1 **Isolation of diverse amoebal grazers of freshwater cyanobacteria for the**
2 **development of model systems to study predator-prey interactions**

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1 **Abstract.**

2 A common method for large-scale production of algal crops is growth in outdoor
3 open-air ponds. While this approach is more cost-effective, outdoor open-air
4 ponds are prone to contamination by competing algae, pathogens, and
5 eukaryotic grazers, including ciliates, flagellates, and amoebae. To characterize
6 grazers of cyanobacteria, or blue-green algae, we have performed enrichments
7 and isolations from water samples obtained from environmental sites and from
8 an experimental production pond. We obtained a set of amoebal isolates that
9 show diversity in phylogeny, morphology, and locomotion. After examination of
10 grazing on solid medium and in liquid medium, we found that some amoebal
11 isolates can graze on a range of cyanobacterial species, while other amoebal
12 isolates appear to have a more limited prey range. These prey ranges correlate
13 with observed growth rates and cyst formation, suggesting differing growth and
14 survival strategies for amoebae in the environment. Taken together, this work
15 provides a glimpse into the range of natural amoebal predators of cyanobacteria
16 and establishes model systems of predator-prey interactions. Further
17 characterization of these systems will facilitate development of strategies for crop
18 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

1 can consume the algal crop species, as has been seen with an amoebal predator
2 of the eukaryotic alga *Scenedesmus dimorphus* [12]. Indeed, experimental
3 production ponds have already encountered issues with contamination [13].
4 Monitoring health and productivity of algal ponds, including early detection of
5 contaminants, is essential for achieving biomass production rates necessary for
6 the economic viability of algal biotechnology [8, 14, 15].

7 In the environment, eukaryotic grazers feeding upon prokaryotes shape
8 microbial population structures, playing an influential role in food web dynamics.
9 Natural grazers of cyanobacteria include small crustaceans, such as copepods
10 and *Daphnia* species [16, 17], and protozoan grazers, such as amoebae, ciliates,
11 heterotrophic flagellates, and mixotrophic flagellates [18]. In environmental field
12 studies, ciliates and amoebae were associated with large reductions of
13 cyanobacterial populations, and grazing was confirmed with feeding experiments
14 [19, 20]. Amoebae in trophozoite stages generally crawl along surfaces through
15 eruptive extrusion of pseudopodia, and many species feed by interception and
16 ingestion of prey through phagocytic mechanisms. A notable exception is a group
17 of amoebae called vampyrellids [21], which perforate cells and ingest the
18 cytosolic content of prey species, which include algae and cyanobacteria [22].
19 Some amoebal species can differentiate into a flagellate stage for rapid three-
20 dimensional movement within aquatic environments, and some species can form
21 dormant cysts to withstand harsh conditions [23]. Although amoebal grazing on
22 cyanobacteria has not been extensively characterized, amoebal feeding behavior
23 appears to be complex, with active selection of prey and sometimes rejection of

1 food particles after ingestion [24]. The molecular interactions between amoebae
2 and cyanobacteria likely play a defining role in determining cellular interaction,
3 and outer membrane surface structures of cyanobacteria, toxin production, and
4 secondary metabolite production can influence these outcomes [25-27].

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

1

2 **2. Materials and Methods**

3 **2.1. Cyanobacterial strains and cultivation.** *Leptolyngbya* sp. BL0902,
4 *Synechococcus elongatus* PCC 7942, and *Anabaena* sp. PCC 7120 were
5 obtained from J. W. Golden and S. S. Golden (University of California, San
6 Diego). All cyanobacterial strains were maintained in 50 ml BG11 [28] flask
7 cultures, and grown under 20 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ continuous light at 30°C.

8 **2.2. Amoebal isolation and maintenance.** Water samples were collected from
9 Huntington Gardens (Pasadena, CA), the California Center for Algae
10 Biotechnology (Cal-CAB) Algae Research and Development Facility at the
11 University of California, San Diego Biology Field Station [29], and a residential lily
12 pond (Encinitas, CA). They were serially diluted with exponentially growing
13 cultures of *Leptolyngbya* sp. BL0902 or *Synechococcus elongatus* PCC 7942 as
14 diluent in 24-well Costar cell culture dishes (Corning) and incubated at 30°C
15 under 15 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ continuous light. After visual and microscopic
16 inspection of wells exhibiting yellowing or poor growth of the cyanobacterium,
17 wells containing amoebae were then subjected to serial dilution and growth on
18 *Leptolyngbya* or *Synechococcus*, with subsequent growth onto agar plates
19 seeded with cyanobacterial lawns to allow plaque formation. Clonal populations
20 of amoebae were prepared by limiting dilution followed by serial plaque
21 expansion on solid medium as described [25]. Amoebae were maintained at
22 20°C on cyanobacterial lawns plated on BG11 agar plates and pregrown at 30°C
23 for 3-5 days. C3, HGG1, and *A. castellanii* were maintained on *Leptolyngbya*

1 lawns, while LPG1 and LPG3 were maintained on *Synechococcus* lawns.
2 Amoebal isolates were tested for bacterial contaminants by staining with SYBR
3 Green I (Invitrogen) and examining for the presence of contaminating cells by
4 microscopy. Amoebal isolates were also tested for heterotrophic contaminants by
5 streaking to LB (Miller) agar plates (Fisher Scientific) and incubating at both 30°C
6 and 25°C. All isolates were free of bacterial contaminants following several
7 rounds of limiting dilutions and serial passaging from expanded plaques, with the
8 exception of LPG3. *Acanthamoeba castellanii* strain ATCC 30234 obtained from
9 the American Type Culture Collection (ATCC) was used as a control and was
10 maintained on lawns of *Leptolyngbya* sp. BL0902.

11 **2.3. 18S ribosomal DNA sequencing.** Amoebal cells were harvested from the
12 edge of expanding plaques on agar plates, and PCR template was prepared by
13 boiling in 10 µl water or with a DNeasy Blood and Tissue Kit (Qiagen). Template
14 was used for PCR amplification with GoTaq Hot Start Polymerase (Promega)
15 using a 0.2 µM final concentration of each primer. Thermocycling conditions
16 using 18S MoonA and MoonB primers were as described [30]. Both strands of
17 the PCR products were then directly sequenced with MoonA, MoonB and internal
18 primers (Table S1). Direct sequencing of the PCR product for B1, LPG1, and
19 LPG3 revealed no ambiguities, while to resolve base pair ambiguities for the C3
20 product, the PCR product was cloned into pCR2.1-TOPO vector (Life
21 Technologies), followed by sequencing of both strands of the inserts in three
22 plasmids using M13 F(-20) and M13 R primers. The sequence from one of these
23 plasmids was used for alignments and phylogenetic reconstructions. The

1 sequences were deposited in GenBank with the following accession numbers:
2 LPG3 (KT892699), B1 (KT892700), C3 (KT892701), LPG1 (KT892702).

3 **2.4. Phylogenetic tree of amoebal isolates.** 18S rDNA sequences were
4 compared to the National Center for Biotechnology Information non-redundant
5 (nr) database using Blastn and Megablast and the results were used to generate
6 phylogenetic trees. The LPG3 18S rDNA gene sequence was manually aligned
7 to an existing set of heterolobosean sequences as previously described [25]. The
8 sequences of the 18S rDNAs of LPG1 and C3 were placed into an existing
9 alignment (M139, [31]) of Amoebozoan 18S rDNA sequences and manually
10 aligned to this set. Neighbor-joining phylogenetic trees were constructed as
11 previously described [25].

12 **2.5. Image Acquisition.** Slides were prepared by harvesting amoebal cells from
13 maintenance plates, resuspending in BG11 medium and preparing wet mounts.
14 Coverslips were sealed with clear nail polish. Still images of cells were obtained
15 using fluorescence and Nomarski interference contrast microscopy using an
16 Axioskop microscope (Zeiss) through a 63x objective. Cyanobacterial
17 autofluorescence was imaged using Zeiss filter set 14 (number 487914) with
18 510-560 nm (ex) and 590 nm longpass (em). Videos were acquired using an
19 Axioplan microscope (Zeiss) through a 100x oil immersion objective. Images and
20 videos were acquired using a Spot Pursuit camera and Spot Advanced software,
21 version 5.1 (Spot Imaging Solutions). Agar plates were photographed over a light
22 box with a Sony Cyber-Shot camera.

2.6. Solid medium grazing assay. Cyanobacterial cultures with OD₇₅₀ ranging from 0.3 to 0.6 were concentrated 10-fold, plated onto BG11 agar plates, and grown for one week at 15 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ continuous light at 30°C. Amoebal suspensions were prepared by scraping cells from the edge of plaques on maintenance plates and resuspending into BG11 medium. Lawns were spotted with 10 μl of amoebal suspensions containing approximately 1000 to 7500 amoebal cells per spot and incubated at 15 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ continuous light at 30°C, with the exception of C3 which was assayed at 10 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ continuous light at 25°C. Plates were scored weekly for plaque formation, and the presence of amoebae in or at the edge of a plaque was confirmed by microscopy. Amoebal grazing was also assayed on freshly plated cyanobacterial lawns prepared by omitting the one-week growth period, to determine whether the initial cyanobacterial density affected the grazing rate. Results from these assays are reported if plaque formation occurred on freshly-plated lawns, but did not occur on one week old lawns.

2.7. Grazing assay in liquid medium. Amoebal cell suspensions were prepared as described for the solid medium grazing assay. In 24-well cell culture dishes, 1.5×10^4 amoebal cells were added to 2 ml cyanobacterial cultures normalized to OD₇₅₀ 0.2. Plates were incubated under 7-9 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ continuous light at 30°C or at 20°C under 3-5 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ continuous light. Wells were assayed daily by resuspending and sampling 50 μl for fixation with 2% neutral buffered formalin, with the exception of LPG1 for which live cells were counted due to the difficulty of distinguishing fixed trophozoite cells from cysts. Amoebal

1 cell density was determined through direct counts in triplicate on a Hausser 3200
2 counting chamber (Hausser Scientific) under 10x magnification. Doubling times
3 were calculated using the exponential growth phase of each growth curve with
4 the program Prism 5 v. 5.0b using the following equation: doubling time
5 (days)= $\ln(2)/k$ and $Y=Y_0 \cdot \exp(k \cdot X)$, where Y =cell count, Y_0 =starting cell count,
6 k =growth rate constant, and X =time (days).

7 **2.8. Assays for flagellate differentiation and cyst formation.** Amoebal strains
8 were tested for formation of flagellate stages in BG11 medium, deionized water
9 or 2 mM Tris buffer, pH 7.2 and examined at 0, 2 and 24 hours, as described [25,
10 32, 33]. No flagellated amoebal cells were observed under the conditions tested.
11 Amoebal maintenance plates were inspected for the presence of cysts, which
12 appear as bright round cells clustered at the center of large plaques.

13 **2.9. Freezing protocol for long-term storage of amoebae.** Cyanobacterial
14 cultures with OD_{750} ranging from 0.4 to 0.6 were concentrated 10-fold, plated
15 onto BG11 agar plates, and grown for four days at $10 \mu\text{mol photons m}^{-2} \text{s}^{-1}$
16 continuous light at 30°C. Lawns were inoculated in six spots with amoebal cells
17 harvested from maintenance plates with an inoculating loop. Multiple spots were
18 used to increase the surface area of actively growing amoebae per plate.
19 Amoebae were grown until densities were high or before plaques grew into each
20 other. Cells were then harvested by adding 2 ml of BG11 medium onto the plates
21 and resuspended by spreading with a plate spreader. Approximately 1 ml of
22 liquid suspension was collected, and Hybri-Max grade DMSO (Sigma-Aldrich)
23 was added to a final concentration of 10%. 250- μl aliquots were placed into 1.2

ml cryovial tubes (Nalgene). Cryovials were inserted into a CoolCell (Biocision) and frozen at -80°C. Amoebal cells were checked for viability after one month. Cryovials were thawed quickly in a 37°C water bath and all 250 µl of the cell suspension was added as 20 µl spots to cyanobacterial lawns, as prepared above. All amoebal strains showed evidence of growth within two to three weeks.

6

3. Results

3.1. Isolation and characterization of amoebal isolates. To obtain a diverse set of natural grazers of cyanobacteria, we sampled from three fresh water sources and performed enrichments using two cyanobacterial species, one unicellular and one filamentous, as prey (Table 1). Environmental sampling sites included a pond at Huntington Gardens in Pasadena, CA and a residential lily pond in Encinitas, CA. We also obtained samples from an experimental production pond at the Cal-CAB Algae Research and Development Facility

Table 1
Amoebal isolates that graze on cyanobacteria.

Name	Sampling Location	Isolation Prey	18S rDNA BLAST Results		
			Description	Accession	Identities (%)
B1	Cal-CAB facility ^a , La Jolla, CA	<i>Leptolyngbya</i>	<i>Vrihiamoeba italica</i>	AB513360.1	1490/1609 (93)
C3	Cal-CAB facility ^a , La Jolla, CA	<i>Leptolyngbya</i>	Uncultured Amoebozoa clone Orly22	FJ577822.1	1729/1813 (95)
HGG1	Huntington Gardens, Pasadena, CA	<i>Leptolyngbya</i>	<i>Vrihiamoeba italica</i>	AB513360.1	1498/1617 (99)
LPG1	Lily pond, Encinitas, CA	<i>Synechococcus</i>	<i>Hartmannella</i> sp. GERF2	HM363627.1	1801/1809 (99)
LPG3	Lily pond, Encinitas, CA	<i>Synechococcus</i>	Uncultured heteroloboseal clone LC103_5EP_3	DQ504339.1	701/903 (78)

^a Outdoor pond at the California Center for Algae Biotechnology Research and Development facility

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1 located at the Biology Field Station of the University of California, San Diego.
2 These water samples were used to enrich for grazers of cyanobacteria by
3 incubation with cyanobacterial species *Leptolyngbya* sp. BL0902 (hereafter
4 *Leptolyngbya*) or *Synechococcus elongatus* PCC 7942 (hereafter
5 *Synechococcus*). *Leptolyngbya* is a cyanobacterium that forms long, thin
6 filaments that are approximately 1.5 μm in diameter. It was originally isolated as
7 a contaminant of a production pond and is being developed as a production
8 strain because of its excellent growth characteristics and tolerance of many
9 stress conditions such as high light, high pH, and high temperature conditions
10 [34]. *Synechococcus* is a model laboratory strain that has long been used for
11 studies of photosynthesis and circadian rhythms. It is unicellular and rod-shaped,
12 with cells that are approximately 1 μm wide and 3 μm long. This strain can be
13 easily genetically manipulated and has excellent resources for generation of
14 knock-out mutations, facilitating identification of factors that contribute to
15 interactions with grazers [25, 35].

16 To isolate grazers, the environmental water samples were mixed with
17 either *Leptolyngbya* or *Synechococcus* in 24-well cell culture dishes and
18 incubated under continuous light at 30°C. When wells showing yellowing or poor
19 growth of the cyanobacterium were inspected by microscopy, many of them
20 contained grazers, such as ciliates, nanoflagellates, and amoebae (data not
21 shown). For the present study, we focused on the isolation and characterization
22 of the amoebal grazers (Table 1).

23

Fig. 1. Phylogenetic tree of amoebal isolates. (A) Neighbor-joining small subunit (SSU) rDNA gene tree showing the phylogenetic position of LPG3 relative to that of HGG1 and 15 other heterolobosean amoebae and two outgroup taxa (*Trypanoplasma borreli* and *Euglena gracilis*). Bootstrap values are based on 1,000 trees. Horizontal bar represents the substitutions/site. Accession numbers of each sequence are shown in parentheses. (B) Neighbor-joining small subunit (SSU) rDNA gene tree showing the phylogenetic positions of LPG1 and C3 relative to that of 17 other Amoebozoa with the deeply branching Dictyostelidae *Dictyostelium discoideum* and *Polysphondilium pallidum* as outgroup taxa. Bootstrap values are based on 1,000 trees. Horizontal bar represents the substitutions/site. Accession numbers of each sequence are shown in parentheses. Asterisks indicate the amoebal isolates described in this study.

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2

1 Because it is not possible to identify amoebae by morphology alone, our
2 isolates were characterized by 18S ribosomal RNA gene sequencing (Table 1)
3 and these data were used to construct phylogenetic trees (Fig. 1). Our amoebal
4 isolates clustered to two major phylogenetic lineages within the Eukarya: the
5 Amoebozoa (isolates C3 and LPG1) and the Heterolobosea (isolates B1, LPG3,
6 and HGG1). The 18S rDNA of isolate C3 exhibits a high degree of identity to that
7 of uncultured Amoebozoan clone Orly 22, recovered from finished drinking water
8 produced at a drinking water treatment facility in Orly, France [36], and groups
9 with members of the Vannellidae (Figure 1B), a group of amoebae that are
10 ubiquitous in aquatic environments, both freshwater and marine [37]. The 18S
11 rDNA of isolate LPG1 is 99.6% identical to that of *Hartmannella* sp. GERF2,
12 isolated from the gill tissue of a rainbow trout [38], and groups with *Hartmannella*
13 *vermiformis*, recently renamed *Vermamoeba vermiformis* [39]. *H. vermiformis* is
14 widespread in nature and has been isolated from soils, freshwaters, water
15 processing facilities and water lines; it can also serve as a carrier for the
16 pathogenic bacterium *Legionella pneumophila* [40]. The best match by Blastn for
17 the 18S rDNA of isolate LPG3 is to that of an environmental clone, uncultured
18 heterolobosean LC103_5EP_3, recovered from the Lost City alkaline
19 hydrothermal vent in the mid-Atlantic [41]. Despite being isolated from
20 geographically distant water sources, the 18S rRNA genes of B1 and HGG1 are
21 99.8% identical over 1603 base pairs (data not shown). As is the case for HGG1
22 [25], the closest match to B1 after HGG1 is to the heterolobosean soil amoeba
23 *Vrihiamoeba italica* [33], with 93% identity over 1609 base pairs. Because B1 and

HGG1 are so closely related, are morphologically similar, and had the same prey preferences and plaque expansion rates in solid medium plaque assays, we excluded B1 from further experiments. We also included *Acanthamoeba castellanii* (hereafter *Acanthamoeba*) for comparison because it has been previously described as a grazer of cyanobacteria [42].

3.2. Morphological and locomotive characteristics of amoebal isolates.

Microscopic observation of these amoebae revealed that they are “naked” amoebae that lack shells, and exhibit great diversity in size, shape, and locomotion (Fig. 2 and Video 1). Our amoebal isolates are generally between 10 and 20 μm in size, with the exception of C3, which is approximately 30-50 μm . HGG1, LPG1 and *Acanthamoeba* can form cysts (Fig. 2) which are found at the center of plaques where the cyanobacterial lawn is depleted. Amoeba C3 locomotion occurs through eruptive bursts, although it moves at a much slower rate than the other isolates (Video 1A). HGG1 extrudes multiple eruptive pseudopodia during movement, which is typical of heterolobosean amoebae (Video 1B). LPG1 can be observed as either inactive round cells, or longer monopodial tube-like cells that have slug-like, or limax-type, movement (Fig. 2 and Video 1C) observed in other amoebal species, including closely related *Hartmannella* species [39, 43]. At rest, LPG3 appears flattened and ovoid, while it often adopts a bispherical shape and undergoes a twisting motion during locomotion (Fig. 2 and Video 1D).

Cyanobacteria can be readily visualized by fluorescence microscopy by virtue of their autofluorescent pigments. Fluorescent food vacuoles,

Fig. 2. Nomarski and fluorescence micrographs of amoebal isolates. Wet mounts were prepared from amoebal cells maintained on cyanobacterial lawns. Transmitted light micrographs of amoebae (left) and corresponding fluorescence micrographs (right) showing autofluorescence from cyanobacterial chlorophyll. Locomotive forms of LPG1 and LPG3 are shown on the bottom images. Images of HGG1, LPG1, and *A. castellanii* (*A.c.*) cysts are shown. All images of each amoebal isolate are scaled identically, including cysts. Scale bars, 10 μ m.

corresponding to internalized cyanobacteria in the process of being digested, were found within all amoebal isolates (Fig. 2), indicating that they graze upon their cyanobacteria prey through direct ingestion of cells. We also observed what appeared to be intact cyanobacteria within amoebal cells. *Synechococcus* cells could be observed within LPG3 and *Acanthamoeba*, and interestingly, we could see shortened *Leptolyngbya* filaments and longer coiled filaments within amoeba C3 (Fig. 2).

Video 1. Locomotion of amoebal isolates. (A) C3, (B) HGG1, (C) LPG1, and (D) LPG3. See accompanying video files.

3.3. Prey preference of amoebae grazing on solid medium. To assess the prey range of the amoebal isolates, we performed grazing assays on solid medium with cyanobacterial lawns as a food source (Fig. 3 and Table 2). In addition to *Leptolyngbya* and *Synechococcus*, we included *Anabaena* sp. PCC 7120 (hereafter *Anabaena*) in these assays. *Anabaena* is a cyanobacterium that is studied for its ability to fix atmospheric nitrogen after differentiation of specialized cells called heterocysts [44]. It forms long and thick filaments that are approximately 3 µm wide. We included it in these studies to explore the prey range of the amoebae, and also because it has a well-developed genetic system

Fig. 3. Solid medium plaque assay for amoebal grazing. Amoebal suspensions were spotted onto lawns of *Synechococcus elongatus* PCC 7942, *Leptolyngbya* sp. BL0902, or *Anabaena* sp. PCC 7120. C3 plates were incubated at 25°C and imaged after three weeks, while plates inoculated with HGG1, LPG1, LPG3, or *A. castellanii* were incubated at 30°C and imaged after two weeks. Images marked with asterisks indicate amoebal plates inoculated onto freshly plated lawns.

1
2
3

Table 2

Amoebal grazing on solid medium assayed by plaque formation on cyanobacterial lawns.

Amoeba	<i>Synechococcus</i>	<i>Leptolyngbya</i>	<i>Anabaena</i>
C3 (25°C)	-/+ ^{a, b}	+	-
HGG1 (30°C)	+++	+++	+
LPG1 (30°C)	+++	++	-/+
LPG3 (30°C)	+ ^b	-	-
<i>A. castellanii</i> (30°C)	+ ^b	+++	+

^a Plaque formation on cyanobacterial lawns: - (no plaque formation), -/+ (clearing at inoculation site without further expansion), and +, ++, +++ (plaque spread beyond inoculation site at various rates).

^b Plaque formation scores from freshly plated lawns.

serving as a model organism for studies of the molecular biology of filamentous diazotrophic cyanobacteria.

Some amoebal strains could form plaques on lawns of different cyanobacterial species (Table 2). Both HGG1 and LPG1 formed rapidly expanding plaques on *Leptolyngbya* and *Synechococcus* lawns (Fig. 3), while plaque formation on *Anabaena* lawns occurred to a lesser extent (see below). HGG1 plaque expansion occurred quickly on either *Leptolyngbya* or *Synechococcus*, while LPG1 plaque expansion occurred more quickly on *Synechococcus*. The plaques of HGG1 spread out as a ring from the inoculation site leaving behind amoebal cysts in the center. Some “scouting” amoebal cells were found past the plaque ring but most were at the interface. LPG1 also had more “scouting” amoebal cells spreading throughout the cyanobacterial lawn beyond the plaque ring, but did not cause yellowing of the cyanobacterial lawns beyond the visible plaque (data not shown). *Acanthamoeba* grew on all cyanobacterial strains to varying degrees, with *Leptolyngbya* supporting the

1 greatest plaque expansion, and *Anabaena* the least. Plaques contained
2 widespread yellowing of the lawn compared to other isolates in which plaques
3 were cleared (Fig. 3), and *Acanthamoeba* cells could be seen spread throughout
4 the cyanobacterial lawn (data not shown).

5 C3 and LPG3, appeared to be selective for the cyanobacterial strain used
6 during their initial enrichment and isolation, and these amoebae also had slower
7 rates of plaque expansion compared to HGG1 and LPG1 (Table 2). C3 cleared
8 only the inoculation site on a freshly-plated *Synechococcus* lawn after three
9 weeks (Fig. 3), while a larger plaque was formed by the third week on
10 *Leptolyngbya* and continued to expand. C3 formed clear plaques on lawns of
11 *Leptolyngbya*, but the grazing border is less distinct and appears to be “lacey”
12 (Fig. 3) such that spots are visibly seen within the plaque where amoebal cells
13 surround clumps of *Leptolyngbya* filaments. This could be due to the ingestion
14 mechanism of C3, which appears to involve pulling in a length of *Leptolyngbya*
15 filament before severing it (Fig. 2). We have observed various stages of this
16 process, including part of a *Leptolyngbya* filament engulfed within C3 while the
17 flanking segments of the engulfed filament are external to the cell. In other
18 images, we see shortened *Leptolyngbya* filaments within the cell, sometimes
19 contained within digestive vacuoles. Like *Acanthamoeba*, C3 cells can be found
20 across the area of the plate throughout the cyanobacterial lawn, while cells of the
21 other amoebal isolates are confined to the plaque interface (data not shown).
22 LPG3 did not form plaques on lawns of *Leptolyngbya* or *Anabaena*, but could
23 form plaques on lawns of *Synechococcus* that were freshly plated, but not on

1 ones that were pregrown for one week (Fig. 3). The LPG3 plaque spread slowly
2 across plates, forming well-defined plaques with few individual amoebal cells
3 spreading beyond the plaque rim.

4 One limitation of assaying grazing on agar plates is that some
5 cyanobacterial lawns grow too thick for proper clearance of the lawn, and these
6 thick lawns are also difficult to assess by microscopy. This is the case with
7 *Anabaena* lawns, on which most of the amoebal isolates do not form plaques
8 beyond the inoculation site. *Anabaena* lawns were dense and dark green,
9 making it difficult to determine whether amoebae were replicating or not. HGG1
10 formed a small expanding plaque on a pregrown *Anabaena* lawn (Table 2 and
11 Figure 3), and could form larger plaques when inoculated onto a freshly plated
12 *Anabaena* lawn (data not shown), but plaque spreading slowed after several
13 days as the lawn began to thicken.

14 **3.4. Amoebal growth and prey preference in liquid culture.** To further assess
15 the prey range and feeding characteristics of our amoebal isolates, we assayed
16 grazing in liquid culture. To determine whether different cyanobacterial strains
17 could support amoebal growth, we performed growth curves on each amoeba-
18 cyanobacterium pair and assessed growth through direct cell counts (Fig. 4).
19 Doubling times were calculated from replicate growth assay experiments (Fig 5).

20 In liquid medium at 30°C, HGG1 growth could be supported by the
21 filamentous strains *Leptolyngbya* and *Anabaena*, but not by unicellular

Fig. 4. Amoebal growth curves with cyanobacterial prey in liquid culture. Each amoebal isolate was incubated with *Leptolyngbya* sp. BL0902, *Synechococcus elongatus* PCC 7942, or *Anabaena* sp. PCC 7120 in 24-well cell culture dishes. Growth curves of (A) C3 and (D) LPG3 were performed at 20°C under continuous light, while growth curves of (B) HGG1 and (C) LPG1 were performed at 30°C under continuous light. Values given are counting averages $\pm$ s.e. Growth curves are representative of replicate experiments.

1
2 *Synechococcus* (Fig. 4 and 5), and we saw the same results at 20°C (Table S2).
3 HGG1 was able to replicate quickly while incubated with *Leptolyngbya* at 30°C
4 and at 20°C, with doubling times of 0.43 ± 0.02 days and 0.63 ± 0.002 days,
5 respectively. HGG1 had a similar doubling time when incubated with *Anabaena*
6 at 30°C (0.67 ± 0.36 days), while growth was slower at 20°C (2.39 ± 0.43 days)
7 (Fig. 5 and Table S2). LPG1 growth was supported by *Synechococcus* in liquid
8 culture, with a rapid doubling time of 0.57 ± 0.15 days when assayed at 30°C,
9 and 0.99 ± 0.35 days at 20°C (Fig. 4 and 5, and Table S2). Growth of LPG1 with
10 *Leptolyngbya* or *Anabaena* did not occur at 30°C (Fig. 4), but could occur at
11 20°C, although this growth was inconsistent (Table S2).

Fig. 5. Doubling times of each amoebal isolate grown on cyanobacterial strains in liquid culture. Growth curve experiments with HGG1 and LPG1 were performed at 30°C under continuous light, while experiments with C3, LPG3, and *A. castellanii* (A.c.) were performed at 20°C under continuous light. Amoebal growth did not occur in columns without values. Values given are averages $\pm$ s.d. from duplicate experiments.

Neither C3 nor LPG3 grew on any cyanobacterium at 30°C, while at 20°C C3 growth was supported only by *Leptolyngbya* (1.95 ± 0.36 day doubling time), and LPG3 grew only on *Synechococcus* (1.17 ± 0.21 day doubling time) (Fig. 4 and 5). Both C3 and LPG3 exhibit slower plaque expansion on solid medium, indicative of slower growth rates in general. *Acanthamoeba* growth did not occur on any cyanobacterium at 30°C, but growth was supported by all cyanobacterial strains at 20°C, with *Synechococcus* supporting the fastest doubling time of 1.37 ± 0.20 days, while doubling times on *Anabaena* and *Leptolyngbya* were 2.39 ± 0.52 and 1.6 ± 0.06 days, respectively (Fig. 5).

4. Discussion. This work provides a glimpse into the diversity of natural grazers that are able to prey on cyanobacteria. Our set of amoebal isolates is phylogenetically diverse and showed varied morphology, locomotion, and prey range. The isolates belong to two of the major lineages of Eukarya; the Amoebozoa and the Heterolobosea. As a group, amoebae are a very diverse,

ubiquitous, and polyphyletic group of protists that share a type of surface-associated, or “amoeboid”, movement characterized by cell deformation accompanied by the extension of pseudopodia. Amoeboid organisms are found in all of the major lineages of eukaryotic descent, do not form a single phylogenetically coherent group [45], and can be difficult to classify on the basis of morphology alone. Ecologically, amoebae occupy an important role as their primary food sources are bacteria and small protists which they consume by phagocytosis; they also are known to invade open-air algal ponds [8].

We have developed methods for the straightforward isolation of natural grazers of cyanobacteria. Amoebal grazers can be particularly easy to isolate and made clonal because of their ability to grow on solid medium in addition to growth in liquid medium. Direct isolations on solid medium might discriminate against grazers that are prone to desiccation on agar plates, but the amoebae in this study were first enriched in liquid culture with their cyanobacterial prey. They were then transferred to agar plates, which appeared to inhibit growth of ciliates, flagellates, and other grazers that require a liquid environment. Carryover of heterotrophic bacteria was common, but the expanding amoebal plaques could outpace the growth of bacteria. With the exception of LPG3, which retained a persistent heterotrophic bacterial contaminant, repeated transferring from expanding plaques to fresh cyanobacterial lawns removed bacterial contaminants.

Amoebal grazing on cyanobacteria occurs in the environment and while not much is known about cyanobacterial defenses against grazers, it appears

1 that production of toxic compounds like microcystins and anabaenapeptins may
2 constitute such a mechanism [27]. These molecular interactions between
3 amoebae and cyanobacteria likely play a major role in grazing interactions. Prey
4 preferences and prey range could play a role on different overall lifestyle
5 strategies adopted by our amoebal isolates, and could reflect mechanisms of
6 tolerance or strategies of avoidance of such toxins. For example, HGG1 and
7 LPG1 which form cysts and have more rapid doubling times, have a larger prey
8 range. In contrast, C3 and LPG3 which have slower observed doubling times and
9 do not form cysts, have a limited prey range. This could be reflective of natural
10 survival strategies of our amoebal isolates against potentially toxic
11 cyanobacteria.

12 Cellular and molecular interactions likely play a large role in prey
13 specificity of amoebae. The cyanobacterial strains used in our grazing assays
14 are morphologically diverse, which could account for the different prey
15 preferences of our amoebal isolates. *Synechococcus* is a small unicellular
16 cyanobacterium, while filamentous cyanobacterial strains *Leptolyngbya* and
17 *Anabaena* form thin and thick filaments, respectively. Amoebal isolates HGG1
18 and LPG1 showed little selectivity in cyanobacterial strain as a food source, while
19 isolates C3 and LPG3 showed a strict prey preference on solid medium and in
20 liquid medium. The prey selectivity of C3 and LPG3, however, does not exclude
21 their ability to ingest the other cyanobacterial strains. Prey selection can occur
22 after ingestion has occurred, and some cyanobacterial species are excreted from
23 the amoebal cell after ingestion [24, 46]. For amoebal isolates with greater prey

1 specificity, C3 and LPG3, grazing assays on solid medium and liquid medium
2 were concordant. However, for the non-selective amoebal isolates, HGG1 and
3 LPG1, prey preferences on solid medium did not directly correlate with grazing in
4 liquid medium. This suggests that cellular physiology and physical interaction
5 between amoebae and cyanobacteria, which can differ in liquid and solid media,
6 play an important role in feeding dynamics. Because amoebae often feed on prey
7 encountered on surfaces, their encounter rates in liquid culture are higher with
8 cyanobacteria that settle to the bottom of the assay well, such as *Anabaena* and
9 to a lesser extent *Leptolyngbya*, than with unicellular *Synechococcus* which
10 largely remains in suspension. This can account for HGG1 grazing upon
11 *Synechococcus* on solid medium, but not in liquid medium. In addition, the
12 replication rates and metabolic activity of cyanobacteria could also affect grazing
13 conditions through changes in pH or oxygen production. These factors could
14 contribute to grazing phenotypes and might account for variability in some
15 assays, such as the inconsistent growth of LPG1 in liquid culture with
16 *Leptolyngya* and *Anabaena*, which appears to be facilitated under lower light and
17 temperature conditions. Dense growth can also inhibit grazing, as was observed
18 with thick *Anabaena* lawns on solid medium (Fig. 3 and Table 1) and with
19 cyanobacterial overgrowth at 30°C when assaying slower growing amoebal
20 isolates in liquid culture. This phenomenon of bacterial inhibition of grazing
21 through growth to high density has been observed for other model bacterial-
22 amoebal systems as well [47]. While the mechanism of this inhibition is not

1 currently understood, possibilities include the accumulation of products toxic to
2 amoebae, and the depletion of factors necessary for amoebal growth.

3 Contamination of open-air algal ponds by grazers is a major obstacle to
4 the development of algal biotechnology because efficient biomass production of
5 algal crops is necessary. With the establishment of diverse amoeba-
6 cyanobacterium model systems, using amoebae isolated from both natural
7 environments and a model production pond, we have begun to characterize the
8 cellular and molecular interactions between amoebal grazers and cyanobacterial
9 prey, providing insight into naturally-occurring interactions in the environment.
10 This will allow us to identify not only natural defense mechanisms, but also genes
11 and genetic pathways that could potentially be exploited for use as protective
12 strategies during cultivation of cyanobacterial crops in open-air outdoor ponds
13 [25]. The methods described here are applicable to the isolation of amoebae
14 preying on any eukaryotic alga that can be plated on solid medium such as, for
15 example, *Scenedesmus*, *Chlorella*, and certain diatom species. Recent methods
16 for the generation of *Chlorella* mutants, and their clonal isolation by plating [48],
17 could be used to generate mutants resistant to grazing, as was recently done for
18 *Synechococcus* [25]. For those algae not amenable to plating and for the
19 isolation of other predators that are sensitive to solid medium culture conditions,
20 such as flagellates and ciliates, an initial enrichment and screen in liquid culture
21 in which serial dilutions of a water source are incubated with algal cultures in
22 tissue culture wells and inspected for yellowing or poor growth, can serve to
23 identify those wells containing predators or pathogens. The predators can then

1 be isolated and rendered clonal by serial dilution in liquid, or by picking individual
2 predator cells with a micromanipulator. In fact, this is the approach we used to
3 isolate ciliate and flagellate predators of cyanobacteria (data not shown). These
4 methods allow isolation and characterization of microalgal predators, including
5 but not limited to amoebae, a significant obstacle to development of industrial
6 open-air algal crop production.

7
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