software Modeltest v3.0 (Posada and Crandall 1998) and the most appropriate model then applied (16S rRNA and *cbb*L, GTR+I+G; *puf*M, K81uf+G; *nif*H, TrNef+G). Robustness of furcated branches was tested by bootstrapping (1,000 replications) using PAUP* and also Bayesian posterior probabilities using Mr Bayes v3.0b4 (http://mrbayes.csit.fsu.edu).

Results

The mat comprised five distinctly coloured layers (Fig. 1). The green surface layer was dominated by morphotypes of the form genus *Synechococcus* embedded in a copious extracellular matrix, although occasionally filaments of

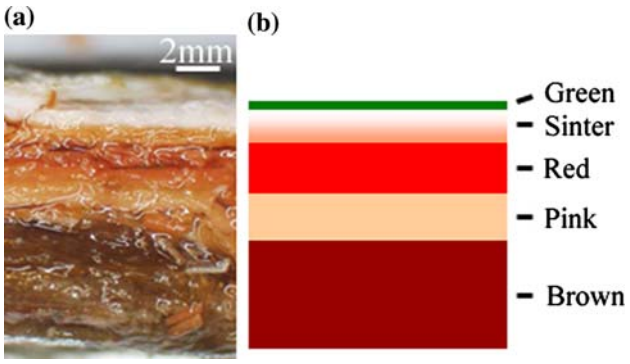

Fig. 1 View of vertical section through mat (**a**) and schematic for labelling of layers used in separate nucleic acid recoveries (**b**)

Roseiflexus castenholzi were also observed. A layer of sinter separated this from lower mat layers and only *Synechocccus* sp. cells were visible in this sinter layer. Beneath this sinter layer red, pink and brown layers occurred successively but all were dominated by *R. castenholzii*-like morphotypes, although within the lowest brown layer strongly pigmented long rods were also observed. The general biotic structure of the mat is typical of those occurring in hot springs throughout western China and other locations such as YNP, where a thin *Synechococcus* layer overlays a relatively thicker layer of predominantly green non-sulphur bacteria. Recovery of environmental DNA from samples allowed an assessment of taxon diversity at the molecular level, as determined by DGGE and sequencing of 16S rRNA genes. This revealed a relatively simple mat composition indicating only four recoverable phylotypes (Supplementary on-line material Figure S1), in general agreement with morphological observations. Phylogenetic analyses revealed that these belonged to a *Synechococcus* sp., *Sphingomonas* sp., *R. castenholzii*, plus an unidentified member of the Thermotogae (Table 1, and Supplementary on-line material Figs 2–5).

This relatively simple assemblage provided an ideal model system with which to investigate the occurrence and expression of key metabolic genes under conditions of thermophily. We resolved the diversity of expressed loci for key enzymes involved in oxygenic photosynthesis (*cbb*L), anoxygenic photosynthesis (*puf*M) and nitrogen fixation (*nif*H) (Fig. 2; Table 1), in order to gain insight into which metabolic processes and taxa were active within the mat structure. For transcribed genes, two *cbb*L loci were detected in the green surface layer and sinter layer but were absent from all lower layers. A *Synechococcus cbb*L with closest similarity to a *cbb*L from *Synechococcus* strain JA-3-3-Ab from YNP was expressed in green and sinter layers whilst a *Nitrobacter*-like *cbb*L showing highest similarity to *cbb*L from *Nitrobacter vulgaris* was expressed

Table 1 Identity of DNA and cDNA phylotypes recovered from a thermophilic mat

Phylotype	Putative group	Identity (closest match in the NCBI GenBank database)			
		Identity	Location	GenBank accession number	Percent nucleotide similarity
16S rRNA genes					
Tibet 16S 1	Proteobacteria (alpha)	*Sphingomonas* sp. V1	Lake Vostok accretion ice, Antarctic	AF324199	99
Tibet 16S 2	Cyanobacteria	*Synechococcus* sp. CR_L35	Geothermal springs, Costa Rica	EF545646	97
Tibet 16S 3	Thermotogae	*Eubacterium* sp. (OS type L)	Yellowstone National Park, US	L04707	94
Tibet 16S 4	Chloroflexi	*Roseiflexus* sp. RS-1	Hot spring, Japan	CP000686	96
cDNA_of metabolic gene transcripts					
*cbb*L 1	Cyanobacteria	*Synechococcus* sp. JA-3-3Ab	Yellowstone National Park, US	CP000239	86
*cbb*L 2	Proteobacteria (alpha)	*Nitrobacter vulgaris*	Unknown	L22885	83
*puf*M 1	Chloroflexi	*Roseiflexus castenholzii* DSM 13941	Hot spring, Japan	CP000804	94
*nif*H 1	Proteobacteria (alpha)	*Azospirillum oryzae* strain N7	Soil	DQ682472	88

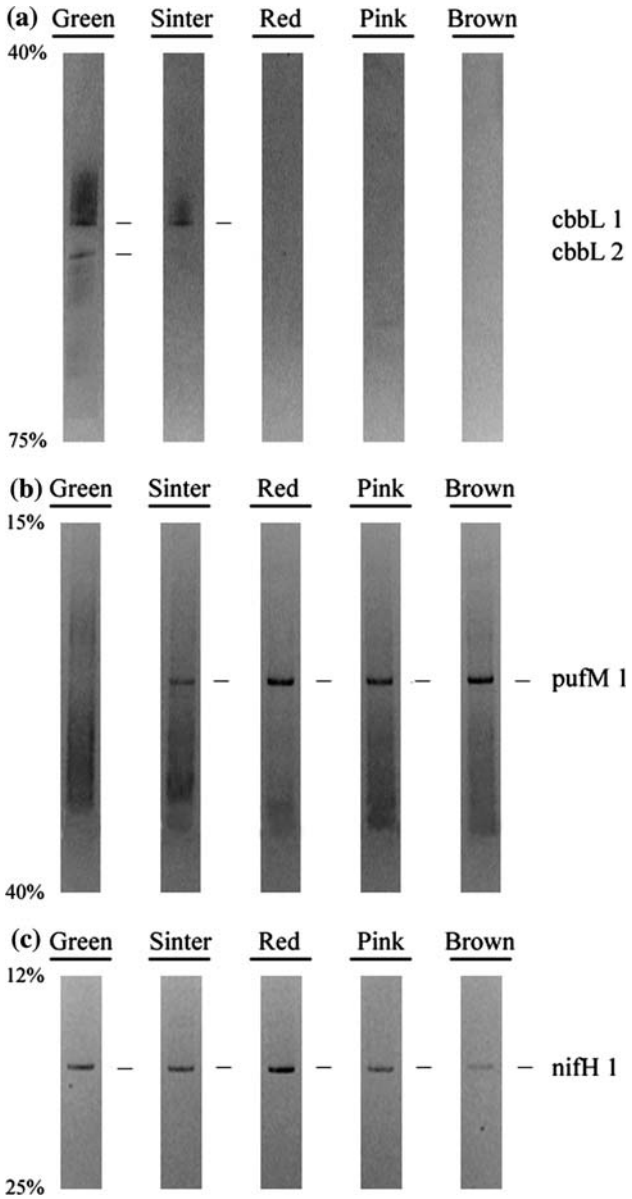

Fig. 2 DGGE profiles of environmental cDNA for key metabolic genes in a thermophilic microbial mat. All treatments were repeated in triplicate but since there was no within-treatment variation a single replicate per treatment is shown

only in the green layer. There was no detectable expression of *puf*M in the surface layer, but expression in all lower layers of the mat was recorded. Transcripts shared highest sequence similarity to those of a cultivated *R. castenholzii puf*M gene from Japan. Only a proteobacterial *nif*H was expressed in the mat, and throughout all layers. No expression of cyanobacterial *nif*H was detected. Phylogenetic analyses of cDNA phylotypes for each functional gene were used to infer the closest identity of the taxon associated with each pathway-specific gene (Figs. 3, 4 and 5).

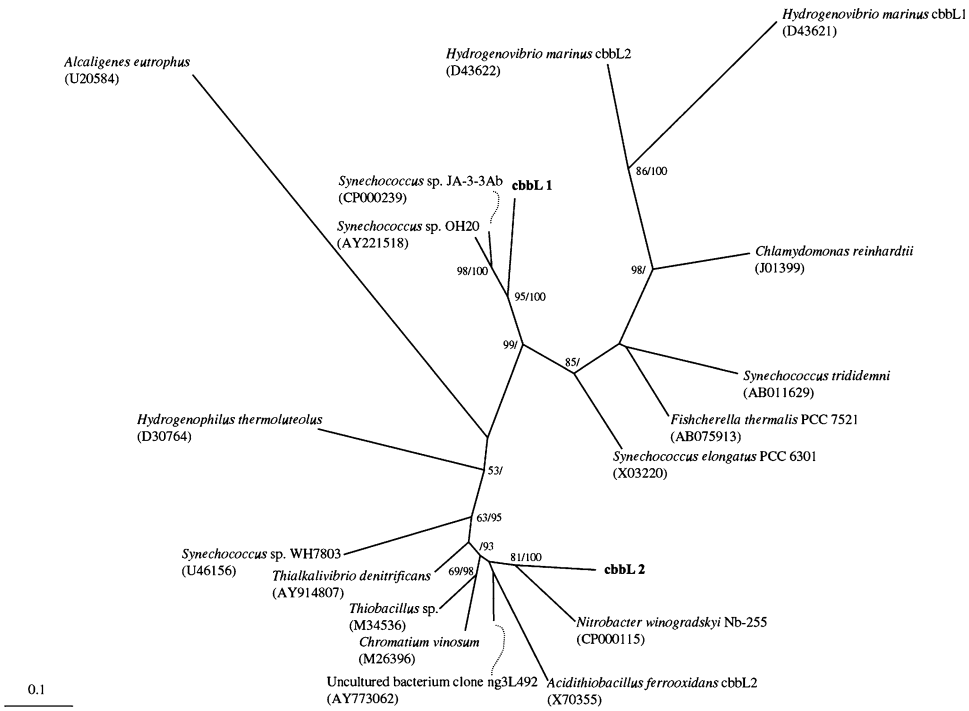

Fig. 3 Phylogenetic relationship for mRNA transcripts of *cbb*L genes for a thermophilic microbial mat. Phylotypes obtained in this study are shown in *bold type*. Tree topologies are supported by bootstrap values for 1,000 replications (*first number*) and Bayesian posterior probabilities (*second number*), shown for branches supported by more than 50% of the trees. *Scale bar* represents 0.1 nucleotide changes per position

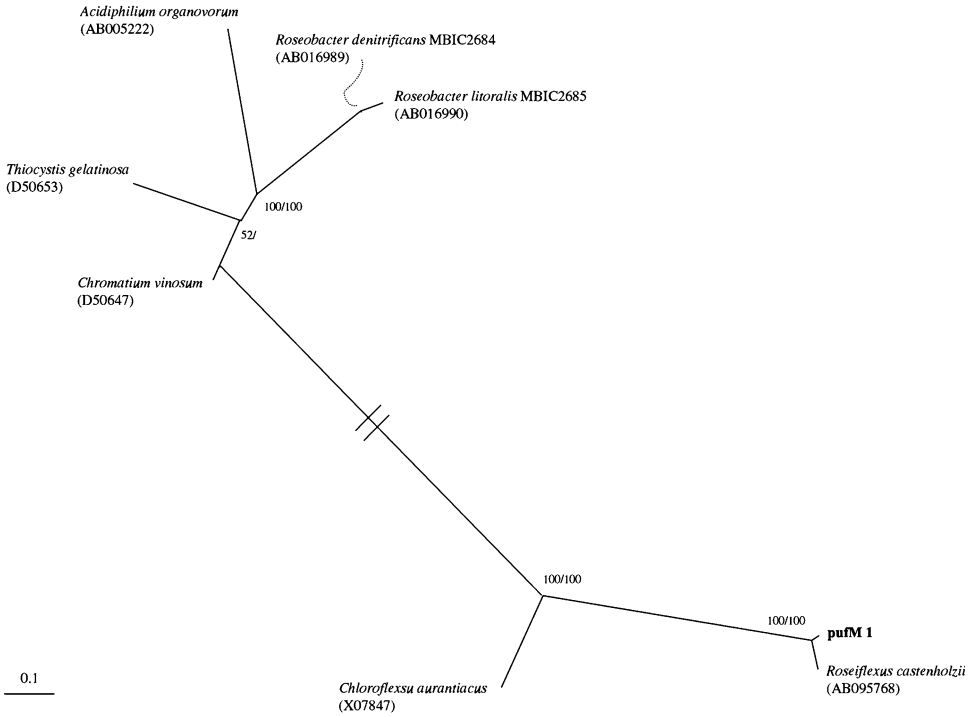

Fig. 4 Phylogenetic relationship for mRNA transcripts of *puf*M genes for a thermophilic microbial mat. Phylotypes obtained in this study are shown in *bold type*. Tree topologies are supported by bootstrap values for 1,000 replications (*first number*) and Bayesian posterior probabilities (*second number*), shown for branches supported by more than 50% of the trees. *Scale bar* represents 0.1 nucleotide changes per position

Discussion

This study provides transcriptomic evidence for partitioning of key physiological processes within a thermophilic microbial mat and closest identity of the taxonomic groups of bacteria involved, and so contributes a novel physiological aspect to the existing body of information on the diversity and ecology of thermophiles. We have shown that the green surface layer of mats largely accounted for by *Synechococcus* sp., supports active transcription of two *cbb*L genes encoding the enzyme RUBISCO which is critical to photoautotrophic carbon fixation. Interestingly only one was from a *Synechococcus* sp. while the other was expressed by a proteobacterium. The identity of this latter

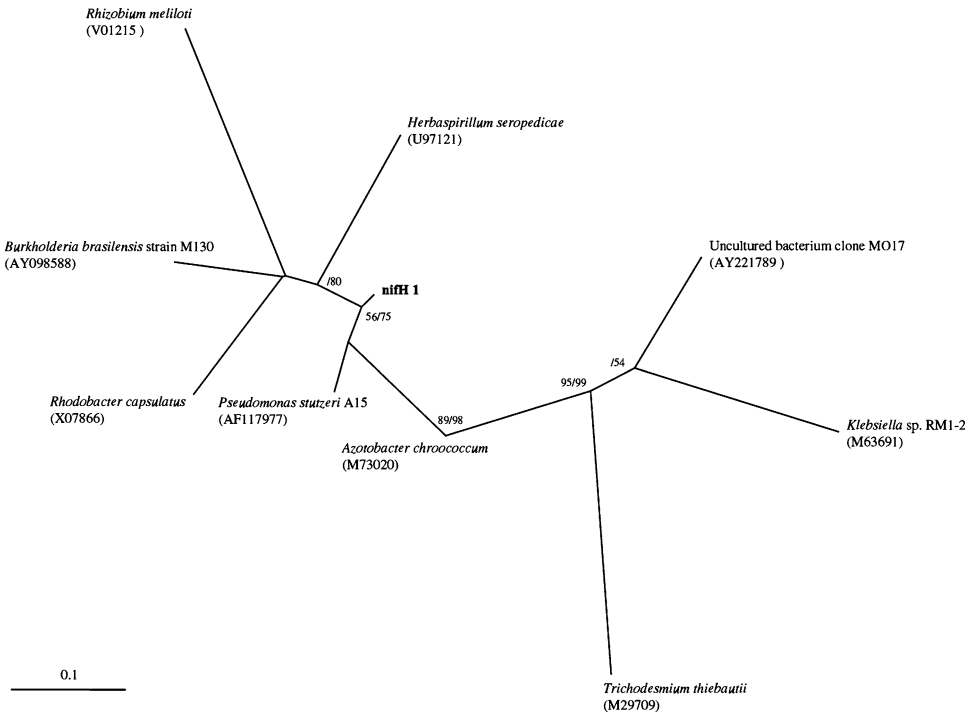

Fig. 5 Phylogenetic relationship for mRNA transcript of *nif*H gene for a thermophilic microbial mat. Phylotypes obtained in this study are shown in *bold type*. Tree topologies are supported by bootstrap values for 1,000 replications (*first number*) and Bayesian posterior probabilities (*second number*), shown for branches supported by more than 50% of the trees. *Scale bar* represents 0.1 nucleotide changes per position

taxon is unclear. The 16S rRNA gene data suggest that it is a *Sphingomonas*-like alpha proteobacterium, and RUBISCO transcripts also support a proteobacterial identity for the diazotroph. The lack of comparable sequences in the NCBI GenBank, however, preclude the definitive association of this gene with the *Sphingomonas* sp. phylotype as its closest BLAST match was with another diazotrophic proteobacterium, *Nitrobacter* sp. Regardless these data are interesting since they indicate two possible sources of primary production in this mat, photautotrophic cyanobacteria and chemolithoautotrophic nitrifiers. It has been demonstrated that a significant portion of photosynthetically fixed carbon in *Synechococcus* mats is stored as polyglucose, which is subsequently used as a fermentation substrate under anaerobic conditions in darkness, which in turn supplies substrates for heterotrophic bacteria (Nold and Ward 1996; Stal and Moezelaar 1997). Diazotrophic proteobacteria produce nitrate via oxidation of ammonium ions during autotrophy in an obligately aerobic process that may also be utilized by heterotrophs. Only the *Synechococcus* sp. *cbb*L was expressed in the sinter layer beneath and since it is unlikely that living cells can tunnel through sinter facies (Lau et al. 2008) this probably represents cyanobacteria that have become partially silicified during a stochastic silica precipitation event, since cyanobacteria may remain metabolically active within silica-depositing niches when this occurs (Phoenix et al. 2000; Lalonde et al. 2005). By extension, the proteobacterium is either not tolerant to such events or colonized the mat after the silica depositional event.

The indication that anoxygenic photosynthesis as evidenced by *puf*M transcription occurred throughout all mat layers below the cyanobacterial surface layer, but that *cbb*L genes were not expressed in lower layers, supports earlier assumptions that photoheterotrophy by *R. castenholzii* is the dominant phototrophic process in these layers and likely supplied with organic substrates derived from *Synechococcus* sp. (Hanada 2003). In our study we show that an autotrophic proteobacterium may be an additional potential source of autotrophically produced organic substrates to *R. casetnholzii*. The sinter layer can be assumed to act as a partial boundary between surface and lower mat layers. It may act as a niche partition favouring growth of *R. castenholzii* below since it is known that photo-pigment production is sensitive to oxygen levels in the anoxygenic phototrophs (Castenholz and Pierson 1995). The different colours of mat layers may reflect variation in certain pigments in response to changing light conditions with depth although this was not measured, and the lower dark layer did not appear senescent. Although anoxygenic phototrophy has also been demonstrated for some cyanobacteria (Cohen et al. 1975; Garlick et al. 1977) this is a photosystem I-mediated process and they do not possess the anoxygenic photosynthetic reaction centre encoded by *puf*M genes.

With regard to nitrogenase genes, their expression profile is interesting since it indicates transcription in all mat layers is mediated by a diazotrophic proteobacterium and transcription occurs during daytime when diazotrophy by cyanobacteria in thermophilic mats is thought to be

inhibited due to photoautotrophic oxygen production (Bebout et al. 1987; Steunou et al. 2006). Diazotrophy by the non-photosynthetic biota in thermophilic mats is known from physiological studies and so this concurs with earlier findings (Wickstrom 1984; Wahlund and Madigan 1993). It is not surprising that cyanobacterial nitrogenase transcripts were not detected in daytime since this process is inhibited by oxygenic photosynthesis. We failed to amplify any cyanobacterial *nif*H loci from genomic DNA using the *nif*H primers and so we conclude the strain in this mat is non-diazotrophic, although certain strains of *Synechococcus* has been shown to be diazotrophic under thermophilic (Steunou et al. 2006) and non-thermophilic (Rippka et al. 1971) conditions. The presence of a proteobacterial nitrogenase transcript throughout mat layers during daytime in our study may indicate day-active diazotrophy, or that nitrogenase is constitutively transcribed but inactivated at the protein level during cellular conditions that do not favour catalysis. It is unfortunate that night-time sampling was not possible due to restrictions on sampling activities in Tibet as this would help to shed light on any possible diurnal patterns particularly with regard to diazotrophy by cyanobacteria and other taxa. Regardless, this report shows for the first time that a proteobacterial nitrogenase is transcribed at a temperature near the previously recorded upper thermal limit for diazotrophy (Belay et al. 1984) in a thermophilic mat.

This study has some limitations related to the resolution powers of DGGE and inference of metabolic activity from mRNA transcripts. The DGGE technique is a qualitative tool (Muyzer et al. 1993) and as such is unable to shed light on the actual level of transcription. In addition, while transcription of a gene may indicate production of an active protein this may not always be the case due to various post-transcriptional controls (Madigan and Martinko 2006). There are nonetheless several findings of importance to thermophilic ecology that emerge. Autotrophy is indicated by additional taxa besides cyanobacteria in mat surface layers. The location of oxygenic and anoxygenic photosynthesis in mats is clearly partitioned between layers, and sinter deposition may play a role in maintaining niche partitions. A proteobacterial nitrogenase is transcribed throughout all mat layers, but transcript levels may not accurately reflect active diazotrophy. Sampling on a diurnal scale was not undertaken and so it is unknown that to what extent any cyanobacterial nitrogenase may also be active in this mat. It is interesting to conclude with a consideration that thermophilic cyanobacteria have been shown to exert a circadian rhythmicity in their metabolism (Onai et al. 2004) and since all the processes considered in this study may be under such control, future studies on a broader temporal scale may yield further interesting data.

Acknowledgments The authors are grateful for financial support from the Hong Kong Research Grants Council (grant number HKU 7573/05M).

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