Acanthamoeba castellanii, a Predator of Cyanobacteria

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Acanthamoeba castellanii was isolated from soil and water as a predator of cyanobacteria. Several strains of *A. castellanii* consumed cyanobacteria but there were differences in predatory activity between strains. The unicellular *Gloeocapsa alpicola* and filamentous *Anabaena flos-aquae* were particularly susceptible to predation. Under optimal conditions the amoebae fed voraciously but they encysted when the cyanobacterial food supply was exhausted. Predation was initiated by engulfment of whole cyanobacteria and, in the case of *Anabaena flos-aquae*, severance of filaments. This was followed by digestion in vacuoles.

INTRODUCTION

Living cyanobacteria (blue-green algae) are attacked by many micro-organisms, including bacteria, viruses, actinomycetes and chytrid fungi (Stewart & Daft, 1976, 1977). The range of antagonists of cyanobacteria has been extended by the isolation of antibiotic-producing filamentous fungi (Redhead & Wright, 1978, 1980). Although there have been reports that some amoebae can feed on cyanobacteria (Leidy, 1879; Comandon & de Fonbrune, 1936; Prowse, 1968; Haberey, 1973*a, b*; Cook *et al.*, 1974; Cook & Ahearn, 1976; Old & Darbyshire, 1978), little attention has been given to soil amoebae, especially the common hartmanellid types. Indeed, Heal & Felton (1970) and Ho & Alexander (1974) indicated that cyanobacteria are unsuitable as food for small amoebae.

Whilst isolating cyanobacterial-lysing microbial agents by plaque formation on lawn cultures (Redhead & Wright, 1978), we frequently noticed that plaques contained small amoebae predatory on the cyanobacteria. The amoebae were identified as *Acanthamoeba castellanii*, and briefly reported by Wright *et al.* (1978). We were surprised to note that apparently only one other worker (Yamamoto, 1978) has made similar observations concerning cyanobacterial lysis plaques, but in this case the amoebae were not identified. Lazcano *et al.* (1977) isolated an *Acanthamoeba* sp. from cultures of the cyanobacterium *Oscillatoria*. We have now made further studies on the distribution and cyanobacteria-predating activity of acanthamoebae and present the findings in this paper.

METHODS

Cultivation of cyanobacteria. Axenic cultures of the following cyanobacteria were obtained from the Culture Centre of Algae and Protozoa (C.C.A.P.), Cambridge: (a) unicellular types – *Anacystis nidulans* 1405/1 and *Gloeocapsa alpicola* 1430/1; (b) filamentous types – *Anabaena cylindrica* 1403/2a, *Anabaena flos-aquae* 1403/13a, *Nostoc muscorum* 1453/12, *Oscillatoria tenuis* 1459/4, *Plectonema boryanum* 1446/2 and *Tolypothrix tenuis* 1482/3a. Batch liquid cultures (100 ml) were grown in 250 ml Erlenmeyer flasks. Medium D plus H₂ micro-elements solution (K & M medium; Kratz & Myers, 1955) was used for the filamentous cyanobacteria *Anabaena flos-aquae*, *N. muscorum*, *P. boryanum* and *T. tenuis*. The modified medium of Hughes *et al.*

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(Allen, 1968) was used for the unicellular cyanobacteria *Anacystis nidulans* and *G. alpicola* and also for *Anabaena cylindrica* and *O. tenuis*. Cultures were incubated at $30 \pm 1^\circ\text{C}$ in an orbital shaker (100 rev. min⁻¹), except for *O. tenuis* which was grown in static culture, and provided with constant fluorescent white illumination (1500 lx). Cyanobacteria were maintained on agar (1.5%, w/v) slants of the same media under the same temperature and light conditions.

Isolation of predatory amoebae from soil and water samples. Surface soil samples were collected from several locations in the winters of 1977, 1978 and 1980. Soil samples (10 g) were dispersed in 1 litre sterile tap water and 0.5 ml samples of further dilutions were gently spread over the surface of 1 d lawn cultures (Redhead & Wright, 1978) of *Anacystis nidulans*, *Anabaena cylindrica*, *Anabaena flos-aquae* and *N. muscorum* in Petri dishes. Alternatively, six soil crumbs (approx. 5 mm diam.) were placed directly on the lawn and each was then eluted with one drop of K & M medium. Freshwater samples were collected in 1976 and in the early months of 1980. Samples (0.5 ml) of the water and dilutions were spread over lawn cultures (2 d) of *Anabaena flos-aquae*, *O. tenuis* and *N. muscorum*. Lawn cultures were incubated at 30°C or at room temperature with constant fluorescent illumination (1500 lx) and examined daily for the appearance of plaques, which were usually present after 4–6 d. Microscopic examination of material removed from the periphery of plaques revealed whether or not predatory amoebae were present. In some cases bacteria and fungi were also detected, but the larger plaques commonly contained vast numbers of amoebae and their cysts, sometimes exclusively so. Material from the edge of plaques containing amoebae was transferred to fresh lawns of the appropriate cyanobacteria and development of further plaques was monitored. The plaques contained apparently identical amoebae with fine, tapering pseudopodia; these amoebae produced cysts with a well-defined polygonal endocyst and rippled ectocyst.

Purification and identification of amoebae. Amoebae from the plaques were examined by Dr F. C. Page, Curator of Protozoa, C.C.A.P., who identified them as the common soil amoeba *Acanthamoeba castellanii*. This identification was subsequently confirmed with axenic cloned material by Dr A. J. Griffiths, University College, Cardiff, and by ourselves using the key of Page (1976). The amoebal clones were obtained by transferring individual cysts from plaques to fresh lawn cultures using a micromanipulator. One clone, designated AC-31, was used extensively in further studies.

Cultivation of amoebae. The following additional axenic amoebal cultures were obtained from the C.C.A.P.: *Acanthamoeba castellanii* 1501/1a, *A. castellanii* 1501/2a, *A. castellanii* 1534/3, *A. palestinensis* 1547/1 and *A. comandoni* 1501/5. *Acanthamoeba castellanii* (Neff strain) was obtained from Dr A. J. Griffiths, University College, Cardiff. The amoebae were grown in batch cultures (10 ml) from 1 ml inocula in 50 ml Erlenmeyer flasks in peptone/glucose/yeast extract (PGY) medium (Chagla & Griffiths, 1974) at 30°C in a reciprocating shaker (80 oscillations min⁻¹).

Predatory activity. This was determined by plaque formation on cyanobacterial lawns to which 0.01 ml samples of exponentially growing amoebal cultures were applied. Drops initially spread to cover an area about 5 mm in diameter without affecting the lawns. Predation was also monitored by the disappearance of cyanobacteria from liquid cultures inoculated with 0.1 vol. amoebal suspension using the wells of divided Petri dishes (Repli dishes, Sterilin). For both experimental systems, the dishes were incubated either at room temperature with 1440 lx illumination or at 30°C with 1500 lx and examined daily up to 12 d. Predation was confirmed by microscopic observations on wet mount and slide culture preparations.

Microscopy. Phase contrast and differential interference contrast (Nomarski) optics on a Leitz Orthoplan photomicroscope were used for observing amoebal predation of cyanobacteria in wet mount preparations and phase contrast for slide culture preparations.

Cyanobacterial cell density; chlorophyll determination. Samples (5 ml) from cultures in liquid media were centrifuged (2000 g, 30 min) and the cells were resuspended in 4 ml boiling methanol for 10 min. The resulting suspension was centrifuged (2000 g, 30 min), and the supernatant was removed and made up to 5 ml with methanol. Absorbance due to the chlorophyll in the extract was determined at 665 nm against a methanol reference.

RESULTS

Distribution of acanthamoebae predatory upon cyanobacteria

Acanthamoebae were detected as cyanobacterial predators in samples of soil and water taken from 30 sites in the vicinity of Bath. The sites included fallow and cultivated soils, lake mud, river sediment and canal, lake and pond waters. Amoebae were more frequently detected as the plaque-forming agent than were fungi, bacteria and actinomycetes combined. Of the species of cyanobacteria used as 'host' lawn cultures, most plaques were formed on *Anabaena flos-aquae* and *Anacystis nidulans*. Although most amoebae fitted the description of *Acanthamoeba castellanii*, some representatives of *A. polyphaga* and *A. palestinensis* were also recognized on the basis of cyst morphology (Page, 1976).

Table 1. Ability of *acanthamoebae* to prey upon a range of cyanobacteria

Cyanobacteria	Plaque diameter*						
	<i>Acanthamoeba castellanii</i> strains					<i>A. palestinensis</i>	<i>A. comandoni</i>
	AC-31 (Griffiths)	Neff	1501/1a	1501/2a	1534/3		
<i>Anacystis nidulans</i>	+++	++	++	+	+++	—	—
<i>Gloeocapsa alpicola</i>	+++	++	+++	++	+++	—	—
<i>Anabaena flos-aquae</i>	+++	+++	+++	+	+++	—	—
<i>Anabaena cylindrica</i>	—	—	—	—	—	—	—
<i>Plectonema boryanum</i>	—(†)	—(†)	—(†)	—(†)	+	—	—
<i>Nostoc muscorum</i>	—	—	—	—	—	—	—
<i>Tolypothrix tenuis</i>	—	—	—	—	—	—	—
<i>Oscillatoria tenuis</i>	—	—	—	—	—	—	—

* Plaque diameter was scored after 12 d incubation at room temperature: —, no plaques; +, ≤ 15 mm; ++, 16–20 mm; +++, > 20 mm. (†) Ingestion followed by rejection.

Prey range of *acanthamoebae*

Several *acanthamoebae* were examined to compare their ability to prey upon a range of cyanobacteria with that of the typical isolate obtained in this laboratory, *A. castellanii* AC-31. Predatory activity was tested in triplicate using cyanobacterial lawn cultures and cyanobacterial liquid cultures in Repli dishes. Predatory activity (Table 1) scored after 2, 5, 7, 9 and 12 d according to plaque formation in lawn cultures and discoloration and clearing in liquid cultures was confirmed microscopically. Similar results were recorded for lawn and liquid cultures and only those for the lawn cultures are given in Table 1. Predatory activity was variable and was confined to the five *A. castellanii* strains.

Both unicellular cyanobacteria were readily predated, but excepting *Anabaena flos-aquae* and to some extent *P. boryanum*, the filamentous species were not. Variation was observed in the plaques formed by different amoebae. Strain AC-31 formed rapidly spreading clear zones fringed by a region of partial cyanobacterial destruction. Most of the other strains produced well-defined plaques which did not increase in size after 7 to 9 d, whilst the clear plaques caused by strain 1501/1a on *G. alpicola* lawns were encircled by a region of smaller cleared zones. In both lawn and liquid cultures *G. alpicola* and *Anabaena flos-aquae* were consistently the most suitable prey. None of the amoebae ingested *Anabaena cylindrica*, *N. muscorum*, *T. tenuis* or *O. tenuis*, even after incubation for 18 h. However, the amoebae were usually closely associated with the cyanobacteria and often moved along their filaments in apparent preference to the free liquid phase.

Acanthamoeba castellanii strains AC-31 and 1501/2a were unable to predate any of a selection of 11 species of unicellular and filamentous eukaryotic algae in lawn culture. However, the strains Neff (Griffiths), 1501/1a and 1534/3 did prey upon the unicellular *Chlamydomonas eugametos*, *C. oblonga*, *C. reinhardtii*, *Chlorella vulgaris* and *Coelastrum microporum* to a limited extent. The amoebae showed greater predation of autoclaved (121 °C, 15 min) *Anabaena flos-aquae* than non-autoclaved filaments, probably due to the liberation of single and short chains of cells by the heating. In all cases where cyanobacteria or algae were not predated, complete encystment of the amoebae was evident 2 d after their inoculation on to such lawns.

Factors affecting amoebal predation of cyanobacteria

These experiments were confined to *A. castellanii* strains AC-31, Neff (Griffiths) and 1501/1a, with *G. alpicola* and *Anabaena flos-aquae*. When suspensions (0.2 ml) of amoebal cells (1501/1a) in the exponential phase of growth were added to *G. alpicola* cultures (2.8 ml) so that the resulting number of amoebae ranged from 1.93×10^4 to 9.67×10^4 ml⁻¹, the

Fig. 1. Predation of *Gloeocapsa alpicola* by *Acanthamoeba castellanii*. *Gloeocapsa alpicola* cells were suspended in fresh medium (95 ml in 250 ml flasks) at three different cell densities giving A_{600} readings in a 1 cm cell of 2.73 (○), 1.20 (●) and 0.94 (□). Each flask was inoculated with 5 ml *A. castellanii* (Griffiths, Neff strain) suspension and incubated statically at 30 °C. At intervals the *G. alpicola* population density was determined by measuring the chlorophyll content of 5 ml samples, as described in Methods.

Fig. 2. Oscillations in *Gloeocapsa alpicola* populations following predation by *Acanthamoeba castellanii*. These were monitored in the system with the lowest initial cyanobacterial population (Fig. 1). At intervals the *G. alpicola* population density was determined by measuring the chlorophyll content of 5 ml samples, as described in Methods.

rate of disappearance of the cyanobacteria was directly related to the number of amoebae present.

The initial rate of disappearance of *G. alpicola* and *Anabaena flos-aquae* from liquid cultures in the presence of *A. castellanii* 1501/1a increased with temperature (13 °C < 23 °C < 30 °C) when incubated in the light, although after 5 d there was little difference in rate. Similar results were obtained with dark incubation. Amoebal encystment did not occur until the prey was virtually exhausted. After this, re-growth of *Anabaena flos-aquae* occurred from surviving cells in the light but not in the dark. Predatory activity of strain AC-31 was generally apparently greater in the dark than in light, probably due to more rapid cyanobacterial proliferation in the light, especially at 30 °C.

Microscopic examination of the *A. castellanii*-induced plaques on *Anabaena flos-aquae* showed that a few isolated amoebae were present 2 to 3 mm beyond the visible plaque periphery but none were beyond this zone. At the leading edge of the plaque there were large numbers of amoebal trophozoites. Most amoebae were actively engulfing filaments or contained several cyanobacterial cells in different stages of digestion. The outermost 5 mm of the plaques contained some amoebal cysts among the trophozoites, but the 'older' centre part was typically devoid of vegetative cyanobacterial cells and comprised many amoebal cysts and debris together with some indigestible heterocysts and akinetes. The non-confluent plaques formed on green algae tended to disappear after 7 d, when the amoebae had encysted.

Predator-prey oscillation and amoebal proliferation

It was repeatedly noticed during maintenance of *A. castellanii* AC-31 in liquid cultures of *Anabaena flos-aquae* that after disappearance of most of the cyanobacteria due to predation, there was re-growth of cyanobacteria. With further incubation oscillations in amoebal and cyanobacterial dominance occurred. More detailed observations of such oscillations were subsequently made with *G. alpicola* and the Neff (Griffiths) strain of *A. castellanii*. The

Fig. 3. Some stages in the predation and digestion of *Anabaena flos-aquae* by *A. castellanii*. (a) Acanthamoebae roaming the surface of an *Anabaena flos-aquae* filament. (b) Extension of amoebal pseudopodium to engulf part of a filament: the amoeba has prominent acanthopodia (A). (c) Ingestion of 11 *Anabaena flos-aquae* cells, with bending of the filament. (d) An amoeba with a vacuole (V_1) containing *Anabaena flos-aquae* cells in an advanced stage of digestion, together with a recently engulfed filament (F) of 9 cells. (e) The same amoeba as in (d) 60 min later: the remains of the filament are now in a vacuole (V_2) adjacent to the nucleus (N). (f) An amoeba containing at least 25 *Anabaena flos-aquae* cells at different stages of digestion. The bar markers represent 20 μm .

decline in cyanobacterial population density in liquid cultures inoculated with *A. castellanii* (Fig. 1) was followed by amoebal encystment. Whilst no further changes were seen in flasks originally containing the higher *G. alpicola* densities (Fig. 1), re-growth of *G. alpicola* occurred in the flask with the lowest initial density after the amoebae had encysted. At this stage *G. alpicola* cells adhered in masses to aggregated amoebal cysts. This was followed by excystment of the amoebae and subsequent decline in the *G. alpicola* cells due to predation

by trophozoites. Such population changes were repeated once again (Fig. 2) upon further incubation although the oscillation amplitude diminished with time.

Extensive replication of the amoebae occurred on lawns of susceptible cyanobacteria. Amoebae feeding upon cyanobacteria were often observed to be dividing and the high number of cysts in the plaques could not be explained by migration from the original inoculum. The numbers of *A. castellanii* AC-31 increased from an initial $3.5 \times 10^3 \text{ ml}^{-1}$ to $3.8 \times 10^4 \text{ ml}^{-1}$ after 5 d incubation at 30 °C in liquid *Anabaena flos-aquae* culture.

The predation and digestion process

The following account describes events in the predation of *Anabaena flos-aquae* filaments. The stages, shown in Fig. 3, were photographed with the Neff (Griffiths) strain of *A. castellanii* and typify those seen with all predatory amoebae examined.

As an amoeba approached a filament and made contact with it (Fig. 3*a*) numerous fine acanthopodia were produced by which the amoeba aligned itself with and often surrounded the filament whilst seeking the terminal cell, to which it became attached. Engulfment of the filament then occurred by the extension of a cylindrical pseudopodium (Fig. 3*b*), the amoeba changing shape markedly. Occasionally amoebae commenced ingestion away from the ends of filaments, bending the filament and folding the two parts together before engulfing the two parallel sections. Pseudopodial extension was often interrupted for a few minutes and occasionally some of the filament re-emerged before engulfment proceeded, but complete escape from amoebae was also seen. Filaments were sometimes engulfed at both ends simultaneously by different amoebae, with one or both achieving ingestion. Usually 4 to 8 cells were engulfed but the number could be up to 15. In the latter cases the amoebae were engulfing filaments three to four times their own length which required considerable contortions by the amoebae. In order to accommodate such a mass of cyanobacteria, typically the first (distal) engulfed cell was turned round when it contacted the opposite interior face of the amoeba, followed by coiling of much of the remaining filament (Fig. 3*c*). The filament was then severed and the portion not ingested was freed (Fig. 3*d*). The time from initiation of engulfment to this stage varied between 15 and 60 min.

The amoeba then made slow movements in the same position, continually re-orientating the ingested filament. After a further 5 to 15 min the filament started to break at several points and cells separated. The initial break was usually between the first and second cell in a filament. Most of the cells had lost their linear arrangement about 30 min after entry and later were grouped in one or more spherical clumps (Fig. 3*e*); it was then evident that they were enclosed in vacuoles. The vacuoles were initially green but became discoloured as digestion proceeded. Complete digestion could take several hours, depending on the number of cells ingested. Undigested cyanobacterial debris was expelled.

Amoebae began to move freely again once the cyanobacterial cells were confined in vacuoles and they were then able to engulf more cells whilst still digesting those captured earlier. Amoebae have been seen with at least 25 *Anabaena flos-aquae* cells in various stages of digestion (Fig. 3*f*).

DISCUSSION

The indirect evidence of wide distribution, frequent isolation from soil and water samples, and voracious appetite for some cyanobacteria including a filamentous form, prompt the suggestion that acanthamoebae may be involved in the natural regulation of cyanobacteria. Whether they operate upon blooms and, if so, whether they could be used for biological control of blooms has yet to be determined.

The present observations illustrate variation in the ability of *A. castellanii* strains to prey upon cyanobacteria (and green algae), which cannot be explained by differences in the size

or shape of the prey. Heal & Felton (1970) and Ho & Alexander (1974) also reported variation in the suitability of prey for this amoeba, but their results concerning the susceptible cyanobacteria differ from those presented here. This may be due to experimental techniques but it is still surprising since some of the same amoebal strains were examined. Heal & Felton (1970) used the strain isolated by Neff which is presumably synonymous with strain 1501/1a in our study, whilst Ho & Alexander (1974) used a strain obtained from Dr A. J. Griffiths, Cardiff. This, also a Neff strain, is identical to that supplied by Dr Griffiths for our own study and may have the same origin as the 1501/1a strain. Ho & Alexander (1974) found that cyanobacteria were unsuitable in promoting growth of this amoeba.

Generally, unicellular cyanobacteria were more susceptible to predation than filamentous forms, with the notable exception of *Anabaena flos-aquae*. Some of the cyanobacteria not ingested included branched filamentous (*Tolypothrix tenuis*) and motile (*Oscillatoria tenuis*) forms. Heterocysts and akinetes resisted amoebal digestion. Although there was no ingestion of several filamentous cyanobacteria, the amoebae tended to move over the filaments in apparent preference to moving freely in the bulk liquid phase. A similar observation was made by Picken (1937).

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