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In-Depth Characterization of Wastewater Bacterial Community in Response to Algal Growth Using Pyrosequencing

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Microalgae have been regarded as a natural resource for sustainable materials and fuels, as well as for removal of nutrients and micropollutants from wastewater, and their interaction with bacteria in wastewater is a critical factor to consider because of the microbial diversity and complexity in a variety of wastewater conditions. Despite their importance, very little is known about the ecological interactions between algae and bacteria in a wastewater environment. In this study, we characterized the wastewater bacterial community in response to the growth of a *Selenastrum gracile* UTEX 325 population in a real municipal wastewater environment. The Roche 454 GS-FLX Titanium pyrosequencing technique was used for in-depth analysis of amplicons of 16S rRNA genes from different conditions in each reactor, with and without the algal population. The algal growth reduced the bacterial diversity and affected the bacterial community structure in the wastewater. The following in-depth analysis of the deep-sequenced amplicons showed that the algal growth selectively stimulated Sphingobacteria class members, especially the *Sediminibacterium* genus population, in the municipal wastewater environment.

Keywords: Algal-bacterial interaction, wastewater, pyrosequencing, Sphingobacteria

Introduction

Sustainable energy and water resource cycles have become significantly more important to address issues resulting from global climate change [1]. Recovery of renewable resources from wastewater could be a solution for improving the sustainability of both energy resources and water resources. Because of their high lipid contents, microalgae have been used to produce biofuels and high-value materials [4, 8, 15]. In addition, microalgae are known to have the capability to fix carbon from the air and to take up nutrient pollutants (N and P species) from aquatic environments and wastewater [2, 11]. Recently, microalgae such as *Selenastrum* were known to grow in real wastewater [12, 22, 25]. Because of this background and other motivations, biorefinery applications for microalgae cultivated from wastewater have attracted the attention of researchers and sustainable biotechnology industries.

When linking microalgal processes with wastewater treatment systems, the ecological interactions between microalgae and wastewater microbes are important factors to consider because of the microbial diversity and complexity in a variety of wastewater conditions [10, 14, 28]. In the literature, ecological interactions between algae and bacteria have been studied in terms of algal eutrophication control in aquatic and marine environments [3, 7]. However, very little is known about the bacterial community response to algal growth in wastewater environments.

Between bacterial and algal populations, their interactions could be competitive or cooperative. Since algal populations take up inorganic carbon to make biomass, heterotrophic bacteria in wastewater may not be competing against the algal population [24]. However, when nitrogen source is limited, bacterial and algal nitrogen utilizers could compete against each other. In addition, nitrogen-deficient algal populations could produce dissolved organic matters that could be either stimulating or inhibiting to a specific group of bacterial population in the wastewater. A stimulated bacterial population would have a growth benefit in algal-bacterial consortia in a wastewater environment. In addition,

bacteria positively interacting with algae that are good at lipid production using wastewater would be useful bioresources for making an algal cultivation in wastewater more effective and stable [24]. Because of these reasons, it is important to search for which bacterial populations positively interact with an algal species of interest in a wastewater environment. However, such question has yet to be examined.

Recently, high-throughput next-generation sequencing (NGS) has attracted attention from researchers of environmental microbiology and sciences. Massive sequencing of 16S rRNA gene amplicons by 454-titanium pyrosequencing (Roche) has become popular in analyzing microbial communities in environmental samples, mainly because of its production of the longest sequence read (~500 bp) among the current high-throughput sequencing techniques [20]. Because of the deep sequencing capability, the 16S rRNA gene amplicon pyrosequencing is potentially useful in screening wastewater bacterial populations positively interacting with algal populations that are effective in biofuel production and wastewater treatment, such as *Selenastrum gracile*. In this study, we characterized wastewater bacterial community responses to the growth of an *Selenastrum gracile* population in a real municipal wastewater condition. For this, an in-depth analysis of bacterial communities was conducted using titanium pyrosequencing of 16S rRNA gene amplicons, which is a high-throughput NGS technique [21].

Materials and Methods

Algal Cultivation Conditions

Low-strength municipal wastewater (COD = 52 mg/l, TN = 32 mg/l, TP = 3.90 mg/l) was obtained from the Seonam Wastewater Treatment Plant in Seoul, South Korea. In the initial wastewater (IWW), indigenous algae, fungi, protozoa, and large particles were eliminated by filtering through 1.2 μm Whatman GF/C glass filters prior to algal cultivation. Because of its abilities to effectively remove nutrients and grow in municipal wastewater environments [23], *Selenastrum gracile* UTEX 325 was selected as the algal population for this study. The algal resource was obtained from the Biological Resource Center, Korea Research Institute of Biology and Biotechnology.

The algal population was inoculated at 24−25°C with the prepared wastewater (IWW, 4.5 L) in 5 L glass batch reactors. The initial algal density was 43 μg-dried weight/l. The algal-wastewater mixture (AWW) was completely stirred with a magnetic stirrer (110 rpm) and aerated with air (0.3 vvm). For algal photosynthesis, light was provided at the intensity of 132 μE. In parallel, a no algal control with only wastewater (WW) was operated in a separate reactor. To explore the effect of the increased biomass of active sludge microbes, activated sludge from the same wastewater plant was filtered through 1.2 μm Whatman GF/C glass filters, and 0.5 L of the filtered activated sludge was added in a separately prepared 4 L algal-wastewater mixture with the same algal density (AWWS). This AWWS reactor was run under the same conditions. In parallel, a no algal control with activated sludge (WWS) was prepared and run under the same conditions (Table 1).

Algal and Bacterial Growth

Algal growth was measured by the concentration of chlorophyll *a* (Chl-*a*) from Sartory and Grobbelaar's protocol [31]. Cultured algal biomass from wastewater samples was filtered by 1.2-μm-pore GF/C glass filters (Whatman, UK), and each glass filter was transferred to a 15 ml BD Falcon Tube (BD Biosciences, USA) that contained 95% ethanol (7.6 ml). The tubes were heated in a warm water bath (70~73°C) for 20 min and cooled for 3 h in dark conditions. The tubes were centrifuged for 2 min at 2,400 rpm, and supernatants in the tubes were analyzed using a T60 UV/VIS Spectrophotometer (PG Instruments Ltd., UK) at 649, 665, and 750 nm. The concentration of Chl-*a* was calculated using the following empirical equation [17]: $\text{Chl } a = [13.7(A_{665}\text{-}A_{750})\text{-}5.76(A_{649}\text{-}A_{750})] \times E/F \times l$, where Chl *a* = concentration of chlorophyll *a* in μg/L; E = volume of ethanol in ml; l = cuvette path length in cm; F = filtration volume in L; A_i = absorbance at wavelength of *i* nm.

To analyze bacterial growth, the standard spread plate method [29] was used in an R2A agar medium. The number of colonies was counted after a 3 day incubation at 20°C [16] .

Pyrosequencing of 16S rDNA and Microbial Community Analysis

From each sample of algae-adapted wastewater, genomic DNA was extracted using a PowerSoil DNA Isolation kit (MOBIO Laboratories, Inc., USA). Barcoded primers were used for sorting

Table 1. Descriptions of the four different reactors.

Reactor	Initial algal density (μg-dried-weight/l)	Filtered wastewater (L)	Filtered activated sludge (L)
AWW	43	5.0	None
WW	None	5.0	None
AWWS	43	4.5	0.5
WWS	None	4.5	0.5

Table 2. Specific growth rates for the algal and bacterial populations.

Sample	Specific algal growth rate, μ (1/h)	Specific bacterial growth rate, μ (1/h)
AWW	0.0268 ± 0.0067	0.0179 ± 0.0057
WW	-	0.0174 ± 0.0139
AWWS	0.0185 ± 0.0120	0.0015 ± 0.0030
WWS	-	0.0147 ± 0.0106

different DNA samples. The following 16S rDNA primers were used for the PCR process: F563/16 (AYTGGGYDTAAAGNG) and BSR926/20 (CCGTCAATTYYTTTRAGTTT) [5]. Each PCR was carried out with 75 μl reaction mixtures containing 50−60 ng of template DNA, 10 μM of each primer (Macrogen, Seoul, Korea), 1.25 U of *Taq* polymerase, 50 mM of $MgSO_4$, and 10× of the PCR buffer AccuPrime *Taq* DNA Polymerase High Fidelity (Invitrogen, WI, USA). A C1000TM Thermal Cycler (Bio-Rad, CA, USA) was used for PCR in the following steps: (i) an initial denaturation step at 94°C for 3 min; (ii) 35 cycles of denaturation, annealing, and extension (94°C for 30 sec followed by 55°C for 30 sec and an extension step at 72°C for 40 sec); and (iii) the final extension at 72°C for 5 min. After the PCR process, the PCR amplicons were purified using a QIAquick Gel Extraction kit and a QIAquick PCR Purification kit (Qiagen). All PCR amplicons were pooled and sequenced by a Roche 454 GS-FLX Titanium instrument (Roche, NJ, USA). Sequencing data were filtered using the AmpliconNoise program in Mothur [27]. Chimera sequences were removed by UCHIME [9]. Filtered reads were aligned and clustered at 97% similarity using the Infernal Aligner and the complete-linkage clustering of the Ribosomal Database Project (RDP) II [6]. The RDP Classifier assigned representative operational taxonomic unit (OTU) reads with a confidence threshold of 50% at the genus level.

Results

Algal Growth Effect on Bacterial Growth

According to the algal growth measured by chlorophyll *a* (Table 2), the algal population grew in the wastewater environment (AWW), and the same algal population could also grow when activated sludge was added to the wastewater (AWWS). Wastewater bacterial populations grew in the WWS at a specific growth rate greater (p-value = 0.001) than in the AWWS, when the bacterial concentration was high. However, when the bacterial concentration was similar, the effect of algal addition seemed to be little (p-value = 0.750).

Algal Growth Effect on Bacterial Diversity

After 9 days of reactor operations in the batch mode, bacterial diversity was measured by 16S rRNA amplicon pyrosequencing. After filtering out low-quality sequences, the lengths of amplicons pyrosequenced were approximately 340 bp regardless of the different reactor samples. However, the numbers of filtered sequences varied between different reactor samples. The WW sample showed the largest number of filtered sequences (12,415), whereas the smallest number of sequences (769) was obtained from the initial wastewater (IWW). Without the extreme cases, the other samples showed similar numbers of filtered sequences (approximately 1,000~1,500). To minimize the effect of different numbers of sequences in diversity comparisons, Shannon Index, which is little affected by the population number, was used to estimate species-level (0.03 distance OTUs) diversities, and the same number of filtered sequences (769) was randomly sampled for the Shannon Index estimation. The smallest Shannon Index value was observed in the reactor showing algal growth (AWW). The other reactors (WW, AWWS, and WWS) showed diversity values similar to IWW (Table 3).

Table 3. Bacterial diversity results.

Sample	Number of sequences (N)	Coverage (%)[a]	Shannon index (H′)[b]
IWW	769	94.7	4.45 ± 0.15
AWW	1,541	95.5	2.60 ± 0.22
WW	12,415	96.8	4.35 ± 0.14
AWWS	1,023	96.1	3.94 ± 0.30
WWS	1,280	94.4	4.15 ± 0.12

[a]The coverage was estimated by Good's coverage for an OTU definition.

[b]Estimated among randomly selected 769 sequences. The average and one standard deviation were calculated from 10 independent random samplings.

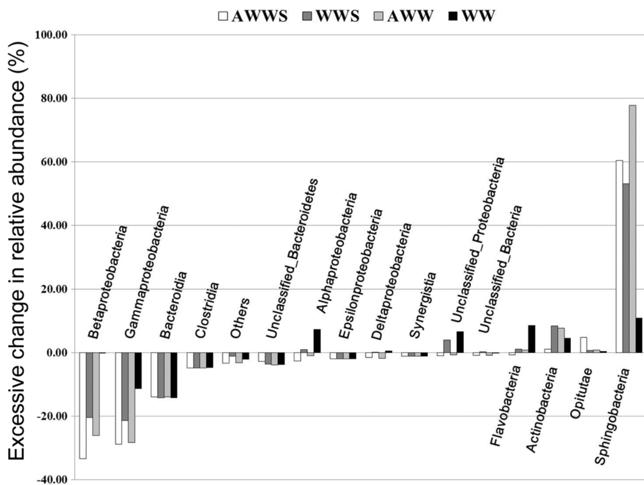

Fig. 1. Bacterial community structure (class level) changes in the different reactors (AWW, WW, AWWS and WWS).
The Y-values indicate excessive change in relative abundance compared with the initial bacterial community.

Algal Growth Effect on Bacterial Community Structure

In the initial wastewater, Gammaproteobacteria and Betaproteobacteria class members were dominant. After the 9 day incubation in the reactors, Sphingobacteria, Betaproteobacteria, and Actinobacteria class members became dominant (data not shown). To identify which class members were selectively stimulated by algal growth, excessive change in the relative abundance ([relative abundance of reactor – relative abundance of IWW]/[relative abundance of IWW]) was calculated (Fig. 1). For this, rare class members (less than 1% relative abundance) were regarded as "others". In AWW exhibiting algal growth (Table 2), Sphingobacteria class members showed 77.5% increase from IWW, which was significantly greater than those in the other reactors (p-value < 0.05). Other class members showed insignificant changes.

Bacterial Genus Populations Responding to Algal Growth

To further explore the effect of algal population on bacterial communities, bacterial genus compositions were compared as shown in Fig. 2A. The following PCA result showed that the genus composition for AWW was distant from IWW and WW (Fig. 2B). This suggests that the algal growth may have had a more significant influence on genus-level population composition than the carbon starvation effect indicated by the PCA distance between the initial wastewater (IWW) and its no algal control (WW). Compared with IWW and WW, the relative abundances of Unclassified Sphingobacteriales and *Sediminibacterium* members were significantly increased in AWW.

Discussion

The effect of the algal population on wastewater bacterial growth was explored with a wastewater-adapted algal population in real wastewater conditions. The algal and bacterial growth rate results (Table 2) showed that the algal growth had an inhibitory effect on the growth of wastewater bacteria. This suggests that *Selenastrum gracile* may be a stable algal species member compared with other algal resources that are vulnerable to bacterial attacks in wastewater environments [24].

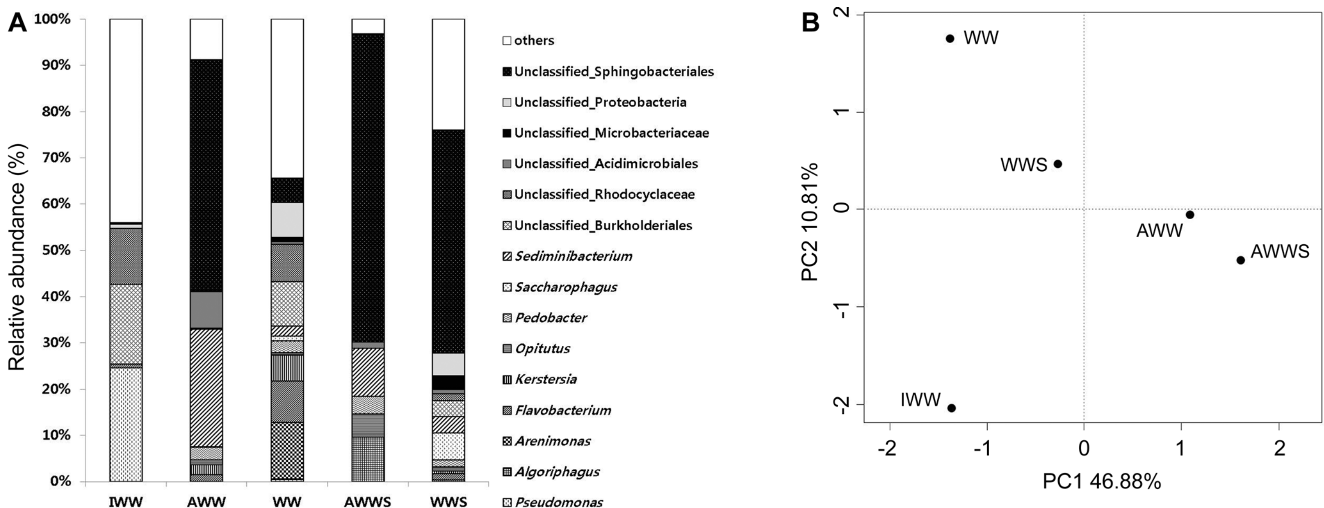

Fig. 2. Comparison of bacterial genus compositions (**A**) and their principal component analysis plot (**B**).

The bacterial community analysis that used 16S rRNA gene-targeted pyrosequencing revealed the algal growth's inhibitory effect on bacterial diversity in the wastewater (Table 3). Similar bacterial diversity of non-algal reactors (WW and WWS) to the initial wastewater (IWW) was expected because bacterial diversity in a batch system may be increased or maintained before long-term carbon starvation [19]. Because of the small effects of an added algal population, no differences in bacterial diversity were observed between WWS and AWWS. The reduced bacterial diversity suggests that the algal population had a selective power for particular members of wastewater bacteria [18].

The bacterial community profiling results led to the conclusion that Sphingobacteria class members were selectively stimulated in response to algal growth in the wastewater environment. In the literature, algal selection of the Sphingobacteria class was observed in marine environments and different wastewater environments [13, 30]. These suggest that the Sphingobacteria class may be a specific taxonomic group that positively interacts with algal growth.

Further bacterial genus composition analysis suggested that the two genus members Unclassified Sphingobacteriales and *Sediminibacterium* may have been stimulated by the algal growth in AWW. However, the possibility of Unclassified Sphingobacteriales was ruled out because the genus population was observed in the no algal growth controls with the addition of activated sludge (WWS). The dominance of Unclassified Sphingobacteriales was likely due to excreted cellular products from algal populations (AWW and AWWS) and bacterial populations (WWS) rather than an algal growth effect. Therefore, *Sediminibacterium* was identified as a genus-level population stimulated by algal growth in AWW. The members of *Sediminibacterium* are reported to inhabit eutrophic reservoirs [26]. Therefore, the dominance of *Sediminibacterium* may be related to the interactions with the *Selenastrum gracile* population.

In the present study, the effect of algal growth on wastewater bacterial communities was explored using 16S rRNA gene amplicon pyrosequencing. The response of the wastewater microbial communities (population diversity, community structure, and selectively stimulated populations) to the algal growth of one of the wastewater-adapted algal species, *Selenastrum gracile*, was observed. Deep sequencing and statistical analysis showed that the algal growth significantly shifted the bacterial community structure and had a reducing effect on bacterial diversity. The phylogenetic analysis of pyrosequenced 16S rRNA gene amplicons revealed that the Sphingobacteria class and *Sediminibacterium* genus group became stimulated selectively in response to algal growth. This study provided information on the ecological characteristics of bacterial communities that cooperatively interacted with algal growth in a municipal wastewater environment.

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