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Isolation of diverse amoebal grazers of freshwater cyanobacteria for the development of model systems to study predator–prey interactions☆

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ABSTRACT

A common method for large-scale production of algal crops is growth in outdoor open-air ponds. While this approach is more cost-effective, outdoor open-air ponds are prone to contamination by competing algae, pathogens, and eukaryotic grazers, including ciliates, flagellates, and amoebae. To characterize grazers of cyanobacteria, or blue-green algae, we have performed enrichments and isolations from water samples obtained from environmental sites and from an experimental production pond. We obtained a set of amoebal isolates that show diversity in phylogeny, morphology, and locomotion. After examination of grazing on solid medium and in liquid medium, we found that some amoebal isolates can graze on a range of cyanobacterial species, while other amoebal isolates appear to have a more limited prey range. These prey ranges correlate with observed growth rates and cyst formation, suggesting differing growth and survival strategies for amoebae in the environment. Taken together, this work provides a glimpse into the range of natural amoebal predators of cyanobacteria and establishes model systems of predator–prey interactions. Further characterization of these systems will facilitate development of strategies for crop protection of open-air algal production ponds.

1. Introduction

Photosynthetic microorganisms are being heavily investigated for renewable production of biofuels, biological materials and valuable co-products. In particular, microalgae show great promise because they have fast growth rates, can grow on non-arable land, and can be grown in a range of water sources, including waste water and seawater [1,2]. Cyanobacteria, or blue-green algae, are a diverse group of photosynthetic bacteria that can potentially be developed for many different industrial applications [3]. They have the added benefit of well-developed methods for genetic manipulation and expression of heterologous genes, such that bioengineering of desired end-products and metabolic engineering for optimal production is possible [4].

Development of algae for renewable production of biofuels and other biological products is hindered by production and refinement costs [5]. To mitigate these prohibitive costs, methods for rapid accumulation of biomass and efficient production of end-products are currently being developed. One production model employs the use of outdoor ponds for growth of algal crops. These outdoor systems make use of ambient air and light conditions, and are relatively inexpensive to operate compared to other systems, such as photobioreactors [6,7]. However, one major disadvantage of outdoor open-air ponds is that they are prone to contamination [8,9]. Competing algae that are able to grow in pond media can exhaust resources, while contamination with heterotrophic microbes, including bacteria and fungi, can hinder growth of the algal crop. Algal crop biomass can be depleted by infectious pathogens such as chytrids and other parasitic fungal pathogens [10,11], while predatory grazers can consume the algal crop species, as has been seen with an amoebal predator of the eukaryotic alga *Scenedesmus dimorphus* [12]. Indeed, experimental production ponds have already encountered issues with contamination [13]. Monitoring health and productivity of algal ponds, including early detection of contaminants, is essential for achieving biomass production rates necessary for the economic viability of algal biotechnology [8,14,15].

In the environment, eukaryotic grazers feeding upon prokaryotes shape microbial population structures, playing an influential role in food web dynamics. Natural grazers of cyanobacteria include small crustaceans, such as copepods and *Daphnia* species [16,17], and protozoan grazers, such as amoebae, ciliates, heterotrophic flagellates, and mixotrophic flagellates [18]. In environmental field studies, ciliates and amoebae were associated with large reductions of cyanobacterial populations, and grazing was confirmed with feeding experiments [19,20]. Amoebae in trophozoite stages generally crawl along surfaces through eruptive extrusion of pseudopodia, and many species feed by

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interception and ingestion of prey through phagocytic mechanisms. A notable exception is a group of amoebae called vampyrellids [21], which perforate cells and ingest the cytosolic content of prey species, which include algae and cyanobacteria [22]. Some amoebal species can differentiate into a flagellate stage for rapid three-dimensional movement within aquatic environments, and some species can form dormant cysts to withstand harsh conditions [23]. Although amoebal grazing on cyanobacteria has not been extensively characterized, amoebal feeding behavior appears to be complex, with active selection of prey and sometimes rejection of food particles after ingestion [24]. The molecular interactions between amoebae and cyanobacteria likely play a defining role in determining cellular interaction, and outer membrane surface structures of cyanobacteria, toxin production, and secondary metabolite production can influence these outcomes [25–27].

To address the problem of protozoan grazing in open-air algal production ponds, we sought to establish model systems of cyanobacteria and their predators to interrogate their interactions at the molecular level. In recent work, we carried out a proof of concept study in which we showed that a model system consisting of a heterolobosean amoebal isolate, HGG1, and the genetically tractable cyanobacterium *Synechococcus elongatus* PCC 7942 could be used to isolate the first cyanobacterial mutants that were resistant to grazing [25]. This amoebal isolate however, did not graze efficiently in liquid, and hence we sought to isolate a wider variety of amoebae to develop model systems that could address conditions representative of open ponds. To obtain a larger sampling of natural predators of cyanobacteria, we developed methods to enrich and isolate predatory species that consume diverse cyanobacteria. We obtained a set of amoebal isolates that are diverse in morphology and phylogeny. These amoebae have different prey preferences and growth rates, which may reflect natural survival strategies in the environment. Our amoebal isolates exemplify the great diversity of grazers of cyanobacteria in the environment. Characterizing eukaryotic grazers and interactions with their cyanobacterial prey are a first step in understanding and counteracting algal crop contamination, a major hindrance to the development of algal biotechnology.

2. Materials and methods

2.1. Cyanobacterial strains and cultivation

Leptolyngbya sp. BL0902, *S. elongatus* PCC 7942, and *Anabaena* sp. PCC 7120 were obtained from J. W. Golden and S. S. Golden (University of California, San Diego). All cyanobacterial strains were maintained in 50 ml BG11 [28] flask cultures, and grown under 20 μmol photons m^{-2} s^{-1} continuous light at 30 °C.

2.2. Amoebal isolation and maintenance

Water samples were collected from Huntington Gardens (Pasadena, CA), the California Center for Algae Biotechnology (Cal-CAB) Algae Research and Development Facility at the University of California, San Diego Biology Field Station [29], and a residential lily pond (Encinitas, CA). They were serially diluted with exponentially growing cultures of *Leptolyngbya* sp. BL0902 or *S. elongatus* PCC 7942 as diluent in 24-well Costar cell culture dishes (Corning) and incubated at 30 °C under 15 μmol photons m^{-2} s^{-1} continuous light. After visual and microscopic inspection of wells exhibiting yellowing or poor growth of the cyanobacterium, wells containing amoebae were then subjected to serial dilution and growth on *Leptolyngbya* or *Synechococcus*, with subsequent growth onto agar plates seeded with cyanobacterial lawns to allow plaque formation. Clonal populations of amoebae were prepared by limiting dilution followed by serial plaque expansion on solid medium as described [25]. Amoebae were maintained at 20 °C on cyanobacterial lawns plated on BG11 agar plates and pregrown at 30 °C for 3–5 days. C3, HGG1, and *Acanthamoeba castellanii* were maintained on *Leptolyngbya* lawns, while LPG1 and LPG3 were maintained on *Synechococcus* lawns. Amoebal isolates were tested for bacterial contaminants by staining with SYBR Green I (Invitrogen) and examining for the presence of contaminating cells by microscopy. Amoebal isolates were also tested for heterotrophic contaminants by streaking to LB (Miller) agar plates (Fisher Scientific) and incubating at both 30 °C and 25 °C. All isolates were free of bacterial contaminants following several rounds of limiting dilutions and serial passaging from expanded plaques, with the exception of LPG3. *A. castellanii* strain ATCC 30234 obtained from the American Type Culture Collection (ATCC) was used as a control and was maintained on lawns of *Leptolyngbya* sp. BL0902.

2.3. 18S ribosomal DNA sequencing

Amoebal cells were harvested from the edge of expanding plaques on agar plates, and PCR template was prepared by boiling in 10 μl water or with a DNeasy Blood and Tissue Kit (Qiagen). Template was used for PCR amplification with GoTaq Hot Start Polymerase (Promega) using a 0.2 μM final concentration of each primer. Thermocycling conditions using 18S MoonA and MoonB primers were as described [30]. Both strands of the PCR products were then directly sequenced with MoonA, MoonB and internal primers (Table S1). Direct sequencing of the PCR product for B1, LPG1, and LPG3 revealed no ambiguities, while to resolve base pair ambiguities for the C3 product, the PCR product was cloned into pCR2.1-TOPO vector (Life Technologies), followed by sequencing of both strands of the inserts in three plasmids using M13 F(−20) and M13 R primers. The sequence from one of these plasmids was used for alignments and phylogenetic reconstructions. The sequences were deposited in GenBank with the following accession numbers: LPG3 (KT892699), B1 (KT892700), C3 (KT892701), LPG1 (KT892702).

2.4. Phylogenetic tree of amoebal isolates

18S rDNA sequences were compared to the National Center for Biotechnology Information non-redundant (nr) database using Blastn and Megablast and the results were used to generate phylogenetic trees. The LPG3 18S rDNA gene sequence was manually aligned to an existing set of heterolobosean sequences as previously described [25]. The sequences of the 18S rDNAs of LPG1 and C3 were placed into an existing alignment (M139, [31]) of Amoebozoan 18S rDNA sequences and manually aligned to this set. Neighbor-joining phylogenetic trees were constructed as previously described [25].

2.5. Image acquisition

Slides were prepared by harvesting amoebal cells from maintenance plates, resuspending in BG11 medium and preparing wet mounts. Coverslips were sealed with clear nail polish. Still images of cells were obtained using fluorescence and Nomarski interference contrast microscopy using an Axioskop microscope (Zeiss) through a 63× objective. Cyanobacterial autofluorescence was imaged using Zeiss filter set 14 (number 487914) with 510–560 nm (ex) and 590 nm longpass (em). Videos were acquired using an Axioplan microscope (Zeiss) through a 100× oil immersion objective. Images and videos were acquired using a Spot Pursuit camera and Spot Advanced software, version 5.1 (Spot Imaging Solutions). Agar plates were photographed over a light box with a Sony Cyber-Shot camera.

2.6. Solid medium grazing assay

Cyanobacterial cultures with OD_{750} ranging from 0.3 to 0.6 were concentrated 10-fold, plated onto BG11 agar plates, and grown for one week at 15 μmol photons m^{-2} s^{-1} continuous light at 30 °C. Amoebal