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Isolation, characterization and implications of the bacterial communities associated with established cultures of *Chattonella marina* (Raphidophyceae) and *Skeletonema costatum* (Bacillariophyceae)

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Abstract

Cultivable bacteria coexisting in the cultures of two microalgal species, *Chattonella marina* (Raphidophyceae) and *Skeletonema costatum* (diatom, Bacillariophyceae), which have been maintained in the laboratory for several years, were examined in this study. Forty-eight and thirty-four cultivable bacterial strains were isolated from different growth stages of *C. marina* and *S. costatum* cultures, respectively. A total of twelve unique bacterial phylotypes were isolated. These bacterial phylotypes belonged to Alphaproteobacteria, Gammaproteobacteria, Bacteroidetes, and Actinobacteria. Alphaproteobacteria predominated in phycospheres of both microalgae, and Rhodobacteraceae was the most common family. Bacterial phylotypes were more diversified in cultures of *S. costatum* than in those of *C. marina*. Bacterial concentrations increased remarkably after the late stationary phase of *C. marina*, which might account for the decline in algal cells. One phylotype of *S. costatum*-associated bacteria had inhibitory effects on *Chaetoceros curvisetus* (Bacillariophyceae). However, most bacterial phylotypes from cultures of *C. marina* showed significant inhibition of the growth of *C. curvisetus*. The results suggested that bacteria associated with *C. marina* might have some ecological roles in its competition with diatoms.

Key words: phycosphere, marine bacteria, *Chattonella marina*, *Skeletonema costatum*, *Chaetoceros curvisetus*, algal growth

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1 Introduction

Many algal isolates are obtained by micropipetting a single algal cell from an environmental sample to produce a clonal culture. In the absence of treatment to render the culture axenic, bacteria that were initially present in the phycosphere have the ability to grow in association with algal cultures through successive transfers (Jasti et al., 2005). The phycosphere is the region immediately surrounding and influenced by the algal cells where bacteria feed on extracellular products of the algae (Bell and Mitchell, 1972). Many bacterial strains have been isolated from microalgal cultures that have been maintained in the laboratory for many years (Hold et al., 2001; Green et al., 2004; Jasti et al., 2005; Sapp et al., 2007; Abby et al., 2014; Schwenk et al., 2014), and some were isolated after an extensive antibiotic treatment (Amaro et al., 2005). It was reported that specific bacterial phylotypes were identified as being associated with different microalgae and even associated with the growth and physiological status of algae (Hold et al., 2001; Grossart et al., 2005). However, some reports demonstrated that compositions of bacterial communities were not necessarily specific to algae species (Sapp et al., 2007; Schwenk et al., 2014).

Many studies have reported evidence for interactions between bacteria and phytoplankton, which has led to the conclusion that bacteria can play an important role in controlling phytoplankton dynamics and bloom development (Fukami et al., 1991; Liu et al., 2008; Park et al., 2010; Teeling et al., 2012; Meyer et al., 2017). For example, bacteria isolated during a multi diatom bloom in the Seto Inland Sea (Japan) are algicidal against dinoflagellates and raphidophytes rather than diatoms (Park et al., 2010). However, natural bacterial communities collected during a dinoflagellate (*Gymnodinium nagasakiense*) bloom inhibited the growth of *Skeletonema costatum* (Fukami et al., 1991), while bacterial communities collected during the later phases of algal blooms promoted the bloom collapse (Liu et al., 2008; Park et al., 2015). Given the complexity of natural phytoplankton communities, it is hard to know which bacterial strains are associated with which phytoplankton species (Jasti et al., 2005). Another approach, therefore, is to characterize bacteria associated with microalgae in culture and study their algicidal or growth-promoting effects.

In this study, cultivable bacteria coexisting in the cultures of the microalgae *Chattonella marina* (Raphidophyceae) and *Skeletonema costatum* (diatom, Bacillariophyceae) were examined. Comparison of the associated bacterial communities was per-

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formed during different growth phases of the algae. Bacterial phylotypes were analyzed by comparison of 16S rDNA sequences to the closely related sequences in GenBank. The effects of the associated bacteria on the growth of a typical diatom species, *Chaetoceros curvisetus*, were investigated. The purpose of this study is: (1) to understand the differences between the associated bacteria in the cultivated diatom (*S. costatum*) and harmful flagellate species (*C. marina*), (2) to discuss the differences between cultivable bacterial communities in relation to the algal growth stages, and (3) to reveal the effects of algae-associated bacteria on the growth of the diatom *C. curvisetus*, and thus to discuss their roles in phytoplankton competition.

2 Materials and methods

2.1 *Isolation of bacterial strains from microalgal cultures*

2.1.1 *Microalgal cultures*

The two microalgal species, *C. marina* and *S. costatum*, were originally isolated from the Daya Bay in the South China Sea in July 2005. The cultures were maintained in sterile *f*/2 medium (Guillard, 1975), which was previously autoclaved at 121°C for 20 min. Algal cells at the exponential growth phase were incubated in 250 mL Erlenmeyer flasks containing 100 mL of sterilized *f*/2 medium. The medium was made with artificial sea salt (Red Coral Sea, nutrient free formula) with salinity of 30–31 and a pH of 7.9±0.1. Microalgal cultures were maintained in an incubator at (20±1)°C and illuminated with 100 μmol photon m^{-2} s^{-1} of cool-white fluorescent illumination with a dark:light cycle of 12 h:12 h. The initial cell densities were 600 and 2 000 cell/mL for *C. marina* and *S. costatum*, respectively. The experiment was run for 53 d for *C. marina* and 19 d for *S. costatum* until growth reached a declining stage. Cultures were aseptically sampled over 2 d to 4 d intervals for cell counting.

The specific growth rate (μ, division/d) was calculated using the following equation (Wood et al., 2005):

$$\mu = (\ln N_2 - \ln N_1) / (t_2 - t_1), \quad (1)$$

where N_2 and N_1 are cell densities at time t_2 and t_1.

2.1.2 *Isolation of cultivable algae-associated bacteria*

Samples were aseptically taken at six growth stages of *C. marina* and five stages of *S. costatum* (Fig. 1). One milliliter of each culture was suspended in 9 mL of Zobell 2216E marine medium (Su et al., 2007) diluted to 10^{-1}. Zobell 2216E marine medium was prepared using artificial seawater. These suspensions were further diluted in the same way to 10^{-2}–10^{-6}. A 0.1 mL aliquot of the dilutions of 10^{-3}–10^{-6} was spread onto Zobell 2216E agar plates, and the plates were incubated at 28°C for 5–7 d. Visually distinct bacterial colonies were subcultured on fresh Zobell 2216E agar plates and incubated for 7 d. The step was repeated three to four times to obtain purified bacterial colonies.

2.1.3 *16S rDNA analyses of bacteria*

Single bacterial colonies were transferred to centrifuge tubes and suspended in 100 μL distilled water. The tube was frozen (–20°C for 7 min) and transferred to a water bath (100°C for 7 min), followed by centrifugation at 10 000×*g* for 10 min at room temperature after freezing and thawing three times. The supernatant served as the template DNA in the PCR. Primers for 16S rDNA V3-V5 region were 341F (5′-CCTACGGGAGGCAGCAG-3′) and 907R (5′-CCGTCAAATCMTTGAGTTT-3′) (Muyzer et al., 1997). All primers used were obtained from Sangong Biotech (Shanghai) Co., Ltd., China.

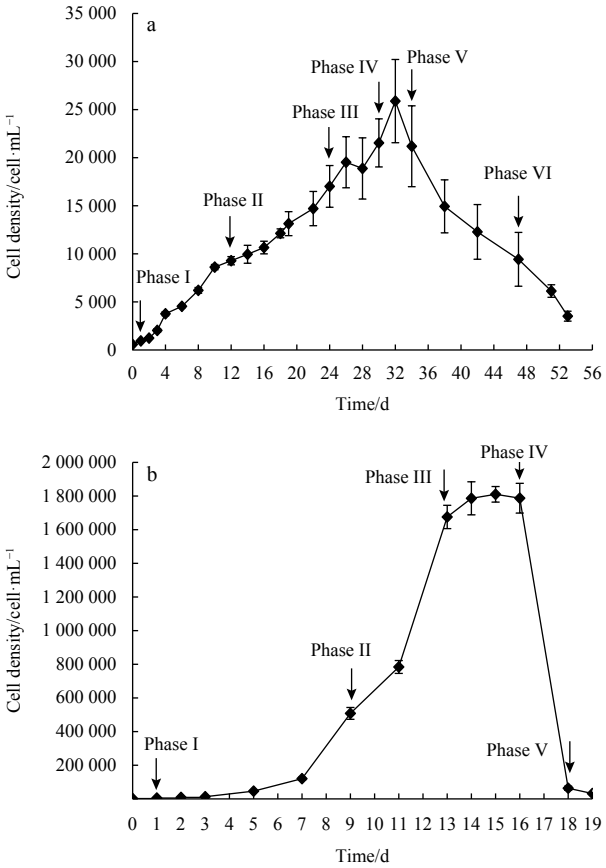

Fig. 1. Growth curves of *Chattonella marina* (a) and *Skeletonema costatum* (b) indicating the sampling stages for bacteria isolation.

PCR mixtures with a volume of 30 μL containing 4 μL of template DNA, 3 μL of 10× Taq buffer (500 mmol/L KCl, 100 mmol/L Tris-HCl, 15 mmol/L Mg (pH 8.3); Eppendorf, Germany), 2.5 μL of 2 mmol/L each dNTP, 0.3 μL of 10 μmol/mL each primer, 0.4 μL of 2.5 U/μL Taq DNA polymerase, 0.6 μL of 10 mg/mL bovine serum albumin (BSA), and mixed with ddH_2O. The PCR was performed using an initial denaturation at 95°C for 4 min; followed by 35 cycles of 95°C for 30 s, 57°C for 30 s and 72°C for 40 s; followed by 72°C for 7 min for a final extension. The PCR was carried out on a Veriti® 96-Well Thermal Cycler (ABI, USA). The specificity of the primers and size of the PCR product were estimated by electrophoresis with a 1.0% agarose gel in TBE buffer, followed by staining with ethidium bromide and visualization under UV light. Purification and sequencing were carried out by an external service (Sangong Biotech (Shanghai) Co., Ltd., China) using the internal primers and an ABI PRISM 3730 Autosequencer (Applied Biosystems).

The sequences generated in the present study were submitted to the National Center for Biotechnical Information (NCBI) at the National Library of Medicine (GenBank). Resulting DNA sequences were compared to the closely related DNA sequences in the NCBI databases (Basic Local Alignment Search Tool, nucleotide blast) using the ClustalX package.

2.1.4 *Estimation of bacterial concentration*

The bacterial concentrations were determined by measuring the colony-forming unit (CFU) number on Zobell 2216E agar me-

dium. Each distinct bacterial colony was counted on a plate of suitable colonies (30–300), and the concentrations of each bacterium and total bacteria in one milliliter algal culture (CFU/mL) was obtained according to the dilution factor.

A cluster analysis based on Euclidian distances was used to compare bacterial community structures after natural logarithm (ln) standardization of the data. All analyses were performed using the program SPSS 19.0 for Windows (SPSS Inc., Chicago, Illinois).

2.2 *Effects of bacteria on the growth of Chaetoceros curvisetus*

2.2.1 *Bacterial isolates*

Bacterial isolates were maintained in Zobell 2216 marine agar plates after removing the repeated isolates by cross-comparison with the results of the rDNA sequences. Before the experiment began, bacterial colonies were transferred to the liquid Zobell 2 216 medium, cultivated at 28°C on a 200 r/min shaking table for 8–10 h, and then gradually acclimated to 20°C within 2 h.

2.2.2 *Antibacterial treatment of Chaetoceros curvisetus*

Chaetoceros curvisetus was isolated from the Daya Bay in the South China Sea in 2005 and maintained in *f*/2 medium. The stock culture was treated with a mixture of antibiotics to destroy any external bacteria. First, 10 μg/mL penicillin (final concentration) was added to the 100 mL culture of *C. curvisetus* in exponential phase and then incubated for 24 h. Then, 10 mL of the penicillin-treated culture was inoculated into 100 mL fresh *f*/2 medium with 10 μg/mL streptomycin sulphate and incubated for another 24 h, followed by treatment with 10 μg/mL kanamycin sulfate. Bacterial presence in the algal cultures was determined by measuring plate counts (CFU) on Zobell 2216 agar plates, and antibiotic treatment was repeated until the bacterial concentration in the algal culture was <100 CFU/mL. The antibacterial treatment culture was maintained in sterilized *f*/2 medium for the experiment.

2.2.3 *Growth of Chaetoceros curvisetus cocultured with bacteria*

Liquid bacterial cultures in exponential phase were added to the exponential *C. curvisetus* cultures. The inoculation rate was 0.1% (v/v), and the initial bacterial concentration was 7.5×10^7 CFU/mL. Cultures in *f*/2 medium (*f*/2) and with same volume of liquid Zobell 2216E medium (BC) were set as the negative controls. Algal cultures were cultivated under the same conditions as Section 2.1.1. All experiments were carried out in sterile 24-well tissue culture plates. The experiment was run for 5 d, and cell numbers were counted everyday. All experiments were performed in triplicate.

3 Results

3.1 *The growth curve of algal cells*

Figure 1 illustrates the growth of *C. marina* and *S. costatum* in *f*/2 medium. The cell numbers of *C. marina* increased quickly after inoculation and almost doubled after one day of incubation (Fig. 1a). Cell numbers kept increasing until Day 26, followed by a slight decrease at Day 28, increasing to the maximum cell number of 2.59×10^4 cell/mL at Day 32. Arrows pointed out the six stages for bacterial isolation: Phase I (Day 1, with the specific growth rate μ=0.50 division/d) for the early exponential stage, II (Day 12, μ=0.04 division/d) and III (Day 24, μ=0.07 division/d) for the mid exponential stage, IV (Day 30, μ=0.06 division/d) for the late exponential stage, V (Day 34, μ=–0.10 division/d) for the early decline stage, and VI (Day 47, μ=–0.05 division/d) for the late decline stage.

Cell numbers of *S. costatum* increased after incubation until Day 13 and then experienced a four-day stationary stage (Fig. 1b). The maximum number of 1.81×10^4 cell/mL occurred at Day 15. The five phases marked in Fig. 1b for bacterial isolation were indicated for the early exponential (Phase I, Day 1, μ=0.90 division/d), mid-exponential (Phase II, Day 9, μ=0.72 division/d), stationary (Phase III, Day 13, μ=0.38 division/d), early decline (Phase IV, Day 16, μ=–0.01 division/d), and late decline (Phase V, Day 18, μ=–1.67 division/d) stages, respectively.

3.2 *16S rDNA analyses of cultivable algae-associated bacteria*

Forty-eight and thirty-four bacterial isolates were collected from cultures of *C. marina* and *S. costatum*, respectively. Six and seven different phylotypes were obtained from cultures of *C. marina* and *S. costatum*, respectively, after removing the duplicate rDNA sequences. Isolated bacteria were labeled according to their algal culture origin: for example, C1–C6 were bacteria isolated from *Chattonella* culture, while S1–S7 were isolated from *Skeletonema* culture. The sequences were submitted to the International Nucleotide Sequence Database Collaboration at NCBI (GenBank accession Nos KF928338-KF928340 and KF953940-KF953942 for C1–C6 and KF953933-KF953939 for S1-S7). As C3 and S2 are the same phylotype (*Roseobacter denitrificans*), twelve different phylotypes were obtained in this study.

The majority of the bacterial phylotypes in this study belong to the Proteobacteria phylum (Table 1). Rhodobacteraceae is the most common family, in which *Ruegeria* (C1, S3) and *Roseobac-*

Table 1. Similarities between bacteria in this study and the closest relative sequences from GenBank based on V3–V5 region of 16S rDNA

Bacterial phylotypes	Phylum or sub-phylum	Order	Family	The closest sequence	Similarity/%
C1 (KF928338)	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	*Ruegeria* sp. (NC 008044)	100
C2 (KF928339)	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	*Citromicrobium bathyomarinum* (NZADAE01000015)	99
C3 (KF928340)	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	*Roseobacter denitrificans* (NC008209)	98
C4 (KF953940)	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	*Labrenzia aggregata*, (NZAAUW01000037)	100
C5 (KF953941)	Bacteroidetes	Flavobacteriales	Flavobacteriaceae	*Robiginitalea biformata* (NC 013222)	92
C6 (KF953942)	Alphaproteobacteria	Rhodobacterales	Hyphomonadaceae	*Hyphomonas johnsonii* (NZARYK01000001)	95
S1 (KF953933)	Gammaproteobacteria	Alteromonadales	Alteromonadaceae	*Marinobacter santoriniensis* (NZAPAT01000001)	98
S2 (KF953934)	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	*Roseobacter denitrificans* (NC008209)	98
S3 (KF953935)	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	*Ruegeria pomeroyi* (NC 003911)	97
S4 (KF953936)	Bacteroidetes	Cytophagales	Cytophagaceae	*Flexibacter elegans* (NZAUMD01000008)	86
S5 (KF953937)	Alphaproteobacteria	Rhizobiales	Phyllobacteriaceae	*Mesorhizobium opportunistum* (NC015675)	93
S6 (KF953938)	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	*Oceanicola batsensis* (NZCH724131)	98
S7 (KF953939)	Actinobacteria	Actinomycetales	Dermabacteraceae	*Brachybacterium faecium* (NC013172)	99

ter denitrificans (C3, S2) were present in both cultures. *Labrenzia aggregata* (C4) and *Oceanicola batsensis* (S6) in Rhodobacteraceae were found associated with *C. marina* and *S. costatum*, respectively. The other two Alphaproteobacteria phylotypes, C6 and S5, had low similarities (93%–95%, Table 1) and high genetic distances (0.058–0.079, Table 2) to *Hyphomonas johnsonii* and *Mesorhizobium opportunistum*, respectively. The only Gammaproteobacteria, *Marinobacter santoriniensis* (S1), was present in *S. costatum*. The two *Bacteroidetes phylotypes* (C5, S4) showed low similarities (86%–92%) and wide distances (0.077–0.172) to the corresponding closest sequences *Robiginitalea biformata* (NC 013222) and *Flexibacter elegans* (NZAUMD01000008), respectively. The only Actinobacteria, *Brachybacterium faecium* (S7), was present in *S. costatum*.

Table 2. Genetic distances among bacterial phylotypes isolated from *Chattonella marina* and *Skeletonema costatum* and their closest sequences from GenBank (the closest distance is indicated in bold font)

Bacterial phylotypes	C1	C2	C3	C4	C5	C6	
Ruegeria sp.	**0.000**	0.163	0.025	0.123	0.397	0.141	
Citromicrobium bathyomarinum	0.160	**0.015**	0.157	0.137	0.351	0.147	
Roseobacter denitrificans	0.053	0.175	**0.015**	0.136	0.380	0.161	
Labrenzia aggregata	0.122	0.120	0.121	**0.000**	0.325	0.147	
Robiginitalea biformata	0.377	0.320	0.357	0.330	**0.077**	0.356	
Hyphomonas johnsonii	0.134	0.151	0.146	0.134	0.375	**0.058**	
Bacterial phylotypes	S1	S2	S3	S4	S5	S6	S7
Marinobacter santoriniensis	**0.025**	0.321	0.342	0.545	0.261	0.310	0.355
Roseobacter denitrificans	0.327	**0.024**	0.047	0.504	0.162	0.040	0.399
Ruegeria pomeroyi	0.318	0.028	**0.035**	0.476	0.170	0.059	0.433
Flexibacter elegans	0.452	0.418	0.443	**0.202**	0.361	0.395	0.426
Mesorhizobium opportunistum	0.204	0.137	0.132	0.364	**0.049**	0.114	0.262
Oceanicola batsensis	0.321	0.038	0.059	0.463	0.151	**0.022**	0.383
Brachybacterium faecium	0.339	0.420	0.456	0.483	0.285	0.365	**0.002**

3.3 *Bacterial community structure in different growth stages of algal cells*

Three to six different phylotypes of bacteria were isolated from each growth phase of *C. marina*; four to five, from *S. costatum* (Fig. 2). Bacterial concentrations in cultures of *C. marina* were 1–2 orders of magnitudes higher than those of *S. costatum*. Bacterial concentrations increased greatly after Phase II in *C. marina* and reached the maximum of 1.35×10^9 CFU/mL in Phase VI (Fig. 2a). Bacterial concentrations in the *S. costatum* culture ranged between 3.01×10^6 CFU/mL and 1.39×10^7 CFU/mL, with the minimum occurring in Phase I and maximum occurring in Phase IV.

Bacterial phylotypes were more diversified in *S. costatum* than in *C. marina*, while bacterial diversity was higher in later growth stages (Fig. 3). Alphaproteobacteria dominated the phycosphere of *C. marina*, which contributed to 100% of the phylotypes in Phases I to IV as well as almost 90% in Phases V and VI (Figs 3a–c). Alphaproteobacteria dominated in the phycosphere of *S. costatum* as well (42.9%–66.7%, Figs 3d–h). Bacteroidetes were the second most common bacterial phylotypes in the phycospheres and occurred in all phases of *S. costatum* and phases V and VI of *C. marina*, occupying 12.5%–16.7% of the phylotypes of *S. costatum*-associated bacteria. Gammaproteobacteria were not present in *C. marina*; however, they were present in all growth phases of *S. costatum*, whereas Actinobacteria occurred in only Phase V of *S. costatum*.

Bacterial phylotypes C1 (*Ruegeria* sp.), C2 (*Citromicrobium bathyomarinum*), and C3 (*Roseobacter denitrificans*) occurred abundantly in all growth phases of *C. marina* (Fig. 4a). C4 (*Labrenzia aggregata*) and C6 (*Hyphomonas* sp.) occurred in all phases except for Phase I. In contrast, C5 (a phylotype in the family Flavobacteriaceae) was present in only Phases V and VI. C1 and C3 dominated in all growth phases, and the percentage of C5 increased greatly in Phase VI (Fig. 4c).

In the phycosphere of *S. costatum*, S1 (*Marinobacter san-*

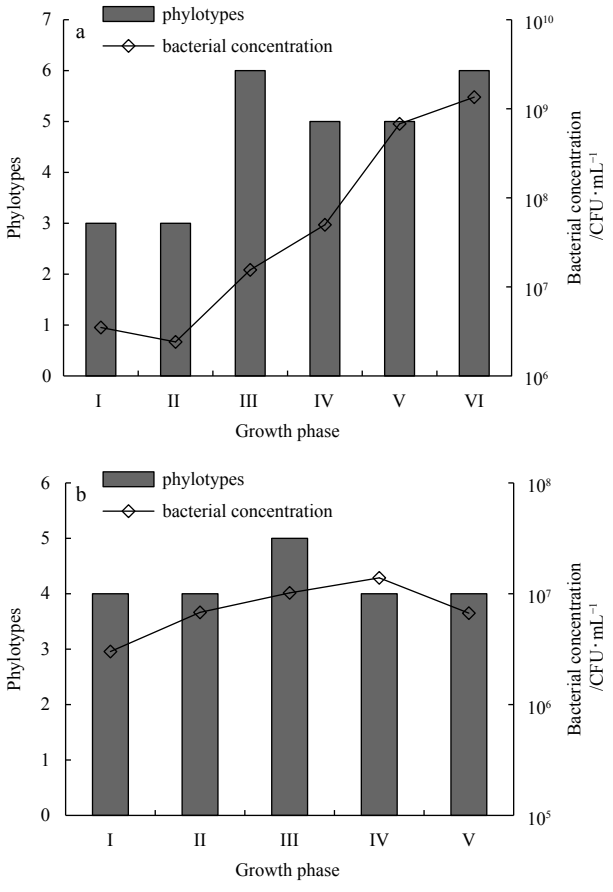

Fig. 2. Phylotypes and abundances of bacteria in phycospheres of *Chattonella marina* (a) and *Skeletonema costatum* (b).

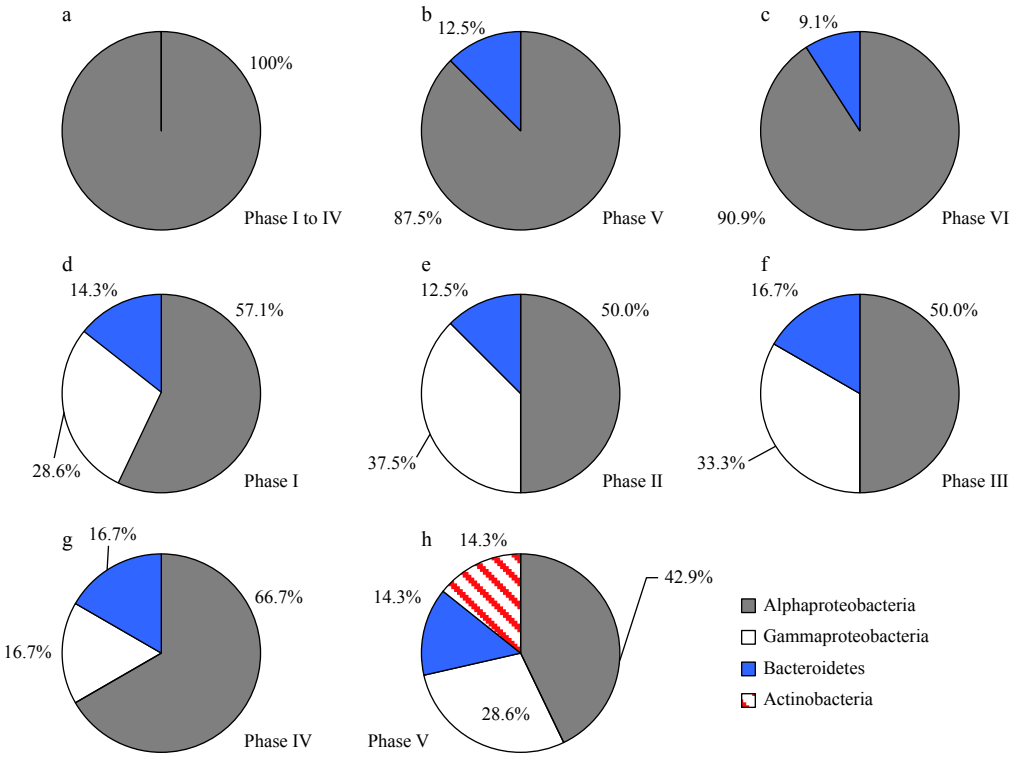

Fig. 3. Percentage profiles of various bacterial phyla in different growth phases of *Chattonella marina* (a–c) and *Skeletonema costatum* (d–h).

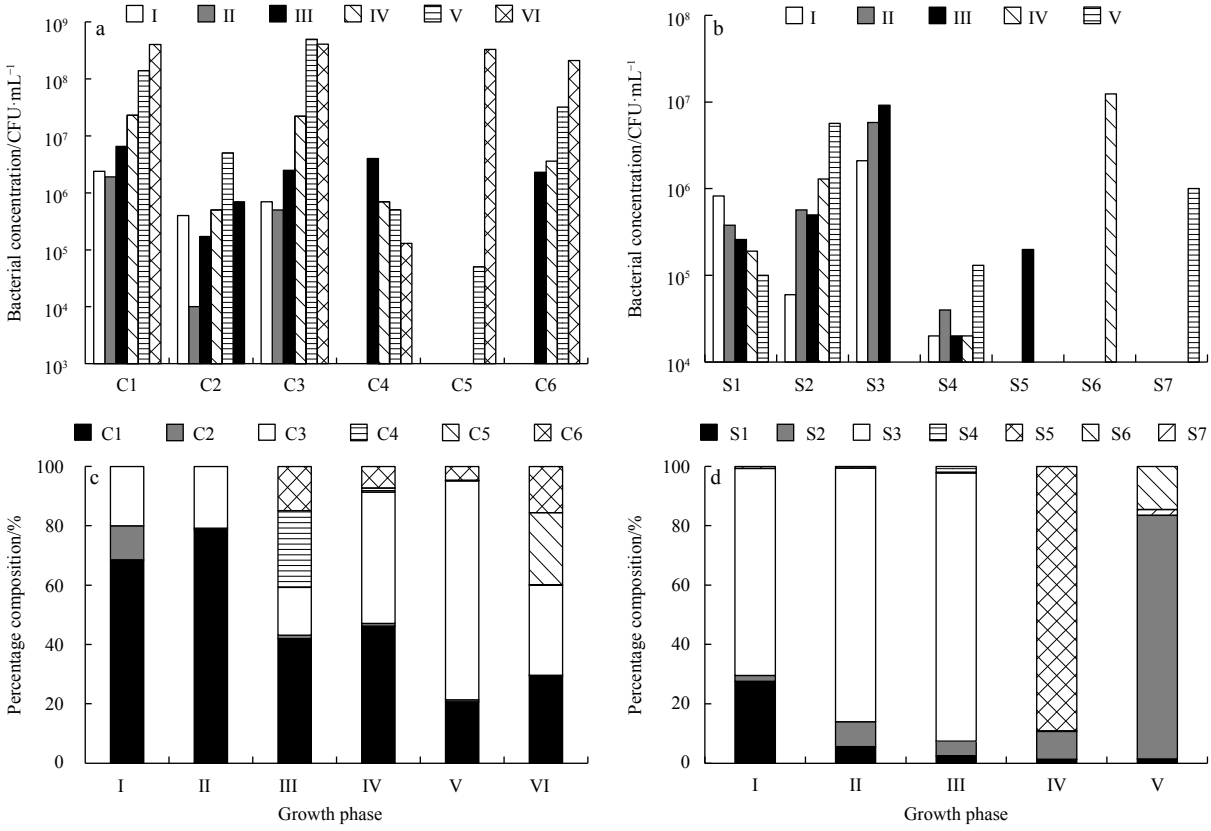

Fig. 4. Concentrations and percentages of each bacterial phylotype in different growth stages of *Chattonella marina* (a, c) and *Skeletonema costatum* (b, d). C1–C6: *C. marina*-associated bacteria; S1–S7: *S. costatum*-associated bacteria.

toriniensis), S2 (*Roseobacter denitrificans*), and S4 (a phylotype in Bacteroidetes) occurred in all growth phases (Figs 4b, d). However, some phylotypes occurred in only a particular growth stage, for example, S5 (*Mesorhizobium* sp.) in Phase III, S6

(*Oceanicola batsensis*) in Phase IV, S2 and S7 (*Brachybacterium faecium*) in Phase V. Bacterial compositions were quite different between the early and late growth phases of *S. costatum* (Fig. 4d). S3 dominated in Phases I to III, and S6 predominated in Phase IV. The percentage of S2 increased after Phase IV and was over 80% in Phase V.

3.4 *Cluster analyses of the bacterial community in different growth stages of algal cells*

Bacteria associated with *S. costatum* and *C. marina* were clustered separately into two large groups (Fig. 5). Bacteria associated with *C. marina* were separated into two subgroups, corresponding to early (Phases I–III) and late (Phases IV–VI) stages. Similarly, bacteria in *S. costatum* during Phases I–III were grouped together, while those from Phases IV and I were ungrouped.

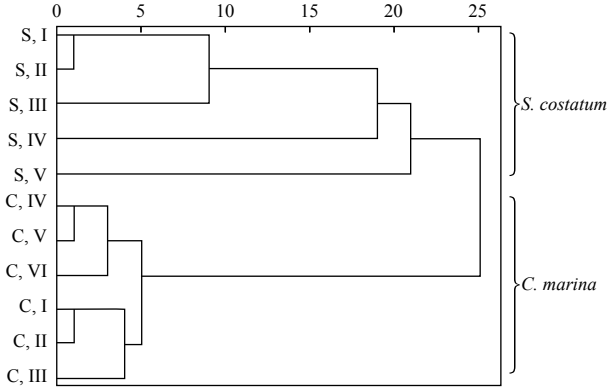

Fig. 5. Cluster analysis of bacterial community structure in different growth stages of *Chattonella marina* (C, I–C, IV) and *Skeletonema costatum* (S, I–S, V).

3.5 *Effects of algae-associated bacteria on the growth of Chaetoceros curvisetus*

The maximum cell numbers of *C. curvisetus* (Fig. 6) in Cultures C3, C4, S2 and S6 were significantly higher than those in *f*/2 and BC cultures (p<0.05). The maximum cell number in S4 was significantly higher than that in the BC culture (p<0.05) but showed no significant differences from the *f*/2 medium culture. Therefore, four bacterial phylotypes (C3/S2, C4, S6 and S4, with C3 and S2 being the same phytotype) had growth stimulation effects on *C. curvisetus*. All these bacterial phylotypes belong to the family Rhodobacteraceae (Table 1). The maximum cell numbers in five bacterial phylotypes, C1, C2, C5, C6 and S3 (Fig. 6), were significant lower than those in *f*/2 and BC cultures (p<0.05), indicating their inhibitory effects on algal growth.

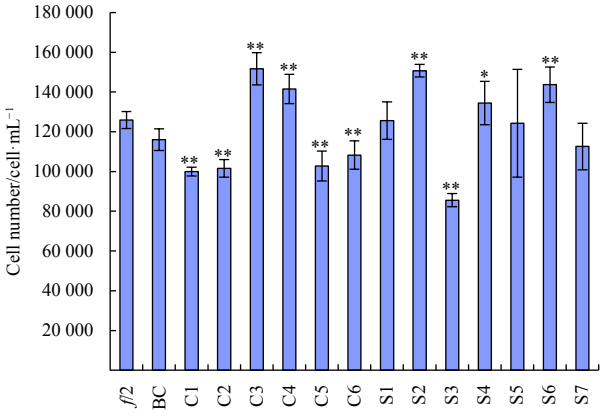

Fig. 6. Maximum cell numbers of *Chaetoceros curvisetus* in the cocultures of bacteria in phycospheres of *Chattonella marina* (C1–C6) and *Skeletonema costatum* (S1–S7). * Significant differences with BC culture (p<0.05); ** significant differences with both BC and *f*/2 cultures (p<0.05).

4 Discussion

As the algal cultures have been maintained in the laboratory for approximately ten years, the bacteria isolated in this study were those that had coexisted with the algal strains for a long time. These bacteria are either favored or suppressed in this artificial setting, feed on the extracellular products of the algae, and reach a relatively balanced situation through years of continual subcultivation (Schwenk et al., 2014). The two microalgal species in this study were isolated in the same oceanic location at the same time, and the associated bacteria of both algae were dominated by Alphaproteobacteria, including one pair of identical phylotypes (C3 and S2). Alphaproteobacteria are a common bacterial group in natural seawater (Eilers et al., 2001; Buchan et al., 2005) and the dominant algae-associated bacteria (Sapp et al., 2007; Schwenk et al., 2014). However, differences were observed between bacteria associated with the two microalgal species. Most *C. marina*-associated bacteria were phylotypes in Rhodobacterales of Alphaproteobacteria, while bacteria associated with *S. costatum* were more diversified, particularly in phase V, during which four groups of bacteria were isolated. Four of the twelve phylotypes (C5, C6, S4 and S5) showed low similarity and wide genetic distance from the closest sequences, which belonged to a new potential species or genus. Goecke et al. (2013) phylogenetically analyzed associated bacteria from 42 algal species, and 101 bacterial taxa corresponding to 71 genera were obtained, of which 36 were newly described. These results suggested that algae were an important environment for the discovery of new bacterial taxa.

Bacterial concentrations increased remarkably after the stationary phase (Fig. 2), and bacterial communities were more diversified in the stationary and declining stages (Fig. 3). The results from the cluster analysis showed that the earlier and later growth phases were grouped separately (Fig. 5). The results suggested that the bacterial community structures were quite different at various growth stages of the algal cells. Furthermore, some specific phylotypes, such as C5 (one phylotype in Bacteroidetes) and C6 (one phylotype in the genus *Hyphomonas*) in *C. marina* culture, and S6 (*Oceanicola batsensis*) and S7 (*Brachybacterium faecium*) in *S. costatum* culture, increased greatly in the late period of growth (Fig. 4). Changes in the bacterial communities depend on the physiological status of algal cells in different growth stages (Verity et al., 1988). It was suggested that maximum bacterial numbers occurred during the stationary phase in microalgal batch culture (Verity et al., 1988; Gallacher and Smith, 1999). Studies on harmful algal blooms have revealed that major changes have been observed in bacterial communities during blooms and can shift from being dominated by oligotrophic Alphaproteobacteria to Flavobacteria that grow on senescent algae (Zubkov et al., 2001; Teeling et al., 2012; Park et al., 2015). The considerable increase in some particular bacteria during the end of blooms might account for the quick decline in the algal blooms (Verity et al., 1988). Therefore, the massive increases in bacterial concentration and in the occurrence of some particular bacterial phylotypes may be associated with the decline in algal cells.

C. marina is a fragile naked raphidophyte, and its cells breakdown shortly after death. Organic matter is then released, and bacteria feed on it. The concentrations of all bacterial phylotypes increased greatly in the declining Phase VI of *C. marina* (Fig. 4a), which was 2–3 orders of magnitude higher than those in Phases I and II. The same remarkable increases in bacterial numbers were observed in the declining phase of the naked dinoflagellate *Gyrodinium instriatum* (Wang et al., 2014) and haptophyte *Phaeocystis pouchetii* (Verity et al., 1988). However, no evident increase in bacterial number occurred for the senescent *S. costatum*. The thick siliceous wall of *S. costatum* may prevent the release of organic matter from the dead cells.

Most of the bacteria in the phycosphere have been classified as plant growth-promoting bacteria (Ramanan et al., 2015). Within the twelve bacterial phylotypes in this study, four phylotypes promoted the growth of *C. curvisetus*, five inhibited the growth, and the other three had no significant effects on algal growth. Within the five inhibitory phylotypes, four belong to Alphaproteobacteria (C1, C2, C6 and S3), and the other one (C5) belongs to Bacteroidetes. Most known algicidal bacteria belong to either the phylum Bacteroidetes or Gammaproteobacteria (such as *Alteromonas*, *Pseudomonas* and *Pseudoaltermonas*), but algicidal Alphaproteobacteria are also reported (Kirchman, 2002; Goecke et al., 2013; Meyer et al., 2017). The two bacterial phylotypes in the genus *Ruegeria* (C1 and S3) demonstrated significant inhibition of algal growth. These are the most common and predominant bacteria in cultures of *C. marina* and *S. costatum*. Ruegeria belongs to the Roseobacter clade, which is one of the most abundant lineages of marine bacteria that occur globally in marine ecosystems (Buchan et al., 2005), and one of the most common types of algae-associated bacteria (Goecke et al., 2013). Bacteria from the Roseobacter clade have been observed as the predominant prokaryotic species, accounting for more than half of the total bacterial community during blooms of *Cochlodinium polykrikoides* (Park et al., 2015). Species in *Ruegeria*, such as *R. pomeroyi* (Riclea et al., 2012) and *R. atlantica* (Amaro et al., 2005), have been reported to release algicidal compounds.

Chaetoceros curvisetus is one of the dominant diatoms observed in the coastal areas of southern China (Wang et al., 2009). In this study, *C. curvisetus* was selected as the test organism as a representative of typical diatoms. Four of the six bacterial phylotypes from cultures of *C. marina* showed significant inhibition of the growth of *C. curvisetus*. The results suggested that *C. marina*-associated bacteria might have some ecological roles in competition with diatoms. It was reported that bacteria collected during a harmful dinoflagellate bloom (*G. nagasakiense*) inhibited the growth of *S. costatum* and played an important role in its competition with diatoms as well as in the development of the algal bloom (Fukami et al., 1991). Within the four growth-inhibitory bacterial phylotypes, C1 (*Ruegeria* sp.) dominated in all growth stages of *C. marina*, which might pose detrimental effects to other microalgal cells. Additionally, C5 and C6, which occurred abundantly in the late phase of *C. marina* (Figs 4a and c), may also relate to *C. marina's* own decline. Association between bacteria and the raphidophyte bloom showed that algicidal bacteria might be an important limiting factor in bloom sustenance and could promote bloom decline (Liu et al., 2008).

Only one strain of *S. costatum*-associated bacteria had an inhibitory effect on the growth of *C. curvisetus*. Most of the bacteria associated with *S. costatum* may have established a positive relationship with their host or with species similar to the host, i.e., other diatoms such as *C. curvisetus*, while these bacteria may be infectious to other hosts. Whether the bacteria associated with *S. costatum* have inhibitory effects on the growth of *C. marina* remains to be further investigated.

5 Conclusions

Cultivable bacteria were isolated in cultures of *C. marina* and *S. costatum* in this study. Forty-eight and thirty-four bacterial isolates were collected from cultures of *C. marina* and *S. costatum*, respectively. Six and seven different phylotypes were obtained from cultures of *C. marina* and *S. costatum*, respectively, after removing the duplicate rDNA sequences. The majority of the algae-associated bacteria in this study belonged to Proteobacteria, and Rhodobacteraceae was the most common family identified. Bacterial phylotypes were more diversified in *S. costatum* than in *C. marina*. Bacterial community differed across different growth phases, and bacterial concentrations and diversities were higher in the later growth stages. One phylotype of *S. costatum*-associated bacteria had inhibitory effects on the diatom *C. curvisetus*. However, most bacterial phylotypes from cultures of *C. marina* showed significant inhibition of the growth of *C. curvisetus*. The results suggested that bacteria associated with *C. marina* might have some ecological roles in its competition with diatoms.

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