Inducible defence against a ciliate grazer, *Pseudomicrothorax dubius*, in two strains of *Phormidium* (cyanobacteria)

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SUMMARY

Experiments were done with two strain of filamentous, mat-forming *Phormidium* and their ciliate grazer *Pseudomicrothorax dubius*, to explain why the ciliates remain hungry in an apparent surplus of food, except for the first 24 hours after feeding. Under grazing pressure, both strains of cyanobacteria showed statistically significant increases in the number of filaments terminating in an empty sheath, compared to the control. Direct observations revealed that the mechanism behind this effect was active withdrawal of the trichomes inside the sheaths when disturbed by grazers. As *P. dubius* is unable to ingest trichomes with such endings, we conclude that cyanobacteria are not limited to chemical means of defence against grazers but can also defend themselves by means of movement and changes in filament morphology. This is apparently the first report on behavioural defence observed in cyanobacteria.

1. INTRODUCTION

For millions of years, cyanobacterial mats—one of the oldest communities of living organisms on Earth—have been under constant pressure from different groups of grazers such as ciliates, cladocerans and copepods. Consequently, cyanobacteria have evolved defences against grazers. These include a filamentous morphology, production of toxins (Brabrand *et al.* 1983; Kirk & Gilbert 1992), production of mucilaginous sheath material (Goarant *et al.* 1994) and the formation of mats or colonies (Dodds *et al.* 1995).

Among the grazers, several strains of ciliates are highly specialized in feeding on filamentous blue-green algae. They have a cytopharyngeal basket adapted for attachment, ingestion and cutting long filaments (Hausman & Peck 1979; Peck 1985). The structure and function of the cytopharyngeal basket in some strains of *Nassula* (Tucker 1968) and *Pseudomicrothorax dubius* (Hausmann & Peck 1978; Hauser & Hausmann 1982) have been described in detail.

Our observations during cultivation of ciliates suggested that some cyanobacteria have a mechanism of defence against ciliates, even highly specialized ones. *P. dubius* fed on pieces of cyanobacterial mats were satiated for only 20–30 hours after being presented with food. Then, despite the fact that food was still apparently plentiful, they became transparent, which indicated that they were starving. However, they were still alive and in good condition, which helped us to rule out the possible role of cyanobacterial toxins. Looking more closely, we noticed that almost every filament protruding from the mat ended with a long, stiff, empty sheath sticking out.

We carried out a set of experiments designed to find out whether the increasing number of filaments ending in an empty sheath, resulting from active escape of cyanobacteria, could be regarded as a form of induced defence.

2. MATERIAL AND METHODS

Two cyanobacteria strains of the genus *Phormidium*, later called Ph1 and Ph2 (table 1) were used. Clonal populations of them were obtained from single short filaments taken from an aquarium maintained at the Department of Hydrobiology, Jagiellonian University, Krakow. The maintenance regime of the aquarium permits algae and protozoans to develop fairly abundantly. A clone of the ciliate *Pseudomicrothorax dubius* was obtained from a single cell taken from the same aquarium. All cultures were kept at constant 23 °C and under a 12L:12D light regime. The algae were grown on blue-green algae medium BG11 (Stanier *et al.* 1971) prepared according to a formula obtained from CCAP, Ambleside. The ciliates were cultured in Volvic mineral water and fed on the *Phormidium* strains mentioned above.

For each strain of *Phormidium*, 24 approximately equal-sized (2 × 2 mm) pieces cut from a mat formed in the culture were taken and were each placed in a Cell Wells[®] (Corning) well containing 50 µl of mineral water. After 24 h the number of trichomes with empty sheaths among 100 randomly chosen filament endings along the edge of the mat was counted. Then 100 *P. dubius* individuals were released into each of 12 wells. The remaining 12 wells were controls. After a further 24 and 72 h the number of trichomes with protruding empty sheaths were counted again in four different wells each time to obtain independent data.

Analysis of variance (ANOVA) was used to check whether there were statistically important effects of grazer pressure and day of experiment on the proportion of filaments ending

Table 1. *Basic characteristics of the two strains of Phormidium*

	<i>Phormidium</i> sp. 1 (Ph1)	<i>Phormidium</i> sp. 2 (Ph2)
width of trichome	7.5–8.5 μm	5.0–5.5 μm
length of cells	3.5–5.0 μm	2.0–2.5 μm
apical cells	rounded	capitated
mat habitus	filaments loosely entangled with gas bubbles	filaments densely entangled, mat coriaceous

in empty sheaths. The data were ($\sqrt{\arcsin}$) transformed before the analysis.

To test the hypothesis that empty protruding sheaths were the result of trichome escape, 50 direct observations of attacked trichomes were made for each strain of cyanobacteria. Small pieces of cyanobacterial mats were placed in wells in 50 μl of mineral water. After 24 h, starved ciliates were released into the wells. Observations were made immediately, and in the case of Ph2 repeated after 48 h of ciliate pressure. Simultaneously, observations were made on the efficiency of an attack by a ciliate on an exposed trichome and on one protected by an empty sheath. One hundred observations were made on each type of filament. For all observations, an OLYMPUS IMT-2 inverted microscope with Nomarski contrast was used.

3. RESULTS

In the case of Ph1, the mean number of protruding sheaths increased during the experiment, almost doubling 72 h after the release of the ciliates. The effect of time was significant (ANOVA, $F[2, 9] = 4.38$, $p < 0.05$). However, the difference in numbers between the 24th and 72nd h was not significant (Tukey HSD test, $p = 0.698$), whereas the difference between numbers at the beginning of the experiment and after 24 h was significant ($p = 0.001$). The pattern was different in the control wells, where the mean number of protruding sheaths decreased slightly (figure 1a). The differences between days were not significant. The effect of the interaction between day and treatment was highly significant ($F[2, 9] = 33.41$, $p < 0.05$). The mean numbers of protruding sheaths in the experimental and control wells did not differ significantly at the start of the experiment (Tukey HSD test, $p = 0.988$). The differences were significant after 24 hrs ($p = 0.0003$) and after 72 h ($p = 0.0002$; figure 1a).

Figure 1b shows the results obtained for Ph2. The mean number of protruding sheaths increased throughout the experiment. After 72 hrs the mean number of filaments ending in empty sheaths exceeded the number recorded at the start of the experiment almost seventy-five fold. The effect of day was significant (ANOVA, $F[2, 9] = 130.64$, $p < 0.05$). The effect of grazers was significant through the first 24 h ($p = 0.0002$) and increased during the next 48 h (Tukey HSD test, $p = 0.0001$). In the wells without ciliates, the mean number of protruding sheaths remained very low and almost constant through the whole experiment; the effect of time was not significant ($p = 1.0$ between the beginning and 24th h, $p = 0.644$ between the 24th and 72nd h). As with Ph1, there was no significant difference ($p = 0.988$) between the numbers of sheaths

Figure 1. Mean percentage of filaments ending in an empty sheath under constant pressure from grazers (dark bars) and without grazer pressure (light bars) for *Phormidium* 1 (a) and *Phormidium* 2 (b).

sticking out in the grazed and control wells at the start of the trial. However, the differences on the other days were significant at $p = 0.0002$ (figure 1b).

The two strains of cyanobacteria differed markedly in their numbers of protruding sheaths (figure 1). At the start of the experiment the mean number of protruding sheaths in Ph1 was 43 in the grazed treatment and 46 in the control, whereas the respective values for Ph2 were 1.25 and 1.0. The mean number of sheaths sticking out in Ph1 after 24 h was higher (77.75) than in Ph2 (25.75), but the increase was much higher in Ph2 (20.6 times) than in Ph1 (only 1.8 times).

Figure 2. (a) Reaction of Ph2 trichome attacked by a ciliate. From left to right: ciliate starts to ingest a trichome, which begins withdrawing immediately after being broken; position of trichome inside the sheath after 5 s; trichome safely hidden inside the stiff sheath 45 s after being broken by a ciliate. (b) Withdrawal of exposed Ph2 trichome at the beginning of direct observations. From left to right: trichome ingested by a ciliate; position of trichome 5 s after ciliate detachment; position of trichome after 15 s. (c) Ciliate sucking a trichome out of the sheath. This is observed only when the trichome is so short that the ciliate is able to suck it out. From left to right: a ciliate has just started to suck a trichome out of the sheath; position of trichome after 5 s; after 15 s the whole trichome is sucked out of the sheath.

After 72 h the mean number of protruding sheaths in Ph1 (84.75) was slightly less than in Ph2 (93.5); the mean number sheaths in Ph1 at 72 h was only 1.09 times higher than the results after 24 h, but 3.63 times higher in Ph2.

Direct observations revealed marked differences in the defence reaction between the two strains. The

situations occurring after the filaments were attacked can be grouped into five categories: (i) the trichome escaped inside the sheath (figure 2a), (ii) the exposed trichome moved away from the grazer (figure 2b), (iii) the trichome was sucked out of the sheath (figure 2c), (iv) the whole trichome was ingested, and (v) no reaction of the trichome was observed.

Table 2. *Types of reactions by cyanobacterial trichomes to the attack of a ciliate—the results of direct observations*

	withdrawing trichomes	escaping trichomes	trichomes eaten whole	trichomes sucked from sheaths	no reaction
Ph1 immediately after the release of ciliates	45	0	0	3	2
Ph2 immediately after the release of ciliates	0	44	5	0	1
Ph2 after 48 hours of ciliate pressure	50	0	0	0	0

In Ph1, where stiff sheaths were present from the very beginning of the experiment, 90% of the trichomes reacted immediately after being cut. They withdrew inside the sheaths, leaving their ends empty. The remaining 10% were sucked out of the sheath or did not show any reaction (table 2). Even though stiff sheaths were not visible at the beginning of the trial in Ph2, in almost 90% of the cases we observed that trichomes move in the opposite direction. The situation markedly changed when the mat was under ciliate pressure for 48 h. The trichomes withdrew inside their sheaths every time they were observed (table 2). There were also rare times when short filaments were ingested whole, and others when the cut filaments showed no reaction.

As far as the efficiency of trichome protection by an empty sheath is concerned, our observations revealed that 100% of the ciliates confronted with a trichome that ended in an empty sheath gave up; without the obstacle of such a sheath, 83% of the ciliates were able to ingest the cyanobacterium.

4. DISCUSSION

These results suggest that both strains of *Phormidium* are able to defend themselves actively against grazers, but with differences in the timing and strength of their reactions.

In our cultures, Ph1 (unlike Ph2) had strong and inflexible sheaths that were not clearly visible until the mat was disturbed. Cutting a mat undisturbed by grazers revealed that about 40% of the filaments ended in protruding, empty sheaths. The proportion of

such filaments dropped slightly in the control wells (figure 1*a*), thus increasing the proportion of exposed endings of trichomes. This suggests that ungrazed Ph1 do not 'need' protruding sheaths. The phenomenon could indicate that Ph1 may be sensitive to any mechanical stimulus, not only the stimulus of being eaten. Ph2 reacted in a different way: just after the mat was cut, stiff protruding sheaths were observed in very low numbers in the wells without grazer pressure, and these did not change throughout the trial (figure 1*b*).

Comparing the results from Ph1 and Ph2, we noted that the defence reaction of Ph2 trichomes was both stronger and later. At the beginning of the experiment, Ph2 filaments not covered with sheaths were easily accessible and abundant food for *P. dubius*, enabling almost unlimited divisions of the ciliates. The very high number of sheaths sticking out after 72 h could be interpreted as the reaction of the filaments to increasing pressure from the grazer.

In the case of Ph1, where food availability was limited from the beginning of the experiment (figure 1*a*), ciliate pressure probably increased less than in Ph2 and reached maximum after 24 h. After that the mean number of protruding sheaths remained constant (figure 1*b*).

The increasing proportion of protruding sheaths in conditions of constant grazer pressure can be explained in two ways. The first possibility is a behavioural reaction: the escape of trichomes inside the sheaths in response to grazer attack. Direct observations of Ph1 support this hypothesis. A similar situation occurred in Ph2 after 48 h of increasing ciliate pressure, when stiff sheaths were formed. The second possibility is a

Figure 3. Trichome reaction when a ciliate tries to attach at the middle of a filament. From left to right: ciliate attempts to attach at the middle of the filament; the trichome has split into two pieces, which have started to move in opposite directions; position of trichomes after 50 s.

biochemical form of defence accelerating the production of sheath-forming material in response to the presence of ciliates. This could explain the large difference in the number of protruding sheaths between the Ph2 experimental and control wells (figure 1*b*).

The fact that all ciliates confronted with a trichome ending in an empty sheath gave up shows that withdrawing inside the sheath is a very effective defence against the grazer. We also often saw a ciliate swimming around the spot where a trichome ended, as though it were able to detect the presence of the trichome deeper inside the sheath.

Peck & Duborgel (1985) found that effective attachment of a ciliate led to ingestion of a trichome by *Oscillatoria formosa*. In our observations, when a ciliate attached itself to a filament it abandoned the filament if the trichome escape reaction was instant and what the grazer actually touched was an empty sheath. Another interesting situation occurred when *P. dubius* tried to fold a filament in the middle. Exactly at the spot where the ciliate tried to attach, the trichome divided and its two parts started to escape in opposite directions, forcing the ciliate to give up (figure 3).

One can argue that a defence strategy involving trichome movement may risk another danger: gliding inside the sheath causes the other end of the trichome to leave its shelter and protrude outside. The exposed end is more vulnerable. However, the investigated strains form mats, and most often the emerging ends are hidden deep inside the mat structure. If such a situation concerns a filament living separately, the trichome will be withdrawing as well. It may happen that the trichome emerging at the opposite end of the sheath will be disturbed by a ciliate. In such a case the trichome will start withdrawing again, stopping in the middle of the piece of sheath; empty sheaths will stick out at both ends of the trichome, making it completely inaccessible to grazers.

At this stage it is not clear whether a non-specific physical impulse, cell-to-cell contact leading to recognition of a grazer, a chemical signal or a combination of these is behind the defence reaction of cyanobacteria. In any case, the observed mechanism of active escape seems effective enough to starve highly specialized ciliates even when they are 'swimming in food'.

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