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The source of inoculum drives bacterial community structure in *Synechocystis* sp. PCC6803-based photobioreactors

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ABSTRACT

Cyanobacteria, which grow using solar energy and carbon dioxide, provide an attractive avenue for sustainable production of biomass and biofuel feedstock. Heterotrophic bacteria can colonize photobioreactor (PBR) cultures of cyanobacteria and may affect the productivity of the system. However, little is known about the types of heterotrophic bacteria associated with PBR cultures of cyanobacteria. The objective of this work was to evaluate the heterotrophic communities in PBR cultures of the cyanobacterium *Synechocystis* sp. PCC6803 using terminal-restriction fragment length polymorphism (T-RFLP) and high-throughput sequencing. To improve the resolution of the heterotrophic bacterial genomic signatures in the PBR communities, we utilized a targeted T-RFLP strategy to remove the T-RFLP signal of *Synechocystis* sp. PCC6803 from the analysis. For all experiments, the results of high-throughput sequencing were consistent with T-RFLP results. In particular, the inocula contained heterotrophic bacteria despite appearing to be pure cultures by light microscopy or non-targeted T-RFLP. Furthermore, the heterotrophic communities in the PBRs were strongly influenced by the microbial community in the inoculum used to start the PBR.

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1. Introduction

Cyanobacteria are promising candidates for large-scale production of renewable biofuels to replace petroleum resources, including biodiesel made from lipids extracted from biomass [1,2]. Cyanobacteria perform oxygenic photosynthesis, gaining energy from sunlight and carbon from carbon dioxide. Thus, biofuels derived from cyanobacteria represent a renewable fuel source that can have a reduced carbon footprint compared to conventional diesel. Cyanobacteria have many advantages over terrestrial plants, including higher areal yields, superior photosynthetic efficiencies, faster growth rates, and no requirement for arable land for cultivation [3–5].

Heterotrophic bacteria grow by oxidizing organic molecules produced by cyanobacteria, including proteins, lipids, nucleic acids, and sugars [6]. Associations between cyanobacteria and heterotrophic bacteria are common, and a number of studies have examined the heterotrophic bacteria associated with cyanobacteria in natural settings [7–10]. Yet, few studies to date have addressed the structure and function of heterotrophic communities in photobioreactor (PBR) cultures of cyanobacteria [11]. In large-scale PBRs, sterilizing the culture medium and maintaining axenic culture conditions are economically and practically prohibitive [12]. Thus, a complete understanding of PBRs as engineered biological systems demands knowledge of the structure and dynamics of the microbial community, including the heterotrophic bacteria.

While the functions of the heterotrophic bacteria that associate with cyanobacteria are not fully understood, several important beneficial interactions have been documented. Primarily, heterotrophic bacteria can provide CO_2 to the cyanobacteria by oxidizing organic compounds released by the cyanobacteria. Additionally, heterotrophic bacteria can recycle macronutrients, such as nitrogen (N) and phosphorus (P), or increase the availability of micronutrients, such as iron, to the cyanobacteria with which they associate [13]. Heterotrophic metabolism also may reduce O_2 super-saturation in cyanobacterial cultures [14]. Heterotrophic bacteria also can be detrimental to PBR operations by causing lysis of cyanobacterial cells [15], competing for key macronutrients, or consuming a desired product produced by the cyanobacteria such as excreted fatty acids [16]. Since the purpose of the PBR is to produce cyanobacteria biomass, exclusion of specific heterotrophic bacteria may be difficult, as the best growth conditions for the cyanobacteria also will be suitable for an array of heterotrophic bacteria. Thus, elucidating the common heterotrophs and key microbial interactions is critical for successful photobioreactor operation.

Synechocystis sp. PCC6803 (hereafter referred to as *Synechocystis*) is one of the most extensively studied cyanobacteria and was the first phototrophic organism to have a completely sequenced genome,

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making it an ideal model organism for further studies leading to large-scale use of cyanobacteria to produce renewable feedstock [5,17,18]. However, little is known about the types and temporal dynamics of the heterotrophic bacteria that can associate with *Synechocystis*-based PBRs. Molecular methods targeting the 16S rRNA gene are useful to uncover the structure of microbial communities associated with PBR cultures of *Synechocystis*, and to monitor these communities through time [19]. Terminal-restriction fragment length polymorphism (T-RFLP) is a rapid, robust, and cost-effective method that is widely used to analyze microbial communities [20–22] and provides a useful avenue to compare and contrast the structures of different PBR microbial communities. Similarly, high-throughput sequencing of 16S rRNA gene libraries can be used to assess the structures of microbial communities [23]. High-throughput sequencing is beneficial because it is highly sensitive and provides phylogenetic information not afforded by T-RFLP; however, it is not accessible to every researcher and requires significant time for sequencing and data analysis.

Here, we used T-RFLP and high-throughput sequencing of 16S rRNA gene libraries to monitor the microbial communities in PBR cultures of *Synechocystis*. We demonstrate that communities of heterotrophic bacteria exist in the PBRs and show that the structure of the PBR microbial communities can be different for each PBR. We also provide evidence that the starter cultures used to inoculate the various PBR experiments play an important role in determining the structure of the resulting PBR microbial communities. Finally, we demonstrate strong agreement between T-RFLP and high-throughput sequencing data, showing that these techniques can be used in tandem or separately to provide a detailed understanding of PBR microbial communities.

2. Materials and methods

2.1. Cell cultures, strains, media and preparation of inocula

The laboratory of Dr. Willem Vermaas (School of Life Sciences, Arizona State University) provided stock cultures of *Synechocystis* sp. PCC6803, which were maintained in BG-11 medium [24]. To make freezer stocks, 0.5 mL of a *Synechocystis* sp. PCC6803 stock culture was mixed with 0.5 mL of sterile 40% glycerol and stored at −80 °C. To prepare fresh PBR inoculum, we spread a small amount of the freezer stock on a sterile BG-11 plate with 1.5% *(w/v)* Bacto Agar (BD, Sparks, MD) using a sterile inoculation loop. We grew the inoculated plates at 30 °C under 200 μE m^{-2} s^{-1} constant incident light intensity until a lawn of cells was evident (5–7 days). We then scraped cells from the lawn using a 10 μL inoculation loop and transferred the cells into 100 mL sterile liquid BG-11 medium in a 250 mL Erlenmeyer flask bubbled with air filtered through a 0.2 μm filter (Pall). We grew this starter culture at 30 °C under 200 μE m^{-2} s^{-1} constant incident light intensity until cells grew to an optical density at a wavelength of 730 nm (OD_{730}) of 0.5–1 (3–5 days). For the purposes of this work, each separate starter culture can be considered as originating from a separate BG-11 agar plate.

2.2. PBR operational parameters

We used a Photobioreactor FMT-150 (Photon Systems Instruments, Czech Republic) equipped with a temperature/pH probe and bubble interrupter for all experiments. We autoclaved the cultivation chamber to ensure that it was sterile prior to inoculation. All PBR experiments were operated with a constant incident light intensity of 200 μE m^{-2} s^{-1}, maintained at 30 °C, and bubbled with air that was humidified by passing through sterile water and filtered through a 0.2-μm filter (Pall, Ann Arbor, MI). We measured optical density (OD) at a wavelength of 730 nm using a Cary-50-Bio UV–Visible spectrophotometer (Varian, Palo Alto, CA) and pH directly using a pH probe integrated with the Photobioreactor FMT-150 and calibrated according to the manufacturer's directions. We inoculated four PBRs at a starting OD_{730} of 0.1 and ran each experiment for a total of 168 h (7 days). Two of the PBRs (PBR-A and PBR-A2) were inoculated from the same starter culture originating from the same agar plate and culture flask, and the other two PBRs (PBR-B and PBR-C) were inoculated using different starter cultures, which originated from different agar plates and liquid culture flasks.

2.3. Light microscopy

For light microscopy, we took 1-mL samples of PBR cultures daily, added 0.2 mL 37.5% formaldehyde (Sigma Aldrich) to fix the cells, and stored these samples at 4 °C. Cells were imaged later by light microscopy using an Olympus BX61 light microscope (Olympus Inc., Center Valley, PA) equipped with differential interference contrast (DIC) using a 100× oil-immersion objective. To evaluate the presence or absence of heterotrophic bacteria, we counted at least 200 total cells. Images were captured with an Olympus DP72 color camera.

2.4. DNA extraction

For DNA extraction we used a previously described method [25]. Briefly, 1-mL samples of the PBR culture were taken daily with a sterile syringe and transferred to a sterile microcentrifuge tube and centrifuged (13 *g*, 3 min) to concentrate the biomass, which was stored at −80 °C prior to DNA extraction. We extracted total genomic DNA from PBR samples using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA) with the following modifications to enhance lysis. We resuspended cell pellets in 200 μL lysis buffer (30 mM Tris·HCl, 10 mM EDTA, 200 mM sucrose, pH 8.2) and incubated the mixture at 65 °C for 10 min. We then added chicken egg white lysozyme (Sigma Aldrich, St. Louis, MO) to a final concentration of 10 mg/mL and incubated the samples for 1 h at 37 °C. Next, we added SDS at 1% (*w*/*v*) and incubated the samples at 56 °C for 10 min. Finally, we added 25 μL proteinase K and 200 μL buffer AL (Qiagen) and incubated that mixture at 56 °C for 30 min. After these additional lysis steps, we completed the DNA extraction according to the manufacturer's (Qiagen) instructions.

2.5. Terminal restriction fragment length polymorphism (T-RFLP)

To improve the resolution of the heterotrophic bacteria, we applied a strategy to remove *Synechocystis* from the T-RFLP analyses. We achieved this goal by choosing a primer and restriction enzyme combination that produced a *Synechocystis* terminal-restriction fragment (T-RF) outside the detectable range of the assay, thereby excluding the *Synechocystis* signal from the analysis and increasing sensitivity towards 16S rRNA gene fragments belonging to other bacteria present in the PBR. We performed T-RFLP analysis using a previously described method [25]. Briefly, we amplified 16S rRNA genes using the universal bacterial primers 8F (5′-AGAGTTTGATCCTGGCTCAG-3′) with a 5′-HEX fluorescent dye and 1392R (5′-ACACACCGCCCGT-3′) [21,22,25]. PCR conditions were as follows: 1× *Taq* PCR Master Mix (Qiagen), 250 nmol L^{-1} of each primer, 1 mmol L^{-1} Mg^{2+}, and 10 ng template DNA in a total of 50 μL. PCR reaction temperature profiles were the following: 94 °C for 6 min, followed by 30 cycles of 94 °C for 45 s, 55 °C for 45 s, 72 °C for 2 min and a final extension period at 72 °C for 10 min. Following PCR, we checked for amplicons of the proper size (approximately 1400 bp) on a 1% (*w*/*v*) agarose gel. We then digested the amplified 16S rRNA genes using the restriction enzymes *Hha*I, *Mse*I, *Hae*III, or *Hpa*II (New England Biolabs, Ipswich, MA). Next, we analyzed the sizes of the T-RFs produced on an ABI 3730 DNA Analyzer using the GeneScan500 ROX Size Standard (Applied Biosystems).

2.6. Analysis of T-RFLP data

We gathered raw T-RFLP data using the free Peak Scanner Software v1.0 (Applied Biosystems) and analyzed the data using previously described methods [26]. We determined total fluorescence intensity

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