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ORIGINAL ARTICLE

Novel *Clostridium* populations involved in the anaerobic degradation of *Microcystis* blooms

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Understanding the microbial degradation of *Microcystis* biomass is crucial for determining the ecological consequences of *Microcystis* blooms in freshwater lakes. The purpose of this study was to identify bacteria involved in the anaerobic degradation of *Microcystis* blooms. *Microcystis* scum was anaerobically incubated for 90 days at three temperatures (15 °C, 25 °C and 35 °C). We used terminal restriction fragment length polymorphism (T-RFLP) analysis of bacterial *16S rRNA* genes, followed by cloning and sequencing of selected samples, to reveal the community composition of bacteria and their dynamics during decomposition. *Clostridium* spp. were found to be the most dominant bacteria in the incubations, accounting for 72% of the sequenced clones. Eight new clusters or subclusters (designated CLOS.1–8) were identified in the *Clostridium* phylogenetic tree. The bacterial populations displayed distinct successions during *Microcystis* decomposition. Temperature had a strong effect on the dynamics of the bacterial populations. At 15 °C, the initial dominance of a 207-bp T-RF (*Betaproteobacteria*) was largely substituted by a 227-bp T-RF (*Clostridium*, new cluster CLOS.2) at 30 days. In contrast, at 25 °C and 35 °C, we observed an alternating succession of the 227-bp T-RF and a 231-bp T-RF (*Clostridium*, new cluster CLOS.1) that occurred more than four times; no one species dominated the flora for the entire experiment. Our study shows that novel *Clostridium* clusters and their diverse consortiums dominate the bacterial communities during anaerobic degradation of *Microcystis*, suggesting that these microbes' function in the degradation process.
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Introduction

Cyanobacteria are a diverse and widely distributed group of organisms common in soil, marine and freshwater environments. In shallow eutrophic water bodies, they can reproduce rapidly under favorable conditions, forming dense concentrations called 'blooms' in aquatic environments (Paerl *et al.*, 2001; Paerl and Huisman, 2008). *Microcystis* spp. are the dominant species of *Cyanobacteria* in many shallow eutrophic lakes (for example, Chen *et al.*, 2003; Conley *et al.*, 2009). In these lakes, *Microcystis* blooms can be driven by the wind to accumulate near the shore or become trapped in emergent macrophytes, forming a dense scum that can be 10–30 cm in thickness (typical chlorophyll *a* concentration $>10\,000\,\mu g/l$). Dissolved oxygen (DO) is rapidly exhausted below the surface of the bloom, and the microenvironment quickly becomes anoxic, especially in the summer (Wang *et al.*, 2006). Abundant *Microcystis*-derived organic metabolites, anoxic conditions and moderate temperatures facilitate the decomposition of the *Microcystis* biomass below the air–bloom interfaces.

This anaerobic decomposition of organic material generally includes hydrolytic, homoacetogenic and methanogenic processes (for example, Rui *et al.*, 2009). Many obligate or facultative anaerobic microbes may be involved in the anaerobic fermentation. The growth and survival of cyanobacterial–bacterial mat consortia under extreme environmental conditions was reviewed in detail by Paerl *et al.* (2000). In addition, a few bacterial species that can lyse *Microcystis*, including *Alcaligenes denitrificans* and strains from the family *Cytophagaceae*, have been isolated from the polysaccharide capsule of *Microcystis* cells and even from water around *Microcystis* blooms (Manage *et al.*, 2000; Rashidan and Bird, 2001). However, little is known about which microbes participate in *Microcystis* degradation under anoxic conditions and their community dynamics during this process. To identify the bacterial communities associated with *Microcystis*

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hydrolysis and to characterize any variations among the dominant species during different phases of decomposition, *Microcystis* blooms were collected from Lake Taihu, China, and the anaerobic degradation process was allowed to proceed in airtight glass bottles for approximately 90 days at three different temperatures (15 °C, 25 °C and 35 °C), mimicking the seasonal fluctuations in subtropical Lake Taihu. The bacterial community compositions and their dynamics were determined by terminal restriction fragment length polymorphism (T-RFLP) analysis of bacterial *16S rRNA* genes, followed by cloning and sequencing of selected samples.

Materials and methods

DO dynamics in Microcystis *mats*
A near-shore study site with dense *Microcystis* blooms (Supplementary Figure S1) was selected to monitor the DO attenuation process in *Microcystis* mats. The blooms were thoroughly disturbed and allowed to stabilize before the first measurement. An YSI 550A DO meter was used to log data from the air–bloom interface to the algae–water interface at 15-min intervals for 2 h.

Anaerobic incubation of Microcystis *blooms*
A *Microcystis* bloom slurry was collected from Lake Taihu (120.19067°E, 31.51317°N; Supplementary Figure S1) and transported to the lab on ice. Airtight glass bottles (60 ml capacity; CNW Technologies GmbH, Düsseldorf, Germany) were filled with 30 ml of the *Microcystis* bloom slurry (equivalent to 4.6 g dry weight, initial pH 6.87). Eighty bottles were prepared for each temperature (15 °C, 25 °C and 35 °C), and the experiment lasted for more than 90 days in the dark. At each sampling point, triplicate bottles of each treatment were analyzed (Table 1). The *Microcystis* slurries were mixed thoroughly before taking samples, and 10 ml of the mixed slurry was put into a sterile tube, which was immersed in liquid nitrogen for 10 min and then frozen at −80 °C until DNA extraction. A pH meter was used to measure the pH of each slurry sample.

Measurement of methane gas
Gas samples (0.5 ml) were taken with syringes from the headspace of the incubation bottles before the caps were opened. CH_4 concentrations were analyzed using a gas chromatograph (VARIAN CP-3380; Varian Analytical Instruments, Palo Alto, CA, USA) equipped with flame ionization detector.

DNA extraction, PCR and T-RFLP analysis
One of the three parallel samples at each sampling point was randomly selected for genomic DNA extraction following the procedures described by (Groâkopf *et al.*, 1998). The final DNA pellets were resuspended in 50 μl of TE buffer (10 mM Tris, 1 mM EDTA; pH 8.0). The DNA concentration was checked by agarose gel electrophoresis.

The PCR amplification and terminal restriction fragment polymorphism (T-RFLP) analyses of bacterial *16S rRNA* gene fragments followed protocols described previously (Liu *et al.*, 1997). Briefly, the amplification of *16S rRNA* genes was performed using the primers 27f and 907r. The 5′ end of the forward primer was labeled with Cy5. Each 20-μl PCR reaction mixture contained 1 μl of DNA template (from a 1:100 dilution of the extracted genomic DNA), 10 μl of 10 × buffer (Fermentas, Burlington, Canada), 1.6 μl of 25 mM $MgCl_2$, 2 μl of 2.5 mM dNTPs (Fermentas), 1 μl of each 50 μM primer (Invitrogen, Shanghai Branch, China) and 1 U *Taq* DNA polymerase (Fermentas). The amplification program was 5 min at 94 °C; 30 cycles of 30 s at 94 °C, 45 s at 55 °C and 60 s at 72 °C; and finally 10 min at 72 °C. The Cy5-labeled PCR product was purified using an agarose gel DNA extraction kit (Takara, Takara Biotechnology (Dalian) Co. Ltd, Dalian, China) and digested at 37 °C for 5 h using *Hha*I (Fermentas). The digestion products were further purified using Sigma Spin Post-Reaction Clean-Up Columns (Sigma-Aldrich, St Louis, MO, USA) and were then size separated using a CEQ 8000 Genetic Analyzer (Beckman Coulter, Fullerton, CA, USA).

For each sample, the relative peak height over a threshold of 1.0% was determined. Then, T-RFs less than 55 bp or greater than 700 bp were discarded. The relative contribution of each T-RF peak area to the total peak area of each sample was calculated,

Table 1 All the revealed *Clostridium* clusters and their respective closest related taxonomically described species

New cluster ID	*Sequence number*	*T-RF length (bp)*	*Closest related taxonomically described species (EMBL accession number)*	*Maximum identity (%) (coverage, %)*
CLOS.1	14	231	*Clostridium lundense* strain DSM 17049 (AY858804)	93 (100)
CLOS.2	8	227	*Clostridium pascui* strain DSM 10365 (NR026322)	93 (100)
CLOS.3	6	223	*Clostridiaceae* bacterium A4d (AB081585)	97 (99)
CLOS.4	3	387	*Clostridium* sp. NML 04A032 (EU815224)	96 (98)
CLOS.5	3	747	*Oscillibacter valericigenes* (AB238598)	95 (100)
CLOS.6	14	386	*Oscillospiraceae* bacterium NML 061048 (EU149939)	97 (99)
CLOS.7	3	588	*Veillonellaceae* bacterium WK011 (AB298743)	99 (97)
CLOS.8	3	198	*Megasphaera paucivorans* strain VTT E-032341 (DQ223730)	95 (100)

Abbreviations: EMBL, European Molecular Biology Laboratory; T-RF, terminal restriction fragment.

and analysis thresholds were set at 1.0% of total peak area (Zhang *et al.*, 2008). T-RFs with an area less than the new threshold value for a sample were removed from the data set. The percentage values of the remaining T-RFs were used to set up data matrices for statistical analysis.

Cloning, sequencing and phylogenetic analysis of selected samples

Four libraries of *16S rRNA* genes were constructed using materials from a 0-day sample (F0), a 55-day sample at 25 °C (B21), a 12-day sample at 35 °C (C9) and a 60-day sample at 35 °C (C24). These time points were chosen to encompass a relatively high diversity of T-RF lengths and sampling times (Supplementary Table S1). PCR amplification of bacterial *16S rRNA* genes was performed using the primer set Bac27f/Bac1492r as described by Weisburg *et al.* (1991). PCR products were purified and ligated into the pGEM-T vector (Promega, Madison, WI, USA) following the manufacturer's instructions. Plasmids were transformed into *Escherichia coli* cells, and clones were randomly selected for sequencing. Sequencing was performed by Invitrogen (Shanghai Branch) using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and an ABI Prism 3730-Avant Genetic Analyzer (Applied Biosystems).

Sequences with a high probability of being chimeric were identified using the Mallard (Ashelford *et al.*, 2006) and Ribosomal Database Project II Chimera Check Program (Cole *et al.*, 2003), and all suspicious sequences were excluded from further analysis. The remaining sequences were compared with GenBank entries using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) to select reference sequences. A Ribosomal Database Project classifier was applied to assign our *16S rRNA* gene sequences to the taxonomical hierarchy proposed in Bergey's Manual of Systematic Bacteriology, 2nd Edition (Wang Q *et al.*, 2007). The coverage index of the clone libraries was estimated at 97% sequence similarity cutoff by the equation $C=(1-n_i/N) \times 100$, where N represents the number of sequences in the sample and n_i is the number of operational taxonomic units classified.

The reference sequences of *Clostridium* cluster I were obtained from Supplementary Figure S2 (web.biosci.utexas.edu/.../SUPPLEMENTARY%20FIG%20S2%20OCT%2029.pdf) in Iyer *et al.* (2008). The sequences affiliated with other *Clostridium* clusters were obtained from Additional file 3 (http://www.biomedcentral.com/content/supplementary/1471-2180-9-68-S3.pdf) in Krogius-Kurikka *et al.* (2009). The online MAFFT version 6.803b software (http://align.bmr.kyushu-u.ac.jp/mafft/online/server/) was used for the multiple alignments of our *Firmicute* sequences and the above references. The Ribosomal Database Project II online program was also used to compare the quality of these alignments, indicating only very minor differences between these different methods. Poorly aligned and very variable regions in the alignments were automatically removed using Gblocks (Castresana, 2000), with previously reported parameters (Xing *et al.*, 2009). A maximum-likelihood tree was built for *Firmicutes* using Paup 4.0b10 (Swofford, 1998). The model selected by Modeltest was (GTR + I + G), and the settings given by Modeltest were used to perform the maximum-likelihood analysis. Maximum parsimony with 1000 bootstrap replications (Felsenstein, 1984) and neighbor-joining with Jukes–Cantor distance correction (Kuhner and Felsenstein, 1994) were used to confirm the robustness of tree topology. When there was discrepancy between the maximum-likelihood topology and the neighbor-joining and maximum parsimony bootstrap analyses, the maximum-likelihood topology was favored as it is considered more robust (Tateno *et al.*, 1994). The complete picture of the genealogical relationships between the *Clostridia* was in accordance with previous reports (Collins *et al.*, 1994; Stackebrandt *et al.*, 1999).

Non-*Firmicute* sequences obtained in this work were aligned separately using Clustal X 1.8 (Thompson *et al.*, 1997). A neighbor-joining tree with Jukes–Cantor distance correction (Kuhner and Felsenstein, 1994) was built using MEGA version 4.0 (Tamura *et al.*, 2007).

Statistical analysis

To investigate the relationship between the incubation temperature, pH and major T-RF dynamics, a redundancy analysis was performed using CANOCO 4.0 for Windows (ter Braak and Verdonschot, 1995). The matrices generated from the relative peak areas of total samples were log $(x+1)$ transformed. Detrended correspondence analysis with detrending by segments was used to test the suitability of the weighted averaging techniques as opposed to linear methods. The results showed that the gradient length of the first axis in standard deviation units always was 2.0, confirming the suitability of weighted averaging-based techniques for analyzing our data. The significance of the relationship between the incubation temperature, pH and bacterial community composition was tested using Monte Carlo permutation tests (499 unrestricted permutations, $P<0.05$).

Nucleotide sequence accession numbers

The nucleotide sequences of the *16S rRNA* genes obtained in this study have been deposited in the GenBank database under accession numbers GU559750–GU559855. Detailed descriptions of these sequences were provided as well (Field *et al.*, 2008).

Results

DO profiles within Microcystis *blooms*

The DO concentration at the air–bloom interface decreased from 1.5 mg/l to approximately 0.2 mg/l

over 1 h of measurement. However, the DO at the bloom–water interface remained at 0.17 mg/l during the measurement period. A typical anaerobic environment formed rapidly in the mats (Supplementary Figure S2).

T-RFLP analysis. In total, 72 T-RFLP profiles were analyzed (Supplementary Table S1). Only those with a relative area percent (*Ap*) greater than 1% in at least one of 72 profiles were selected as the signature T-RFs for the analyses of bacterial dynamics. In total, 114 T-RFs were detected across the profiles.

Two types of significant successions of bacterial populations were identified at the three different temperatures during the 90-day incubations (Supplementary Figure S3). This first type was a single succession at 15 °C (Figure 1a). The early succession group 61- and 207-bp T-RFs (accounting for approximately 66% (*Ap*) at its maximum) experienced a rapid increase in relative abundance at the beginning of the experiment, followed by a sharp decrease over the later stages (Figure 1a). The relative abundance of the late succession 227-bp group gradually increased until approximately day 31, and then remained at high level until the end of the experiment. Other T-RFs (61, 223 and 231 bp) were relatively stable (around 10% of combined relative contribution) during the succession.

In contrast, an alternating succession occurred at 25 °C and 35 °C (Figures 1b and c). Unlike what was observed at 15 °C, two T-RFs, 227 and 231 bp, repeatedly dominated the communities (Figures 1b and c). Moreover, at 25 °C, we observed that a 386-bp T-RF always fluctuated concomitantly with the 227-bp T-RF, and we designated these two fragments 'group I'. When group I dropped to a low relative contribution, the 231-bp fragment peaked. However, the length of 231-bp T-RF dominance was obviously shorter than that of the 227-bp T-RF. At 35 °C, the 231-bp T-RF dominated in the first 5 days. Then, the 227-bp T-RF became dominant, followed by the alternating succession described above. The dominance time of the 231-bp T-RF at 35 °C was longer than that at 25 °C.

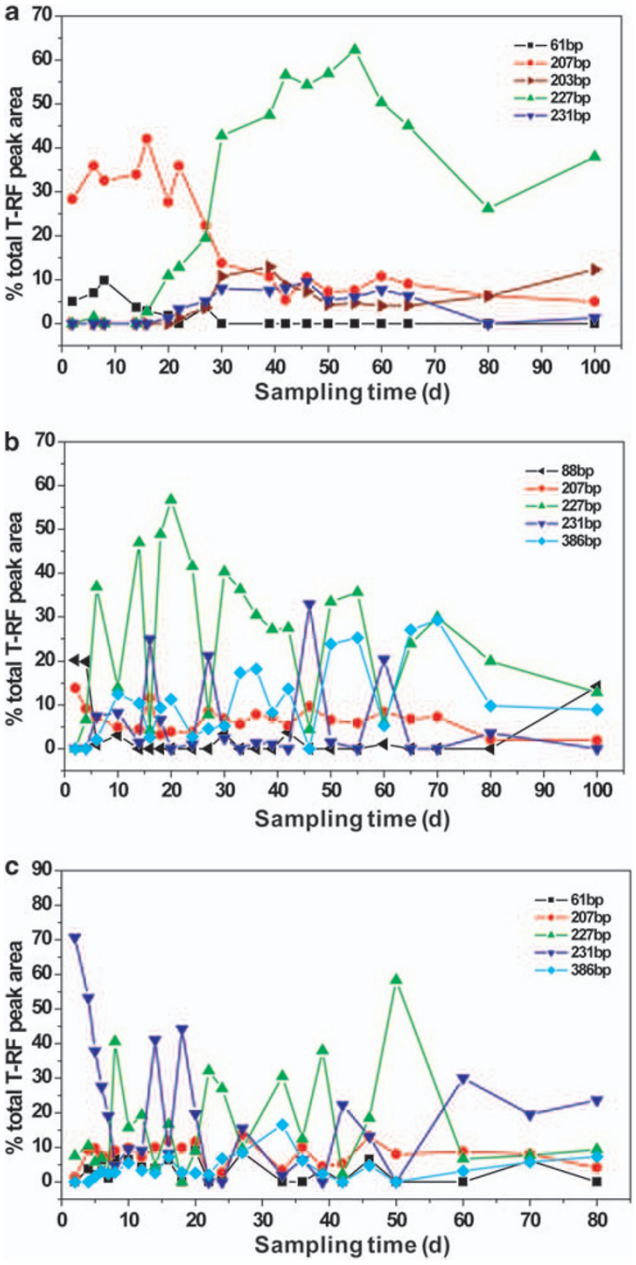


Figure 1 The relative abundance dynamics of major T-RFs in anaerobically incubated *Microcystis* blooms at 15 °C (**a**), 25 °C (**b**) and 35 °C (**c**), as a measure of the composition of the bacterial community by T-RFLP method.

Phylogenetic analysis and assignment of T-RFs

In total, 145 nearly complete *16S rRNA* gene sequences (about 1400 bp) were obtained from the four clone libraries (F0, B21, C9 and C24; Supplementary Table S1). The F0 library (starting point) contained mostly *Microcystis* sequences (Supplementary Table S2), with few sequences affiliated to *Clostridium* and *Proteobacteria*. The last three clone libraries were dominated by *Clostridium* sequences and had in general a good coverage of bacterial diversity (Supplementary Table S3). Thirty-six sequences, mainly from the F0 sample, were affiliated with *Microcystis* sp. As the *Hha*I restriction fragment was 978-bp long for this *Microcystis* sp., its sequences did not influence the relative contribution of the other phyla in the T-RFLP results. Furthermore, *Microcystis* sequences were excluded from the analyses because our aim was to determine heterotrophic bacterial dynamics during the incubation. Phylogenetic analyses of the remaining 105 sequences showed that *Firmicutes* was the most abundant group (76 out of 105 sequences), followed by *Proteobacteria* (22 sequences), *Bacteroidetes* (six sequences) and *Gemmatimonadetes* (one sequence) (see Figure 2 and Supplementary Figure S4). Both *in silico* analysis of 105 sequences and the T-RFLP fingerprinting

of representative clones was used to assign T-RFs to individual bacterial lineages. All the abundant T-RFs can be assigned from the three established clone libraries, confirming a good coverage of diversity by these libraries despite their limited sequence numbers. The 15 most abundant lineages are listed in Supplementary Table S4. Both the cloning method and T-RFLP analysis indicated a high proportion of *Clostridium* organisms in the incubation systems.

All of the *Firmicutes* sequences belonged to the genus *Clostridium*. Classification clusters were inherited from Collins *et al*. (1994). The vast majority of our *Clostridium* sequences (72.4%) fell into eight novel groups (CLOS.1–8; Figure 2), and each group represented 4–18% of the clone sequences. The 227-bp T-RF sequences (CLOS.2) formed a novel subcluster in cluster IF. Bootstrap values highly supported two novel clusters, CLOS.1 (231 bp) and CLOS.3 (223 bp), which were closely related to many existing subclusters in clusters I and II. Three novel subclusters, CLOS.4 (387 bp), CLOS.5 (747 bp) and CLOS.6 (386 bp), were embedded in *Clostridium* cluster IV. CLOS.7 (588 bp) and CLOS.8 (198 bp) were most closely affiliated with *Clostridium* cluster IX. As the clones in the eight major groups had less than 97% *16S rRNA* gene identity with their closest taxonomically named relatives (Table 1), all eight groups likely represent new bacterial species. The remaining 22 *Clostridium* sequences were distributed separately among the reference tree. Three clones belonged to *Clostridium* group IA, and three clones belonged to *Clostridium* group XIV. Eight non-clustered sequences belonged to *Clostridium* cluster IV. Another eight clones could not be assigned to any existing clusters or subclusters.

Although no obvious novel clusters could be identified from the non-*Clostridium* sequences (Supplementary Figure S4), there were still some representative T-RFs in each phylum. Six T-RFs (59, 61, 341, 513, 515 and 582 bp) belonged to *Alphaproteobacteria*. Two T-RFs (67 and 207 bp) were affiliated with *Betaproteobacteria* and *Gammaproteobacteria*, respectively. A 94-bp T-RF was the major representative of *Bacteroidetes*. Only one clone was affiliated with *Gemmatimonadetes*, and its corresponding T-RF length was 229 bp.

Statistical analysis

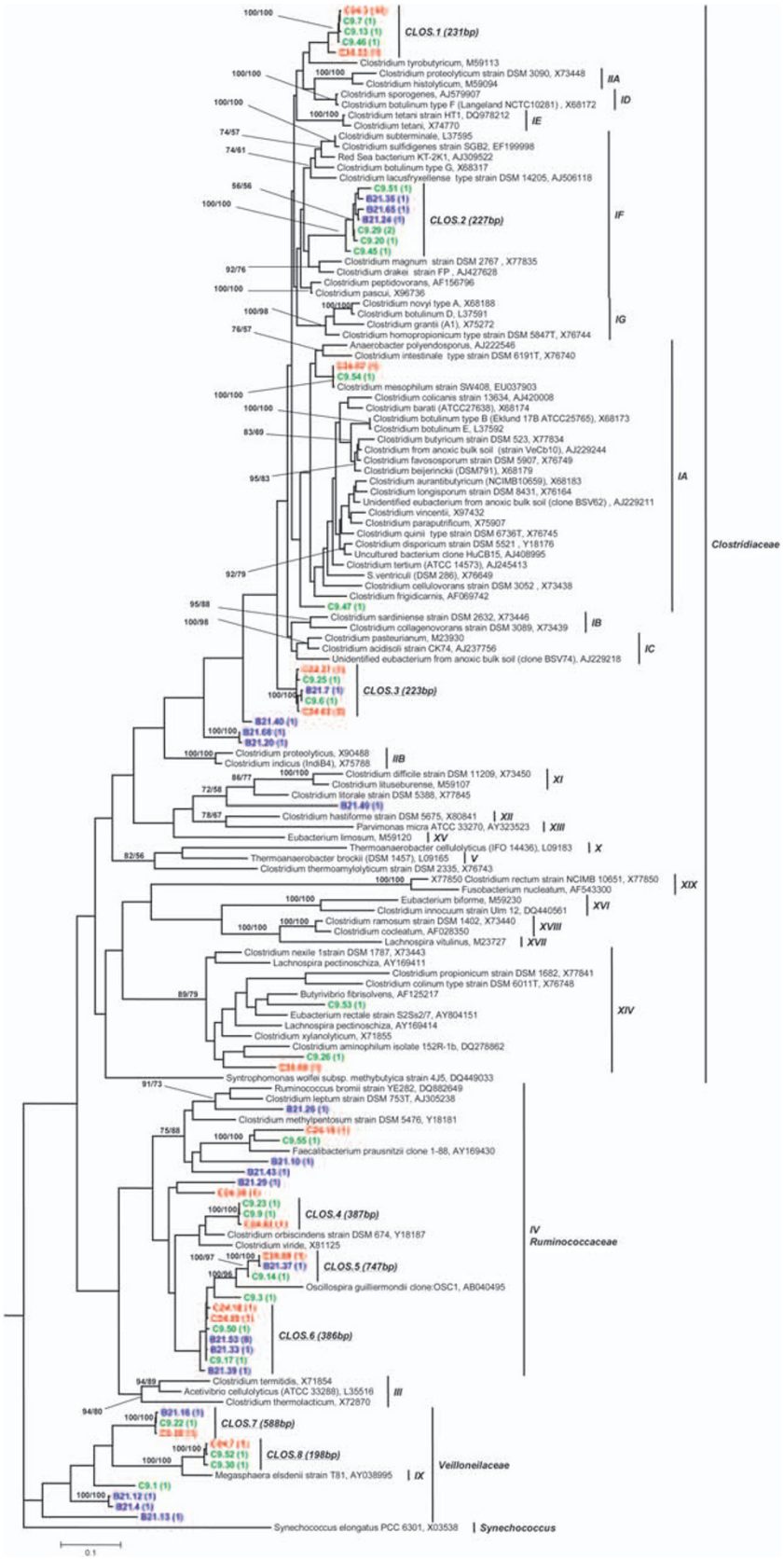
The pH dynamics were similar at 25 °C and 35 °C. After 20 days of incubation, the pH of the 15 °C system dropped from 6.87 to 5.43, while the pH of the 25 °C and 35 °C systems dropped to 5.86 and 5.88, respectively (Supplementary Figure S5).

Without considering the effect of time during the incubation, the influence of temperature and pH was evaluated for 19 abundant T-RFs using redundancy analysis methods (Figure 3a). The results indicated that the two factors accounted for 13.5% of species variation. Moreover, temperature had a significant effect on the fermentation flora ($P<0.01$). Among the abundant T-RFs, different heterotrophic bacteria appeared to be adapted to different conditions. Four of the new clusters (CLOS.1, 2, 3 and 8) seemed to be adapted to low temperature and low pH systems, whereas CLOS.4 (387 bp) and CLOS.7 (588 bp) responded actively to higher temperatures and higher pH. The major representative of *Proteobacteria* (207 bp) was obviously sensitive to temperature and pH changes. In addition, samples of three temperatures formed distinct groups in the two-dimensional space shaped by temperature and pH (Figure 3b). Samples obtained from the 15 °C incubation formed one cluster, while the 25 °C and 35 °C samples grouped together in a separate cluster.

Discussion

We designed experiments to investigate the composition of bacterial communities and their dynamics under anaerobic conditions at different temperatures. The results of our phylogenetic analysis indicated that 72% of the bacterial *16S rRNA* gene sequences obtained in this work was affiliated with genus *Clostridium*, which was also supported by the high relative contribution of their corresponding T-RFs (Supplementary Tables S1 and S4). The predominance of *Clostridium* suggests that these organisms are closely associated with the decomposition of *Microcystis* biomass. As members of the genus *Clostridium* are capable of forming endospores, they are ubiquitous and can be found in many extreme environments, such as the deep sea, hot springs and the intestinal tracts of animals (Jin *et al*., 1988; Tajima *et al*., 2000; Van Dyke and McCarthy, 2002; Verberkmoes *et al*., 2009). Furthermore, microorganisms affiliated with *Clostridium* have been found to be major players in the fermentation of organic substances. The absolute dominance of this genus was detected during the anaerobic fermentation of cellulose in a landfill leachate bioreactor (Burrell *et al*., 2004) and the

Figure 2 Maximum-likelihood dendogram of *Clostridium* with representative members of clusters I–XIX, based on *16S rRNA* gene sequences. Sequences from this work were in bold faces (numbers of identical sequences from the same library were shown within the parentheses), with red labels for C24 (35 °C 60-day sample) clone library, blue and green labels for C9 (35 °C 12-day sample) and B21 (25 °C 55-day sample) library, respectively. Clusters and subclusters were indicated by the vertical bars. Eight new clusters and subclusters were named by CLOS (with their corresponding T-RF length within the parentheses). Sequence of *Synechococcus* PCC6301 was used as outgroup. Bootstrap values (percentage of 1000 replicates) with corresponding significant (>50) were shown on the internal branches obtained from neighbor-joining and maximum parsimony methods or a '—' if not significant (<50). The bar represents 0.1 change per nucleotide pair.


CLOS.1 (231bp)
CLOS.2 (227bp)
CLOS.3 (223bp)
CLOS.4 (387bp)
CLOS.5 (747bp)
CLOS.6 (386bp)
CLOS.7 (588bp)
CLOS.8 (198bp)
Clostridium tyrobutyricum, M59113
Clostridium proteolyticum strain DSM 3090, X73448
Clostridium histolyticum, M59094
Clostridium sporogenes, AJ579907
Clostridium botulinum type F (Langeland NCTC10281) , X68172
Clostridium tetani strain HT1, DQ978212
Clostridium tetani, X74770
Clostridium subterminale, L37595
Clostridium sufidigenes strain SGB2, EF199998
Red Sea bacterium KT-2K1, AJ309522
Clostridium botulinum type G, X68317
Clostridium lacusfryxellense type strain DSM 14205, AJ506118
Clostridium magnum strain DSM 2767 , X77835
Clostridium drakei strain FP , AJ427628
Clostridium peptidovorans, AF156796
Clostridium pascui, X96736
Clostridium novyi type A, X68188
Clostridium botulinum D, L37591
Clostridium grantii (A1), X75272
Clostridium homopropionicum type strain DSM 5847T, X76744
Anaerobacter polyendosporus, AJ222546
Clostridium intestinale type strain DSM 6191T, X76740
Clostridium mesophilum strain SW408, EU037903
Clostridium colicanis strain 13634, AJ420008
Clostridium barati (ATCC27638), X68174
Clostridium botulinum type B (Eklund 17B ATCC25765), X68173
Clostridium botulinum E, L37592
Clostridium butyricum strain DSM 523, X77834
Clostridium from anoxic bulk soil (strain VeCb10), AJ229244
Clostridium favososporum strain DSM 5907, X76749
Clostridium beijerinckii (DSM791), X68179
Clostridium aurantibutyricum (NCIMB10659), X68183
Clostridium longisporum strain DSM 8431, X76164
Unidentified eubacterium from anoxic bulk soil (clone BSVI62) , AJ229211
Clostridium vincentii, X97432
Clostridium paraputrificum, X75907
Clostridium quinii type strain DSM 6736T, X76745
Clostridium disporicum strain DSM 5521 , Y18176
Uncultured bacterium clone HuCB15, AJ408995
Clostridium tertium (ATCC 14573), AJ245413
S.ventriculi (DSM 286), X76649
Clostridium cellulovorans strain DSM 3052 , X73438
Clostridium frigidicarnis, AF069742
Clostridium sardiniense strain DSM 2632, X73446
Clostridium collagenovorans strain DSM 3089, X73439
Clostridium pasteurianum, M23930
Clostridium acidisoli strain CK74, AJ237756
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Clostridium proteolyticus, X90488
Clostridium indicus (IndiB4), X75788
Clostridium difficile strain DSM 11209, X73450
Clostridium lituseburense, M59107
Clostridium litorale strain DSM 5388, X77845
Clostridium hastiforme strain DSM 5675, X80841
Parvimonas micra ATCC 33270, AY323523
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Thermoanaerobacter cellulolyticus (IFO 14436), L09183
Thermoanaerobacter brockii (DSM 1457), L09165
Clostridium thermoamylolyticum strain DSM 2335, X76743
X77850 Clostridium rectum strain NCIMB 10651, X77850
Fusobacterium nucleatum, AF543300
Eubacterium biforme, M59230
Clostridium innocuum strain Ulm 12, DQ440561
Clostridium ramosum strain DSM 1402, X73440
Clostridium cocleatum, AF028350
Lachnospira vitulinus, M23727
Clostridium nexile 1strain DSM 1787, X73443
Lachnospira pectinoschiza, AY169411
Clostridium propionicum strain DSM 1682, X77841
Clostridium colinum type strain DSM 6011T, X76748
Butyrivibrio fibrisolvens, AF125217
Eubacterium rectale strain S2Ss2/7, AY804151
Lachnospira pectinoschiza, AY169414
Clostridium xylanolyticum, X71855
Clostridium aminophilum isolate 152R-1b, DQ278862
Syntrophomonas wolfei subsp. methybutyica strain 4J5, DQ449033
Ruminococcus bromii strain YE282, DQ882649
Clostridium leptum strain DSM 753T, AJ305238
Clostridium methylpentosum strain DSM 5476, Y18181
Faecalibacterium prausnitzii clone 1-88, AY169430
Clostridium orbiscindens strain DSM 674, Y18187
Clostridium viride, X81125
Oscillospira guilliermondii clone:OSC1, AB040495
Clostridium termitidis, X71854
Acetivibrio cellulolyticus (ATCC 33288), L35516
Clostridium thermolacticum, X72870
Megasphaera elsdenii strain T81, AY038995
Synechococcus elongatus PCC 6301, X03538
IA
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Veilloneilaceae
Synechococcus
0.1

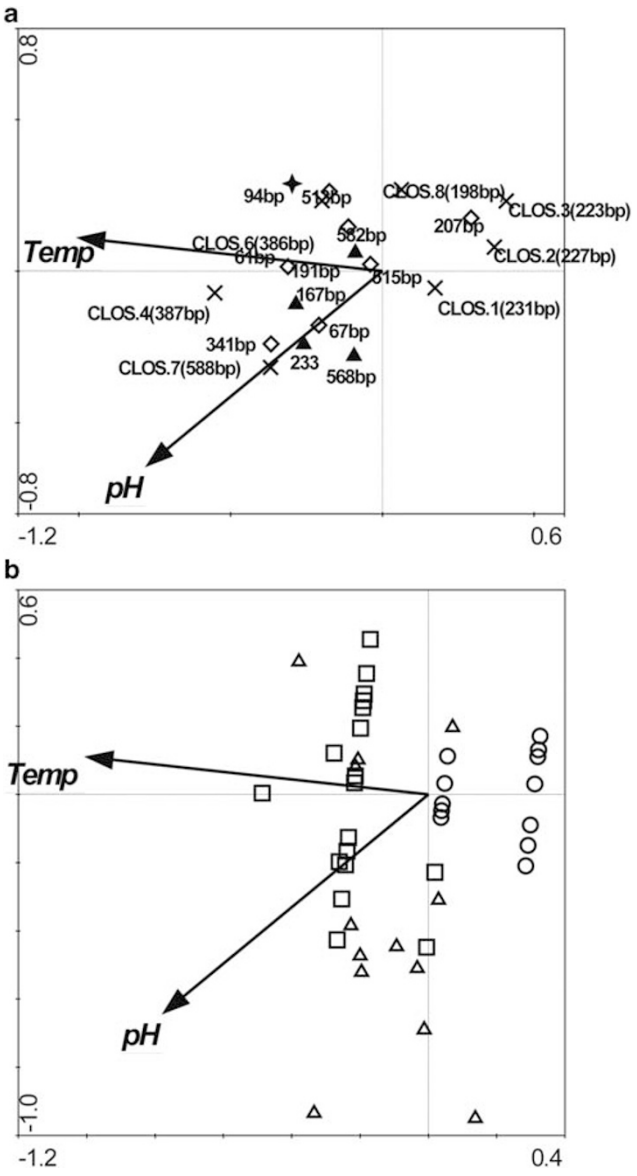

Figure 3 Temperature and pH adaptations for the 19 identified abundant T-RFs (**a**), and for samples (**b**) under three temperature specified by a redundancy analysis (RDA). Four groups of T-RFs were classified, '×'standing for new clusters in *Clostridium*, '▲' for other *Clostridium* found in work, '□' for *Proteobacteria* and '×' for *Bacteroidetes*. Samples were also specified by different symbols, '○' for 15 °C samples, '□' for 25 °C samples and '△' for 35 °C samples.

production of hydrogen from heat-treated activated sludge (Wang X *et al*., 2007).

It is interesting that so abundant *Clostridium* spp. could coexist in the *Microcystis* hydrolysis system. We speculate that the divergence of niches, such as substance utilization and adaptation to environmental conditions (Macalady *et al*., 2008), could allow for this situation. Generally, genotypic diversification (Schauer *et al*., 2005) and even only phenotypic diversification (Acinas *et al*., 2009) can lead to bacterial niche divergence. Our phylogenetic analysis showed that genetic polymorphisms were evident among the *Clostridium* sequences, in which eight new clusters and subclusters (CLOS.1–8) and 18 singletons could be identified. This polymorphism could be the genetic basis for niche divergence, which may be reflected as diversified substrate demands. Moreover, members of the genus *Clostridium* are originally metabolically versatile. Previous studies have shown that isolated *Clostridium* species are able to utilize various substrates, ranging from those with a high molecular weight and a complex structure (Lynd *et al*., 2002; Murashima *et al*., 2002) to those with a low molecular weight and a simple structure (Ren *et al*., 2006; Seedorf *et al*., 2008). For example, *Clostridium acetireducens* sp. is an amino-acid-oxidizing, acetate-reducing anaerobic bacterium (Örlygsson *et al*., 1996), whereas *Clostridium thermocellum* is a cellulose-hydrolyzing anaerobic bacterium (Johnson *et al*., 1982). Furthermore, *Microcystis* cells contain abundant intracellular and extracellular polysaccharides, proteins, cellulose and nucleic acids, all of which could meet the needs of various *Clostridium* populations.

Besides the potential divergence of substance utilization, the novel clusters displayed distinct temperature and pH adaptabilities, with significant differences in temperature fitness. This variation is another important characteristic that could permit the coexistence of so close relatives. Temperature adaptability is likely the result of regulation of enzymatic activity, which might directly influence the efficiency of the biochemical pathways involved in *Microcystis* decomposition (Sridhar *et al*., 2000; Yokoyama *et al*., 2007).

During the anaerobic incubation of the *Microcystis* slurry, *Clostridium* species appear to have the collaborative ability to hydrolyze initial substrates and produce low molecular weight intermediates, such as acetate and ethanol, and even directly generate biohydrogen gas (Chang *et al*., 2006; Yokoyama *et al*., 2007; Seedorf *et al*., 2008). The occurrence of these processes in the fermentation systems was confirmed by the presence of methanogenic Archaea (our unpublished data), which anaerobically utilize low molecular weight intermediates (for example, acetate or H_2/CO_2) to produce CH_4 (Supplementary Figure S6). Furthermore, in this work, two dominant clusters succeeded each other more than four times during the incubation (at 25 °C and 35 °C), which is uncommon for bacterial communities in closed systems (Rui *et al*., 2009). The diverse *Clostridium* clusters could also have some degree of competition, especially at higher temperatures.

Our study shows that novel *Clostridium* clusters and their multiple, diverse consortiums dominate the bacterial communities during anaerobic degradation of *Microcystis*. These results indicate that the novel *Clostridium* clusters are likely involved in the hydrolysis of *Microcystis* biomass. This involvement differs from that of *Clostridium* in single cellulose degradation in terrestrial environments and manually controlled bioreactors. We were also able

to conclude that these microbes play a crucial role in the degradation process that leads to the metabolites CH_4. However, further studies are needed to characterize in detail the intermediate products and potential hydrolysis pathway for anaerobic fermentation of *Microcystis*. Pure cultures of the *Clostridium* strains present in these anaerobic systems will be isolated to facilitate these studies.

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