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ORIGINAL PAPER

Differential microbial communities in hot spring mats from Western Thailand

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Abstract The microbial communities of freshwater hot spring mats from Boekleung (Western Thailand) were studied. Temperatures ranged from over 50 up to 57°C. Green-, red-, and yellow colored mat layers were analyzed. In order to detect the major components of the microbial communities constituting the mat as well as the microorganisms showing significant metabolic activity, samples were analyzed using DNA- and RNA-based molecular techniques, respectively. Microbial community fingerprints, performed by denaturing gradient gel electrophoresis (DGGE), revealed clear differences among mat layers. Thermophilic phototrophic microorganisms, *Cyanobacteria* and *Chloroflexi*, constituted the major groups in these communities (on average 65 and 51% from DNA and RNA analyses, respectively). Other bacteria detected in the mat were *Bacteroidetes*, members of the Candidate Division OP10, *Actinobacteria*, and *Planctomycetes*. Differently colored mat layers showed characteristic bacterial communities and the major components of the metabolically active fraction of these communities have been identified.

Keywords Microbial fingerprints · Hot spring · Microbial mat · Molecular survey · *Cyanobacteria* · *Chloroflexi* · Pigment profile · Metabolicaly active bacteria

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Introduction

Thermophilic mat communities develop in geothermal springs at temperatures of up to about 65°C (Ward et al. 1998). *Cyanobacteria* are the most commonly reported microbial group constituting these mats and they are considered the major primary producers in these habitats (Castenholtz 1973). Other bacteria share these environments with the *Cyanobacteria* and have important roles within these microbial communities (Ward et al. 1990; Weller et al. 1992; Moyer et al. 1995).

Current results based on molecular methods are showing a high complexity in the microbial communities forming these hot spring mats (Ward et al. 1990). *Synechococcus*-like representatives are the *Cyanobacteria* usually found at the highest temperatures (near 60°C) (Hongmei et al. 2005). Members of the bacterial division *Chloroflexi* are also commonly retrieved from thermophilic mats (Weller et al. 1992; Moyer et al. 1995). While some studies underline the high abundance of *Chloroflexus*-like bacteria in some mat communities (Polerecky et al. 2007), others have proposed a limited role of these bacteria and a dominance of *Cyanobacteria* in other high-temperature mats (Roeselers et al. 2007). At present, there is controversy on the presence and activity of *Chloroflexi* and *Cyanobacteria* in different mat systems.

A large fraction of microorganisms in natural systems have been reported to be in dormant stages, so that they are inactive members of the community waiting for better conditions to arrive (Nold and Ward 1996). RNA-based molecular methods are being introduced to unambiguously detect the metabolically active microorganisms within a community (Mills et al. 2004; Nogales et al. 1999; Portillo et al. 2008). Recent studies have emphasized the importance of microbial immigration and dispersion in current

estimates of microbial diversity and community structure (Sloan et al. 2005; Portillo and Gonzalez 2008a). Thus, a differential distribution of microorganisms in distinctive layers of the mats could be proposed. Distinct colored mats might be a result of the composition and structure of the microbial communities or the metabolic activity of their microbial components (Bauld and Brock 1973; Castenholtz 1973; Ward et al. 1998). Nevertheless, previous studies have been unable to report changes in microbial communities of hot spring mats (Ferris and Ward 1997; Ward et al. 1998).

This study aims to determine the major members of the microbial communities participating in the activity of different microbial mat layers at Boekleung Hot Springs, Western Thailand. Different colored mat layers (green, yellow and red) were observed and studied using molecular methods based on both DNA and RNA. The results confirmed the uniqueness of the microbial communities integrated in each mat layer.

Materials and methods

Sampling sites and sample collection

Samples were collected from microbial mats at a hot spring in Boekleung (Ratchaburi Province), Thailand, located at 13° 31.015′N 099° 14.682′E (Fig. 1). Temperature was measured with a digital thermometer and a thermopar microprobe. Water showed temperatures up to 57°C at the source. A pH of 6.8 was measured at the studied mats. Mats collected at 56.5°C showed two clear layers, an exterior (upper) layer showing green color (a) and an interior (inner) layer with a bright red color (c) in contact with the stone substrate (Fig. 1). A monolayer, green-yellowish colored mat (b) was collected at 50.8°C (Fig. 1) in the surroundings of the previous mat.

Samples were collected using sterile tubes containing RNAlater (Ambion, Austin, USA) to preserve microbial RNA, maintained on ice until arrival to the laboratory, and then stored at −80°C until processed. Samples collected for the culture of microorganisms were stored in sterile tubes and maintained on ice until processed.

Nucleic acid extractions

DNA was extracted using the Nucleospin Food DNA Extraction Kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's recommendations. Total RNA was extracted using the RNAqueous4PCR kit (Ambion Inc., Austin, USA). The protocol for total RNA extraction includes DNaseI treatment (37°C for 1 h) to remove any DNA present in the final RNA extract.

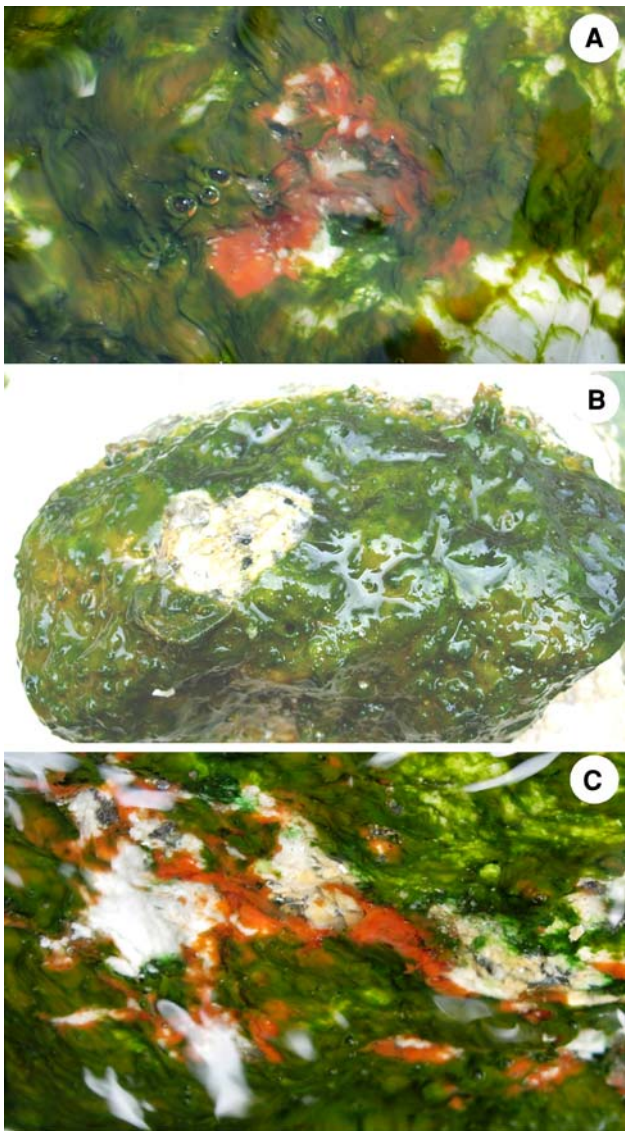

Fig. 1 Detail of three different layers of microbial mats from Beokleung Hot Springs analyzed during this study. **a** *Green* (external layer); **b** *yellowish* mat; **c** *bright red* (internal layer). Temperature was 56.5°C for **a** and **c**, and 50.8°C for **b**

Controls without sample (as controls for tubes and reagents) were carried out in parallel to the samples.

Reverse transcription and DNA amplification

A reverse transcriptase reaction was carried out to obtain the complementary DNA (cDNA) to the 16S rRNA genes to be amplified. The reverse transcriptase Thermoscript (Invitrogen, Carlsbad, USA) was used in this study with a 16S rRNA gene-specific primer, 518R (5′-ATT ACC GCG GCT GCT GG; Muyzer et al. 1993), at an annealing temperature of 55°C for 1 h. Controls lacking reverse transcriptase were also carried out to check for the presence of DNA traces in the extracted RNA. A standard

amplification reaction by PCR followed production of cDNA. Amplification of 16S rRNA fragments from cDNA was performed by PCR using as forward primer 27bF (5′-AGA GTT TGA TYM TGG CTC AG; Portillo et al. 2008) for Bacteria or Cya106F (5′-CGG ACG GGT GAG TAA CGC GTG A; Nübel et al. 1997) for *Cyanobacteria* and 518R as reverse primer. ExTaq (Takara, Shiga, Japan) was the DNA polymerase used for PCR, following the manufacturer's recommendations. Thermal conditions for the amplification reaction consisted on the following steps: 95°C for 2 min; 30 cycles of 95°C for 15 s, 55°C for 15 s and 72°C for 1 min; and a final incubation at 72°C for 10 min.

Amplifications from DNA were performed following a similar protocol although the primers 27bF and 907R (5′-CCC CGT CAA TTC ATT TGA GTT T; Lane 1991) for Bacteria and Cya106F and a combination of Cya781Ra (5′-GAC TAC TGG GGT ATC TAA TCC CAT T) and Cya781Rb (5′-GAC TAC AGG GGT ATC TAA TCC CTT T) for *Cyanobacteria* (Nübel et al. 1997) were used. Amplification of rRNA gene fragments from Archaea and Eukarya DNA was performed using the primer pairs 20bF (5′-YTC CSG TTG ATC CYG CSR GA) and 1492bR (5′-GGY TAC CTT GTK WCG ACT T), and EukA (5′-AAC CTG GTT GAT CCT GCC AGT) and EukB (5′-TGA TCC TTC TGC AGG TTC ACC TAC), respectively (Gonzalez and Saiz-Jimenez 2005).

Microbial community fingerprints

Fingerprints of the total and active microbial communities were obtained by denaturing gradient gel electrophoresis (DGGE) following the method described by Muyzer et al. (1993). Extracted DNA and the generated cDNA were directly amplified by using the primer pair 341F-GC (5′-CCT ACG GGA GGC AGC AG with a GC-rich tail attached to its 5′ end; Muyzer et al. 1993) and 518R for bacterial DGGE analysis. Cyanobacterial-specific fingerprints were obtained by a nested PCR approach from extracted DNA. A first amplification using cyanobacterial-specific primers (Cya106F and Cya781Ra + b; Nübel et al. 1997) was followed by a PCR amplification using the primer pair 341F-GC and 518R. Only bacterial PCR-DGGE analyses were performed following quantitative requirements (Portillo et al. 2008; Portillo and Gonzalez 2008b) while cyanobacterial PCR-DGGE, obtained after a nested PCR were not considered quantitative. Gels obtained by DGGE were digitalized using Kodak 1D image analysis software (Kodak, New Haven, CT). Images were analyzed with the program tnimage (http://entropy.brneurosci.org/tnimage.html) using its densitometry function. Relative quantification of bands from each bacterial community profile was performed following the quantitative procedure described by Portillo and Gonzalez (2008b). According to the described procedure, product amplification was only considered during the exponential phase of PCR. Comparisons between community fingerprints were performed as described by Portillo and Gonzalez (2008b) calculating a Cramér-von Mises-type statistic through a Monte-Carlo test procedure to determine the significance of differences between microbial community fingerprints from different samples and between DNA- and RNA-based analyses.

Environmental clone library construction and screening

PCR products from bacteria and *Cyanobacteria* were obtained with the 27F-907R and Cya106F-Cya781Ra + b primer pairs, respectively, purified with the PCR purification kit (JetQuick, Germany), and cloned using a TOPO-TA cloning kit (Invitrogen). The 16S rRNA libraries obtained were used to identify some of the microbial components of the community. A previously described screening procedure (Gonzalez et al. 2003) based on discrimination of clones using PCR-DGGE was followed with these libraries to identify the major DNA bands observed in DGGE analyses.

Nucleotide sequence analysis

Sequence data were edited using the software Chromas, version 1.45 (Technelysium, Tewantin, Australia). Homology searches from the nucleic acid sequences were performed using the Blast algorithm (Altschul et al. 1990) at the NCBI (National Center for Biotechnology Information; http://www.ncbi.nlm.nih.gov/Blast/). Sequences were checked for chimeras using the program Ccode as described by Gonzalez et al. (2005). Sequence alignments were carried out with the program ClustalW (Thompson et al. 1994). Aligned sequences were processed with the program Treepuzzle (Strimmer and von Haeseler 1996) to obtain tree topologies, using a maximum likelihood quartet puzzling method, showing the phylogenetic relationships between retrieved sequences and their related homologs. Reliability values were calculated for internal branches indicating the number of times (as percentages) a cluster has been reconstructed during the puzzling steps (generally from 5,000 estimations). In order to obtain a conservative tree topology, multifurcations are drawn where the support value for a bifurcation is lower than 50% (Stott et al. 2008). The number of nucleotides for tree construction ranged from 740 to 860 bp depending on the bacterial group. *Thermotoga neapolitana* (Accession Number AB039768) was used as outgroup for tree construction.

Nucleotide sequence accession numbers

Nucleotide sequences obtained in this study have been deposited in the GenBank database under the accession numbers reported in Table 1.

Results

Cyanobacterial fingerprints

DNA-based PCR-DGGE analysis with *Cyanobacteria*-specific primers revealed differences between the mat layers described above. Cyanobacterial community fingerprints showed the presence of dominant phylotypes in each of the studied layers (Fig. 2). The green layer (a) of the mat was dominated by members of the Order Chroococcales showing 93% similarity in its 16S rRNA gene sequence with its closest relative. The red layer (c) of the mat was dominated by closed relatives of *Thermosynechococcus elongatus* BP-1, a cyanobacterium belonging to the Order Chroococcales, with percentages of similarity in their 16S rRNA gene sequences ranging from 95 to 99%. In the yellowish zone (b), the dominant phylotype was related to thermophilic, uncultured *Cyanobacteria* (i.e. EF032786) (93% similarity) and was clearly different of the phylotypes dominating the green and red layers (a and c).

Bacterial fingerprints

Both DNA- and RNA-based PCR-DGGE analysis of the bacterial communities in Boekleung Hot Spring mats (Fig. 3) confirmed the dominance of the *Cyanobacteria* detected using cyanobacterial specific primers as reported above. Nevertheless, a relative quantification of the major bands detected in the community fingerprints showed other bacterial groups, besides the *Cyanobacteria*, as important constituents of these communities, both metabolically active members of the communities (analysis based on RNA) and microorganisms present in these communities (analysis based on DNA). Banding patterns of bacteria confirmed that the three studied mat layers were represented by different bacterial communities which was corroborated by statistical comparisons ($P < 0.001$). Comparisons of banding patterns from DNA- and RNA-based analyses (Fig. 3) also showed significant differences ($P < 0.05$) between the structure of the bacterial communities when the total and the metabolically active bacterial profiles were compared. However, most bands detected by DNA analysis were also significant constituents of the metabolically active microbial communities (detected by RNA-based analysis) in the three mat layers under study. The quantitative proportion of these bands in the community differ between the DNA- and RNA-based analyses leading to significant differences between the community structure of the total (DNA-based) and metabolically active (RNA-based) microbial communities (Table 1).

Results of the quantification of identified bands from DGGE analyses (Fig. 3) is presented in Table 1 for the green (a), green-yellowish (b), and red (c) mat layers. Based on RNA analyses, the phylotype showing the highest metabolic activity in the green mat layer (a) belonged to the *Cyanobacteria,* Orden Chroococcales, and was related to the genus *Thermosynechococcus* representing 15.2% of the quantified RNA (Table 1). Based on DNA, PCR-DGGE profiles showed *Roseiflexus*-related *Chloroflexi* as the most representative components of the total bacterial community (DNA-based) of the green mat layer (a) followed by a member of the Candidate Division OP10 and a *Synechococcus*-related sequence (*Cyanobacteria*).

PCR-DGGE profiles from the yellow mat layer (b) showed a *Thermosynechococcus*-related cyanobacterium and a member of the *Bacteroidetes* as the most important metabolically active components of this mat (Table 1) with over 10% of the total quantified bands from RNA-based profiles. Based on DNA, the total bacterial community was mainly represented by *Thermosynechococcus* and *Synechococcus*-related *Cyanobacteria* (over 15 and 12% of total quantified DNA) and different members of the phylum *Chloroflexi*.

The red mat layer (c) was dominated by different metabolically active *Cyanobacteria* and a *Roseiflexus*-related *Chloroflexi* (Table 1) as judged by RNA-based PCR-DGGE profiles. Based on DNA, PCR-DGGE profiles showed a major band corresponding to the genus *Roseiflexus* (*Chloroflexi*) which represented over 21% of the quantified DNA.

Table 2 summarizes the composition of the studied communities classified in major bacterial groups. These results confirm *Cyanobacteria* as the dominant phylum in the studied mat layers with average percentages of 37 and 31% of total quantified DNA and RNA, respectively. *Chloroflexi* was the second major component averaging 28 and 20% of DNA and RNA, respectively. Members of the Candidate Division OP10 and *Bacteroidetes* represented significant fractions of DNA and RNA (between 5 and 11%) while bacteria within the *Planctomycetes* and *Actinobacteria* constituted lower fractions in these community fingerprints. Archaea and Eukarya sequences were not detected through PCR amplification from extracted DNA and RNA.

Phylogenetic relationships

Phylogenetic trees showing the relationship between the retrieved sequences and their closest relatives were constructed for the major bacterial groups detected during this study. Thermophilic *Cyanobacteria* detected in Boekleung Hot Spring mats grouped in three major distinctive clusters (Fig. 4a). Cluster I was related to *Cyanobacteria* from

Table 1 Estimates of relative intensity for the major bands obtained by denaturing gradient gel electrophoresis (DGGE) analysis and the corresponding bacteria detected during this study from green, yellowish, and red mat layers

Migration[a]	Phylotype accession no.	Taxonomic affiliation	Green mat (%)[b]		Yellow mat (%)[b]		Red mat (%)[b]	
			DNA	RNA	DNA	RNA	DNA	RNA
41					2.05		4.70	5.51
57			2.70	2.57				
67				3.42		2.02	1.36	6.91
84			0.32				2.04	0.84
94	EU376449	*Cytophagales* (*Bacteroidetes*)	0.27	0.26	1.66	10.59	4.49	0.31
101						6.82		
110			0.27	2.65	0.62			1.46
121			0.19					
128					0.28	2.72		
133				0.23				
142	EU376433	*Chroococcales* (*Cyanobacteria*)	1.04		0.26		3.02	2.34
155	EU376422	*Chroococcales* (*Cyanobacteria*)		2.17		0.62		1.26
173	EU376430	*Roseiflexus* (*Chloroflexi*)	1.84	2.64	2.99	1.33	0.53	1.76
189						0.44		
193	EU376419 EU376425 EU376441 EU376447	*Thermosynechococcus* (*Cyanobacteria*)	0.97	2.40			5.31	4.16
207					0.19	0.63		
217	EU376453	*Thermosynechococcus* (*Cyanobacteria*)	0.25	7.85	0.50		11.50	7.71
227					0.57	1.57		
234	EU376442	*Synechococcus* (*Cyanobacteria*)		0.34			2.05	1.28
247	EU376409	*Bacteroidetes*	0.25	0.35		0.56		
257	EU376431 EU376437	*Cyanobacteria*			3.60			
263	EU376421	*Bacteroidetes*	2.72	7.84			2.61	2.47
276	EU376406 EU376407 EU376420 EU376436	*Thermosynechococcus* (*Cyanobacteria*)			15.53	10.47		
286	EU376446	Candidate Division OP10	12.45	7.82				
292	EU376414 EU376440 EU376450	*Cyanobacteria*			1.13	2.86	6.84	7.40
305	EU376432	*Planctomycetes*	5.11	4.67	6.17	2.97		
317	EU376448	*Cyanobacteria*					1.79	3.07
334	EU376412	*Chroococcales* (*Cyanobacteria*)	8.23	15.21	3.80	7.81	11.90	8.82
348	EU376411	*Bacteroidetes*		3.45				3.82
369	EU376439	Candidate Division OP10	0.11	1.12			0.32	2.50
379	EU376418 EU376444	*Bacteroidetes* *Chloroflexi*	0.10	0.54	5.81	2.90	0.26	1.42
388	EU376416	*Chloroflexi*	0.56	2.59			0.90	

Table 1 continued

Migration[a]	Phylotype accession no.	Taxonomic affiliation	Green mat (%)[b]		Yellow mat (%)[b]		Red mat (%)[b]	
			DNA	RNA	DNA	RNA	DNA	RNA
394	EU376434	*Chloroflexi*	2.46		11.54	6.16	1.67	3.88
410	EU376426	Candidate Division OP10	2.30	2.03	6.05	7.34	0.90	0.88
	EU376429							
423			0.13					
431				0.18	0.29	1.84	1.18	0.19
439						0.58		
449					4.12	1.35		
455			3.28	2.41			0.87	3.65
463					1.51	3.17		
475	EU376417	*Synechococcus* (*Cyanobacteria*)	11.56	4.19	12.38	4.05	9.05	
485			5.11	4.79				2.32
505	EU376428	*Propionibacterium* (*Actinobacteria*)	1.31	0.68	1.19	2.04	2.78	2.41
515					0.26			0.86
525				1.70	0.43	4.35		
536	EU376424	Candidate Division OP10		0.79			0.17	2.97
548	EU376435	*Roseiflexus* (*Chloroflexi*)		3.09	0.33	3.75	0.25	4.94
553			10.41				0.40	
564				1.27	4.43	2.51	0.72	2.00
574				1.86				
586	EU376438	*Roseiflexus* (*Chloroflexi*)	25.04	7.08	11.21	8.54	21.13	7.81
	EU376445							
597				2.32	0.88			

Bacterial identifications were carried out in base to DNA and RNA analyses of the microbial communities

[a] DGGE bands are defined in base to their migration (a.u.) during electrophoresis (see Fig. 2)

[b] Percentages corresponding to DNA- and RNA-based analyses

other thermophilic sites (i.e. EF032786; 92–94% similarity in the 16S rRNA gene sequence) and relatively divergent from the *Cyanobacteria* at hot springs in Northern Thailand (i.e. EF452004). This cluster I was constituted mainly by *Cyanobacteria* from the yellow zone (b) of the mat suggesting a differential distribution of Cyanobacterial phylotypes at the different colored mat layers. Some cluster II and III sequences were only retrieved from the green (a) and red (c) layers matching the cyanobacterial community profiles by DGGE (Figs. 2, 3) although some representatives of the yellow mat layer (b) were also detected. Cluster II was closely related to *Cyanobacteria* from hot springs (i.e. DQ131174; 95–99% similarity in their 16S rRNA gene sequences) in Asia and America and were grouped with *Thermosynechococcus elongatus* BP-1. Cluster III represented a divergent group of *Cyanobacteria* found at Boekleung Hot Spring showing 92–94% similarity with their closest homolog. Sequences EU376442 and EU376417 represented independent lineages of *Cyanobacteria* within *Synechococcus*-like clades.

Within the *Chloroflexi*, the detected sequences were grouped in two lineages (Fig. 4b). One group is closely related to the genus *Roseiflexus*. These sequences showed a 95% similarity in the 16S rRNA gene sequences with respect to their closest homolog *Roseiflexus castenholzii*. *Chloroflexi* corresponding to this cluster were found in the three studied mat layers. The phylotypes corresponding to this cluster, especially EU376438 and EU376445, were the most representative components of the *Chloroflexi* in the studied mat layers. A second group includes sequences related (96% similarity in their 16S rRNA gene sequences) to *Chloroflexi* from geothermal springs at the Tibet area (i.e. DQ001387) and a thermophilic bioreactor (i.e. AY297975).

Closely related sequences belonging to the Candidate Division OP10 have been mainly detected in the green (a) and red (c) mat layers. Their closest homologs (96–97% similarity) are uncultured bacteria from hot springs at Yellowstone National Park (i.e. AF445745 and AF445740) and other thermophilic bacteria within this division (Fig. 4c).

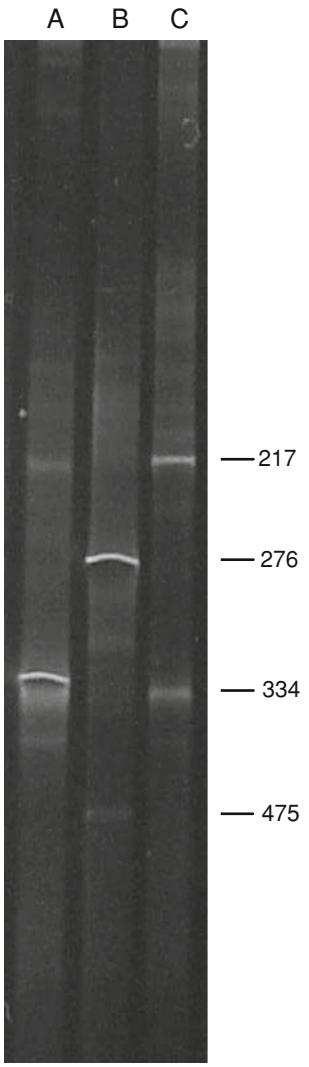

Fig. 2 DGGE molecular fingerprints of cyanobacterial 16S rRNA genes from the analyzed mat samples: *A green* (external layer) (56.5°C); *B yellowish* mat (50.8°C); *C bright-red* (internal layer) (56.5°C) underneath layer *A*. Migration distances correspond to those reported on Table 1

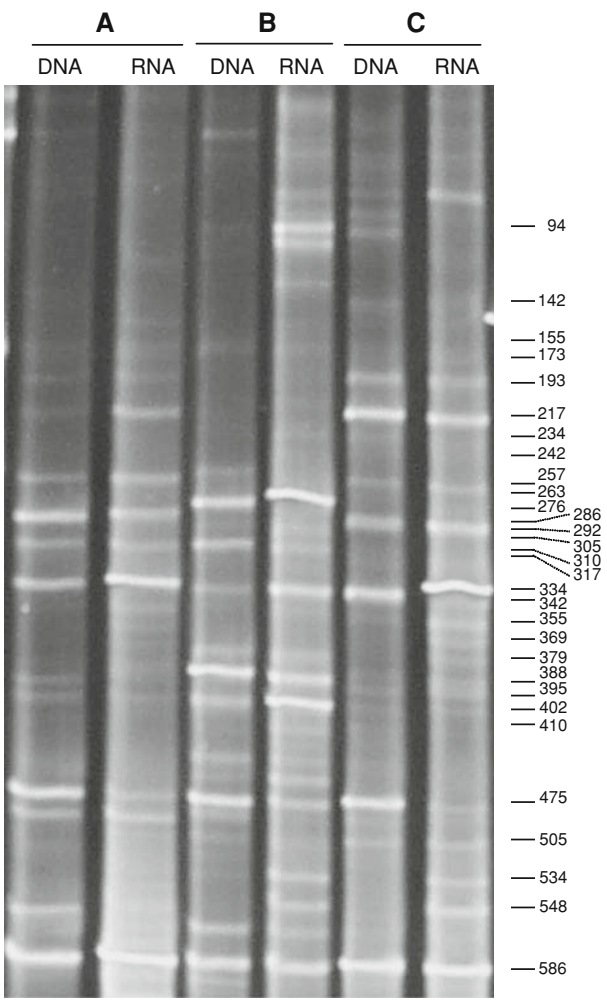

Fig. 3 Comparative DNA and RNA based molecular fingerprints of bacteria obtained by DGGE based on 16S rRNA genes from the analyzed mat samples: *A green* (external layer, 56.5°C); *B yellowish* mat (50.8°C); *C bright-red* (internal layer, 56.5°C) underneath layer *A*. Position of migration markers are indicated by *arrows*. Migration distances correspond to those reported on Table 1

Sequences belonging to the Phylum *Bacteroidetes* from the studied mat layers clustered with others from geothermal zones at Yellowstone National Park (i.e. AF027008; 91% similarity), China (i.e. DQ340754; 98% similarity), Japan (i.e. AB113584; 96% similarity), Austria (i.e. AM902639), and a hot spring in Ratchaburi Province in Thailand (AY555776) (Fig. 4d).

Discussion

Community fingerprinting by PCR-DGGE provides a representation of the major components of a microbial community (Roeselers et al. 2007; Portillo et al. 2008). In this study, bacterial communities from freshwater hot spring mats were analyzed based both on DNA and RNA aiming to determine the most abundant microbial groups and those actively participating in the development of the different mat layers, respectively.

Several authors have commented on the dominance of *Cyanobacteria* or *Chloroflexi* in different thermophilic mats (Ward et al. 1998; Roeselers et al. 2007; Polerecky et al. 2007). During this study, phototrophic bacteria, *Cyanobacteria* and *Chloroflexi*, represented the two most abundant and metabolically active members of the analyzed mat communities. Besides, different mat layers were characterized by distinctive bacterial communities, including the members of these two phyla (*Cyanobacteria* and *Chloroflexi*). Direct interactions among light-

Table 2 Composition of the bacterial communities in the green, yellow, and red layers of the studied mat based on analysis of banding patterns obtained by PCR-DGGE based on DNA and RNA

The fraction corresponding to unidentified DNA bands is also indicated

ND Not detected

Phylum	Percentages in							
	Green mat		Yellow mat		Red mat		Total	
	DNA	RNA	DNA	RNA	DNA	RNA	DNA	RNA
Actinobacteria	1.3	0.7	1.2	2.0	2.8	2.4	1.8	1.7
Bacteroidetes	3.3	12.2	4.6	12.6	7.2	7.3	5.0	10.7
Candidate Division OP10	14.9	11.8	6.1	7.3	2.7	11.4	7.9	10.2
Chloroflexi	30.0	15.7	29.0	21.2	25.5	22.8	28.2	19.9
Cyanobateria	22.1	32.2	37.2	25.8	51.5	36.0	36.8	31.3
Planctomycetes	5.1	4.7	6.2	3.0	ND	ND	3.8	2.6
Unidentified	23.3	22.7	15.7	28.1	10.3	20.1	16.5	23.6

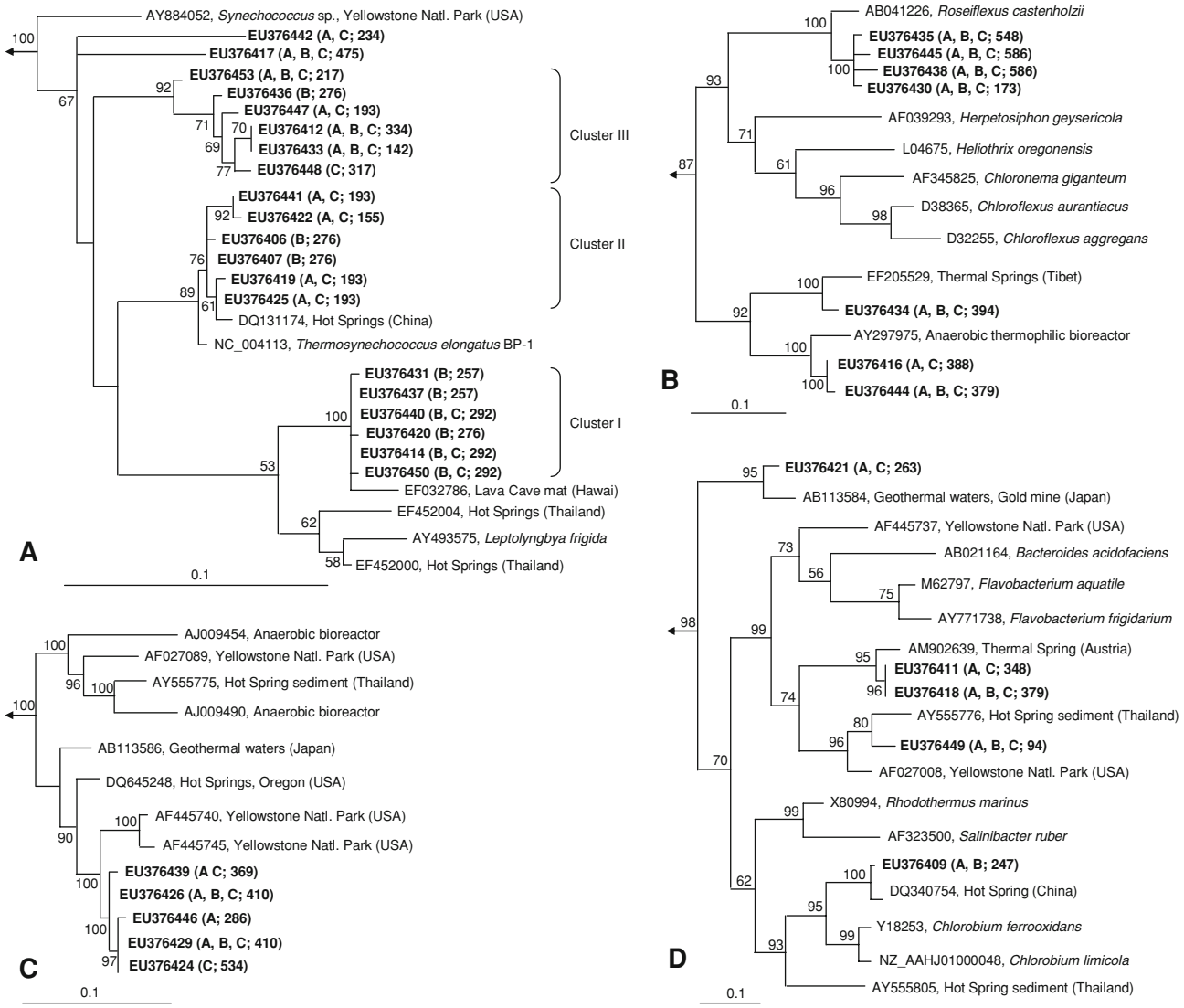

Fig. 4 Phylogenetic tree showing the relationships between sequences obtained from Boekleung Hot Spring mats and closely related *Cyanobacteria* (**a**), *Chloroflexi* (**b**), Candidate Division OP10 (**c**), and *Bacteroidetes* (**d**). The mat layer of origin for each OTU is reported between *brackets* together with its migration distance during DGGE analysis. *Bar* indicate changes per nucleotide

dependent microorganisms, primary producers (*Cyanobacteria*) and photoheterotrophs or chemoheterotrophs (*Chloroflexi*), could be hypothesized for Boekleung Hot Spring mats. A coexistence of *Cyanobacteria* and *Chloroflexi* at the studied mat layers (Table 2) could represent a preliminary evidence for this mutualism.

Previous studies on the cyanobacterial components of thermophilic mats from Northern Thailand have reported on the presence of members of the genus *Thermosynechococcus* detected by culturing techniques (Hongmei et al. 2005; Sompong et al. 2005). In Boekleung Hot Spring mats, *Thermosynechococcus*-related *Cyanobacteria* were an abundant group at the three studied mat layers. Besides, clear differences in the distribution of cyanobacterial phylotypes were observed. At this respect, for instance, Allewalt et al. (2006) suggested the existence of clear preferences for the level of light intensity and temperature by thermophilic *Cyanobacteria* belonging to different *Synechococcus*-like clades, which could explain those differences in cyanobacterial distribution. Kanokratana et al. (2004) showed a scarce representation of *Cyanobacteria* in sediments from a hot spring in Ratchaburi Province. This information together with the detection during our study of *Cyanobacteria* through RNA-based analyses suggested that these thermophilic *Cyanobacteria* are adapted to thrive in hot spring mats rather than the sediments or surrounding waters and the thermophilic mats are where they optimally develop and find their preferred habitat.

Chloroflexi also require specific conditions for growth depending on oxygen, sulfide, and light intensity (Pierson and Castenholz 1974; Hanada et al. 2002). The present study shows dominance of metabolically active *Chloroflexi* and *Cyanobacteria* in three mat layers at Boekleung Hot Springs. Results from Kanokratana et al. (2004) studying the sediments of a hot spring at Ratchaburi Province suggested the presence of a low proportion of *Chloroflexi*. Some previous works have reported a high abundance of *Chloroflexi* (Polerecky et al. 2007) in thermophilic mat communities while others suggested a scarce representation of this bacterial group (Roeselers et al. 2007). The present study confirms *Chloroflexi* as a major group of metabolically active bacteria in Boekleung Hot Spring mats and reports on the preferential distribution within the mat of specific members of the Phylum *Chloroflexi* which tend to avoid the surrounding waters and sediments. A preferential and distinctive distribution of *Cyanobacteria*, *Chloroflexi*, and other bacteria confirms the existence of differential community structures between the studied mat layers.

Kanokratana et al. (2004) detected the presence of 5% of phylotypes from DNA analysis belonging to the Candidate Division OP10 while our results suggested the presence on average of around 8–10% of bacteria belonging to this phylum based on DNA and RNA analyses. Since the OP10 sequences detected in both studies belonged to two different clusters (i.e. AY555775) within the phylum (Fig. 4c), a differential distribution between mat and sediments for distinct phylotypes could be suggested. In the present study, the green mat layer (a) represented the preferred environment for members of the Candidate Division OP10 where they constituted up to 15% of the quantified nucleic acids (Table 2). Besides showing a differential distribution of phylotypes belonging to the Candidate Division OP10 in Boekleung Hot Spring mats, we have demonstrated their significant contribution to mat activity since they were metabolically active members of the microbial communities in the analyzed mat layers.

Most *Bacteroidetes* detected during our study were related to others previously reported in thermophilic environments. From those detected by Kanokratana et al. (2004) in hot spring sediments only one of the sequences (i.e. AY555776) was closely related to the *Bacteroidetes* related sequences (i.e. EU376449) detected in Boekleung mats. Results confirmed a significant and characteristic representation of *Bacteroidetes* in hot spring habitats from Ratchaburi Province.

Planctomycetes was another bacterial phylum well represented in our study in mats and that of Kanokratana et al. (2004) in sediments; both studies suggest a differential distribution of phylotypes of this phylum between mats and sediments and the present study indicates they might constitute 5 or 10% of DNA or RNA-based molecular surveys, respectively. The presence of Crenarchaeota, related to the low-temperature clusters, in hot spring sediments (Kanokratana et al. 2004) and their non-detectable presence in mats (this study) also constitutes evidence supporting the existence of differences in the distribution of Archaea in hot spring sediments and mats.

Some studies on hot springs have reported the presence of the *Thermus* group (e.g., *Meiothermus*, *Thermus*) as a significant component of these communities (Ward et al. 1998; Skirnisdottir et al. 2000). For instance, Kanokratana et al. (2004) reported 11% of sequences belonging to this group in sediments from hot springs in Thailand. In the mats analyzed in this study, this group was not detected using molecular techniques although representatives of the genus *Meiothermus* could be obtained by culturing methods (data not shown). These results suggest the *Thermus* group is present in these hot springs (i.e. sediments) but represents only a minor component of the mat communities. The strict physico-chemical and biological conditions existing in this environment govern its microbial diversity resulting in communities typically characterized by relatively low diversity dominated by a few microbial groups (Skirnisdottir et al. 2000).

Heterotrophic bacterial communities in the studied hot spring mats showed relatively low phylum diversity

although each of these bacterial groups were constituted by numerous, distinctive, phylotypes. The number of bacterial divisions (6; Table 2) found at Boekleung Hot Spring mats is slightly below the values previously reported in similar systems. For instance, Kanokratana et al. (2004) detected up to 11 bacterial phyla in sediments of nearby hot springs. Ward et al. (1998) mentioned about nine bacterial divisions in Octopus Spring mat community (Yellowstone National Park). Interestingly, *Proteobacteria* and *Firmicutes* remained undetected through nucleic acid analysis (both DNA and RNA) in this study. Other studies reported over 80% *Proteobacteria* based on DNA analysis in geothermal communities (Moyer et al. 1995). In hot spring sediments from Ratchaburi Province, *Proteobacteria* represented about 12% of 16S rDNA sequences (Kanokratana et al. 2004) and their presence was considered as potential contamination from surrounding systems. *Proteobacteria* and *Firmicutes* generally constitute a major component of bacterial communities in soils (Fierer et al. 2007). The absence of *Proteobacteria* and *Firmicutes* within the detected microorganisms through DNA and RNA analyses suggests Boekleung Hot Spring mats as a system with low influence from external bacterial communities. Thus, Boekleung Hot Spring mats appeared as a relatively closed system as previously suggested for some thermophilic *Cyanobacteria* (Papke et al. 2003) and opposed to open volcanic environments presenting high influence from allochthonous microorganisms (Portillo and Gonzalez 2008a).

This study confirms the existence of differential bacterial communities constituting green, yellow, and red mat layers at Boekleung Hot Springs. *Cyanobacteria* and *Chloroflexi* clearly dominated these communities. Through DNA- and RNA- based analyses the major components present and participating in the activity of the microbial mat have been identified representing diverse communities of bacteria belonging to a relatively low number of phyla.

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References

Allewalt JP, Bateson MM, Revsbech NP, Slack K, Ward DM (2006) Effect of temperature and light on growth and photosynthesis by *Synechococcus* isolates typical of those predominating in the Octopus Spring Microbial Mat Community of Yellowstone National Park. Appl Environ Microbiol 72:544–550

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215:403–410

Bauld J, Brock TD (1973) Ecological studies of *Chloroflexis*, a gliding photosynthetic bacterium. Arch Microbiol 92:267–284

Castenholtz RW (1973) Ecology of blue-green algae in hot springs. In: Carr NG, Whitton BA (eds) The biology of blue-green algae. University of California Press, Los Angeles, pp 379–414

Ferris MJ, Ward DM (1997) Seasonal distributions of dominant 16s rRNA-defined populations in a hot spring microbial mat examined by denaturing gradient gel electrophoresis. Appl Environ Microbiol 63:1375–1381

Fierer N, Bradford MA, Jackson RB (2007) Toward an ecological classification of soil bacteria. Ecology 88:1354–1364

Gonzalez JM, Saiz-Jimenez C (2005) Application of molecular nucleic acid-based techniques for the study of microbial communities in monuments. Int Microbiol 8:189–194

Gonzalez JM, Ortiz-Martinez A, Gonzalez-delValle MA, Laiz L, Saiz-Jimenez C (2003) An efficient strategy for screening large cloned libraries of amplified 16S rDNA sequences from complex environmental communities. J Microbiol Methods 55:459–463

Gonzalez JM, Zimmermann J, Saiz-Jimenez C (2005) Evaluating putative chimeric sequences from PCR amplified products and other cross-over events. Bioinformatics 21:333–337

Hanada S, Takaichi S, Matsuura K, Nakamura K (2002) *Roseiflexus castenholzii* gen. nov., sp. nov., a thermophilic, filamentous, photosynthetic bacterium which lacks chlorosomes. Int J Syst Evol Microbiol 52:187–193

Hongmei J, Aitchison JC, Lacap DC, Peerapornpisal Y, Sompong U, Pointing SB (2005) Community phylogenetic analysis of moderately thermophilic cyanobacterial mats from China, the Philippines and Thailand. Extremophiles 9:325–332

Kanokratana P, Chanapan S, Pootanakit K, Eurwilaichitr L (2004) Diversity and abundance of Bacteria and Archaea in the Bor Khlueng Hot Spring in Thailand. J Basic Microbiol 44:430–444

Lane DJ (1991) 16S/23S rRNA sequencing. In: Stackebrandt E, Goodfellow M (eds) Nucleic acid techniques in bacterial systematics. Wiley, Chichester, pp 205–248

Mills HJ, Martinez RJ, Story S, Sobecky PA (2004) Identification of members of the metabolically active microbial populations associated with *Beggiatoa* species. Appl Environ Microbiol 70:5447–5458

Moyer CL, Dobbs FC, Karl DM (1995) Phylogenetic diversity of the bacterial community from a microbial mat at an active, hydrothermal vent system, Loihi Seamount, Hawaii. Appl Environ Microbiol 61:1555–1562

Muyzer G, de Waal EC, Uitterlinden AG (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl Environ Microbiol 59:695–700

Nogales B, Moore ERB, Abraham WR, Timmis KN (1999) Identification of the metabolically active members of a bacterial community in a polychlorinated biphenyl-polluted moorland soil. Environ Microbiol 1:199–212

Nold SC, Ward DM (1996) Photosynthate partitioning and fermentation in hot spring microbial mat communities. Appl Environ Microbiol 62:4598–4607

Nübel U, Garcia-Pichel F, Muyzer G (1997) PCR primers to amplify 16S rRNA genes from *cyanobacteria*. Appl Environ Microbiol 63:3327–3332

Papke RT, Ramsing NB, Bateson MM, Ward DM (2003) Geographical isolation in hot spring *cyanobacteria*. Environ Microbiol 5:650–659

Pierson BK, Castenholz RW (1974) Studies of pigments and growth in *Chloroflexus aurantiacus*, a phototrophic filamentous bacterium. Arch Microbiol 100:283–305

Polerecky L, Bachar A, Schoon R, Grinstein M, Jorgensen BB, De Beer D, Jonkers HM (2007) Contribution of *Chloroflexus* respiration to oxygen cycling in a hypersaline microbial mat from Lake Chiprana, Spain. Environ Microbiol 9:2007–2024

Portillo MC, Gonzalez JM, Saiz-Jimenez C (2008) Metabolically active microbial communities of yellow and grey colonizations on the walls of Altamira Cave, Spain. J Appl Microbiol 104:681–691

Portillo MC, Gonzalez JM (2008a) Microbial communities and immigration in volcanic environments of Canary Islands (Spain). Naturwissenschaften 95:307–315

Portillo MC, Gonzalez JM (2008b) Statistical differences between molecular fingerprints from microbial communities. Antonie van Leeuwenhoek 94:157–163

Roeselers G, Norris TB, Castenholz RW, Rysgaard S, Glud RN, Kühl M, Muyzer G (2007) Diversity of phototrophic bacteria in microbial mats from Arctic hot springs (Greenland). Environ Microbiol 9:26–38

Skirnisdottir S, Hreggvidsson GO, Hjorleifsdottir S, Marteinsson VT, Petursdottir SK, Holst O, Kristjansson JK (2000) Influence of sulfide and temperature on species composition and community structure of hot spring microbial mats. Appl Environ Microbiol 66:2835–2841

Sloan WT, Lunn M, Woodcock S, Head IM, Nee S, Curtis TP (2005) Quantifying the roles of immigration and chance in shaping prokaryote community structure. Environ Microbiol 8:732–740

Sompong U, Hawkins PR, Besley C, Peerapornpisal Y (2005) The distribution of *cyanobacteria* across physical and chemical gradients in hot springs in Northern Thailand. FEMS Microbiol Ecol 52:365–376

Stott MB, Crowe MA, Mountain BW, Smirnova AV, Hou S, Alam M, Dunfield PF (2008) Isolation of novel bacteria, including a candidate division, from geothermal soils in New Zealand. Environ Microbiol 10:2030–2041

Strimmer K, von Haeseler A (1996) Quartet puzzling: a quartet maximum likelihood method for reconstructing tree topologies. Mol Biol Evol 13:964–969

Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix-choice. Nucleic Acids Res 22:4673–4680

Ward DM, Weller D, Bateson MM (1990) 16S rRNA sequences reveal uncultured inhabitants of a well-studied thermal community. FEMS Microbiol Rev 75:105–116

Ward DM, Ferris MJ, Nold SC, Bateson MM (1998) A natural view of microbial biodiversity hot spring cyanobacterial mat communities. Microbiol Mol Biol Rev 62:1353–1370

Weller R, Batterson MM, Heimbuch BK, Kopczynski ED, Ward DM (1992) Uncultivated *cyanobacteria*, *Chloroflexus*-like and spirochete-like inhabitants of a hot spring microbial mat. Appl Environ Microbiol 58:3964–3969