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Short communication

# Phycosphere bacterial diversity in green algae reveals an apparent similarity across habitats

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ABSTRACT

Phytoplankton and bacteria play the foremost role in primary production and often act in unison in biogeochemical cycling. Studies conducted so far are inconclusive on species specificity of phycosphere bacteria as the overarching function of specific clades of algae-associated bacteria, for instance *Roseobacter* in sulfur cycling, is widely held. In this study, we attempt to demonstrate the diversity of phycosphere bacteria in phylogenetically divergent unialgal green algae from vastly different environmental samples like soil, freshwater, marine, and wastewater with diatom and cyanobacteria as an outgroup. Diversity analyses using Differential Gel Gradient Electrophoresis (DGGE) revealed the predominant presence of bacteria belonging to Bacteroidetes phylum (46% of all strains). 454 pyrosequencing of selected strains from different habitats not only confirmed the presence of Bacteroidetes (33.1% of total reads) but also revealed the presence of bacteria belonging to α-Proteobacteria (52.6%), all in close association with their host. Majority of those symbiotic bacteria have been classified as Plant Growth Promoting Bacteria (PGPB) including prominent Sphingomonads and Rhizobacter. Results suggest that although host algae might encourage species specific interactions, specific functional traits are prerequisite for proximal adhesion in nutrient-rich phycosphere. While Bacteroidetes is known to have significant role in nutrient cycling through degradation of plant and algal macromolecules and for its attached growth, PGPB have proven symbiosis with plants and the overwhelming presence of these bacteria in green algae points to possible co-evolution.



## 1. Introduction

Axenic cultures only exist under strict laboratory conditions and even in relatively non-sterile environment, algae would get contaminated, in other words, find some associates to live with [2,4]. In nature, algae depend on bacteria and bacteria exploit algae resulting in a dynamic role in biogeochemical cycling [3]. In specific cases, algae completely depend on bacteria for growth and division [4,11]. Most algae, especially green algae, can survive on their own but depend on bacteria to grow faster as the growth rates of axenic cultures are much slower than xenic cultures [6]. Noticeably, in each study, the mechanism of interaction has been found to be different. This leads us to question the nature of algal–bacterial interactions. Some studies reveal that algal–bacterial interactions are species specific [10,12], while other studies indicate that certain communities like Bacteroidetes and Proteobacteria are dominant among diatoms and dinoflagellates [1,13,25]. Amidst the inconclusiveness of the nature of interactions, either species specific or generic, in diatoms and dinoflagellates, there is little evidence of nature of bacterial diversity in green algae, considering their commercial importance.

Furthermore, most studies on bacterial diversity in algae are rightly focused on marine environment, considering their importance to biogeochemical cycling [1,28]. In studies on marine algae alone, there have been suggestions of species specific interactions yet clades like *Roseobacter* have been identified as dominant clade [25,28]. Our recent studies on bacterial assemblages in phycosphere of green algae from freshwater sources revealed their role in algal growth and metabolism, and in wastewater treatment, and thereby their applicability to microalgal biotechnology [6,7,16,17]. Hence in this study, we have focused on the phycosphere bacterial diversity of phylogenetically and ecologically diverse green algae, isolated from lentic and lotic freshwater systems, soil, wastewater, and marine waters. The endeavor is to investigate whether these interactions are species specific and habitat influenced or dominated by certain functional or phylogenetic clades, irrespective of the ecosystem they have been isolated from.

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Furthermore, this study might throw light on some important questions on a possible co-evolution by bacteria with both algae and land plants as the latter also harbor bacteria in its roots for nutrient exchange [24].

## 2. Materials and methods

### 2.1. Strain isolation and culture conditions

The strains used in this study, their source and their accession numbers have been provided in Table 1. Each strain was cultivated in specific medium in 75 ml culture flasks at 100 μmol $m^{-2}$ $s^{-1}$ photosynthetically active radiation (PAR) at 25 °C and allowed to grow till stationary phase [2,6]. Once the strains reached stationary phase, the cultures were collected and centrifuged.

### 2.2. Denaturing gel gradient electrophoresis (DGGE)

After centrifugation, the cultures were washed twice with TE buffer (Tris 10 mM, EDTA 1 mM, pH 8.0) followed by centrifugation at 4800 ×*g* for 5 min. To eliminate free living bacteria, the cultures were subjected to a mild centrifugation at 1000 ×*g* for 3 min which pellets algae with attached bacteria but not free living bacteria. This step was repeated thrice and followed by microscopic observation [7]. The biomass was resuspended in 1.5 ml distilled water and was centrifuged at 10,000 ×*g* for 3 min at room temperature. DNA extractions were carried out in accordance with eukaryotic microalgal nucleic acids extraction (EMNE) method [15]. The dried DNA samples were resuspended in 60 μl diethyl pyrocarbonate (DEPC) $H_2O$. The isolated DNA samples were analyzed on a 1.0% agarose gel. DGGE and identification of the bands were performed as described earlier [16].

### 2.3. 454 pyrosequencing

The PCR products of 16S rRNA gene were analyzed using pyrosequencing with a 454 Genome Sequencer FLX Instrument (Roche 454 Life Sciences, Branford, CT, USA). The raw reads were deposited into the NCBI short-reads archive database. The sequences obtained in this study were compared using Silva rRNA database.

### 2.4. Phylogenetic analysis

The 16S rRNA gene sequences were aligned with published sequences retrieved from EMBL using CLUSTAL X and edited using BIOEDIT. Phylogenetic trees were reconstructed using the neighbor-joining algorithm in the MEGA 5 software. The percentages for the replicate trees in which the associated taxa were clustered together in a bootstrap analysis were based on a 1000 resampled data set.

**Table 1**
Information on the strains used in this study.

| S. no. | Strains | KCTC accession no. | Source | Medium |
|---|---|---|---|---|
| 1. | *Botryococcus braunii* | AG30114 | Freshwater | BG11 |
| 2. | *Nannochloris* sp. | AG20447 | Seawater | ESM |
| 3. | *Chlamydomonas reinhardtii* | AG30169 | Laboratory strain — freshwater | TAP |
| 4. | *Chlamydomonas moewusii* | AG20713 | Soil | TAP |
| 5. | *Cymbella* sp. | AG20316 | Brackish water | F2 |
| 6. | *Ettlia texensis* | AG40020 | Freshwater | BG11 |
| 7. | *Chlorella vulgaris* | AG10191 | Freshwater | BG11 |
| 8. | *Stigeoclonium* sp. | AG20296 | Wastewater | BG11 |
| 9. | *Scenedesmus quadricauda* | AG10003 | Freshwater | BG11 |
| 10. | *Arthrospira platensis* | AG20590 | Freshwater | BBM |
| 11. | *Tetraspora gelatinosa* | AG10149 | Freshwater | BG11 |

## 3. Results and discussion

### 3.1. Phycosphere bacterial diversity using DGGE

Studies on algal–bacterial interactions have focused on either model organisms such as *Alexandrium* [14] or on specific ecosystems such as marine or freshwater [10,12]. Here, we explore the bacterial assemblages associated with algae isolated from a variety of ecosystems. Bacterial assemblages harbored in the phycosphere of 11 algal strains isolated from different habitats were analyzed by DGGE (Table 1). A total of 44 bands were cut and sequenced (Fig. 1A). Sequence analysis of DGGE bands yielded 15 different strains mostly belonging to Bacteroidetes [7] and Proteobacteria [5] phylum (Supplementary Table 1). The strains belonging to the Bacteroidetes phylum were not only dominant in each alga (46%) but also prevalent as they were detected in 8 out of 11 algal strains studied. The only omissions were *Botryococcus braunii*, *Nannochloris* sp., and *Scenedesmus quadricauda*. Within the Bacteroidetes phylum, *Flavobacterium* was present in the phycosphere of two algal strains namely *Chlorella vulgaris* and *Tetraspora gelatinosa* (green algae, freshwater), and in the only Cyanobacterium, used as an out-group in this study, *Arthrospira platensis*. The other strains belonging to the Bacteroidetes phylum included *Terrimonas rubra* present in *Ettlia texensis* and *C. vulgaris* (green algae, freshwater), *Sediminibacterium goheungense* in *Chlamydomonas reinhardtii* and *Chlamydomonas moewusii* (green algae, freshwater/soil) *Chryseobacterium* sp., in *Stigeoclonium sp.*, (green algae, wastewater) *Roseivirga ehrenbergii* and *Adhaeribacter aerophilus* in *Cymbella* sp., (diatom, brackish water).

Beta-proteobacteria is the dominant sub-phylum, within the Proteobacteria phylum, with *Methylophilus* genus present in *E. texensis* and *C. vulgaris*, while *Acidovorax radicis* was found in *B. braunii* (green algae, freshwater). Similarly, α-Proteobacteria, *Porphyrobacter neustonensis*, was found in *Nannochloris* sp., (green algae, seawater) and *Cymbella* sp., (Diatom, brackish water), while the only γ-Proteobacteria identified in DGGE, *Pseudomonas toyotomiensis*, was present in *Scenedesmus quadricauda* (green algae, freshwater). Apart from these strains, a Firmicutes bacterium, two uncultured bacteria and an uncultured Cynaobacterium were also observed (Fig. 1B & Supplementary Table 1).

Among the Bacteroidetes strains, *Flavobacterium* has been recently isolated from a variety of algal strains in freshwater and marine ecosystems [5,6,23]. Genome sequencing of *Formosa agariphila*, a genus of class Flavobacteria isolated from green algae, has revealed the presence of 129 proteases and 88 glycoside hydrolases with an extraordinary ability to degrade a wide range of algal macromolecules complete with transporters and polysaccharide utilization loci (PUL). Other Bacteroidetes strains detected are also usually present in green algae and diatoms [1,21,22,29]. The most abundant β-Proteobacteria was *Methylophilus*, a methanol oxidizing genus, and has not been reported to be present in algal phycosphere so far. As algae excrete a series of macromolecules, it is likely that they also secrete single carbon compounds, which are metabolized by methylotrophs, similar to plant–methylotroph interactions [19]. *Porphyrobacter*, an α-Proteobacteria, is known to exert dominance in cyanobacterial biofilms and surface water samples [8].

Taken together, the dominance and prevalence of Bacteroidetes in the DGGE analysis only indicate the versatility of phylum in degradation of algal macromolecules. Most genera of Bacteoidetes phylum host PULs specific to each polysaccharides and are switched on in the presence of respective polysaccharides [26]. Considering the diversity of polysaccharides secreted by phytoplankton, it would be unrealistic to host genes specific for each polysaccharide. Hence, apart from *Flavobacterium* and *Terrimonas*, other genera are not prevalent among all algal strains as each phycosphere offers a different microenvironment and hence encourages specific bacteria strains. Nevertheless, role of collection of microorganisms grouped together by phylogeny