

Enrichment and characterization of an anaerobic cellulolytic microbial consortium SQD-1.1 from mangrove soil

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Abstract Enrichment of microbial consortia provides an approach to simulate and investigate microbial communities in natural environments. In this study, a cellulolytic microbial consortium SQD-1.1 was enriched from mangrove soil of Qinglan port (Hainan, China) by 27 times continuous subcultivation under anaerobic static conditions. The consortium could completely degrade 0.2 % (w/v) filter paper within 3 days and utilized it as the sole carbon source. PCR-denaturing gradient gel electrophoresis analysis revealed a stable microbial community structure in the incubation process of 10 days and in the procedure of subcultivation. Twenty-four operational taxonomic units belonging to seven phyla were obtained from the full-length 16S rRNA gene library. Five clones, closest related to the genera *Alkaliflexus*, *Clostridium*, *Alistipes*, *Spirochaeta*, and *Trichococcus*, were the predominant ones. Among them, M117, phylogenetically showing high similarity (16S rRNA gene identity, 95.3 %) with the cellulolytic anaerobic bacterium *Clostridium straminisolvens* CSK1^T, was the potential key cellulolytic bacterium. Using the plate cultivation method, 12 strains, including one potential new species and four potential new species of new genera, were isolated. The strain P2, corresponding to the most frequently detected clone (M05) in the

16S rRNA gene library, showed both CMCase and xylanase activity and may be another important cellulolytic bacterium. The findings of cellulase activity in cell pellet and cohesion and dockerin domains in metagenome data further suggested the potential of utilization of cellulosomes by the consortium to degrade cellulose. Consortium SQD-1.1 provides a candidate for investigating the mechanism of cellulose degradation under anoxic conditions in natural environments.

Keywords Microbial consortia · Cellulolytic bacterium · Mangrove · Cellulosome · Cellulase

Introduction

Lignocellulose is a primary product of photosynthesis in terrestrial environments and one of the most abundant carbon resources over the world. Because of the high worldwide demand for energy and the uncertain petroleum sources, development of alternative energy has been resurged. In response, many countries have initiated extensive research and programs in bioconversion of lignocellulosic biomass into energy such as bio-ethanol (Faaij 2006). Effective conversion of lignocellulose to fermentable sugars for energy production mainly requires three steps: size reduction, pretreatment, and enzymatic hydrolysis. Among them, hydrolysis of lignocellulose by microorganisms and cellulases represents the greatest challenge (Percival Zhang et al. 2006).

Bio-hydrolysis and utilization of cellulose are carried out mainly by microorganisms including bacteria and fungi (Lynd et al. 2002). A wide spectrum of fungi, such as *Trichoderma* spp. and *Aspergillus* spp., can secrete all of three types of cellulases to hydrolyze cellulose (Mach and Zeilinger 2003; Villena and Gutiérrez-Correa 2006). Most of the aerobic bacteria only secreted endoglucanases to hydrolyze amorphous cellulose (Lynd et al. 2002). Alternatively, some anaerobic

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bacteria, such as *Clostridium thermocellum* and *Clostridium cellulolyticum*, can hydrolyze cellulose using their complex cellulase system, known as cellulosome (Doi and Kosugi 2004). However, cellulose is decomposed through the cooperation of many microbes in natural environments (Warnecke et al. 2007; Hess et al. 2011), which could keep stable and show higher cellulolytic efficiency than the individual strains. Cellulolytic microbial consortia functioning similarly to those in natural environments can be enriched by incubation and successive subcultivation. Haruta et al. (2002) bred a stable microbial community using composting materials, which could degrade rice straw with high efficiency. Using the similar method, Wongwilaiwalin et al. (2010) enriched another thermophilic lignocellulose-degrading microbial consortium. Feng et al. (2011) also constructed a microbial consortium from woodland soils using filter paper as substrate, which could efficiently degrade raw corn stover powder. These examples as well as other studies describing enriched consortia now have been reviewed by Zuroff and Curtis (2012).

Mangrove ecosystems are diverse intertidal wetlands commonly situated in tropical, subtropical, and temperate coastal systems. The diversity of microorganisms in these systems is expected to be high due to a combination of the mixing of seawater and freshwater and the resuspension of sediments and particles from many sources (Hyde and Lee 1995; Yan et al. 2006; Hong and Yan 2008). The unique swampy, saline, and partially anaerobic environment makes it a special source for isolating microorganisms as well as mining enzyme and gene resource (Jiang et al. 2006). Cellulolytic microbes are important members in these systems because of the presence of lignocellulose such as fallen leaves of mangrove trees (Pointing et al. 1999; Jiang et al. 2006; Gao et al. 2010). In this study, an anaerobic cellulolytic microbial consortium, SQD-1.1, was enriched using mangrove soils collected from Qinglan port (Hainan, China) by 27 times continuous subcultivation. Structure dynamics of the consortium was analyzed by denaturing gradient gel electrophoresis (DGGE) to assess its stability during the cellulose degradation procedure, and microbial diversity was checked by construction of 16S rRNA gene library and isolation of single strains to find the key cellulolytic microbes. Metagenomic analysis was further employed for mining cellulolytic enzyme resources and deeply understanding cellulolytic mechanism of the anaerobic consortium.

Materials and methods

Samples and media

Mangrove soil samples were collected from the Qinglan and Dongzhai ports in Hainan province of China. Polyvinyl

chloride pipes (400×Ø60 mm) were pushed deep into mangrove soil. After soil filled the pipes, both terminuses of the pipes were sealed, which could keep soil under almost the same condition with that in the mangrove environment. The pipes were then immediately transported back to the laboratory and anaerobically stored at 4 °C. The cellulose substrate, filter paper slurry, was prepared by wet-ball milling Whatman No. 1 filter paper (Sigma, USA) according to the method mentioned previously by Leschine and Canale-Parola (1983). S-FP medium for enrichment and subcultivation of cellulolytic consortia was composed of the follows (per liter): 1.0 g yeast extract, 1.0 g tryptone, 5 g NaCl, 0.7 g KCl, 6.3 g MgSO₄·7H₂O, 4.4 g MgCl₂·6H₂O, 1.2 g CaCl₂·2H₂O, 0.2 g NaHCO₃, 2 g filter paper slurry, 0.5 g Na₂S, 0.5 g Cys-HCl, and 1.0 mg Resazurin. S-CB medium for isolation of single strains was modified S-FP medium, in which filter paper was replaced with cellobiose. 4S medium was as follows (per liter): 0.5 g Na₂S, 0.5 g Cys-HCl, 1.0 mg Resazurin, and 250 ml artificial sea water [2 % NaCl, 0.3 % MgCl₂·6H₂O, 0.6 % MgSO₄·7H₂O, 0.1 % (NH₄)₂SO₄, 0.02 % NaHCO₃, 0.03 % CaCl₂, 0.05 % KCl, 0.042 % K₂HPO₄, 0.005 % NaBr, and 0.003 % SrCl₂·6H₂O]. 4SF was modified 4S medium, in which 2 g filter paper slurry was added. All the media were adjusted to pH 6.7, dispensed into serum bottles or Hungate's tubes under N₂ gas flow, sealed with butyl rubber stoppers, and autoclaved at 121 °C for 20 min.

Consortium enrichment and cellulose degradation efficiency evaluation

The whole operations for enrichment and subcultivation of microbial consortia were performed under anoxic conditions in a Forma 1025/1029 Anaerobic Chamber (Thermo Scientific, Marietta, OH, USA) with the gas phase N₂/CO₂/H₂=85:5:10. Approximately 5 g of mangrove soil sample at each site was transferred into a serum bottle containing 50 ml 4S medium and shaken for 1 h. Five milliliters of the suspension was inoculated into 50 ml S-FP medium and incubated statically at 30 °C for 2 weeks. A volume of 0.5 ml of each positive culture, in which filter paper was completely decomposed, was subinoculated into 5 ml S-FP medium in Hungate's tubes. Positive samples were continuously subinoculated using fresh S-FP medium in order to construct stable cellulolytic consortia. A stable cellulolytic consortium SQD-1.1 was enriched after subcultivation for 27 times. For evaluation of the cellulose degradation efficiency of the consortium, the culture of SQD-1.1 was inoculated into 10 Hungate's tubes with S-FP medium. Time-course degradation dynamics of filter paper slurry were observed in each day. Meanwhile, consortium SQD-1.1 was inoculated into 4SF medium and subcultivated for seven times to assess the efficiency of

the filter paper degradation when using cellulose as the sole carbon source.

Structure dynamics of the microbial consortium

Total genomic DNA from the 1-week-incubation cultures in the subcultivation procedure and from the cultures in each day of the 27th subcultivation were extracted using the modified cetyltrimethylammonium bromide/NaCl (CTAB/NaCl) method (Syn and Swarup 2000): 3 ml of cultures was collected and centrifuged to discard the suspension; 567 μ l ELB buffer (20 mM Tris-HCl, 12 mM EDTA, 1.2 % Triton X-100, and 0.5 % lysozyme) was added to the pellet and the mixture was incubated at 37 °C for 1 h; another 30 μ l of 10 % sodium dodecyl sulfate and 3 μ l of 20 mg/ml proteinase K were added; the same protocol of the CTAB/NaCl method was used in the following procedure. The DNA samples were used to amplify V3 regions of the 16S rRNA gene sequence corresponding to positions 341–534 in *Escherichia coli* (Muyzer et al. 1993) using PrimerSTAR HS DNA polymerase (TaKaRa, Japan) according to the manufacturer's protocol. The primers were V3-F with GC clamp (5'-CGCCCCCGCGCGCGGCGGGCGGGGCGGGGGCA CGGGGGGCTACGGGAGGCAGCAG-3') and V3-R (5'-ATTACCGCGGCTGCTGG-3'). The temperature profile consisted of 20 cycles of 10 s of denaturation at 98 °C, 30 s of annealing at 65 °C for the first cycle with a touchdown of 0.5 °C per cycle, and 60 s of extension at 72 °C, followed by 10 cycles of 10 s at 98 °C, 30 s at 55 °C, 60 s at 72 °C, and ended by a final extension at 72 °C for 10 min. PCR product (200 μ l) of each sample was concentrated to 15 μ l, which was examined by electrophoresis on 2 % agarose gel and adjusted to be equal. The quantified products were loaded on 8 % (w/v) polyacrylamide gel. DGGE was performed with a DCode Universal Mutation Detection System (Bio-Rad, Hercules, CA, USA). The denaturing gradient range was 30–60 %. It initially ran at 30 V for 30 min and then 130 V for 4.5 h. The gels were stained with ethidium bromide for 15 min. The band patterns on gels were checked to analyze the dynamics of the microbial consortium structures in the incubation process of 10 days and in the procedure of subcultivation.

Analysis of microbial consortium diversity

16S rRNA gene library was constructed according to the method described by McKew et al. (2007). Nearly complete 16S rRNA gene sequences were amplified by PCR from genomic DNA of the microbial consortium using universal primers 27F and 1492R (Weisburg et al. 1991). They were purified and ligated into pMD18-T (TaKaRa, Japan). The ligation mixture was transformed into *E. coli* DH5 α (TaKaRa, Japan) to generate a gene library. V3 regions of

16S rRNA gene were amplified from clones in the library and analyzed by DGGE. Clones, which were assigned to different locations on DGGE gels, were chosen to be sequenced. Meanwhile, 100 clones were selected randomly and sequenced for calculating the occurrence frequency of each ribotype. The nearly full-length 16S rRNA gene sequences were compared with sequences from GenBank using the BLASTN program (<http://blast.ncbi.nlm.nih.gov/Blast/>) to identify their closely related sequences. Chimeric sequences were preidentified by Mallard and Pintail programs (Kevin et al. 2005; Kevin et al. 2006). Pairwise alignments were carried out using the EzTaxon-e program (Kim et al. 2012). To test the evolutionary relationships, phylogenetic analysis was performed with the program MEGA 4.0 (Tamura et al. 2007). Multiple alignments of the sequences were performed using CLUSTAL W (Thompson et al. 1994). Distance matrices were calculated according to the Kimura's two-parameter correction model (Kimura 1980). Phylogenetic trees were inferred using neighbor joining (Saitou and Nei 1987). Bootstrap values were determined based on 1,000 replications.

Isolation of single strains and enzyme activity evaluation

Isolation of single strains was performed under anoxic conditions as mentioned above. When filter paper was partially degraded, the cultures of microbial consortium were diluted with S-CB medium and plated onto solid S-CB plate. The plates were incubated at 30 °C for 1–7 days. Colonies that showed different phenotypic characters were chosen and subinoculated for five times. The purified strains were phylogenetically identified by the 16S rRNA gene sequences. The isolated strains were inoculated into S-FP medium in Hungate's tubes and incubated for 7 days. The suspensions of cultures were used for enzyme activity evaluation. CMCase activity was assayed by Congo red overlay method using carboxymethylcellulose sodium as substrate (Teather and Wood 1982). For xylanase activity analysis, the cultures were mixed with equal volume 1 % oat spelt xylan in 50 mM phosphate buffer (PB, pH 7.0) and reducing sugar was detected by the dinitrosalicylic acid method (Miller 1959).

Validation of the existence of cellulosome-like complex

Cultures of the consortium incubated for 1 week were collected and centrifuged to discard the suspension. The residual cells were repeatedly washed for four times with 50 mM PB (pH 7.0). The discarded suspension, washing buffers, and the residual cells were dropped onto agar plate containing carboxymethylcellulose sodium and assayed for cellulase activity using Congo red overlay method (Teather and Wood 1982). Strain G21, which only produced an

extracellular endoglucanase, was used as the negative control (Gao et al. 2010).

Mining of cohesion and dockerin domains from metagenome sequences

Total metagenomic DNA was extracted from consortium SQD-1.1 and purified as described above. DNA library with insert sizes of 100–200 bp was constructed and subjected to Illumina GA for paired-end sequencing (2×75 bp). The reads were trimmed with a minimum quality score of 20 and a minimum read length of 50 bp using the toolkit SolexaQA (Cock et al. 2010). Then, paired-end reads were overlapped into environment gene tags (EGTs) with a minimum match length of 5 bp and minimum read length of 100 bp. The EGTs were annotated by Blastx search against Pfam database with e value lower than $1E-1$ (Punta et al. 2012). Cohesin and dockerin domains were mined from the annotation results, which were further certified by hmmscan search against Pfam-A (Finn et al. 2011). Only sequences that matched the best hits with e value lower than $1E-4$ by hmmscan were considered as cohesin and dockerin domains. The confirmed domains were further analyzed by phmmer to find the closest related proteins in the PFAM database (Finn et al. 2011).

Nucleotide sequence accession numbers

16S rRNA gene sequences from the gene library and isolated strains have been submitted to GenBank database under accession numbers HQ697913, HQ697914, and JN688020 to JN688053, respectively.

Results

Enrichment of consortium SQD-1.1 and evaluation of cellulose degradation efficiency

For enrichment of cellulolytic consortia, 14 soil samples were collected. After incubation in S-FP medium for 2 weeks under anaerobic static conditions, 11 cultures showed the capability of decomposing filter paper. These

positive cultures were chosen for successive subcultivation using the same medium. After subcultivation for 27 times, three of them still showed stable cellulolytic activity. Consortium SQD-1.1, which exhibited the highest efficiency on filter paper degradation, was chosen for further study. Time-course dynamics of cellulose degradation by consortium SQD-1.1 was performed using 0.2 % of filter paper slurry as substrate (S-FP medium). Filter paper was obviously decomposed after incubation for 2 days and almost completely degraded after 3 days (Fig. 1), which implied its high efficiency for cellulose degradation. Meanwhile, cellulose utilization by consortium SQD-1.1 was also analyzed in 4SF medium, in which cellulose was the sole carbon source. Filter paper could be degraded efficiently even after successive subcultivation for seven times, but more than 1 week was needed for complete decomposition. The degradation of filter paper in 4SF medium further revealed that consortium SQD-1.1 could utilize cellulose as the sole carbon source.

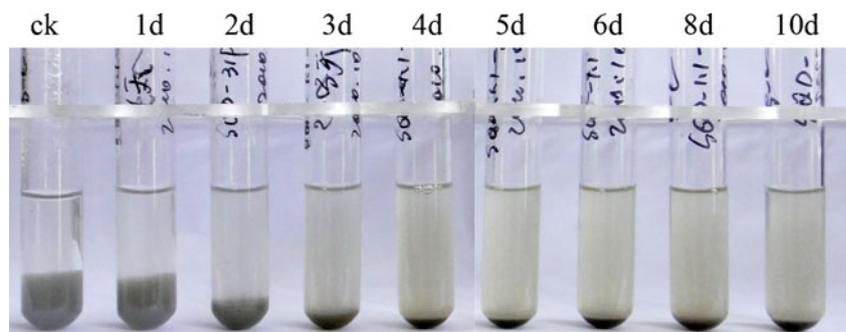
Dynamics of the microbial community structure of consortium SQD-1.1

Time-course dynamics of the microbial structure of consortium SQD-1.1 were analyzed by DGGE gels (Fig. 2a). More than 20 bands were found on the gels through the whole cultivation procedure, indicating a complex microbial community. However, DGGE patterns of each gel lane corresponding to each-day samples of the 27th subcultivation were similar, and no obvious differences were found. Meanwhile, dynamics of the microbial structure in the subcultivation procedure were also analyzed using the samples from the 8th, 19th, 21th, 22th, and 27th subcultivations (Fig. 2b). Several weak bands among these samples were changing. However, the main bands of each sample, which stood for the dominant members of the consortium, were almost the same. These results indicate that consortium SQD-1.1 was structurally stable even after continuous subcultivation for 27 times.

Microbial diversity of consortium SQD-1.1

By construction of 16S rRNA gene library and screening distinct clones on the DGGE gels, 24 operational taxonomic

Fig. 1 Time-course degradation of filter paper slurry by consortium SQD-1.1. *ck* the control S-FP medium that was not inoculated with consortium SQD-1.1; *1d–10d* the S-FP media incubated for 1–10 days after being inoculated with consortium SQD-1.1



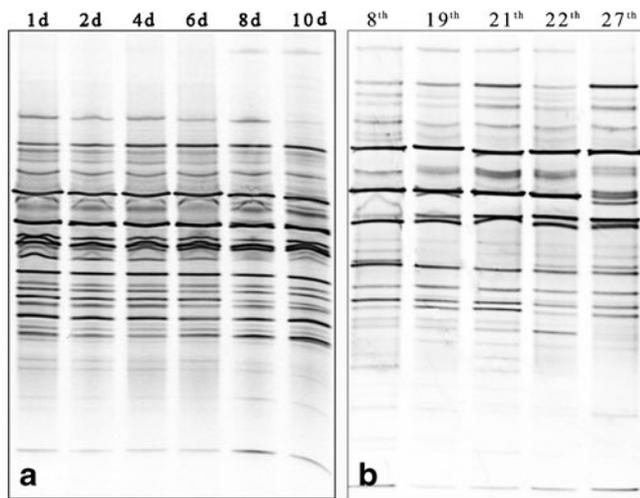


Fig. 2 Dynamics of the microbial structures of consortium SQD-1.1 performed by DGGE using the V3 regions of 16S rRNA gene. **a** Time-course dynamics of the structures of consortium SQD-1.1 within 10 days. 1d–10d the samples of consortium SQD-1.1 incubated for 1–10 days. **b** Dynamics of the structures of consortium SQD-1.1 in the procedure of successive subcultivation. 8th–27th the samples of consortium SQD-1.1 that has been sub-inoculated for 8–27 times

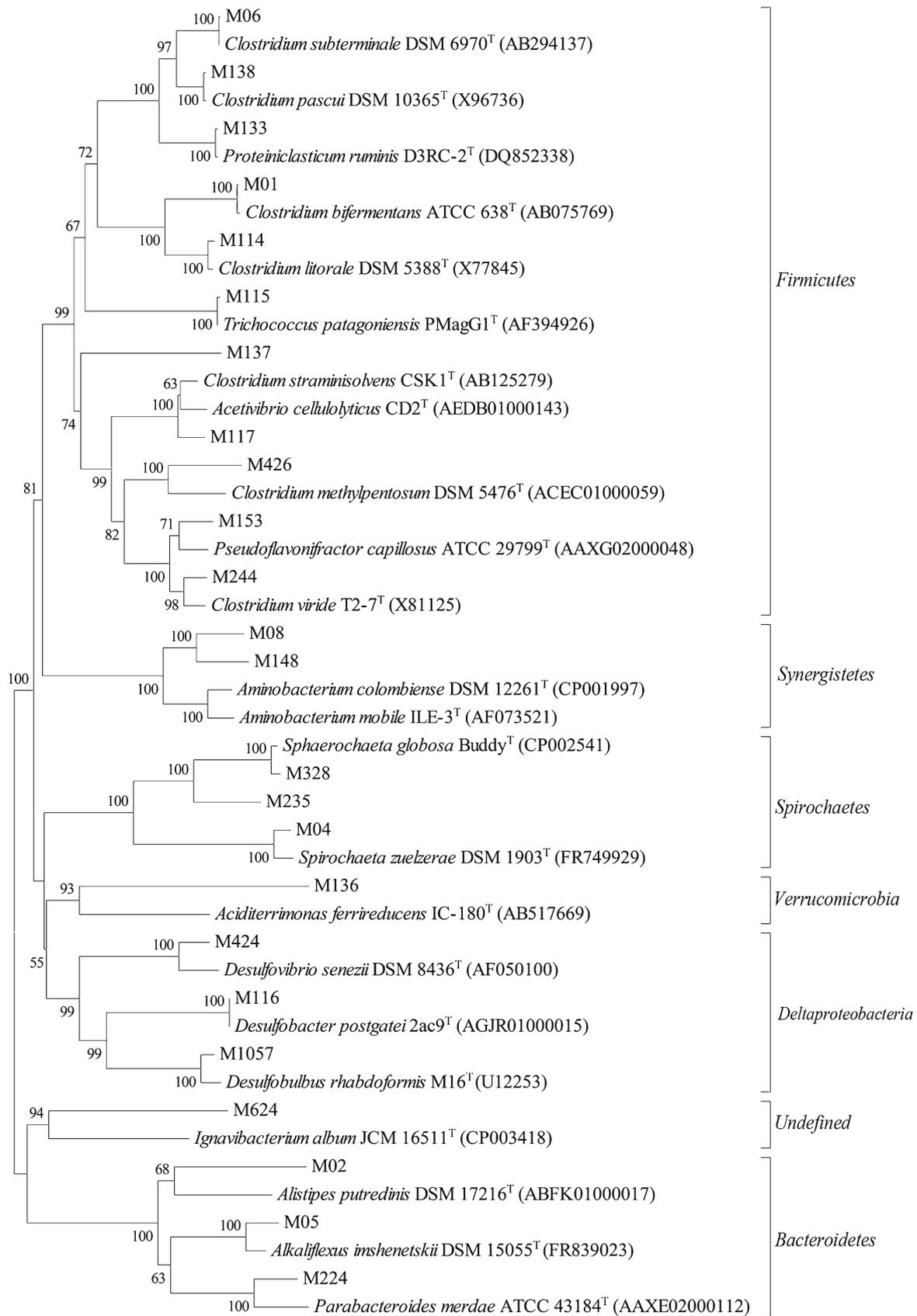
units (OTUs, 99 % 16S rRNA gene sequence identity as the threshold) were obtained (Table 1). Phylogenetic analysis based on the nearly full-length 16S rRNA gene sequences showed that these OTUs were assigned to 7 phyla and 11 of them belonged to the phylum *Firmicutes* (Fig. 3). On the other hand, 100 of randomly selected clones in the library were sequenced, of which 92 positive clones were obtained (Table 1). Among them, M05, occupying 22.8 % (21 of 92 clones) of the microbial consortium, was the predominant member. It showed the highest 16S rRNA gene sequence similarity with bacterium *Alkaliflexus imshenetskii* DSM15055^T (94.9 % identity) isolated from a cellulose-degrading enrichment culture. Another dominant clone M117, occupying 13.0 % of the microbial consortium, was most closely related to cellulolytic bacteria *Clostridium straminisolvans* CSK1^T and *C. thermocellum* ATCC 27405^T with 16S rRNA gene identities of 95.3 and 92 %. The other frequently detected ones included M02 (16.3 % of the consortium), M115 (13.0 %), and M04 (12.0 %). They showed the highest 16S rRNA gene sequence similarities to *Alistipes putredinis* DSM 17216^T, *Spirochaeta zuelzeriae* DSM 1903^T,

Table 1 Microbial diversity of consortium SQD-1.1 identified by the 16S rRNA gene library

Band number	The closest type strains (accession number)	Similarity (%) ^a	Frequency ^b
M01	<i>Clostridium bif fermentans</i> ATCC 638 ^T (AB075769)	99.7	1/92
M02	<i>Alistipes putredinis</i> DSM 17216 ^T (ABFK01000017)	85.5	15/92
M04	<i>Spirochaeta zuelzeriae</i> DSM 1903 ^T (FR749929)	96.4	11/92
M05	<i>Alkaliflexus imshenetskii</i> DSM 15055 ^T (FR839023)	94.9	21/92
M06	<i>Clostridium subterminale</i> DSM 6970 ^T (AB294137)	99.7	4/92
M08	<i>Aminobacterium colombiense</i> DSM 12261 ^T (CP001997)	90.9	1/92
M114	<i>Clostridium litorale</i> DSM 5388 ^T (X77845)	99.0	1/92
M115	<i>Trichococcus patagoniensis</i> PMagG1 ^T (AF394926)	99.7	12/92
M116	<i>Desulfobacter postgatei</i> 2ac9 ^T (AGJR01000015)	99.9	4/92
M117	<i>Clostridium straminisolvans</i> CSK1 ^T (AB125279)	95.3	12/92
M133	<i>Proteiniclasticum ruminis</i> D3RC-2 ^T (DQ852338)	99.2	0/92
M136	<i>Aciditerrimonas ferrireducens</i> IC-180 ^T (AB517669)	80.0	0/92
M137	<i>Acetivibrio cellulolyticus</i> CD2 ^T (AEDB01000143)	85.1	1/92
M138	<i>Clostridium pascui</i> DSM 10365 ^T (X96736)	99.0	1/92
M148	<i>Aminobacterium colombiense</i> DSM 12261 ^T (CP001997)	90.1	0/92
M153	<i>Pseudoflavonifractor capillosus</i> ATCC 29799 ^T (AAXG02000048)	94.6	0/92
M224	<i>Parabacteroides merdae</i> ATCC 43184 ^T (AAXE02000112)	93.4	1/92
M235	<i>Sphaerochaeta globosa</i> Buddy ^T (CP002541)	89.8	0/92
M244	<i>Clostridium viride</i> T2-7 ^T (X81125)	95.4	0/92
M328	<i>Sphaerochaeta globosa</i> Buddy ^T (CP002541)	98.6	2/92
M424	<i>Desulfovibrio senezii</i> DSM 8436 ^T (AF050100)	94.36	3/92
M426	<i>Clostridium methylpentosum</i> DSM 5476 ^T (ACEC01000059)	90.6	1/92
M624	<i>Ignavibacterium album</i> JCM 16511 ^T (CP003418)	82.3	0/92
M1057	<i>Desulfobulbus rhabdiformis</i> M16 ^T (U12253)	96.3	1/92

^a Similarities were calculated using nearly full-length 16S rRNA gene sequences by pairwise alignment program of EzTaxon-e server

^b Occurrence frequency of OTUs in the 16S rRNA gene library (92 random clones in total)



0.02

◀ **Fig. 3** Phylogenetic tree based on the nearly full-length 16S rRNA gene sequences derived from the 16S rRNA gene library of consortium SQD-1.1 and their closest relatives using the neighbor-joining method. Based values were expressed based on 1,000 replications, and only values above 50 % were shown. Bar 0.02 % estimated sequence divergence

and *Trichococcus patagoniensis* PMagG1^T with 85.5, 96.4, and 99.7 % identities, respectively.

Single strains from consortium SQD-1.1 and enzyme activity analysis

Using the plate cultivation method, 12 strains were isolated from consortium SQD-1.1 (Table 2). Among them, 16S rRNA gene sequences of seven strains were the same with the clones in the 16S rRNA gene library, including the frequently detected M05 and M115 (corresponding to strain P2 and N1). Phylogenetic analysis showed that seven of the isolated strains were homologous or closely related to the validated type species of genus *Clostridium*, *Proteiniclasticum*, *Trichococcus*, and *Desulfomicrobium*. Other five strains shared 16S rRNA gene sequence similarities of 85.4 to 96.3 % with the most closely related type species and could be proposed as new species or new species of new genera. Enzyme activity analysis showed that strain P2 showed both CMCase and xylanase activity and strain N1 showed only xylanase activity.

Cellulosome-like complex in consortium SQD-1.1

A cellulosome is the complex cellulase system adhered to the cell surface of anaerobic bacteria such as *C. thermocellum* (Doi and Kosugi 2004). Consortium SQD-1.1 was enriched under anoxic conditions and maybe contain similar cell-surface complex. An experiment to validate the hypothesis was designed (Fig. 4). The suspension and the first washing buffer of the control strain *Vibrio* sp. G21 showed cellulase activity, whereas its cell pellet did not, which was consistent

with the previous report that G21 only produced an extracellular endoglucanase (Gao et al. 2010). In contrast, both the suspension and cell pellet of consortium SQD-1.1 showed cellulase activity. The cellulases in the suspension of consortium SQD-1.1 maybe come from extracellular endoglucanase-producing bacteria and/or were released from cellulosomes of anaerobic cellulolytic bacteria by mechanical force in the experiment procedure, whereas the cellulase activity in the its cell pellet definitely came from the cells themselves. The result suggested the adhesion of cellulosome-like complex to the cell surface of microbes in the consortium.

Cohesin and dockerin domains from the metagenomic data

For consortium SQD-1.1, 8 million paired-end reads, 1.2 Gbp in total, were sequenced. 5.46 million environmental gene tags (EGTs), longer than 100 bp, were assembled by overlapping the qualified paired-end reads. The EGTs were annotated by BlastX program and further certified by hmmscan, and 24 of cohesin domains and 60 of dockerin domains were mined from the annotation results (Fig. S1 in the Supplementary Material). Phmmer search further showed that the dockerin domains were most closely related to those of the bacteria in the genus *Clostridium*, including *C. thermocellum*, *Clostridium papyrosolvans*, *Clostridium cellulovorans*, and *C. cellulolyticum*, whereas the cohesin domains were assigned to the genus *Clostridium*, *Acetivibrio*, and *Bacteroides* (Table S1 and S2 in the Supplementary Material). However, all of closest relatives were anaerobic cellulolytic bacteria containing cellulosomes according to the previous reports (Doi and Kosugi 2004; Fontes and Gilbert 2010).

Discussion

Enrichment of microbial consortia provides an approach to simulate and investigate cellulose degradation by microbial

Table 2 Bacteria isolated from consortium SQD-1.1

Isolates	The closest type strains (accession number)	Similarity ^a (%)	Relative clones ^b
N1	<i>Trichococcus patagoniensis</i> PMagG1 ^T (AF394926)	99.7	M115
N2	<i>Proteiniclasticum ruminis</i> D3RC-2 ^T (DQ852338)	99.5	M133
A1	<i>Clostridium subterminale</i> DSM 6970 ^T (AB294137)	99.7	M06
C1	<i>Clostridium glycolicum</i> DSM 1288 ^T (X76750)	99.3	
B1	<i>Clostridium bifermens</i> ATCC 638 ^T (AB075769)	99.7	M01
E2	<i>Clostridium lituseburense</i> ATCC 25759 ^T (M59107)	99.9	
P1	<i>Desulfovibrio senezii</i> DSM 8436 ^T (AF050100)	94.4	M424
P2	<i>Alkaliflexus imshenetskii</i> DSM 15055 ^T (FR839023)	94.9	M05
R2	<i>Christensenella minuta</i> YIT 12065 ^T (AB490809)	85.4	
R9	<i>Christensenella minuta</i> YIT 12065 ^T (AB490809)	85.4	
S2	<i>Desulfomicrobium norvegicum</i> DSM 1741 ^T (AJ277897)	98.6	
S4	<i>Desulfobulbus rhabdiformis</i> M16 ^T (U12253)	96.3	M1057

^aSimilarities were calculated using nearly full-length 16S rRNA gene sequences by pairwise alignment program of EzTaxon-e server

^bThe corresponding clones in the 16S rRNA gene library that was consistent with the isolates

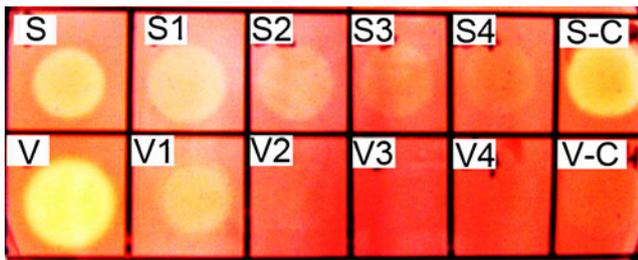


Fig. 4 Cellulase activity analysis of different portions of cultures from consortium SQD-1.1. *S* the suspension; *S1–S4* the first, second, third, and fourth washing suspension of the residual cells with phosphate buffer; *S-C* cell pellets of SQD-1.1 after being washed for four times; *V*, *V1–V4*, and *V-C* the relative controls using endoglucanase-producing strain *Vibrio* sp. G21

communities in natural environments. Several cellulolytic consortia have been enriched from composts, soils, decayed straws, and so on (Haruta et al. 2002; Guevara and Zambrano 2006; Lv et al. 2008; Wongwilaiwalin et al. 2010; Feng et al. 2011; Okeke and Lu 2011; Wang et al. 2011; Zuroff and Curtis 2012). These studies mainly focused on microbial communities in terrestrial ecosystems. As one of the most productive system and a partially anaerobic ecosystem, mangrove soils have not yet been received enough attention. In this report, mangrove soils were collected from Hainan province of China, and a stable anaerobic cellulolytic consortium, SQD-1.1, was enriched. The consortium showed high efficiency for cellulose degradation and maintained the cellulolytic ability after successive subcultivation for 27 times. PCR-DGGE analysis further revealed the stability of its microbial community structure in the incubation process of 10 days and in the procedure of subcultivation. These results implied that consortium SQD-1.1 was functionally and structurally stable.

Based on the 16S rRNA gene library study, M117 was one of the predominant clones (13.0 % of the consortium) and was most closely related to the anaerobic cellulolytic bacteria *C. straminisolvans* and *C. thermocellum* (Kato et al. 2004; Doi and Kosugi 2004). *C. thermocellum* is well known as a typical anaerobic cellulolytic species and because of using cellulosomes on their cell surface for cellulose degradation (Doi and Kosugi 2004). Cellulase activity assay of cell pellets indicated the adhesion of a cellulosome-like complex to the cells surface of microbes in consortium SQD-1.1. The key domains in cellulosomes, cohesin and dockerin, were further found in the metagenomic data of consortium SQD-1.1 and were mainly homologous to those in the genus *Clostridium*. These results suggested that the consortium contained cellulolytic bacteria closely related to species in the genus *Clostridium* and utilized cellulosomes to degrade cellulose. As the predominant *Clostridium* clone in consortium SQD-1.1, M117 was speculated to use cellulosomes for more efficient cellulose degradation and the bacterium was most likely to be the key cellulolytic species. Another clone *Alkaliflexus* sp. M05,

corresponding to the isolated strain P2, was the most frequently detected one (22.8 %), which showed both CMCase and xylanase activity but no activity against avicel. M05 may be another key cellulolytic bacterium, which hydrolyzed the cello-oligosaccharides produced by bacteria such as M117. Besides, M115 in the genus *Trichococcus* (corresponding to strain N1) showed xylanase activity and may also play roles in lignocellulose degradation. Multiple-species source of lignocellulolytic enzymes was the internal mechanism of high efficiency cellulose degradation of microbial consortia. In consortium SQD-1.1, combination of cellulosomes from genus *Clostridium* and extracellular cellulolytic enzymes from *Alkaliflexus* sp. and *Trichococcus* sp. may be the key factors for the highly efficient degradation of cellulose.

Other predominant clones in consortium SQD-1.1 included M02 (16.3 %) and M04 (12.0 %). M04 was most closely related to *S. zuelzeriae* DSM 1903^T and fell into the phylum *Spirochaetes* (Table 1). Metagenomic analysis of hindgut microbiota of a wood-feeding higher termite revealed the abundance of bacteria in genus *Treponemes* of the phylum *Spirochaetes* and *Treponemes*-encoded glycoside hydrolase gene modules (Warnecke et al. 2007). Another metagenomic study on cow rumen also demonstrated that bacteria of order *Spirochaetales* in the phylum *Spirochaetes* were adherent to and degraded the plant fiber materials (Hess et al. 2011). These studies suggest that bacteria in the phylum *Spirochaetes* are important members of cellulolytic microorganisms. M04 may be also important for cellulose degradation in consortium SQD-1.1, yet as an uncultured bacterium, further study on the metagenome data will give us the answer. M02 was phylogenetically distinct from the validated type species, and no direct relationship with cellulose degradation could be proposed. A previous study implied that bacteria in microbial communities synergistically coexist and keep the stability of the consortium by promotion or inhibition relationships (Kato et al. 2005). As a predominant clone, M02 may also play an important role in keeping the stability of the microbial structure of the consortium, yet its actual roles is unclear now.

Consortia enriched previously from terrestrial ecosystems, in general, were composed of anaerobes and aerobes (Haruta et al. 2002; Wongwilaiwalin et al. 2010; Feng et al. 2011; Zuroff and Curtis 2012). Aerobes eliminated oxygen in the beginning. Then, some anaerobes began to degrade cellulose under anaerobic conditions. Other anaerobes played synergistic roles on cellulose degradation or keeping the stability of the consortia (Kato et al. 2005). Distinguished from them, consortium SQD-1.1 was enriched under anoxic conditions. Its microbial structure was stable and consistent through the degradation procedure, and anaerobes were always the predominant members because of the anaerobic conditions in our study. Besides of the potential cellulolytic bacteria from the genus *Clostridium*, new potential cellulolytic bacteria such as

Alkaliflexus sp. M05 may also play important roles in consortium SQD-1.1. This represented a novel anaerobic cellulolytic consortium with distinct microbial community structure and different cellulolytic mechanism from the consortia reported previously. Further detail research on this anaerobic consortium will reveal the detail mechanism of cellulose degradation under anoxic conditions in natural environments, especially in mangrove ecosystems.

Most microbes have resisted cultivation in the laboratory, despite or perhaps because of the tremendous diversity in the physiology capabilities that microbes possess. Kinds of methods have been used for isolation of previously uncultivable bacteria (Kaeberlein et al. 2002; Zengler et al. 2003; Bomar et al. 2011). Enrichment of consortium using modified media was proposed as one of the useful choices (Sizova et al. 2012). In our study, five novel strains were isolated. Among them, the strains P1 and P2 have been validated as new species of new genera (Zhao et al. 2012a; Zhao et al. 2012b). Strain R2 and R9, which were phylogenetically distinguished from known bacteria and showed the highest 16S rRNA gene similarities of 85.4 % with validated type species, could be proposed as new species in a new family (Table 2). The isolation of novel species is another important finding of our study, which provides a new insight into the microbial diversity of the mangrove ecosystems and also promotes the understanding of the uncultured bacteria in anaerobic natural environments.

In conclusion, a stable microbial consortium SQD-1.1 was constructed in this study. The consortium could degrade filter paper efficiently under anaerobic conditions, which represents a novel anaerobic cellulolytic consortium. The finding of potential anaerobic cellulolytic clone M117 and isolated strain P2, as well as of the cohesin and dockerin domains in the metagenomic data, suggests a typical anaerobic cellulose-degradation system. The novel new strains isolated from consortium SQD-1.1 also provide a new insight into the microbial diversity of anaerobes in mangrove ecosystems. Further study of consortium SQD-1.1 provides a candidate for investigating the mechanism of anaerobic cellulose degradation in natural anaerobic environments.

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