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Microorganisms colonizing surface in coastal marine water as revealed by 16S rRNA gene clone library analysis

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To investigate the community structure and dynamics of surface-associated microbial cells, sterile strengthened glass sheets were immersed in near-shore marine water of Xiamen, China. The organisms colonizing the glass surface were sampled after 1 h, 7 and 14 days. Three 16S rRNA gene clone libraries were constructed using primers universal for the domain bacteria. 40 clones were selected randomly from each clone library for sequencing, and the sequences were submitted for homology and phylogenetic analyses. All cloned sequences fell into 6 major groups: Sequences of clones originating from chloroplasts of eukaryotic Bacillariophyta were abundant in all three samples (ranging from 16 to 64%) and predominated in 7 and 14-day samples and occupied 45% of the total cloned sequences; γ-Proteobacteria came next and occupied 42% of the total sequences and the proportion of γ-Proteobacteria in each clone library decreased with immersion time. An entophytic bacterium Serratia proteamaculans involving biofilm formation was predominant within the γ-Proteobacteria and took up more than half of the clone library of 1 h sample. Other bacterial groups, Bacteroidetes, α-Proteobacteria, Firmicutes and Cyanobacteria occupied 5 to 1%.

Key words: Microorganisms colonization, dynamics, 16S rRNA, clone library, marine coastal water.

INTRODUCTION

The majority of microorganisms, including numerous microalgae, diatom species and members of bacteria can be attach to any substratum, whatever its nature (inert or living), forming a biofilm (Kumar and Prasad, 2006). When an object is immersed in the sea, microbial cells are attached to its surface immediately and spontaneously, and produce extra-cellular products that connect microbial cells to each other and the substratum so that, they form a biofilm. Biofilms play important roles in ecology, environment and economy. The settlement and metamorphosis of marine pelagic larvae is important to marine invertebrates. Some secretions of attached bacteria attract and induce the settlement and metamorphosis of some invertebrate larvae (Unabia and Hadfield, 1999). Therefore, we can screen bacteria with the ability or extract effective substances from those bacteria to accelerate breeding of marine invertebrate such as escallop or abalone. By contrast, some bacteria in biofilms prevent the settlement of larvae and spores, thus hindering settlement of macrofauna (Dobretsov et al., 2006). This provides a new approach to anti-fouling using biofilms. Finally, biofilms in nature are a reservoir for pathogenic bacteria, which makes it difficult to prevent diseases (Kumar and Prasad, 2006; Parsek and Singh, 2003). Furthermore, biofouling and marine fouling originates from the formation of biofilms, so when studying microbiological corrosion it is important to

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determine the abundance and groups of attached bacteria as well as, the formation and properties of biofilms (Dhanasekaran et al., 2009).

The formation of a biofilm is a highly complex and dynamic process (Kumar and Prasad, 2006). Marine bacteria are very important part of biofilm, and play important roles in formation and succession of biofilm. Traditional methods for the study of marine bacteria are mainly based on culture. This has its limitations, since the majority (typically, ≥95%) of bacteria in nature cannot be cultured, and isolated bacteria are not representative of in situ diversity (Amann et al., 1995). By contrast, culture-independent molecular biology techniques based on 16S rRNA gene analysis are very useful for studying microorganism community structure in various habitats (Rappé and Giovannoni, 2003). Using culture-independent techniques, studies revealed that microbial communities mainly consist of bacteria and diatoms in marine biofilms developed on unpainted and painted artificial surfaces (Railkin, 2004; Dang et al., 2011; Briand et al., 2012). Proteobacteria, especially α-Proteobacteria, appear dominant among these bacterial communities (Dang and Lovell, 2000; 2002; Jones et al., 2007; Dang et al., 2008), but the population dynamic depends on several complex factors. In some cases, γ-Proteobacteria or microalgae seemed to be more important as the pioneering population (Lee et al., 2008; Briand et al., 2012).

This study thus seeks to determine the community structure, dominant groups, and dynamics of marine attached bacteria in the coastal water of Xiamen based on 16S rRNA gene clone library analysis, with the aim to better characterize the microbial composition and dynamic during biofilm formation, hoping that the data present here will provide a foundation for further studies on attached microorganisms dynamics and their functions in biofilm formation.

MATERIALS AND METHODS

Sample collection

Nine pieces (each piece the size of 9 × 5 cm) of sterile strengthened glass sheets were immersed in marine water near Xiamen Bridge (24°34′N, 118°05′E) at the depth of about 1.5 m for 14 days, in March, 2009. The surface water temperature was 24.6°C, and salinity was 23.8. The attached organisms on the glass surface were sampled after 1 h, 7 and 14 days immersion. Each sample was taken from 3 same pieces separately using sterile cotton swabs immediately after the glass plate had been removed from the water. The cotton swabs were then placed in a 5-ml sterile disposable centrifuge tube, and the samples were stored in a freezer at −20°C before DNA extraction.

DNA extraction, PCR amplification and clone library construction

Cotton swab samples were incubated in 1.5 ml of GTE buffer (0.75 M sucrose, 50 mM Tris [pH 8.0], 40 mM EDTA [pH 8.0]) containing lysozyme at a concentration of 2 mg/ml. After incubation at 37°C for 2.5 h, lysates were transferred to another sterile centrifuge tube and the cotton swabs were rinsed with 0.5 ml of GTE buffer. The lysates were pooled and cells were further lysed by the addition of proteinase K (0.5 mg/ml) and sodium dodecyl sulfate (1%) for 1 h at 53°C. The cell lysates were extracted twice with phenol–chloroform–isoamyl alcohol (25:24:1) and once with chloroform–isoamyl alcohol (24:1) before nucleic acids were precipitated with 0.8 volume of isopropanol at room temperature. Nucleic acids were recovered by centrifugation, washed once with 70% ethanol, and resuspended in TE. The primers used for amplification of bacterial 16S rDNA were 27F (5′-AGAGTTTGATCCTGGGCTCAG-3′) and 1492R (5′-GTTACCTTGTAGGACCTT-3′). The amplification reaction mixture consisted of 1 μl of each primer, 200 μM dNTPs, 2 μl 10×PCR buffer, 1 unit Taq DNA polymerase (TaKaRa Biotechnology Co., Dalian, China) and 1 μl of DNA solution, with sterilized MilliQ water added to the total volume of 20 μl. The amplification conditions were 94°C for 5 min, 35 cycles at 94°C for 1 min, 58°C for 1 min, and 72°C for 2 min, with a final extension for 10 min at 72°C to facilitate the TA cloning. The amplified products were gel-purified and ligated into the pMD18-T vector (TaKaRa Biotechnology Co., Dalian, China) and then transformed into competent cells of E. coli DH5α. The ampicillin-resistant clones were randomly picked and screened for inserts by performing colony PCR with M13 primers for the vector (Invitrogen, Shanghai, China).

DNA sequencing and phylogenetic analysis

Clones with correct inserts identified by colony PCR were selected for sequencing, which was carried out on an ABI model 3730 automated DNA sequence analyzer (GenScript Corporation, Nanjing, China) using the sequencing primer 27F. All the nucleotide sequences were checked for putative chimeras by the RDP CHIMERA_CHECK (Maidak et al., 2001) and the reliable sequences were grouped as operation taxonomic unit (OTU) by 97% or greater sequence similarity using the software DNAmAn. Sequences (one representative sequence out of one OTU) were compared to known 16S rDNA sequences in the database by using the BLASTN search (http://www.ncbi.nlm.nih.gov/BLAST/). A phylogenetic tree was generated using the neighbor-joining algorithm by the software MEGA4 (Tamura et al., 2007). Bootstrap values were obtained with 1000 resamplings.

Nucleotide sequence accession number

Clone sequences were deposited in GenBank under the accession numbers HQ122367-HQ122387.

RESULTS

OTU analysis of the constructed 16S rDNA clone libraries

Clones with correct inserts were sequenced and the potential chimeras were removed. Totally there were 89 reliable sequences derived from the samples. All sequences were grouped into 23 OTUs by DNAmAn software. The two most abundant OTUs contained 66 clones (74% in total clones), suggesting significant dominance. Other OTUs were observed only once or twice in the clone libraries reflecting significant genetic
diversity of the attached bacteria in the coastal water of Xiamen. The number of OTUs detected through immersion time remained approximately constant, from 28% in the 1 h sample (9 OTUs/32 clones) to 31% in the 7 day sample (9/29) and 32% in the 14 day sample (9/28).

Phylogenetic analysis of the 16S rDNA sequences in the three clone libraries

One sequence of a unique OTU was selected and compared to known 16S rDNA sequences in GenBank, and a phylogenetic tree was constructed using the neighbor-joining method. The main branches of the tree possess a high bootstrap value indicating high confidence of the tree topology (Figure 1). All cloned sequences fell into six major groups: γ-Proteobacteria (42%), Bacteroidetes (5%), Firmicutes (2%), α-Proteobacteria (2%), Cyanobacteria (1%), and some other clones originating from chloroplasts of eukaryotic Bacillariophyta (45%), and a few unidentified sequences. A total of 37 sequences (42%) grouped with the γ subdivision of the Proteobacteria, were assigned to nine OTUs, and were dominated by S. proteamaculans (28/37). S. proteamaculans is an endophytic bacterium and has a growth-promoting effect for their plant host. These bacteria decreased with immersion time, comprising 59.38% of the 1 h sample, 20.69% of the 7d sample and 10.71% of the 14d sample. The high percent S. proteamaculans counted on 1h sample indicated they might have important implications for microorganism primary colonization. The other nine clones, classified into eight OTUs, had no significant dominance. Only two of these clones came from the 14d sample, while the other seven clones came from the 1h sample. Six clones were most closely related to Aestuariibacter, Pseudoalteromonas, Arenicella xantha, Vibrio sp., Haliea sp., with a sequence similarity of over 95%, and three clones were most closely related to Thiohalocapsa, Thiopseudomonas lithotrophic, and Amphritea balenae, with a sequence similarity ranging from 87 to 92%.

Four OTUs (four clones) were grouped with Bacteroidetes; they were retrieved in the 7 and 14 days samples. Their sequence similarity to the most similar sequence in Genbank was lower than 95%. Two OTUs (two clones) were clustered into α-Proteobacteria; they were all retrieved in the 7d sample, and were affiliated with Sulfitobacter lithoralis (99% sequence similarity) and Oceanica marinus (93.25% sequence similarity). Two OTUs (two clones) were clustered into Firmicutes, and all of them were from the 14day sample, and were clustered tightly with Clostridium sp. and Trichococcus floculiformis, with a sequence similarity > 99% (Figure 1).

Only one clone (1 h-35) from the 1 h sample was detected to be related to the cyanobacterium Dermocarpa sp. (96% sequence similarity). Three clones, 7day-31, 7day-32, and 14day-34, contained inserts that were closely related (with more than 95% sequence similarity) to environment sequences. These similar sequences respectively came from the South China Sea, the epibiote of a hydrothermal vent galatheid crab and activated sludge (GenBank description). 40 sequences (45%) in all the three clone libraries related to the chloroplasts of eukaryotic Bacillariophyta were detected. These Eukaryota included Haslea salstonica and Phaeodactylum tricornutum (> 97% sequence similarities), accounting for 95 and 5% in total chloroplasts, respectively.

Dynamics of marine attached microorganisms in coastal water of Xiamen

Sequences were assigned to major groups based on BLAST similarities and phylogenetic analysis, and the species-composite clone libraries are shown in Figure 2. In the 1 h sample, bacterial structure was relatively simple, and dominated by γ-Proteobacteria. The number of OTUs increased slightly with immersion time, indicating that bacterial groups increased with time. The proportion of γ-Proteobacteria decreased from 81% in the 1 h sample, to 21% in the 7 days sample, and 18% in the 14 days sample. α-Proteobacteria accounted for 7% in the 7 days sample, while no α-Proteobacteria was detected in the 1 h and 14 days samples. We detected Bacteroidetes in the 7 and 14 days samples (both 7%) and Firmicutes in the 14days sample (7%). The 7 and 14days samples contained a few uncultured bacterial sequences. Moreover, Cyanobacteria were detected in the 1h sample but not in the 7d and 14d samples (Figure 2).

We detected numerous sequences of the chloroplasts of eukaryotic Bacillariophyta in all samples, and these sequences increased with immersion time, from 16% after 1h immersion to over 50% after 7 and 14 days immersion.

DISCUSSION

Comparison of the microorganism community structure on biofilms among this study and others

Previously, α-Proteobacteria were recognized as the pioneering organisms in marine biofilm formation (Dang and Lovell, 2000; 2002; Jones et al., 2007; Dang et al., 2008), while results of this study revealed that α-Proteobacteria only occurred in the 7 day sample. Although the abundance of this group might be underestimated by insufficient sampling and low clone numbers, γ-proteobacteria were more abundant and dominant in our case, especially in the 1 h-immersed surfaces.
Figure 1. Neighbor-joining phylogenetic tree generated from an alignment of 16S rDNA sequences of attached microorganisms from coastal water of Xiamen. Clones from this study are indicated in bold and designated as 1h-n, 7d-n and 14d-n, in which 1h, 7d, 14d represent sequences derived from 1h, 7d, 14d samples respectively, and n represents the number of different clones. Accessions numbers are shown in parentheses, and numbers that follow the accession numbers indicate the frequency of occurrence of the OTUs in the three libraries. The remaining sequences were obtained from GenBank. Clones in brackets share the same OTU patterns with the corresponding representative clone. The tree is rooted using *Cenarchaeum symbiosum*, a kind of Archeae, as the outgroup. Bootstrap values above 50 (1000 iterations) are shown at each node. Scale bar represents the nucleotide substitution percentage.

Similar results can be seen on artificial coral surfaces (Sweet et al., 2011), bivalve shell (Gillan et al., 1998), and artificial surfaces in the eastern shore of Korean Peninsula (Lee, et al., 2008), which indicated that γ-proteobacteria dominated the biofilms, especially in the early stage of biofilm formation.
Figure 2. Percentage of abundance of the bacterial phylotypes as determined by 16S rDNA clone library statistics and phylogenetic analysis.

Notwithstanding the discrepancy resulting from the use of different molecular techniques, the composition and the dynamic of marine bacteria in biofilms depends on physicochemical properties of solid surfaces (Dang and Lovell, 2000; Jones et al., 2007; Lee et al., 2008), environmental factors, such as temperature (Lau et al., 2005), nutrient availability (Chiu et al., 2008), etc. The bacteria on the biofilms come from the water column and the communities may be also affected by the bacteria in the water and change during the course of succession.

In this study, γ-proteobacteria were predominant and occupied 42% of the total cloned sequences. Further analysis revealed that the high percent was mainly due to the high abundance of *Serratia proteamaculans*. It comprised about 31.5% of the total cloned sequences. Since *Serratia proteamaculans* is an entophytic bacterium and is able to promote plant growth, and a great amount of eukaryotic diatom (45%) were detected in the samples, we supposed that the *Serratia proteamaculans* may associate with the eukaryotic microalgae. Furthermore, Serratia species were reported to have the quorum sensing (QS) systems involved in various physiological processes in bacteria, such as conjugation, symbiosis and biofilm formation (Waters and Bassler, 2005; Liu et al., 2011). The bacteria rely on QS system to govern various aspects of biofilm development, including adhesion, motility, maturation, and dispersion (Rice et al., 2005). A set of homologous genes encoding a putative quorum sensor was identified in the *Serratia proteamaculans* (Christensen et al., 2003). Thus the *Serratia proteamaculans* in the present study may promote the biofilm formation.

Sequences of chloroplasts of eukaryotic Bacillariophyta were detected frequently in this study, and a recently published paper (Briand et al., 2012) also reported that two diatom species dominated in the marine biofilm communities, suggesting that this taxon is essential for the biofilm formation. Although many studies that have been conducted to investigate the microorganism on biofilms have focused on bacteria, microalgae present in biofilms have largely been ignored. More attention in the future should be paid to this group to elucidate their functions in biofilm formation.

Comparison of the community structure between the attached and free-living microorganisms in the coastal water of Xiamen

The community structure of the attached and free-living microorganisms in the coastal water of Xiamen was different. Ma et al. (2009) studied on the free-living bacteria in the Xiamen Port (24°29'N, 118°04'E). They found that α-Proteobacteria was dominant, and γ-Proteobacteria was the second dominant group. In our experiment, however, attached bacteria were dominated by γ-Proteobacteria, while α-Proteobacteria accounted for only a small fraction. Chloroplasts of eukaryotic Bacillariophyta and Cyanobacteria were detected in both marine attached and free-living bacteria. However, eukaryotic chloroplasts were more abundant in the former, while Cyanobacteria were more abundant in the latter. Furthermore, the species components within the Bacillariophyta and Cyanobacteria differed between the attached and free-living bacteria. *H. salstonica* and a small number of *P. tricornutum* affiliated sequences were detected in the attached bacterial libraries, while *Skeletonema pseudocostatum* affiliated sequences were
detected in the free-living bacterial library. *Democarpa* sp. affiliated sequences were detected in the attached 16S rDNA clone libraries; while *Synechococcus* affiliated sequences were detected in the free-living bacterial library. Bacteroidetes were detected in both marine attached and free-living bacteria, while no other bacteria of *Cytophaga-Flavobacterium-Bacteroides* (CFB) pylum were detected.

β-Proteobacteria, δ-Proteobacteria and Actinobacteria contained clones from the free-living bacterial libraries, and Firmicutes contained clones from the attached bacterial libraries, indicating a lower level of bacterial diversity in the attached bacteria of coastal water of Xiamen. Previous studies also indicated that, even in the same marine water, the community structure between attached and free-living microbial assemblages were different. Attached bacteria contained very little diversity, and most clones belonged to the γ-Proteobacteria, while the free-living bacteria were highly diverse and dominated by α-Proteobacteria (Delong et al., 1993; Sweet et al., 2011), at least near the surface water (Acinas et al., 1999). Bacteria on biofilms may come from their surrounding water, the communities do not appear to arise from passive settlement from the water column, but instead appear to have become established through a selection process (Sweet et al., 2011). The ability to select certain species from the water column and deny settlement of others may depend on physiochemical properties of the settlement surfaces, antimicrobial activity of the host and/or the resident microbial community (Ritchie and Smith, 1995; Kooperman et al., 2007; Sharon and Rosenberg, 2008), also, the environmental factors, such as temperature (Lau et al., 2007), nutrient availability (Chiu et al., 2008), etc., may also influence the microbial communities and dynamics on biofilms.

**Future prospects**

We sampled at three immersion times (1h, 7, 14 days) only, and the number of sequenced clones was limited. This has resulted in insufficient data to define community structure and dynamics of marine attached bacteria in the coastal water of Xiamen. Future investigations that consider additional samples, more cloned sequences and a combination of the molecular approach presented here and other techniques such as DGGE will help us to better understand bacterial community structure and dynamics. However, we have determined the dominant groups of marine attached bacteria, and found that microalgae, especially diatom, and some plant entophytic bacteria involving in biofilm formation are important members on biofilms in our case. All the data present here will be good supplements to the current understanding of microbial community on biofilms and will provide references for further studies on the dynamics of attached bacteria, and their functions in biofilm formation.

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