Community characteristics and ecological roles of bacterial biofilms associated with various algal settlements on coastal reefs

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ABSTRACT

Bacterial biofilms, which are a group of bacteria attaching to and ultimately forming communities on reefs, perform essential ecological functions in coastal ecosystems. Particularly, they may attract or repulse the settling down of opportunistic algae. However, this phenomenon and the interaction mechanism are not fully understood. This study investigated reefs from the Changdao coastal zone to determine the structures and functions of bacterial biofilms symbiosing with various algae using high-throughput sequencing analysis. The Shannon diversity index of microbiota with algal symbiosis reached 5.34, which was higher than that of microbiota wherein algae were absent (4.80). The beta diversity results for 11 samples revealed that there existed a separation between bacterial communities on reefs with and without attached algae, while communities with similar algae clustered together. The taxa mostly associated with algae-symbiotic microbiota are the Actinobacteria phylum, and the Flavobacteriia and Gammaproteobacteria classes. The Cyanobacteria phylum was not associated with algae-symbiotic microbiota. As revealed by functional analysis, the bacteria mostly involved in the metabolism of sulfur were represented by brown and red algae in the biofilm symbiosis. Bacteria related to the metabolism of certain trace elements were observed only in specific groups. Moreover, phototrophy-related bacteria were less abundant in samples coexisting with algae. This study established the link between bacterial biofilms and algal settlements on coastal reefs, and revealed the possible holobiont relationship between them. This may provide new technical directions toward realizing algal cultivation and management during the construction of artificial reef ecosystems.

1. Introduction

The formation of bacterial biofilm in the marine ecosystem is ubiquitous and characterized by a complicated and integrated process. Originally, a small group of planktonic bacteria adhere together by secreting protective extracellular polymeric substances consisting of polysaccharides, proteins, and nucleic acids (Gutierrez-Pacheco et al., 2018). The bacterial assembly gradually proliferates and attaches to specific substrata, owing to the effects of external conditions that include environmental changes (Hošťáká et al., 2010), competition with other strains, and stimulation of algae on the substrata (Oliveira et al., 2015). As a result of diverse chemical and biological activities, the adhesion of communities has various consequences on the environment of substrates, including the decomposition of inorganic substances (Xu et al., 2015), metabolism of organic substances, and circulation of various nutrient elements (Butturini et al., 2000). Because the micro-environment tends to be stable and suitable, biofilms can induce the attachment of additional organisms (Koo et al., 2017) and the settlement of algae (Seyedsayamdost et al., 2011).

Reciprocal relationships between bacteria and algae have been widely reported and accepted. In one example, Phaeobacter gallaeciensis (a species of Roseobacter) is attracted to marine algae by producing auxin phenylacetic acid, which is a potent broad spectrum antibiotic tropodithietic acid (TDA), and a valence tautomer thiotropocin of TDA.
Fig. 1. Workflow of processes used in this study, including sample collection, data processing, analysis of community characteristics, and microorganism ecological function prediction. a. Sampled location and sampled reefs; samples were collected from reefs with brown algae (labeled as B), red algae (labeled as R), green algae (labeled as G) and without algae (labeled as NO). b. Characteristic analysis of community: alpha diversity analysis, community composition analysis, LEfSe analysis, beta diversity analysis, and evolutionary analysis. c. Microorganism ecological function prediction based on FAPROTAX database. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)
In turn, the algae can contribute nutrients and a suitable surface for the colonization of the Roseobacter (Buchan et al., 2005). Similarly, on coastal reef systems, bacterial biofilms can induce the attachment of aquatic eukaryotes, including algae, to form a complex symbiotic system between the algae and the bacteria (Amin et al., 2015; Ramanan et al., 2016; Koo et al., 2017). In this system, algae benefit from the supply of nutrients (Makino et al., 2003; Wang et al., 2007), release of specific auxin (Le Bail et al., 2010; TAPIA et al., 2016; De Mesquita et al., 2019), inhibition of fouling organisms (Rao et al., 2007; Egan et al., 2008), and prevention of harmful substances (Serra et al., 2016; Levy et al., 2011). In turn, algae improve the system environment by serving as a habitat for many epibiotic species, and also create substrata Favoring the attachment of microorganisms and the production of many organic substances functioning as nutrients for bacterial multiplication and bacterial biofilm production (Singh et al., 2013).

The interactions between algae and bacterial biofilm are not always beneficial. The abovementioned reciprocal relationships between the rosebacter and algae can shift to a negative phase. Emiliania huxleyi (an alga) can generate p-coumaric acid (pCA) to indicate an elevated algal population density or algal senescence, and signal Phaeobacter gallaeciensis (the Roseobacter species) to produce roseobacticides from phe-nylacetic acid and a tropone precursor in TDA biosynthesis. This shift from symbiosis to pathogenesis may allow P. gallaeciensis to access food resources from aging algae, and make the bacteria disassociate from dying algae and re-associate with other healthy algae (Seyedsayyamdost et al., 2011). Moreover, the lack of nutrients may lead to the deterioration of a reciprocal relationship, such as that wherein adhered bacteria and algae compete with each other for phosphorus, when bacteria are limited by the scarcity of phosphorus during the metabolic process of nutrients (Wang et al., 2007). Moreover, pathogenic bacteria can exert disastrous effects on algae (Vairappan et al., 2008; Sun et al., 2012). Climatic changes exert stress on marine habitat formers and their associated microbiota, and produce an inflow of harmful pollutants. This deterio-rates the environmental conditions and may make the bacterial biofilms susceptible to potential opportunistic pathogens, which has a negative effect on algae (Gachon et al., 2010).

Changdao Island is located at the northeastern region of Shandong Province and is the largest island in North China (Chi et al., 2016). This area belongs to the East Asian monsoon climatic region and is characterized by moderate rain, humid air, and mild climate. Owing to the rapid development of marine fisheries in Changdao Island, the urbanization process and economic level of the island have effectively improved, whereas damages to the resources and the environment have become more extreme. For the purpose of ecological protection and sustainable development, since 2013, artificial reefs have been used to promote the settlement of algae and exert fish-gathering effects (Wang and Li, 2013). Under similar environmental conditions, various algae have settled onto various immersed reefs, but visible algae do not exist in some reefs, and this affects the reef's fish-gathering function. The underlying cause of this phenomenon has been inferred as the relationship between the reef algae and the bacterial biofilms. However, direct evidence clarifying the precise mechanism of these interactions is still ambiguous. Therefore, we investigated bacterial biofilms associated with algae-attached and algae-free reefs from Changdao Island by carrying out diversity estimation and community composition analysis using high-throughput sequencing. The specific objectives of this study were to investigate the community characteristics of bacterial biofilms associated with various algal settlements on coastal reefs, access and compare the ecological functions of microbiota in the biofilms using the FAPROTAX database, and link the compositions and functions of bacterial biofilms with the settlement of reefs by algae.

2. Materials and methods

2.1. Sample collection

Field sampling was conducted in a small area of no more than 25 square meters (37.92° E, 120.76° N) at the coast of Changdao Island. Samples were collected in triplicate from adjacent reefs settled with brown (Sargassum thunbergii; labeled as B), red (Gelidium; labeled as R), green (Ulva lactuca; labeled as G), or no (labeled as NO) algae in August 2018 (Fig. 1A). The sampling environmental parameters were as follows: water depth of 0.5–1.5 m; salinity of 31.96; temperature of 32.0°C; conductivity of 47.21 mS cm⁻¹. The bacterial biofilms were cautiously scratched with sterile scalpel blades, following the methods described by Pootakham et al. (2017). The collected microbiota were placed in 2-mL screw-capped tubes filled with sterile seawater and transported back to a vehicle within 2 h under low light inside a container with a temperature of 4°C. Upon returning to the laboratory, the bacterial biofilm samples were stored at −20°C for use in experiments to be conducted as soon as possible.

In the laboratory, after thawing, ultrasonic oscillation was promptly applied to the centrifuge tubes containing various samples to remove any impurities. Each separated seawater was filtered through a 0.22-µm (47-mm) polycarbonate membrane and stored at −80°C to conduct the DNA extraction experiments described below.

2.2. DNA extraction, PCR, and sequencing

DNA was extracted using the Fast DNA SPIN Kit (MP BIO, USA) as described by the manufacturer. Then, the Nano Drop 2000c spectrophotometer (Thermo Fisher, USA) and agarose gel electrophoresis were used to determine the concentration and quality of the extracted DNA, which was subsequently purified using the methods specified in Ceh et al. (2011). To amplify the sample genes, the Polymerase Chain Reaction (PCR) process was conducted according to the protocol of Yu et al. (2017). The primers 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GACTACHVGGGTWTCTAAT-3') flanking the V3–V4 regions of the 16S gene were used for environmental DNA amplification while attached with a unique 6-nt barcode at the end of the forward primer. Using 20 ng of purified DNA as a template, the PCRs were carried out using the ABI GeneAmp 9700. In the PCR cycling, the DNA was first pre-denatured at 95°C for 5 min, and then again after 35 cycles of denaturation at 94°C for 30 s. After the denaturation had been completed, annealing was performed at 55°C for 30 s, followed by extension at 72°C for 40 s, and finally by extension at 72°C for 7 min.

The amplified products were combined and subsequently gene-cleaned using the AxyPrep DNA Gel Extraction Kit (Axygen, USA). The resulting amplicon libraries were used for quantification through agarose gel electrophoresis and the QuantiFluor-ST fluorometer (Promega, USA). The Majorbio Co., Ltd. (Shanghai, China) 468-bp PE300 Illumina MiSeq sequencing platform was used to perform high-throughput sequencing for the PCR products. However, the B_2 sample was not successfully sequenced (the detailed processing methods used for the B_2 sample are described in S1).

2.3. Data processing

The FastQC software was used to control the quality of the raw FASTQ data obtained from the high-throughput sequencing products (Brown et al., 2017). Sequences shorter than 1000 nt were removed prior to downstream analyses based on the Quantitative Insights into Microbial Ecology (QIIME) pipeline, as described by Caporaso et al. (2010). Non-repetitive sequences from optimized sequence facilitates were extracted (Bokulich et al., 2013). Then, operational taxonomic units (OTU) clustering was performed on the non-repetitive sequences (excluding single sequences) according to a similarity of 97%, and chimeras were removed during the clustering to obtain the
representative OTU sequences (Edgar et al., 2011). All optimized sequences were mapped to the OTU representative sequence, and the similarities of the representative sequence exceeding 97% were selected to generate an OTU table (Caporaso et al., 2009). To obtain the taxonomic group information corresponding to each OTU, the Silva database was used for comparison. Similarly, based on the Ribosomal Database Project (RDP) pyrosequencing pipeline, a Bayesian classification algorithm was used to classify the 97% similarity level of the OTU representative sequence and calculate the community composition of each sample at each taxonomic hierarchy (Desantis et al., 2006). The libraries belonging to non-bacteria taxa (Archaea, Chloroplast, and unassigned taxa), or accounting for <0.01% of the total sequences, were removed to ensure the validity of the bacterial community estimation (Bokulich et al., 2013).

2.4. Community characteristics and functional analysis

The Mothur v.1.30.1 software package and the QIIME pipeline were used to analyze the alpha diversity, wherein the Chao1 estimator (predicted OTU) and Shannon diversity index were selected to evaluate the richness and diversity of the community (Fig. 1B; Leray and Knowlton, 2016). Analysis of Variance (ANOVA) with a post-hoc test (Tukey) was conducted to detect whether the index values between multiple groups had any significant differences. Based on the taxonomic data sheet, the community distribution bar map of each sample and the Venn diagram at the OTU level were plotted using the R programming data (Pathak, 2014). Moreover, the vegan package in R was used to map the community heatmap. The Circos-0.67-7 software was used to map a visual circle describing the correspondence between the samples and species (An et al., 2014; krzywinski et al., 2009). Linear Discriminant Analysis Effect Size (LEfSe) analysis was carried out, and the results are presented in visual graphs (Segata et al., 2011). The QIIME pipeline and R language were used to calculate the beta diversity distance matrix, and draw the hierarchical clustering tree and principal coordinate analysis (PCoA) statistical map. According to the maximum likelihood method, the phylogenetic tree was constructed by selecting sequences corresponding to the classification information at a certain hierarchy using the FigTree v1.4.3 software. The sequencing data were functionally annotated using the FAPROTAX database (Louca et al., 2016). The results are presented as graphs created using the PermutMatrix software (Fig. 1C).

2.5. Data availability

The sequence data of this study were deposited to the Sequence Read Archive of NCBI (https://www.ncbi.nlm.nih.gov/). A bio-project associated with this study was applied for and processed in NCBI with the accession number PRJNA532757. The metatranscriptome sequences for all 11 samples were stored in a sequence reading archive (accession number SRR9705613-SRR9705623).

3. Results

3.1. Sample information statistics

The PCR products of the 16 S rRNA gene’s V3-V4 region were sequenced. Hence, the barcoded high-throughput sequencing generated 529,132 quality sequences from 11 samples, with an average of 48,103 sequences per sample (after optimization; Table 1).

Table 1

|           | B       | R       | G       | NO      |
|-----------|---------|---------|---------|---------|
| Number of sequences | 54,507  | 47,688  | 49,194  | 43,157  |
| Goods coverage (%)   | 98.78   | 98.72   | 98.54   | 98.41   |
| Pan OTU             | 1588    | 1486    | 1333    | 1241    |
| Predicted OTU (Chao1)| 1652    | 1421    | 1375    | 1263    |
| Shannon diversity    | 5.69    | 5.43    | 4.91    | 4.80    |

3.2. Alpha diversity analysis

Only the denoised sequences of the QIIME analyses are discussed in this paper. The Chao index (predicted OTUs) for each sample in groups B, R, and G ranged from 1375 to 1652, which is higher than that in the NO group (1263). The B-group samples had the highest Chao index, followed by the R-group samples and the G-group samples. Most bacterial Shannon diversity indices associated with any of the B, R, and G groups (highest at HZ_1, 5.72) were also higher than those observed in the NO group, with a range from 4.91 to 5.65 (Table 1).

The ANOVA with a post-hoc test (Tukey) results revealed that the Shannon and Chao index in different groups had various degrees of difference (Fig. S1). Significant Shannon index differences between groups B, R, and NO were detected (B–NO, p = 0.002; R–NO, p = 0.009). Significant differences amongst groups B, R, and G were also observed (B–G, p = 0.005; R–G, p = 0.024). Similarly, significant differences in the Chao index were observed between B and NO (p = 0.025).

3.3. Community composition analysis

The B-group samples were dominated by ten phyla (using the class level for the Proteobacteria phylum): Alphaproteobacteria (31.6%), Bacteroidetes (29.5%), Gammaproteobacteria (12.8%), Cyanobacteria (6.7%), Verrucomicrobia (3.5%), Actinobacteria (3.3%), Deltaproteobacteria (2.9%), Parcubacteria (2.2%), Fusobacteria (1.5%), and Firmicutes (1.4%). Approximately 96.0% of bacterial sequences in the R-group could be classified into twelve phyla: Alphaproteobacteria (35.7%), Bacteroidetes (22.0%), Gammaproteobacteria (12.0%), Fusobacteria (8.0%), Actinobacteria (5.8%), Verrucomicrobia (3.2%), Deltaproteobacteria (2.2%), Cyanobacteria (2.0%), Saccharibacteria (1.9%), Deinococcos-Thermus (1.2%), Parcubacteria (1.1%), and Firmicutes (1.0%). Eight phyla accounted for more than 1% in the G group: Alphaproteobacteria (53.8%), Bacteroidetes (20.6%), Cyanobacteria (7.8%), Gammaproteobacteria (6.4%), Fusobacteria (3.6%), Deltaproteobacteria (1.6%), Verrucomicrobia (1.4%), and Actinobacteria (1.3%). In the NO group, 97% of the bacterial sequences could be classified into six phyla: Alphaproteobacteria (32.7%), Cyanobacteria (26.6%), Bacteroidetes (22.2%), Fusobacteria (8.4%), Gammaproteobacteria (6.2%), and Deltaproteobacteria (1.3%). The abundant phyla/classes could cluster all groups into two parts: one part for the NO group and another part for the algae-attached groups (B, R, and G), which indicates two distinctive distances of microbial communities (Fig. 2; Fig. S2).

3.4. Community LEfSe analysis

The LEfSe can analyze differences in the species composition between groups and identify different microbial species (http://huttenhower.sph.harvard.edu/galaxy/). The LDA results (Fig. 3; Table S1) revealed that the B group was discriminatingly associated with the phylum Chlorobi (including class Chlorobria, order Chlorobiales and family OPB56), Firmicutes (including class Firmicutes bacilli), Parcubacteria, Planctomycetes (including class OM190, order Phycisphaerales, family Phycisphaeraceae), class Flavobacteriia (including order Flavobacteriales, family Flavobacteriaceae, genera Lutimona, Endoraea, Aquibacter, Actibacter, and Algibacter, and species Algibacter asturiai), Deltaproteobacteria, order Caldilineales (including family Caldilineaceae), Xanthomonadales, families Ferrimonadaceae (including genus Ferrimonas and species Ferrimonas pelagia), Celiivibrionales, Halleaceae (including genus Halicea and the OM60/NOR5
clade), Rhodospirillaceae, and genera Hirschia and Marivita. The discriminative taxa related to the R group were phyla Gracilibacteria, Actinobacteria (including class Actinobacteria order Acidimicrobiales), Saccharibacteria, classes Clostridia (including order Halanaerobiales), and Gammaproteobacteria (including order Chromatiales, family Granulosicoccaceae, genera Granulosicoccus, Coxiella, and Thiobrix), orders Rhodospirillales and Desulfobacterales (including family Desulfobulbaceae), family Helicobacterace (including genus Sulfurovum), genera Portibacter, Limnobacter, Ahrensia, Oricola, Litorimonas, and Hellea, and species Aquisalinus flavus. Discriminative taxa associated with the G group were relatively sparse, and only included class Anaerolineae (including order Anaerolineales and family Anaerolineaceae), and genera Ulvibacter, Winogradskyella, Croceitalea, Sphingomicrobiurn, Tropicimonas, Loktanella, and Boseongicola. The discriminative taxa associated with the NO group were phylum Cyanobacteria (including class member Cyanobacteria, order member Subsection I and Subsection III, Family I of Subsection I, and Family I of Subsection III, and genus Phormidium), unclassified Bacteroidetes, order Bradymonadales, family Shewanellaceae (including genus Shewanella), genus Rubidimonas, species Lewinella agrilytica, Nonlabens sediminis, Tenacibaculum skagerrakense, Vibrio mediterranei, and Pseudoalteromonas ruthenica.

3.5. Beta diversity analysis

The principal co-ordinate analysis of the weighted UniFrac distance revealed that the B, R, G, and NO groups possessed distinctive distances of microbial communities (Fig. 4). In the coordinates of PC1 and PC2, the PC1 and PC2 axis explains 47.27% and 24.24% of the total variation. The samples of the NO group are dispersed on the right side of the graph, along the primary axis and away from the B, R, and G groups. On the left side of the coordinates, the samples of the B and R groups were

**Fig. 4.** Principal co-ordinate analysis results showing the spatial variations of four groups according to the weighted UniFrac distance. The top two PCoA axes (PC1 and PC2) are shown in the graph. Samples collected from reefs with brown algae were labeled as B; samples with red algae were labeled as R; samples with green algae were labeled as G; samples without algae were labeled as NO.
interlaced and separated from the clustered G group. Briefly, the communities on reefs with identical algae settlements were relatively clustered together and separated from those on reefs without algae. Overall, the top two PCoA axes explain 71.5% of the variation in the different communities.

3.6. Prediction of microorganism’s ecological function

FAPROTAX was used to map prokaryotic taxa (e.g., genus and species) to metabolic or other ecologically relevant functions ([Louca et al., 2016]). The bacterial taxa were categorized into 54 types, each with different ecological functions (total of 90 types in the database). The functions of annotated taxa were related to the metabolism of C (methylotrophy, methanotrophy, chitinolysis, cellulolysis, and xylanolysis), N (aerobic ammonia oxidation, nitrate respiration, and denitrification), S (dark sulfide oxidation, sulfite respiration, and respiration of sulfur compounds), and other trace elements (oxidation and respiration of iron and manganese). The relative abundance of each function was processed to generate the bacterial metabolic heat map (Fig. 5), which clearly illustrates the diverse ecological functions and metabolic pathways in each group. The richer diversity of the ecological functions and metabolic types is associated with the B, R, and G groups (40 function types existed in the R group, 45 function types existed in the B group, and 41 function types existed in the G group), rather than with the NO group (only 35 function types were present).

The percentage of microbiota associated with carbon metabolisms was more than 90% amongst all groups (Fig. 6A). A certain percentage of sulfur-metabolism-related communities existed in all groups. The B and R groups had the highest proportion, followed by the G group and NO group, which had the lowest proportion. The proportion of classifiable bacteria associated with the metabolism of nitrogen was relatively constant in all groups. The taxa related to the metabolic processes of trace elements (iron and manganese) had low abundance in all groups (less than 1%). Additionally, bacteria related to the metabolism of certain trace elements were only observed in specific groups.

All functional annotated bacteria were classified as aerobic, facultative, or anaerobic. Additionally, it was found that the bacterial libraries in the B, R, G, and NO groups were dominated by sequences that were most similar to aerobes. However, the percentage in the NO group declined, and the percentage in the B, R, G groups was 67.76%, 70.20%, 74.47%, and 52.04%, respectively, as shown in Fig. 6B. Moreover, the percentage of bacteria related to the facultative class was highest in the NO group (41.11%) and lowest in the R group (12.09%). The percentage of aerobic bacteria in the R group was the highest amongst all groups (17.71%), while the B, G, and NO groups were not very different (~8%). With regard to heterotrophs and phototrophs, the communities in all groups were dominated by the groups most closely related to known heterotrophs. However, the percentage in the NO group declined, while the B, R, and G groups reached 96.78%, and the NO group reached 82.51%. The bacterial communities belonging to the NO group contained more groups related to photoautotrophs (17.48%).

4. Discussion

This study investigated the surface of reefs in Changdao Island and found that they harbored abundant and diverse microbial communities with specific characteristics. Environmental parameters such as temperature, salinity, and electrical conductivity were measured within a sampling area of 25 square meters. However, the difference of hydrological conditions and physical and chemical parameters was much smaller than the diurnal variation and tidal range variation of the station itself. For this reason, we simplified the abovementioned parameters to better investigate the structure and function of bacteria associated with different algae and establish the relationship between them. Eleven communities from different reefs were clustered into two categories: the first category corresponds to the B, R, and G groups, while the second one corresponds to the NO group. This indicates a separation between the bacterial biofilms with and without algal symbiosis. The alpha diversity indices for each of the B, R, and G groups (the Chao index ranged from 1375 to 1652, and the Shannon diversity index ranged from 4.91 to 5.69) were higher than those of the NO group (the Chao index was 1263, and the Shannon diversity index was 4.80). An assay focusing on the microbial diversity on benthic reefs produced similar results, wherein the diversity of microbial communities associated with the reef algae (the Chao index was 266–461, and the Shannon diversity index was 2.84–5.63) was richer than that associated with the reef-building coral (the Chao index was 953–3300, and the Shannon diversity index was 6.22–7.82; Barott et al., 2011). This tendency indicates that more diverse functional microbiota can coexist with algae, and thus produce more complex reactions in the algae-bacterial symbiotic system.

Discriminative taxa were also found amongst the four groups, and their detailed descriptions were presented in Section 3.4. This variability of bacterial communities between the four groups was likely caused by the diverse heterogeneous assemblage of algae and bacteria, which increased the heterogeneity of bacterial communities. Some of these heterogeneous bacteria are widely distributed in other habitats such as extensive sea water (Roush et al., 2014), tidal flat sediment (Kim et al., 2008), marine organisms (Hyun et al., 2015), and even industrial production (Roush et al., 2014). However, an association with algae has been demonstrated for most of these bacteria, which
suggests that the bacteria engage in multiple interactions with algae. The biofilms on the surface of the kelp Laminaria hyperborea exhibit a higher proportion of Planctomycetes compared with other investigated Planctomycete-rich habitats (open water, peat bogs, and sandy sediments; Bengtsson and Øvreås, 2010). A study on bacterial communities associated with the genus Asparagopsis (red seaweed) also found a high abundance of Acidimicrobiales in Asparagopsis armata samples (Aires et al., 2016). Additionally, most species in the Alginibacter genus (Flavobacteria) were isolated from or were close to the algae habitat, which indicates a preference for complex algae polysaccharides (Sun et al., 2016). The adherence of these heterogeneous bacteria on coastal reefs may improve the surrounding environment through various nutrient metabolic and specific reactions to satisfy the requirements of algae. Thus, a settlement of opportunistic algae can be attracted to reefs. Furthermore, various bacteria can protect the algae from adverse external factors, and thereby maintain the steady state of the algal symbiosis system and promote the healthy development of algae. The FAPROTAX results revealed that, in all groups, the communities were dominated by the bacteria most related to carbon metabolism (greater than 93%). This proves that the metabolic activities of carbon are extensive on reef surfaces. This microbial carbon metabolism can maintain the stability of the carbon cycle in the environment, which is the basis for algal attachment. Moreover, compared with the NO group, the settlement of algae in the B, R, and G groups leads to a complex exchange of carbon elements between the algae and bacteria. With the settlement of algae on the reef surface, the algae become primary carbon producers through the discharge of carbon and tissue growth. Additionally, through the absorption of exuded carbon and the degradation of necrotic tissues, bacterial biofilms convert these carbonaceous compounds to specific forms that can be reused by the algae. The various bacteria with carbon-metabolizing functions found on the investigated reefs provide insight to these processes. Marine Flavobacteria (discriminative taxa in the B group) play an important role in the degradation of algal tissues owing to the use of biopolymers such as polysaccharides and proteins (Thomas et al., 2012). The Saccharibacteria found in the R group are capable of degrading cellulose, hemicellulose, pectin, starch, and 1,3-β-glucan. Additionally, they can generate hydrolyase to breakdown salicylic acid and interconvert a variety of isoprenoids. Consequently, Saccharibacteria can generate acetate and lactate by fermenting the algal exudates and tissues to promote the carbon cycle in the system (Starr et al., 2018). Moreover, Anaerolineaceae have been found in the G group, which indicates the existence of fermentation and the oxidation of alkanes to small molecules such as formate, acetate, hydrogen, and carbon dioxide (Liang et al., 2015).

Carbon enrichment in the surrounding environment is considered to promote the settlement of opportunistic algae, while the types of settled algae may be related to the ecological cycle and sulfur supply. The percentage of classifiable bacteria associated with sulfur metabolism was high on reefs with attached algae, particularly in the B and R groups where it reached up to 3.57% and 5.63%, respectively (Fig. 6A). This conclusion is based on the distinct physiological characteristics of these algal groups. Brown and red algae can release sulfur-containing secretions (elicitors) when subjected to abiotic or biotic stress with intense sunlight, wind, or bacterial and viral infections (Hou et al., 2015). Red coralline algae are macroalgal producers of dimethylsulphonio propane (DMSP), which is a vital component of the marine sulfur cycle (Sunda et al., 2002). Porphyran, which is a sulfated galactan, is the main cellular polysaccharide in marine red algae. Oligoporphyran obtained by the acid hydrolysis of porphyrin use their H2O2-inducing abilities as defense responses to Pyropia yezoensis (Hou et al., 2015). Moreover, various algal tissues may contain a sulfolipid compound. For example, fucoidan (polysaccharides containing a substantial amount of sulfate ester groups) has been reported as a constituent of brown seaweed (Bilan et al., 2002) and possesses various biological properties, such as anticoagulant, antithrombotic, and antiviral properties (Li et al., 2008). Planctomycetes (found in the B group) can particularly degrade sulfated polymeric carbon, as proven by the detection of genes involved in the breakdown of sulfated polysaccharides (Glockner et al., 2003). Thiophtrix are colorless sulfur-oxidizing bacteria that may deposit sulfur when grown in the presence of sulphide or thiosulphate (Nielsen et al., 2000). These sulfur-oxidizing and sulfur-metabolizing bacteria cannot only metabolize sulfur-containing substances in the environment, but can also decompose residual sulfur-containing secretions or corroded algal tissues to generate absorbable sulfur molecules, which are consumed again by these algae.
This study found that the inter-group specificity of bacteria is associated with other trace elements, which may also explain the types of settled algae (Fig. 5). Geodermatophilus (Actinobacteria), which is a type of bacteria associated with manganese oxidation, have only been found in the G group. Additionally, iron-associated bacteria have only been discovered in groups with attached algae. For instance, Ferrimonas (found in the B group) are reducing bacteria that can use yeast extract, lactate, pyruvate, casamino acids, and tryptophan as electron donors, and carbon sources to reduce iron(III) oxohydroxide, iron(III) citrate, selenate, arsenate, elemental sulfur, and thiosulfate (Nakagawa et al., 2006).

Furthermore, on reefs with algal settlement, various taxa have been observed to exert protective effects. Acidimicrobials discriminately associate with the R group, and various studies have demonstrated that these bacteria can contribute to the resistance of oxidative stress and toxic compounds (heavy metal detoxification; Ai et al., 2016). The toxic effects of heavy metals and other environmental stresses to algae are produced by reactive oxygen species (ROS), while algae respond to these toxic compounds by producing antioxidants such as glutaredoxins and glutathione. The bacteria effects supporting the resistance of toxic compounds are associated with the synthesis pathway of glutathione, which is used to scavenge the produced ROS (Dring, 2005; Mellado et al., 2012). Various Haliea (Gammaproteobacteria, found in the B group) can generate alkenes monooxygenases to oxidize alkenes, which has multiple effects on algae, such as the inducement of chlorophyll-a loss and the aggravation of mastoparan-induced cell death (Suzuki et al., 2012). Similarly, a few bacteria have been observed to synthesize a diverse array of steroids and cyclic triterpenoids with defensive functions against pests and pathogens. For example, a species of Latimonas (Flavobacteria, found in the B group) can produce two isoorbinal-like lipids, eudoraenol, and adriaticol (Banta et al., 2017). This resistance to toxic substances in the environment and the prevention of pathogenic bacterial invasion are essential for maintaining the steady state of the algae-bacteria symbiotic environment and promoting the healthy development of algae settled on reefs.

In contrast, besides the lack of relevant metabollic bacteria, the absence of algal settlements in the NO group is attributed to the invasion of pathogenic and competitive bacteria and the lack of associated protective microbiota. Pseudoalteromonas has been observed to be pathogenic in group NO. The swarming of Pseudoalteromonas has potent algicidal effects, which can cause rapid cell lysis and the death of gymniodinoids (including Gymnodinium catenatum) and raphidophytes (including Heterosigma akashiwo and Chattonella marina), and the ecdysis of armored dinoflagellates (such as Alexandrium catenella) (Lovejoy et al., 1998). Pseudoalteromonas have pronounced effects against the germination of spores from green algae Ulva lactuca and red alga Polysiphonia through the production of an extracellular component with a specific activity toward algal spores (Egan et al., 2001). Additionally, compared with the B, R, and G groups, discriminative taxa associated with the NO group mostly belong to the phylum of Cyanobacteria, which are widely considered as oxygenic photosynthetic bacteria (Soo et al., 2017). These results correspond to the FAPROTAX results wherein the proportion of bacteria associated with photoautotrophs in the NO group reached 17.48%, which is the highest proportion amongst all groups (Fig. 6B). However, owing to the competition for light or other phototrophic substances, a higher proportion of phototrophs may be detrimental to the attachment of algae. Moreover, algae have been observed to exert allelochemical effects through secretions of antimicrobial metabolites targeting photosynthetic bacteria to prevent the photosynthetic ephibions from competing with macroalgae for light and nutrients (Beam et al., 2016). Owing to the allelochemical effects of algae on phototrophic bacteria, the relationship between them is considered as antagonistic. Furthermore, the breeding of over-dense cyanobacteria can produce inherent cyanotoxins (including cyclic peptides and alkaloids) in toxic quantities in the vicinity of hydrophytes and fishes (Van Apeldoorn et al., 2007).

5. Conclusions

Coastal reefs with and without attached algae harbor abundant and characteristic microbial communities. Groups B, R, G, and NO have significant differences in terms of microbial community structures and ecological functions. The characteristic microbiota related to the B, R, and G groups have richer diversity compared with those found in the NO group. The discriminative taxa in the B group are mostly classified into the Planctomycetes, Parcubacteria, Firmicutes, and Chlorobi phyla, and the Flavobacteria and Deltaproteobacteria classes. Additionally, the bacteria in the R group are mostly associated with the Gracilibacteria and Actinobacteria phyla, and the Gammaproteobacteria, Deltaproteobacteria, and Clostridiales classes. Although discriminative taxa were discovered in other phyla or classes, the most relevant bacteria in the G group belong to the Anaerolineae class. The NO group mainly harbors bacteria belonging to the Cyanobacteria phylum, and unclassified Bacteroidetes. Based on the comparative analysis of community characteristics and the ecological functions of bacterial biofilms in the four groups, it was concluded that the metabolism and supply of nutrients by the bacterial biofilms induced the settlement of opportunistic algae. Particularly, the presence of bacteria associated with sulfur and other elements may explain the settlement of different algae types on reefs. Furthermore, in groups with algal attachment, various discriminative taxa were observed to exert protective effects, including resistance to toxic substances in the environment and prevention against pathogenic bacterial invasion. This was essential to maintain the steady state of the algae-bacteria symbiotic environment and promote the healthy development of algal settlements on reefs. Additionally, for the NO group, the competition of photosynthetic bacteria for light and nutrients and the toxicity of pathogenic bacteria such as Pseudoalteromonas can explain the lack of algal settlements in the NO group.

Therefore, in practical artificial reef applications, microbial agents of bacteria beneficial to the nutrient supply can be introduced to reefs to attract the settlement of opportunistic algae. Additionally, the attraction of specific types of algal attachment should focus on bacteria associated with special elements such as sulfur and trace elements. Moreover, on artificial reefs, phototrophic bacteria (Cyanobacteria) and pathogenic bacteria (Pseudoalteromonas) should be eradicated to maintain the benign development of algae. The influence of various temporal and spatial factors should be considered, and the sample size should be increased to further strengthen the representativeness of this study. However, according to the results, on reefs with different types of attached algae, it is possible to isolate discriminative bacteria and then apply them to the settlement and development of algae on artificial reefs, which will be investigated in future work.

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Appendix A. Supplementary data

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References

Aires, T., Serrão, E.A., Engelen, A.H., 2016. Host and environmental specificity in
Thiel, V., Brinkhoff, T., Dickschat, J.S., Wickel, J., Wagner-Döbler, I., Simon, M., Schulz, S., 2010. Identification and biosynthesis of tropone derivatives and sulfur volatiles produced by bacteria of the marine Roseobacter clade. Org. Biomol. Chem. 8, 234–246.

Thomas, F., Barbeyron, T., Tonon, T., Génicot, S., Czjzek, M., Michel, G., 2012. Characterization of the first alginolytic operons in a marine bacterium: from their emergence in marine Flavobacteria to their independent transfers to marine Proteobacteria and human gut Bacteroides. Environ. Microbiol. 14, 2379–2394.

Vairappan, C.S., Chung, C.S., Hurtado, A., Soya, F.E., Lhonneur, G.B., Critchley, A., 2008. Distribution and symptoms of epiphyte infection in major carrageenophyte-producing farms. J. Appl. Phycol. 20, 477–483.

Van Apeldoorn, M.E., Van Egmond, H.P., SPEIJERS, G.J., BAKKER, G.J., 2007. Toxins of cyanobacteria. Mol. Nutr. Food Res. 51, 7–60.

Wang, J.G., Li, W.Q., 2013. A demonstration area of island reef ecological restoration will be built in Changdao. Shandong Fish. 5 59–59. (in Chinese).

Wang, H., Smith, H.L., Kuang, Y., Elser, J.J., 2007. Dynamics of stoichiometric algae-bacteria interactions in the epilimnion. SIAM J. Appl. Math. 68, 503–522.

Xu, F.L., Duan, J.Z., Lin, C.G., Hou, B.R., 2015. Influence of marine aerobic biofilms on corrosion of 316L stainless steel. J. Iron Steel Res. 22, 715–720.

Yu, S.X., Pang, Y.L., Wang, Y.C., Li, J.J., Qin, S., 2017. Spatial variation of microbial communities in sediments along the environmental gradients from Xiaoqing River to Laizhou Bay. Mar. Pollut. Bull. 120, 90–98.