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Bacterial community composition in thermophilic microbial mats from five hot springs in central Tibet

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Abstract Despite detailed study of selected thermophilic taxa, overall community diversity of bacteria in thermophilic mats remains relatively poorly understood. A sequence-based survey of bacterial communities from several hot spring locations in central Tibet was undertaken. Diversity and frequency of occurrence for 140 unique 16S rRNA gene phylotypes were identified in clone libraries constructed from environmental samples. A lineage-per-time plot revealed that individual locations have evolved to support relatively large numbers of phylogenetically closely related phylotypes. Application of the F_{ST} statistic and P test to community data was used to demonstrate that phylogenetic divergence between locations was significant, thus emphasizing the status of hot springs as isolated habitats. Among phylotypes, only the Chlorobi were ubiquitous to all mats, other phototrophs (Cyanobacteria and Chloroflexi) occurred in most but not all samples and generally accounted for a large number of recovered phylotypes. Phylogenetic analyses of phototrophic phylotypes revealed support for location-specific lineages. The alpha, beta and gamma proteobacteria were also frequently recovered phyla, suggesting they may be abundant phylotypes in mats, a hitherto unappreciated aspect of thermophilic mat biodiversity. Samples from one location indicated that where phototrophic bacteria were rare or absent due to niche disturbance, the relative frequency of proteobacterial phylotypes increased.

Keywords Chlorobi · Chloroflexi · Hot Springs · Proteobacteria · *Synechococcus* · Thermophiles

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Introduction

Terrestrial hot springs support thermophilic prokaryotic communities and significant research attention has centered upon the lithic laminated microbial mats that occur in thermal waters from ~50–75°C (Ward and Castenholz 2000). Studies have largely focused upon the cyanobacterium *Synechococcus* that occurs as a monospecific surface layer on '*Synechococcus* mats' (Ward et al. 1998), although at lower temperatures other unicellular and filamentous cyanobacteria co-occur with this taxon (Norris et al. 2002; Jing et al. 2005). Some heterotrophic mat taxa are also known from cultivation studies (Santegoeds et al. 1996; Nold et al. 1996). Mats have recently been revealed to support a far more diverse range of taxa than previously appreciated throughout all the laminae comprising the total thickness of mats, spanning 12 known archaeal and bacterial phyla (Lau et al. 2006). Biodiversity among thermophiles is largely determined by temperature, although other important abiotic factors include pH and dissolved hydrogen sulphide levels (Ward and Castenholz 2000; Purcell et al. 2007). Recent studies have also

highlighted that other factors such as biogeography (Whitaker et al. 2003) and geological history (Takacs-Vesbach et al. 2008) can also be important in determining thermophilic diversity.

Diversity studies on environmental samples of thermophiles have largely employed a DGGE approach, and so estimates of relative abundance of taxa within mats are generally lacking (Ward et al. 1998). Nonetheless, much useful qualitative data has emerged from studies of DGGE-defined biodiversity at several geothermal locations including extensive work at Yellowstone National Park (YNP) and Great Basin springs in the USA (Ward et al. 1998; Ward and Castenholz 2000), and also relatively less well studied locations in China, Greenland, Japan, New Zealand and Thailand (Lacap et al. 2005; Jing et al. 2005, 2006; Papke et al. 2003; Lau et al. 2006; Roeselers et al. 2006). These studies have focused mainly upon cyanobacteria but have yielded some interesting findings that may be applicable to other thermophilic taxa: phylogenetic lineages of *Synechococcus* identified from several springs worldwide indicate evidence for phylogeographic patterns that indicate distance is a barrier to dispersal (Papke et al. 2003). Diversity among *Synechococcus* and other thermophilic cyanobacteria also show responses to stochastic disturbance and seasonal environmental changes (Wickstrom and Castenholz 1985; Ferris and Ward 1997; Ferris et al. 1997; Norris et al. 2002; Lacap et al. 2007). The focus on cyanobacteria, which are restricted to a relatively thin surface layer of typical *Synechococcus* mats, reflects the perceived importance of primary production to such communities.

Major gaps in our knowledge relate to the overall diversity that exists within mats, and also to a lack of data that may indicate relative abundance for thermophilic taxa in mats. There is therefore a need for studies to elucidate α-diversity in hot springs at the community level. The DGGE approach is not suitable for such studies and so alternatives are necessary. One of the most widely used approaches in microbial ecology has been to construct clone libraries from which relative abundance is inferred from the frequency with which clones of a given phylotype are encountered during a sampling where a rarefaction curve approaches asymptote. Although not without its limitations related to potential for bias in PCR and cloning which may affect the inference of relative frequency for phylotypes, this approach is extensively used in microbial ecology and well-suited to a study of thermophilic mats where attempts at quantitative diversity estimates are lacking. Accordingly, we set out to determine diversity and abundance of thermophilic bacteria from hot springs at five geothermal localities with the hypothesis that bacterial diversity in hot spring microbial mats is greater than previously resolved and displays significant variation between locations. By focusing on overall bacterial diversity using PCR primers that were universal for the domain Bacteria and sequencing a large numbers of clones, we were able to infer diversity and relative abundance of thermophilic phylotypes from 16 phyla and quantify divergence between communities at each location. Samples were obtained from several previously uninvestigated springs in a region that is relatively under-represented in terms of studies on thermophilic mats, and included undisturbed and disturbed mat samples.

Materials and methods

Field locations and sampling

Five sampling locations at three undisturbed geothermal sites located along a ~380 km longitudinal transect (29°36.083′N 85°44.856′E–32°57.745′N 86°35.842′E) north of the Yarlung Tsangpo suture zone were sampled in August 2004. At all localities, the hot springs were associated with normal faults at the front of high mountain ranges. The southernmost location in the Daggyai Tso Geyser field (29°36.083′N 85°44.856′E, 5,070-m altitude) (sample pools DTB, DTM) and an un-named geothermal location 50 km north (30°01.436′N 85°40.810′E, 5,189-m altitude) (sample pool TP) emerged in the Lhasa terrane (Aitchison et al. 2002). The northernmost samples some 380 km north were collected from a geyser field near Yibbug Caka associated with the Qiangtang terrane (32°57.745′N 86°35.842′E, 4,713-m altitude) (sample pools YCB, YCC) (Taylor et al. 2003).

Physico-chemical parameters known to have the greatest influence on thermophilic diversity (temperature, pH and hydrogen sulphide) were tested in the field when selecting sampling sites in order to ensure all samples were taken from within the known optimum temperature and pH range for thermophilic prokaryotic mat diversity using previously described protocols (Lau et al. 2006). Temperature and pH at each sampling location was recorded using a combined temperature/pH electrode (Orion, Boston, MA, USA). Values obtained were within the range 60–65°C and pH 7–7.4 across sites (DTB 65°C, pH 7.4; DTM 60°C, pH 7.1; TP 61°C, pH 7.0; YCB 64°C, pH 7.0; YCC 62°C, pH7.0). Hydrogen sulphide was below the detection limit of 0.1 mg/l (as determined by methylene blue titration, HS-WR, Hach, Loveland, CA, USA) at each location. Vertical sections of mats (approx. 100 mg each) were collected in triplicate from each location as previously described (Lau et al. 2006) and preserved in RNAlater solution (Ambion Inc, Austin, TX, USA) to preserve nucleic acids until processed (approximately 2 weeks) since no refrigeration facilities were available under the remote field conditions. Sample DTM was unusual in that the mat did not support a

surface cyanobacterial layer, and we have frequently observed in the field that this phenomenon is related to senescence and displacement of this surface layer into the water column during stochastic thermal disturbance events. The remaining biomass continues to appear viable and does not senesce, and cyanobacteria sometimes re-colonize the mat surface over time. We therefore included samples from this spring in our study for comparative purposes, together with those from a nearby spring where a cyanobacterial layer was present.

Recovery and cloning of environmental 16S rRNA genes

Community DNA was isolated from ~100 mg (wet weight) mat sections as previously described (Lau et al. 2006), and 16S rRNA genes amplified using 'universal' bacterial primers 27F-1492R corresponding to equivalent nucleotide positions in *E. coli* as previously described (de la Torre et al. 2003). Triplicate environmental amplicons from each location were pooled and used to construct clone libraries (Cloning^{Plus}, Qiagen, Valencia, CA, USA) for each location, and a sample size constraint of 101 clones per location was enforced. Simulation analysis was carried out using the sequence data for site TP to validate the pooling approach for amplicons used in cloning. This approach was demonstrated to yield comparable recovery efficiency as if the library were generated from three separate (un-pooled) samples. A total of 478 clones were screened by RFLP (Msp I, Hae III, Hinf I, Amersham, Bucks, UK), and 151 unique RFLP-defined phylotypes were sequenced (ABI 3730 Genetic Analyzer, Applied Biosystems, Foster City, CA, USA) to generate 140 unique 16S rRNA gene sequences of approximately 1,412 bp each (these sequence data have been submitted to the NCBI GenBank databases under accession numbers EF205440–EF205590).

Sequence analysis and statistics

Multiple alignments were created with reference to selected GenBank sequences using Clustal X v.1.81 (Thompson et al. 1997), and sequences checked for possible chimeric structure using the Chimera_Check software on the Ribosome Database Project website (http://rdp.cme.msu.edu.html). Sequenced phylotypes were delineated based upon 99% sequence similarity in order to account for closely related ecotypes known to occur in thermal habitats (Ward and Castenholz 2000). An approximate phylogenetic affiliation for each phylotype was determined with reference to the NCBI GenBank database (http://www.ncbi.nlm.nih.gov).Non-parametric rarefaction was used to estimate the sufficiency of sampling from the library, and estimates of OTU Richness from clone libraries were made using Chao 1 with the software EstimateS (http://viceroy.eeb.uconn.edu/estimateS). Coverage estimates for libraries were calculated to infer the proportion of phylotypes in a clone library of infinite size that could be represented in a smaller library (C) and the fraction of clones encountered more than once (C_{ACE}) using the equations of Good (1953) and Chao et al. (1993), respectively:

$$C = 1 - \frac{n_1}{N}$$

Where n_1 is the number of singleton phylotypes and N represents the total number of clones retrieved for the library.

$$C_{ACE} = 1 - \frac{F_1}{N_{rare}}$$

Where F_1 is the number of singleton phylotypes and N_{rare} represents the total number of clones for phylotypes that were represented 10 times or fewer. The Shannon–Weiner Index [H(s)] (Lloyd et al. 1968) and Pielou's Evenness Index [J] (Pielou 1966) were used to describe diversity in clone-derived communities as follows:

$$H(\mathrm{s}) = \frac{C}{N}\{(N \log 10N) - \sum_{i=1}^{s} n_i \log 10 n_i\}$$

where $C = 3.321928$ (constant used in converting log10 to log2), N represents the total number of individuals, n_i the number of individuals in the 'ith' species, and S the total number of species.

$$J = \frac{H(\mathrm{s})}{H(\max.)}$$

where H(s) represents the Shannon–Wiener information function and H(max.) the theoretical maximum value for H(s) if all taxa in the sample were equally abundant.

All phylogenetic analysis was performed using PAUP* v4.0b10 (Swofford 2001). Maximum likelihood analysis of 16S rRNA gene datasets within phyla was used to illustrate the relationship of sequences to representative taxa using appropriate models for evolutionary change as described in the results. Bootstrap values for 1,000 replications and Bayesian posterior probabilities were calculated and are shown for branch nodes supported by more than 50% of the trees. Quantification of the degree of phylogenetic differentiation between communities was expressed by the F_{ST} statistic (Martin 2002) using the software Arlequin v3.0 (Excoffier and Schneider 2005). This technique compares the genetic diversity within each community to the total genetic diversity of the combined communities using the equation:

$$F_{ST} = (\varnothing_T - \varnothing_W)/\varnothing_T$$

where $\varnothing_T$ is the genetic diversity for all samples and $\varnothing_W$ is diversity within each community averaged over all the

communities being compared. An alternative approach was also used in which the extent to which unique sequences between communities exhibited significant covariation within the neighbor-joining (NJ) phylogeny was measured. The NJ tree was constructed with the archaeon *Sulfolobus acidocaldarius* as outgroup. Branch length was optimized using a Maximum Likelihood model (GTR + I + G), and subject to the constraint that all sequences were contemporary. Significance testing was carried out by employing a *P* test (Martin 2002) using the software MacClade v4.0.3, (Maddison and Maddison 2001), from which the number of evolutionary steps causing the observed topology was computed and compared to 1,000 topologies generated by randomization. Lineage-per-time plots were also constructed as described by Martin (2002) in order to illustrate evolutionary patterns explaining extant diversity. A best-fit evolutionary model was applied to each community in order to incorporate elements of a molecular clock. All sequences were then enforced as contemporary on dendrograms from which the number of lineages present at arbitrarily-defined yet constant time intervals were assessed.

A BEST analysis (Clarke and Warwick 2001) was used to maximize the rank correlation between phylogenetically determined biotic data and measured environmental variables, with the aim of establishing a ranking and confidence estimate for the effects of temperature and pH on diversity between locations.

Results and discussion

Mat samples all appeared morphologically similar with a reddish-pink predominantly filamentous biomass overlain in all but one sample by a thin green cyanobacterial layer. The mats variously supported 22–47 phylotypes representing 5–16 Phyla (Table 1). Each location supported a distinct community, indeed only approximately 8% of OTU's were shared between mats. Some obvious commonality between mats existed, for example, all mats supported both phototrophic and heterotrophic phylotypes, although taxa and relative abundance varied greatly. Only one mat (DTM) supported known chemolithoautotrophic phylotypes. Some phylotypes, particularly among the proteobacterial phyla were affiliated only to other environmental sequences and therefore identity and putative physiological role could not be estimated. It is possible that some of the phylotypes recovered represent itinerant mesophiles, since we made no attempt to cultivate strains and verify their thermophilic status. This consideration must be borne in mind when interpreting the data, although we argue that mesophilic taxa are unlikely to remain viable or proliferate in a thermally challenging environment such as hot springs.

Table 1 16S rRNA gene-defined diversity of bacterial phylotypes

Percentage of total phylotypes recovered					
Location	DTB	DTM[a]	TP	YCB	YCC
Phylum					
Acidobacteria	4		4		
Aquificae		2			
Bacteroidetes			1	1	
Candidate division BRC1			1		
Candidate division OP7			1		
Candidate division OP8		7	2		
Candidate division OP9		2	2		
Candidate division OP10			11	1	
Candidate division OP11			5		
Chlorobi	19	1	15	12	4
Chloroflexi	10	1	15	10	
Cyanobacteria	31		3	29	24
Firmicutes	3	3	10		
Nitrospirae		8	11		
Planctomycetes			3	1	2
Alpha Proteobacteria	1	2		43	
Beta Proteobacteria	1		3	1	62
Delta Proteobacteria	6	1	10		
Gamma Proteobacteria	25	73	3		8
Spirochaetes				1	
Thermodesulfobacteria			1		
Verrucomicrobia				1	

Phylogenetic identity of sequences from clone libraries was determined by BLAST search of the NCBI GenBank database and phylogenetic analysis. Relative abundance of phylotypes in each library is shown as a percentage of the total for each phylum (a) DTB, clone library $n = 72$; (b) DTM, clone library $n = 100$; (c) TP, clone library $n = 101$; (d) YCB, clone library $n = 93$; (e) YCC, clone library $n = 101$

[a] Disturbed mat sample

Plots of rarefaction for the five clone libraries revealed coverage levels of 68–89% (Supplementary Material Fig. S1). This indicates that all locations probably support greater diversity than indicated by our study. Considerations such as primer and PCR/cloning bias may affect the recovery of phylotypes, plus the RFLP screening stage may underestimate phylotype diversity. These issues must be borne in mind when inferring community structure and relative abundance from clone library data. Estimates of OTU richness using Chao 1 (Supplementary Material Fig. S1) revealed that at 95% CI no significant difference in OTU richness between mats existed (one-way ANOVA, $p = 7.72 \times 10^{-5}$, $p > 0.5$). This may indicate a range for genetic diversity within thermophilic mats. The lineage-per-time plots were step-wise in nature, suggesting a punctuated evolutionary pattern for these thermal habitats. Acceptance of a molecular clock for bacteria allows

construction of lineage-per-time plots for phylogenetic data. The convex nature of these plots implied that a large portion of OTU's for any given location were closely related (Fig. 1). This is of interest, since it concurs with the notion that thermophilic cyanobacteria may exist as closely related ecotypes, each capable of exploiting minutely variable niche conditions (Ferris and Ward 1997; Ward and Castenholz 2000). The concept of ecotypes has been related to periodic selection that purges genetic variation from niches (Cohan 2002), and the step-wise evolutionary increments may reflect such a process.

In support of observed similar levels of OTU richness and apparently similar evolutionary trends at each location, Shannon–Weiner and Evenness estimates (Table 2) were similar when excluding the DTM sample which supported an unusually diverse mat community as compared with all other sites. In order to take advantage of the phylogenetic nature of our data, we also employed the F_{ST} statistic. We established a significant difference between mats at the community phylogenetic level using the F_{ST} statistic (Table 2). Differentiation between mats was significant ($p < 0.00001$ for all comparisons), and values for individual samples were similar indicating that locations contributed in approximately equal measure to phylogenetic divergence for the overall data (F_{ST} approx. 0.24). This was supported by a significant covariance of unique phylotypes with phylogeny using the P test ($p < 0.001$). Indeed, the observed topology required 55 evolutionary steps if determined by location, as opposed to 77–95 changes for random variation (Supplementary Material Fig. S2). No statistically significant relationship between genetic distance and geographic distance could be established, although this was also the case in another study over similar short distances when at larger scales clear distance-related effects were evident (Whitaker et al. 2003).

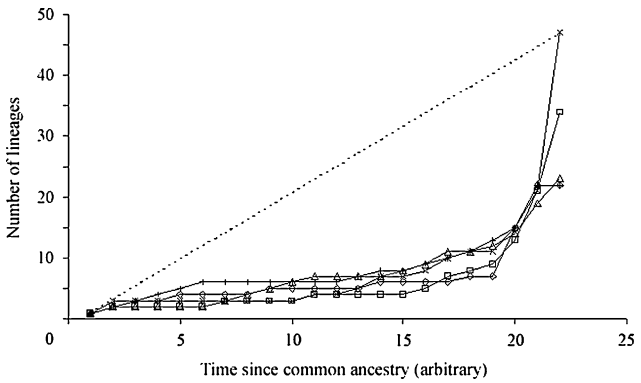


Fig. 1 Lineage-per-time plots for the five geothermal spring communities. The *dashed linear line* represents the expected trend with constant rates of birth and extinction. DTB *plus sign*, DTM *open diamond*, TP *cross sign*, YCB *open square*, YCC *open triangle*

A BEST analysis revealed no significant influence of either temperature or pH on diversity. Our data therefore indicates that for geothermal springs with comparable levels for abiotic variables known to have a major effect on thermophilic diversity, significant variation between communities exists at the phylogenetic level despite similar levels of diversity as indicated by major diversity indices. Whether this is due to some unmeasured niche-determining variable(s) is unknown from our study, since our primary focus was to resolve diversity at the bacterial community level rather than to determine the ecological effects of abiotic influences. Interestingly, the findings of several studies suggest that a complex suite of variables may be at work: a study focusing on thermophilic cyanobacteria revealed no effect for over 20 potentially niche-determining variables on diversity of *Synechococcus* between springs in Japan, New Zealand and the USA (Papke et al. 2003). Another study identified distance-related effects on diversity for the hot spring archaeon *Sulfolobus* (Whitaker et al. 2003), whilst it has recently been shown that geological history rather than contemporary factors or distance can most satisfactorily explain diversity patterns in *Sulfurihydrogenibium* sequences from YNP (Takacs-Vesbach et al. 2008). It is interesting that in our study where we have adopted a whole-community approach, whilst several bacterial phyla show evidence for location-specific

Table 2 Summary of hot spring community data. DTB, DTM, TP, YCB and YCC indicate sampling locations as explained in the text

Community	No. clones	No. RFLP-defined phylotypes sequenced	No. distinct phylotypes	Chao1 richness ± SD.	*C* coverage (%)	C_{ACE} coverage (%)	Avg. BLAST identity[a] for all phylotypes (%)	Shannons diversity	Pielou's evenness (*J*)	F_{ST}
DTB	74	25	22	24.2 ± 7.9	83.3	78.2	98.6	3.73	0.84	0.239
DTM	101	30	22	26.7 ± 11.7	82.0	40.0	96.4	2.10	0.47	0.243
TP	101	65	47	49.5 ± 10.9	68.3	57.9	96.7	4.93	0.89	0.237
YCB	101	40	36	40 ± 11.8	69.3	51.8	94.6	3.86	0.76	0.239
YCC	101	35	24	25.5 ± 6.8	88.7	47.6	95.6	3.37	0.75	0.242

[a] Average percentage similarity among all phylotypes from a given location with closest matches in the NCBI GenBank database

phylogenetic lineages, patterns are most pronounced among the Cyanobacteria thus identifying their status as useful test organisms for such studies.

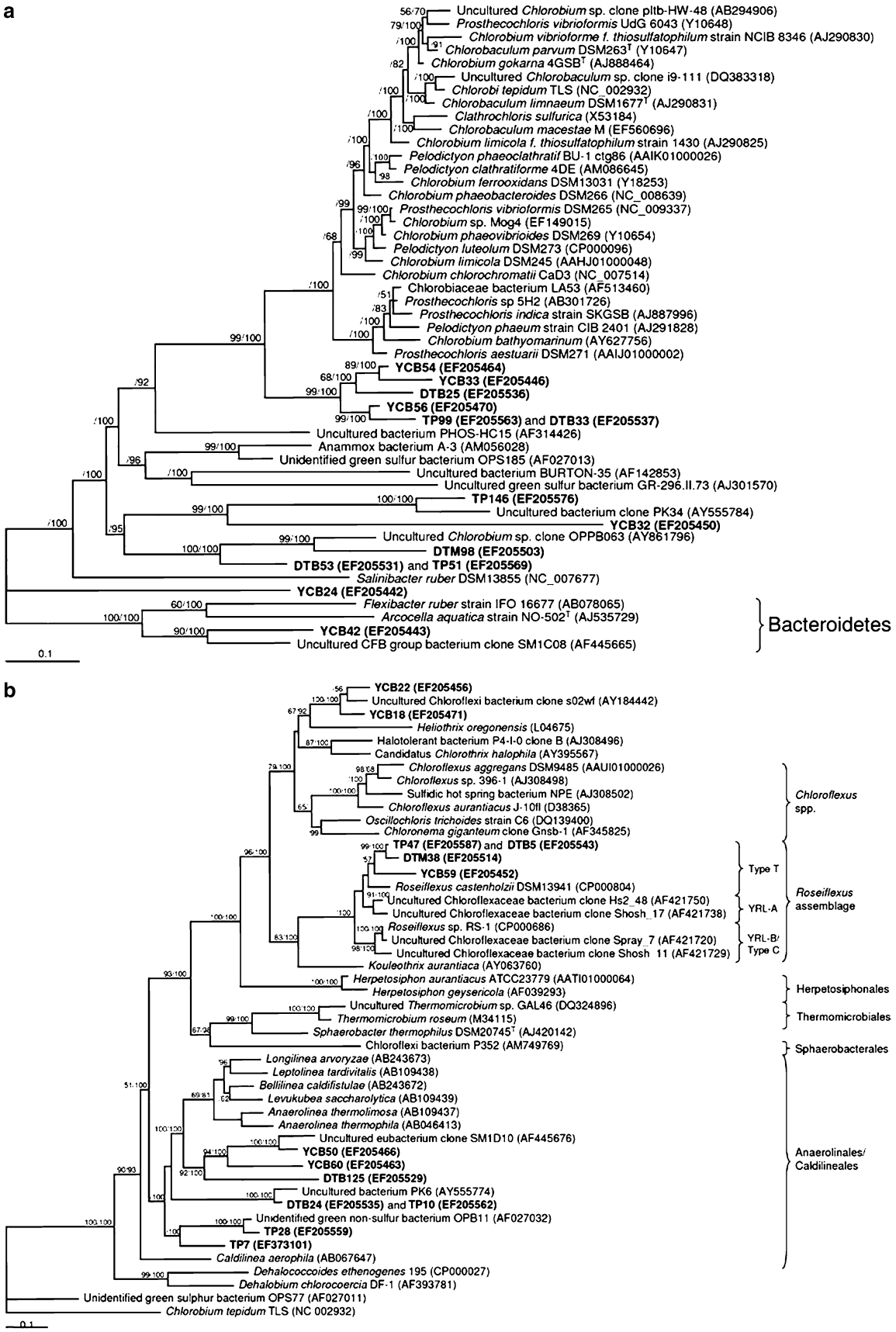
The diversity and relative abundance data from the location-specific clone libraries in our study is novel and offers some interesting insights into thermophilic microbial ecology. Interpretation of phylotype frequency from clone libraries has been identified as the most reliable method available for microbial abundance estimates (Curtis et al. 2001), although it is always important to bear in mind that PCR, ligation and clone selection bias may favor recovery of certain phylotypes over others. Nonetheless, our results resulted in recovery of phylotypes related to thermophiles and in the absence of any comparable data on abundance for thermophiles, we offer the following interpretation as a starting point to establishing frequency of occurrence for bacteria in thermophilic mats whilst acknowledging these limitations. Mats in each location clearly comprised a complex community of phototrophic and heterotrophic taxa, with the former plus a few Aquificales phylotypes also capable of autotrophy. The only phylum to display ubiquity among all mats was the Chlorobi. This indicates that anoxic microniches must exist in each mat since they are obligate anaerobes. These bacteria comprised 4–15% of taxa in typical mats with a significant phototrophic component. In the disturbed mat (DTM), Chlorobi and Chloroflexi accounted for only 1% each of phylotype abundance and no cyanobacterial phylotypes were encountered. Interestingly, the dominant phylotypes in this disturbed mat were heterotrophic gamma proteobacteria. This may reflect niche changes bought about by a lack of oxygenic phototrophic activity. It is not known at this stage to what extent this mat type represented a transient state in response to physical stress or a permanent shift to a different mat type, but it is interesting that autotrophy is indicated only in this mat by the presence of aquificales and these may in part replace the role of photoautotrophic taxa. Other studies of disturbance effects on thermophilic mats are limited; in YNP mats some shift in assemblage occurred within the genus *Synechococcus* if surface cyanobacteria were artificially removed (Ferris et al. 1997), and a recent study of whole-community response to stochastic disturbance in filamentous thermophilic mats showed that initial drastic changes in overall community structure were largely recoverable after several months (Lacap et al. 2007). The current study, thus, provides an opportune comparison between typical and disturbed communities.

Among the cyanobacteria and green non-sulphur bacteria, it was notable that cyanobacteria accounted for a fairly constant 24–31% of mat taxa in mats where they were abundant (only in the highly diverse TP mat were they less abundant). This may reflect a range of abundance for primary producers necessary to support such mats.

Fig. 2 Phylogenetic relationships among thermophilic phototrophic bacterial phylotypes from central Tibet, **a** Chlorobi, **b** Chloroflexi, **c** Cyanobacteria, **d** Proteobacteria. Trees also contain phylotypes from other phyla where these are phylogenetically informative. 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. Sequences from this study are shown in bold type with prefixes DTB, DTM, TP, YCB and YCC indicating different sample locations ▶

Similarly, the Chloroflexi were present as 10–15% of phylotypes in typical mats, and this may also represent their range of abundance necessary to sustain these mats. Our findings highlight that whilst the major focus of research has been on cyanobacteria in such mats, they may not necessarily be the most abundant phylum (c.f. Norris et al. 2002; Jing et al. 2005; Roeselers et al. 2006) and there was no clear relationship between cyanobacterial abundance and diversity/abundance of other taxa, except in disturbed (i.e., cyanobacteria-less) mats where relative abundance of proteobacterial phyla increased. Indeed, relative abundance is not necessarily correlated with activity, and it is possible that small numbers of particularly active cyanobacteria and other autotrophs act as a first trophic level in mats supporting a relative abundance of consumers. One must also accept that a certain degree of metabolic plasticity probably occurs among thermophilic bacteria. The absence of cyanobacteria in mat DTM, however, suggests that cyanobacteria may not be essential for thermophilic mat survival per se as also observed for some Icelandic thermophilic mats (Skirnisdottir et al. 2000) although again the temporal ecological context of the Icelandic mat is unknown. All mats in our study do, however, support at least one autotrophic taxon and this highlights the probable importance of primary production in providing nutrients to other mat taxa via photoexcretion (Bateson and Ward 1988).

An interesting finding was the high frequency of proteobacterial phylotypes in all samples. Most phylotypes recovered were phylogenetically affiliated with heterotrophic (and non-facultatively autotrophic) taxa and so these phylotypes may indicate thermophilic heterotrophy is significant in mats although isolation and cultivation of strains would be necessary to confirm this. A significant role for proteobacteria in mats would be interesting if confirmed, since this has not been previously appreciated. This finding warrants further work on the degree of thermophily and functional role for proteobacteria within thermophilic mats. Significant between-site variation was observed. Daggyai Tso samples (DTB, DTM) supported predominantly gamma proteobacteria, whereas proteobacteria occurring in mats from Yibbug Caka (YCB, YCC) were predominantly alpha and beta proteobacteria. This may reflect variation in niche conditions or a founder effect, where a competitive


a
Uncultured Chlorobium sp. clone pltb-HW-48 (AB294906)
Prosthecochloris vibrioformis UdG 6043 (Y10648)
Chlorobium vibrioforme f. thiosulfatophilum strain NCIB 8346 (AJ290830)
Chlorobaculum parvum DSM263T (Y10647)
Chlorobium gokarna 4GSBT (AJ888464)
Uncultured Chlorobaculum sp. clone i9-111 (DQ383318)
Chlorobi tepidum TLS (NC_002932)
Chlorobaculum limnaeum DSM1677T (AJ290831)
Clathrochloris sulfurica (X53184)
Chlorobaculum macestae M (EF560696)
Chlorobium limicola f. thiosulfatophilum strain 1430 (AJ290825)
Pelodictyon phaeoclathratif BU-1 ctg86 (AAIK01000026)
Pelodictyon clathratiforme 4DE (AM086645)
Chlorobium ferrooxidans DSM13031 (Y18253)
Chlorobium phaeobacteroides DSM266 (NC_008639)
Prosthecochloris vibrioformis DSM265 (NC_009337)
Chlorobium sp. Mog4 (EF149015)
Chlorobium phaeovibrioides DSM269 (Y10654)
Pelodictyon luteolum DSM273 (CP000096)
Chlorobium limicola DSM245 (AAHJ01000048)
Chlorobium chlorochromatii CaD3 (NC_007514)
Chlorobiaceae bacterium LA53 (AF513460)
Prosthecochloris sp 5H2 (AB301726)
Prosthecochloris indica strain SKGSB (AJ887996)
Pelodictyon phaeum strain CIB 2401 (AJ291828)
Chlorobium bathyomarinum (AY627756)
Prosthecochloris aestuarii DSM271 (AAIJ01000002)
YCB54 (EF205464)
YCB33 (EF205446)
DTB25 (EF205536)
YCB56 (EF205470)
TP99 (EF205563) and DTB33 (EF205537)
Uncultured bacterium PHOS-HC15 (AF314426)
Anammox bacterium A-3 (AM056028)
Unidentified green sulfur bacterium OPS185 (AF027013)
Uncultured bacterium BURTON-35 (AF142853)
Uncultured green sulfur bacterium GR-296.II.73 (AJ301570)
TP146 (EF205576)
Uncultured bacterium clone PK34 (AY555784)
YCB32 (EF205450)
Uncultured Chlorobium sp. clone OPPB063 (AY861796)
DTM98 (EF205503)
DTB53 (EF205531) and TP51 (EF205569)
Salinibacter ruber DSM13855 (NC_007677)
YCB24 (EF205442)
Flexibacter ruber strain IFO 16677 (AB078065)
Arcocella aquatica strain NO-502T (AJ535729)
YCB42 (EF205443)
Uncultured CFB group bacterium clone SM1C08 (AF445665)
Bacteroidetes
0.1
b
YCB22 (EF205456)
Uncultured Chloroflexi bacterium clone s02wf (AY184442)
YCB18 (EF205471)
Heliothrix oregonensis (L04675)
Halotolerant bacterium P4-I-0 clone B (AJ308496)
Candidatus Chlorothrix halophila (AY395567)
Chloroflexus aggregans DSM9485 (AAUI01000026)
Chloroflexus sp. 396-1 (AJ308498)
Sulfidic hot spring bacterium NPE (AJ308502)
Chloroflexus aurantiacus J-10fl (D38365)
Oscillochloris trichoides strain C6 (DQ139400)
Chloronema giganteum clone Gnsb-1 (AF345825)
Chloroflexus spp.
TP47 (EF205587) and DTB5 (EF205543)
DTM38 (EF205514)
YCB59 (EF205452)
Type T
Roseiflexus castenholzii DSM13941 (CP000804)
Uncultured Chloroflexaceae bacterium clone Hs2_48 (AF421750)
Uncultured Chloroflexaceae bacterium clone Shosh_17 (AF421738)
YRL-A
Roseiflexus assemblage
Roseiflexus sp. RS-1 (CP000686)
Uncultured Chloroflexaceae bacterium clone Spray_7 (AF421720)
Uncultured Chloroflexaceae bacterium clone Shosh_11 (AF421729)
YRL-B/ Type C
Kouleothrix aurantiaca (AY063760)
Herpetosiphon aurantiacus ATCC23779 (AATI01000064)
Herpetosiphon geysericola (AF039293)
Herpetosiphonales
Uncultured Thermomicrobium sp. GAL46 (DQ324896)
Thermomicrobium roseum (M34115)
Thermomicrobiales
Sphaerobacter thermophilus DSM20745T (AJ420142)
Chloroflexi bacterium P352 (AM749769)
Sphaerobacterales
Longilinea arvoryzae (AB243673)
Leptolinea tardivitalis (AB109438)
Bellilinea caldifistulae (AB243672)
Levukubea saccharolytica (AB109439)
Anaerolinea thermolimosa (AB109437)
Anaerolinea thermophila (AB046413)
Uncultured eubacterium clone SM1D10 (AF445676)
YCB50 (EF205466)
YCB60 (EF205463)
DTB125 (EF205529)
Uncultured bacterium PK6 (AY555774)
DTB24 (EF205535) and TP10 (EF205562)
Unidentified green non-sulfur bacterium OPB11 (AF027032)
TP28 (EF205559)
TP7 (EF373101)
Caldilinea aerophila (AB067647)
Anaerolinales/ Caldilineales
Dehalococcoides ethenogenes 195 (CP000027)
Dehalobium chlorocoercia DF-1 (AF393781)
Unidentified green sulphur bacterium OPS77 (AF027011)
Chlorobium tepidum TLS (NC 002932)
0.1

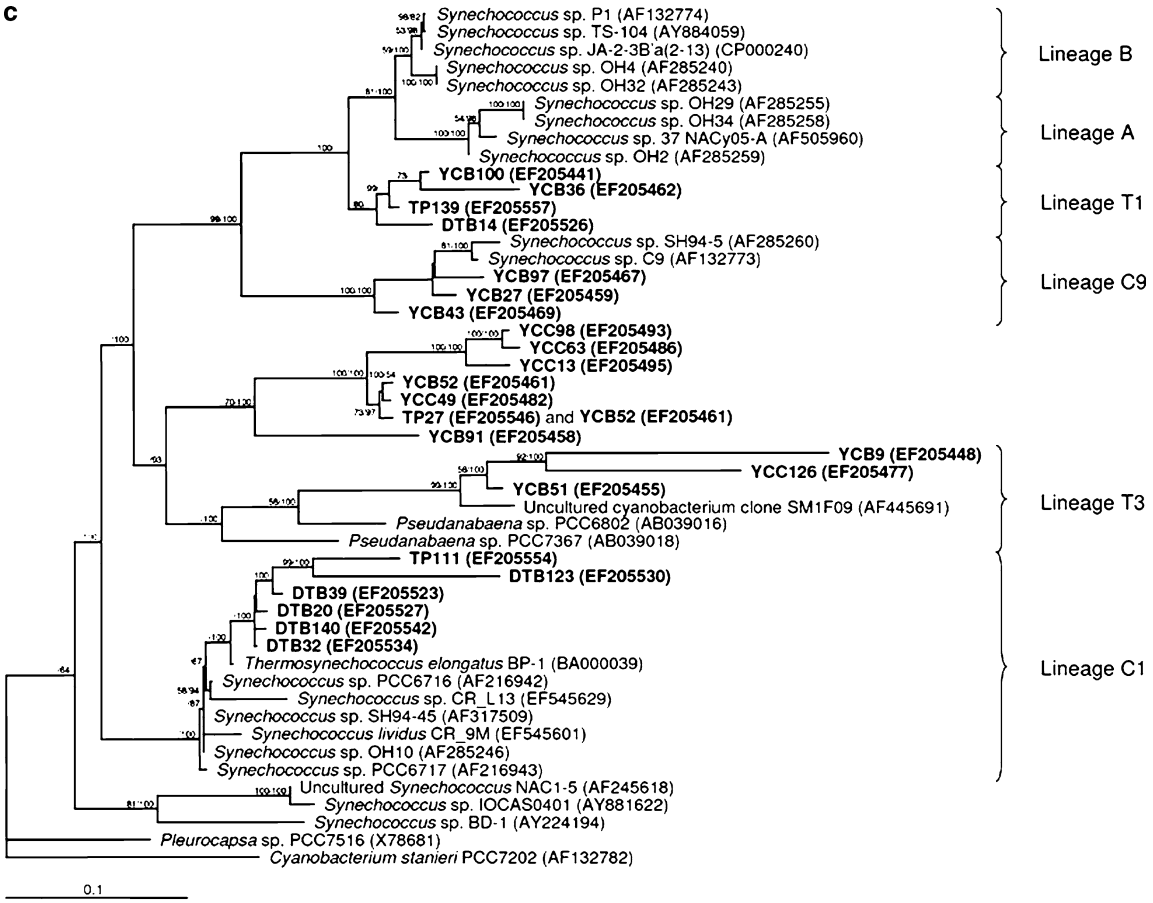


Fig. 2 continued

advantage accrued to early heterotrophic colonists. We discount significant PCR or cloning bias since there was no evidence for a particular taxon or lineage dominating our dataset. It is also notable that in a study of thermophilic bacteria from thermal waters in Indonesia, both cultivation and cloning also indicated a significant proteobacterial component (Baker et al. 2001).

Most of the remaining phylotypes indicated presence of taxa phylogenetically affiliated most closely to Firmicutes and Nitrospirae. The notable point about these remaining phyla is that most of these phylotypes occurred in a single location (TP). With phylotypes spanning 16 bacterial phyla this level of diversity approaches estimates collectively obtained for water, mat and sediment samples of Obsidian Pool in Yellowstone National Park reported by Hugenholtz et al. (1998), widely recognized as a highly diverse collection of thermal niches.

The near-full length 16S rRNA gene sequences were used to construct phylogenetic relationships for the most abundant phylotypes, namely, Chlorobi, Chloroflexi, Cyanobacteria and Proteobacteria. For the Chlorobi, the only ubiquitous phylum ubiquitous to mats, the tree topology illustrates that Tibetan isolates span most known lineages and also indicate possible new lineages (Fig. 2a). The molecular taxonomy of the Chlorobi is unfortunately not well resolved. It is, however, interesting that only two phylotypes were shared between locations. A similar pattern is revealed in the phylogeny for the Chloroflexi (Fig. 2b). Here, the Tibetan lineages span most of the known thermophilic clades. It is interesting that all *Roseiflexus* sequences affiliated only with others from Tibet, thus adding further support to the recently proposed 'T' lineage for this genus (Lau et al. 2006), which may be biogeographically determined. Some evidence for shallow structuring of tree topology with regard to location also existed within the Anaerolineales.

The cyanobacteria have been relatively well studied in terms of thermophilic phylogenies. The Tibetan sequences showed some structuring of tree topology according to location, they spanned all known *Synechococcus* lineages except for the A/B lineage which appears to be unique to YNP (Fig. 2c). It has been previously suggested that Tibetan geothermal springs represent a biodiversity hotspot for thermophiles (Lau et al. 2006), and the phylogenetic

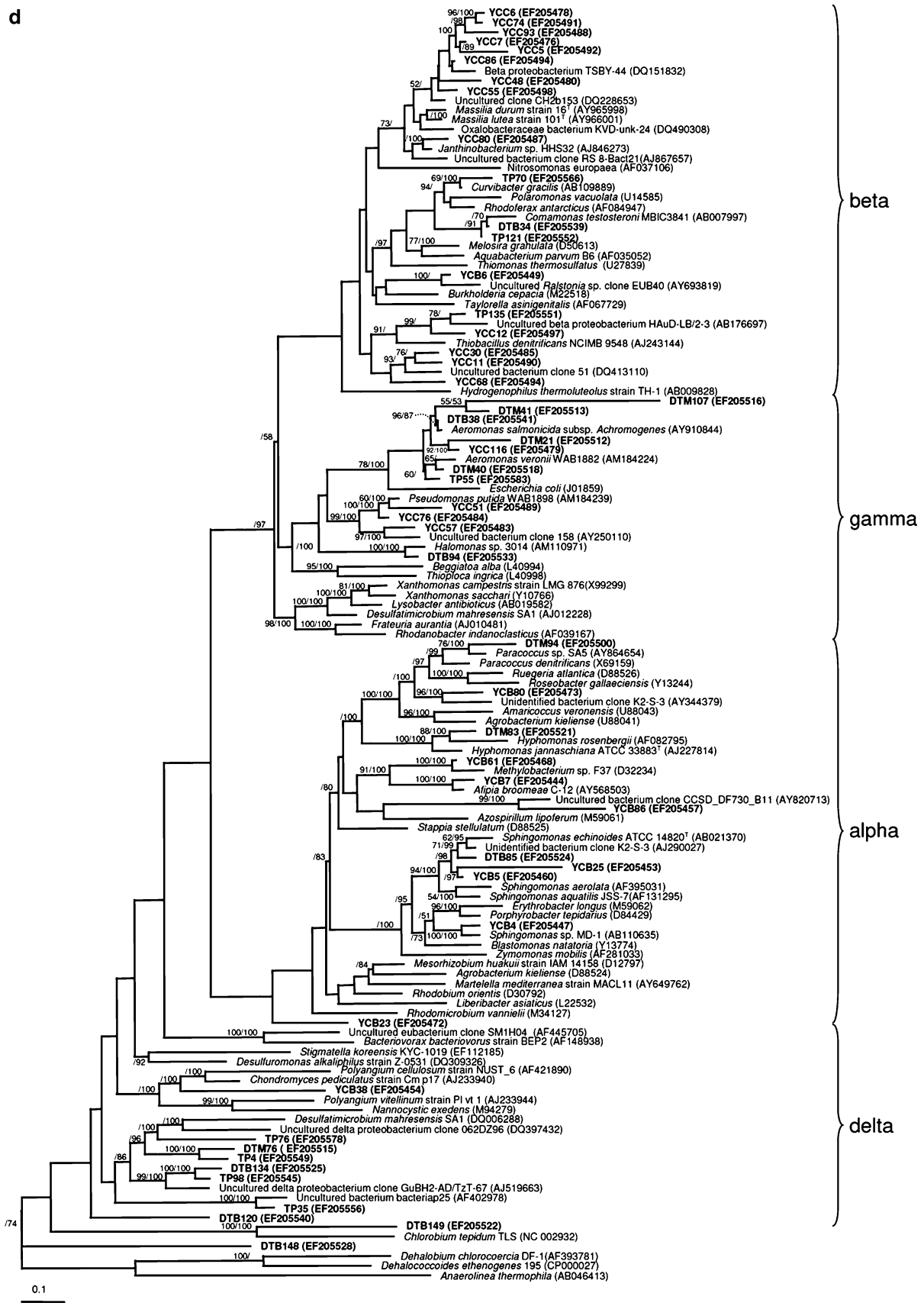


Fig. 2 continued

evidence in this study supports this notion. On a broader scale, the existence of *Synechococcus* phylotypes in hot springs from Tibet and other continents that were unique despite similar geochemical variables supports the existence of distance effects at an inter-continental scale, and so this adds to the growing body of evidence for microbial biogeography (Hughes Martiny et al. 2006).

The phylogeny for proteobacteria revealed considerable diversity among phylotypes (Fig. 2d). Despite some ambiguities related to the lack of sister sequences in the NCBI GenBank, phylotypes shared close affinity to those obtained from other thermal environments where they are known for a given lineage. Whilst it is not possible to conclude their thermophily or mode of metabolism from environmental sequences alone, closest phylogenetic affiliations were found with a number of physiological groups including C1 metabolizing taxa (DTM83), aerobic and anaerobic heterotrophs (most alpha, beta and gamma proteobacterial lineages), denitrifiers (DTM94) and sulphate reducers (delta proteobacterial lineages).

In conclusion, it is notable that whilst numerous qualitative diversity studies have been carried out on thermophilic mats, our study provides the first attempt to elucidate both diversity and frequency of occurrence for all bacterial taxa. This has yielded possible ranges for abundance of photoautotrophs, and also the finding that proteobacteria form a significant component of the mat community and may proliferate in post-disturbance conditions. Whilst abundance cannot be directly extrapolated to activity, it is reasonable to assume that in a stressful thermal environment few taxa are likely to exist in a dormant state for long periods and so these findings have ecological value. Furthermore, the data suggests a conservation imperative for hot springs, since communities in physico-chemically similar springs supporting similar levels of OTU richness were nonetheless phylogenetically distinct. Such heterogeneity is also of applied relevance given the focus of bioprospecting efforts in geothermal springs (Schiraldi and de Rosa 2002; Ferrer et al. 2007).

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