Antagonistic bacteria disrupt calcium homeostasis and immobilize algal cells

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Photosynthetic unicellular organisms, known as microalgae, are key contributors to carbon fixation on Earth. Their biotic interactions with other microbes shape aquatic microbial communities and influence the global photosynthetic capacity. So far, limited information is available on molecular factors that govern these interactions. We show that the bacterium \textit{Pseudomonas protegens} strongly inhibits the growth and alters the morphology of the biflagellated green alga \textit{Chlamydomonas reinhardtii}. This antagonistic effect is decreased in a bacterial mutant lacking orfamides, demonstrating that these secreted cyclic lipopeptides play an important role in the algal–bacterial interaction. Using an aequorin Ca\textsuperscript{2+}-reporter assay, we show that orfamide A triggers an increase in cytosolic Ca\textsuperscript{2+} in \textit{C. reinhardtii} and causes deflagellation of algal cells. These effects of orfamide A, which are specific to the algal class of Chlorophyceae and appear to target a Ca\textsuperscript{2+} channel in the plasma membrane, represent a novel biological activity for cyclic lipopeptides.
Carbon fixation by photosynthetic organisms is a crucial step in the global carbon cycle, converting CO₂ and light into valuable, energy-rich organic molecules. Apart from higher plants, prokaryotic and eukaryotic microalgae in aquatic environments are responsible for approximately 50% of all carbon fixation annually. In addition, these photosynthetic microorganisms are at the base of aquatic food webs, thus playing a key role in diverse ecosystems. In their freshwater and marine habitats, microalgae also naturally coexist with a large variety of other microorganisms. In analogy to the terrestrial plant environment, these complex interactions may influence the fitness and performance of the microalgae and even lead to their death. However, compared to the large body of knowledge on the effects of mutualists or parasites on higher plants, there is limited information on biotic interactions of photosynthetic microbes. Only recently, an increasing number of studies were reported on

![Diagram](image1.png)

**Inoculation ratio 1:1 (algae to bacteria)**

| Time (d) | 0 | 1 | 2 | 3 | 4 |
|----------|---|---|---|---|---|
| Cell density (algae per ml) | 10⁴ | 10⁵ | 10⁶ | 10⁷ | 10⁸ |

**Inoculation ratio 1:100 (algae to bacteria)**

| Time (d) | 0 | 1 | 2 | 3 | 4 |
|----------|---|---|---|---|---|
| Cell density (algae per ml) | 10⁴ | 10⁵ | 10⁶ | 10⁷ | 10⁸ |

![Supplemental Movie 1](image2.png)
the characterization of algicidal bacteria and of natural products or enzymes that directly affect algal fitness\textsuperscript{3–7}. In most cases, diffusible algicidal agents are secreted by the bacteria that inhibit cell growth, disrupt the cell envelope, and/or rapidly lyse target cells\textsuperscript{8}. Other algicidal bacteria require direct contact with the algae to exert their destructive effects. In these cases, they employ enzymes to cleave polysaccharides or proteins that are present on the cell wall of the algae, thereby disrupting cell integrity and causing lysis\textsuperscript{9}. However, most algicidal factors, their effects, and the involved signaling pathways remain to be elusive\textsuperscript{8}. This lack of knowledge is surprising in light of the well-known relevance of microalgae for life on Earth and their emerging importance for biofuel production.

The poor knowledge on the mediators of microalgal–microbial communities may—at least in part—be attributable to the lack of interacting species that are genetically tractable. To evaluate the factors governing the interaction between bacteria and microalgae, we thus focused on a fully sequenced model organism, *Chlamydomonas reinhardtii*, for which molecular tools are available\textsuperscript{9–11}. This biflagellate unicellular green alga is ubiquitously distributed in habitats such as fresh water and moist soil, and has been extensively used to study light perception, photosynthesis, and flagellar function, and also with regard to human diseases\textsuperscript{9, 12, 13}. However, *C. reinhardtii* is usually grown axenically in the laboratory, and only a few studies have explored how this algal genus responds to changes in biotic factors\textsuperscript{14–16}. Here, we report on the biological function of secondary metabolites from the bacteria *Pseudomonas protegens* in their interplay with the motile microalgae *C. reinhardtii*. *P. protegens* (formerly known as *P. fluorescens*)\textsuperscript{17} also lives in aquatic and soil environments, where it either promotes plant growth\textsuperscript{18} or leads to the cell death of the photosynthetic organism\textsuperscript{17}. By means of high-resolution mass spectrometry and a tailor-made reporter system, we found that *P. protegens* employs chemical mediators, including cyclic lipopeptides to deflagellate the *C. reinhardtii* cells and alter cytotoxic Ca\textsuperscript{2+} levels. Immobilization and disruption of algal cells by *P. protegens* and its secondary metabolites appears to provide an advantage to the bacteria when deprived of micronutrients.

**Results**

*P. protegens* Pf-5 arrests the growth of *C. reinhardtii*. To investigate whether heterotrophic bacteria sharing the same habitat as *C. reinhardtii* affect algal growth, we selected *Flavobacterium johnsoniae*, *Xanthomonas campestris* pv. *campestris*, and *P. protegens*. Specifically, we used sequenced strains of bacterial species previously isolated from a microalgal culture\textsuperscript{14} and applied the bacteria to a restricted area on a plate that contains *C. reinhardtii* cells (Fig. 1a). In coculture with *F. johnsoniae* or *X. campestris*, the growth of *C. reinhardtii* appeared to be unaffected, i.e., comparable to that on the medium control. In contrast, *P. protegens* strongly inhibited the growth of *C. reinhardtii*. Likewise, in liquid cultures (inoculation ratio of 1:1 and 1:100 algae to bacteria), only *P. protegens* substantially decreased the cell density of *C. reinhardtii* compared to pure algal cultures (Fig. 1b). Algal growth was stopped within the first day in coculture (1:100 ratio) or starting 1 day after inoculation (1:1 ratio). Photographs taken of the cultures in a replete medium show that the typical green color of the algal culture is absent when cocultivated with *P. protegens* (1:100 ratio) (Fig. 1c), indicating the arrest of the algal growth. Furthermore, in contrast to the other bacteria, *P. protegens* altered the morphology of the algal cells within 1 day in coculture. The usually oval algal cells were enlarged and almost circular, and their inner structure became granular (Fig. 1d).

It is known that the depletion of micronutrients such as iron or zinc can affect the biocontrol properties of *P. protegens*\textsuperscript{19, 20}. To test whether *P. protegens* may benefit from *C. reinhardtii* under nutrient-limiting conditions, we omitted the trace elements (Fe, Zn, Cu, Co, Mn, and Mo) from the growth medium. While bacterial growth was largely unaffected in a replete medium (Supplementary Fig. 1a–b), it was enhanced in coculture in a medium lacking micronutrients, as compared to the axenic bacterial culture grown in the same medium (Supplementary Fig. 2a). Algal cells also showed an altered morphology after 1 day in coculture in the medium depleted of micronutrients, and they had lost their flagella (Supplementary Fig. 2b).

To learn more about the dynamics of the interaction of *C. reinhardtii* with *P. protegens*, we recorded a video (Supplementary Movie 1). Initially, the algal cells swim freely in the medium. After 19 s, the bacteria were introduced from one corner of the slide (Fig. 1e). Within only 1.5 min, the bacteria start to surround the algal cells that already appear to be immobilized. Two minutes later, the intensity of the swarming reaches a maximum, and algal cells are completely encircled by bacteria. The physical contact of the bacteria and the algal was independently confirmed using an acridine orange-stained fluorescent micrograph after a 10-min incubation time (Fig. 1f).

**Orfamide A immobilizes several Chlorophyceae.** *P. protegens* biosynthesizes a wide range of natural products, including medium-size molecular weight compounds such as polyketides and nonribosomal peptides\textsuperscript{21} that could have a role in the bacterial–algal interplay. To study the chemical mediators of the interaction, we monitored two-dimensional patterns of diffusible metabolites between mixed bacterial and algal cultures by MALDI-imaging mass spectrometry (MALDI-IMS)\textsuperscript{22–24}. Organisms were directly grown on disposable indium tin oxide-coated glass slides that were overlaid with a layer of solidified growth medium (Supplementary Fig. 3a). These experiments...
revealed a cluster of very intense ions, in the range of m/z 1300–1360, localized mainly to the area surrounding the *P. protegens* growth (Supplementary Figs. 3b and 4). To identify these ions, we analyzed extracts from culture supernatants of *P. protegens* grown axenically and in coculture with *C. reinhardtii*. A drop of 20 µl culture was applied on the slide. The cells were visualized in a bright-field microscope at ×200 magnification (scale bar: 10 µm); the cell movement was recorded before and after application of 35 µM orfamide A or methanol as control (Supplementary Movies 2–5). To visualize cells that retained or lost their flagella, cells were treated with orfamide A or methanol (control) for 30 s, fixed with 8% potassium iodide and visualized using differential interference contrast (DIC) microscopy at ×630 magnification. The orfamide and control treatments were done in biological triplicates (see also Supplementary Movies 2–5); a representative picture is shown. The experiment was replicated twice.

We next analyzed whether orfamide A (Fig. 2b) alone has an effect on the algal cells. A drop of *C. reinhardtii* cell suspension was placed on a glass slide, and a video was recorded before adding orfamide A or methanol (negative control), which were carefully applied from the top (Supplementary Movies 2–5).
5). Whereas no effect was observed for the negative control, upon the addition of orfamide A, the cells stopped moving within 30–40 s. Light micrographs from orfamide A treated and untreated cells revealed that exposure to orfamide A results in the rapid deflagellation of algal cells (Fig. 2c). These results suggest that the bacteria employ orfamide A as a tool to incapacitate the algal cells, and thus the algal cells lose the ability to evade the bacteria by swimming away.

The specificity of orfamide A-triggered loss of motility was further tested with flagellated algae other than C. reinhardtii.

### Table 1 Loss of motility of exemplary flagellate algae upon treatment with orfamide A

| Species                  | Class      | Phylum    | Habitat | Loss of motility | Supplementary videos |
|--------------------------|------------|-----------|---------|------------------|----------------------|
| Chlamydomonas reinhardtii| Chlorophyceae | Chlorophyta | FW/WS   | Yes              | 2–5                  |
| Chlamydomonas sp.        | Chlorophyceae | Chlorophyta | Marine  | Yes              | 6, 7                 |
| Haematococcus pluvialis  | Chlorophyceae | Chlorophyta | FW/WS   | Yes              | 8, 9                 |
| Gonium pectorale         | Chlorophyceae | Chlorophyta | FW/WS   | Yes              | 10, 11               |
| Pedinomonas minor<sup>a</sup> | Pedinophyceae | Euglenophyta | FW/WS | No              | 12, 13               |
| Euglena gracilis         | Euglenophyceae | Euglenophyta | FW/WS   | No              | 14, 15               |

FW Freshwater, WS wet soil
Orfamide A was applied at a concentration of 35 µM
<sup>a</sup>Non-axenic strain
Therefore, a drop of algal cell suspension was placed in each case on a glass slide, and the cells were visualized using bright-field microscopy. A video was recorded before and after adding orfamide A. Negative controls with methanol that were carried out as well did not influence motility in any case (not shown). Other Chlorophyceae, such as the closely related marine

![Graphs and images](image_url)

**Fig. 4** Orfamides cause a rapid increase in cytosolic Ca\(^{2+}\) and contribute to deflagellation and algal growth arrest. a P. protegens is able to trigger Ca\(^{2+}\) changes in C. reinhardtii. Time course of cytosolic Ca\(^{2+}\) concentrations after addition of bacteria to AEQ34 cells. For the Ca\(^{2+}\) measurements, the bacterial cell density was adjusted to the indicated cell ratios. As control, LB broth was added (black arrowhead). b Effect of P. protegens spent medium on cytosolic Ca\(^{2+}\) levels in C. reinhardtii. Aequorin-expressing cells (AEQ34) were incubated with P. protegens in a ratio of 1:300 and used for the measurement. Further, sterile-filtered spent medium from an overnight culture of P. protegens (black arrowhead) was used. c Orfamide A elicits dose-dependent Ca\(^{2+}\) changes. Orfamide A that was dissolved in methanol and further diluted in TAP medium was added to cells at the indicated concentrations. As control, methanol proportional to that of the highest concentration of orfamide A was used. d Ca\(^{2+}\) measurement upon treatment with 5 μM orfamide A or B. As control, methanol was used. e Ca\(^{2+}\) measurements with wild-type P. protegens or ΔofaA mutant at a ratio of 1:400 of algae to bacteria. f Extracts prepared from supernatants of P. protegens and the ΔofaA mutant cultures, respectively, having similar cell densities (around 3.14 × 10\(^8\) and 3.16 × 10\(^8\) cells ml\(^{-1}\), respectively) were incubated with C. reinhardtii at a 1% concentration for 30 s or 5 min in triplicates. A 1% extract of TAP medium was taken as control. g Liquid co-cultivation of C. reinhardtii together with wild-type P. protegens or ΔofaA mutant compared to axenic algal cultures. A 1:100 ratio of algae to bacteria was used for inoculation with an initial concentration of 10\(^5\) algal cells per ml. The corresponding growth curves are depicted in Supplementary Fig. 12. a–e Each line in the graph represents the mean of three biological replicates, and each biological replicate includes three technical replicates. All experiments were replicated twice except for f that was performed once with one biological and three technical replicates. The experiment in g was performed twice with three biological replicates.
Chlamydomonas sp. SAG 25.89 (Supplementary Fig. 6), Haematococcus pluvialis of the order of Chlamydomonadales, as well as the colony-forming Gonium pectorale of the order of Volvocales lost motility to a large extent upon orfamide A treatment (Table 1; Supplementary Movies 6–11). In contrast, Pedinomonas minor, a Chlorophyte more distantly related to C. reinhardtii from the class of Pedinophyceae as well as the Euglenophyte, Euglena gracilis, retained motility upon orfamide A exposure (Table 1; Supplementary Movies 12–15). These data indicate that orfamide A specifically affects the motility of algae from the Chlorophyceae, whereas tested algae outside this taxonomic class are not vulnerable to this bacterial secondary metabolite.

Establishment of an aequorin reporter in C. reinhardtii. Since it is known that Ca$^{2+}$ spikes can cause flagellar excision in C. reinhardtii, we aimed at monitoring cytosolic Ca$^{2+}$ changes in situ during the microbial interaction. For this purpose, we established an aequorin reporter system in C. reinhardtii. We constructed a gene cassette containing the apo-aequorin gene of Aequoria victoria, which was codon optimized for efficient expression in C. reinhardtii, and introduced it into the algal cells (Fig. 3a and Supplementary Fig. 7). Several transgenic lines (AEQ22, AEQ24, AEQ33, and AEQ34) expressed the 22-kD apoaequorin successfully (Fig. 3b) and were selected for further examination.

As high extracellular Ca$^{2+}$ concentrations reportedly cause an increase of cytosolic Ca$^{2+}$ levels, we initially analyzed aequorin activities by providing external Ca$^{2+}$ to the AEQ cell lines. Notably, upon addition of excess of external Ca$^{2+}$, all four transgenic AEQ lines immediately showed a luminescence spike (Fig. 3c). This response was neither observed in the wild type nor in the negative control containing the vector only. In order to estimate the intracellular Ca$^{2+}$ concentrations, cell lysis was performed at the end of the measurement using an ethanol-containing saturating solution of Ca$^{2+}$ to trigger the maximum luminescence (Supplementary Fig. 8; Fig. 3d). We also compared maximum luminescence values dependent on the age of the cells, which was the highest during the exponential growth phase (Supplementary Fig. 9). To further validate our reporter system, abiotic stimuli (abrupt acidification and salt stress) previously demonstrated to increase intracellular Ca$^{2+}$ levels were applied to the transgenic strains. In both cases, the luminescence increased as expected (Fig. 3e, f).

Orfamides trigger a Ca$^{2+}$ signal and influence algal growth. With a reliable aequorin reporter system at hand, we next studied the biotic interactions of C. reinhardtii. The Ca$^{2+}$ response in aequorin-expressing C. reinhardtii cells was measured in the presence of P. protegens cells at different ratios (algaebacteria 1:100 and 1:400). At a 1:100 ratio, no changes in cytosolic Ca$^{2+}$ levels could be observed within the first 30 min of incubation (Fig. 4a), whereas a strong Ca$^{2+}$ signal occurred at a ratio of 1:400 after just 5 min. These results show that the interaction between C. reinhardtii and P. protegens cells results in a very fast alteration of the intracellular Ca$^{2+}$ level, once a critical number of bacterial cells surrounding the algae (Supplementary Movie 1) is reached. In contrast, when adding X. campestris or F. johnsoniae cells at the same ratios, no Ca$^{2+}$ release was triggered (Supplementary Fig. 10a and b). Even a 4500-fold excess of X. campestris or F. johnsoniae did not trigger a Ca$^{2+}$ signal, demonstrating that the response of C. reinhardtii is not generally caused by the presence of bacteria, but it is specific to P. protegens.

We also found that a direct contact of P. protegens with the algal cells is not required to trigger the Ca$^{2+}$ release. The cell-free supernatant of the bacterial culture also caused a signal in the aequorin reporter line (Fig. 4b), thus showing that a diffusible signal is responsible for the Ca$^{2+}$ release. Next, we tested the capability of orfamide A to cause alterations in cytosolic Ca$^{2+}$ in a manner similar to the coculture and the supernatant of P. protegens. Solutions of pure orfamide A at different concentrations were added to the algal aequorin reporter line. Whereas the control with the solvent alone showed no alteration, we observed a dose-dependent change in the Ca$^{2+}$ levels up to ~5 µM orfamide A within the first 5 min after the addition of orfamide A. Higher levels of orfamide A (10 µM) resulted in saturation (Fig. 4c). These data unequivocally show that orfamide A affects Ca$^{2+}$ homeostasis in the bacterial–microalgal interaction. In addition, the concentration-dependent response up to ca. 5 µM orfamide A mirrors the effect of the bacterial cell density when surrounding the algae, thus causing a high orfamide A concentration in the vicinity of the microalgae.

The extended response of C. reinhardtii to the spent media of P. protegens (Fig. 4b) suggested that chemical mediators other than orfamide A may also contribute to the signal. As orfamide B was also identified in P. protegens (Supplementary Fig. 5) and is commercially available, we analyzed if it has a similar effect as orfamide A. We observed that orfamide B triggers a Ca$^{2+}$ release, whose signature, however, differs from that of orfamide A (Fig. 4d). A sharp peak of low amplitude observed immediately after the addition of orfamide A was also present with orfamide B, but the larger and broader response found with orfamide A was missing. Yet, orfamide B deflagellates the algal cells (Supplementary Fig. 11).

Orfamides are biosynthesized from an operon consisting of three structural genes known as ofaA, ofaB, and ofaC, encoding nonribosomal peptide synthetases. To investigate the in vivo role of orfamides in algal–bacterial mixed cultures, we used a ΔofaA mutant that lacks all orfamides. The observation that this mutant is still able to elicit a Ca$^{2+}$ signal (Fig. 4e) indicates that P. protegens produces inorganic chemicals other than the orfamides with the capacity to influence the cytosolic Ca$^{2+}$ levels in C. reinhardtii. However, a 1% extract from the culture supernatant of the ΔofaA mutant deflagellated algal cells after 30 s only at a relatively low rate compared to the supernatant from a P. protegens culture (Fig. 4f). After a longer incubation time (5 min), the rate of deflagellation was high in both cases (Fig. 4f). These data show that the orfamides contribute to the rapid deflagellation event of algal cells. We also looked at the growth of algal cells in coculture with the mutant. Although the ΔofaA mutant initially suppressed algal growth in a coculture assay, the algae recovered and started to grow after 9–10 days (Fig. 4g; Supplementary Fig. 12). In contrast, wild-type P. protegens suppressed algal growth over a period of 19 days. Although these data provide strong evidence that further secondary metabolites contribute to the complex interplay between P. protegens and the algae, they clearly suggest at the same time that the orfamides are involved in the antagonistic effect exerted by P. protegens on the algae.
orfamides as well as of other chemical compounds is needed for large-scale changes in morphology after 24 h.

We further checked if the deflagellation and the increase in Ca\(^{2+}\) in the algal cells are caused by permeabilization effects by orfamide A using the vital stain Evans blue. In the positive control, 10 \(\mu\)M mastoparan, known as a permeabilization agent\(^{33}\), leads to an efficient staining of approximately 80\% of the *Chlamydomonas* cells with Evans blue (Fig. 5b) and concomitant high rates of deflagellation (Fig. 5c). In contrast, 35 \(\mu\)M (used in Fig. 2c) as well as 10 \(\mu\)M orfamide A, which still causes \~80\% of deflagellation (Fig. 5c) did not result in an increased staining of *Chlamydomonas* cells compared to the control (Fig. 5b). Algal cells treated with 10 \(\mu\)M orfamide B did not show increased permeability to Evans blue either (Supplementary Fig. 11). These data show that orfamides do not provoke a major permeabilization of the algal cells—at least not on a short term—for orfamide-induced deflagellation in Chlorophyceae as observed with orfamide A (Table 1, see Discussion).

To investigate whether the orfamide-mediated rise in cytosolic Ca\(^{2+}\) is due to an influx of Ca\(^{2+}\) from the extracellular medium or if it is released from intracellular Ca\(^{2+}\) stores, we resuspended the cells in a medium lacking Ca\(^{2+}\) ions that had been additionally supplemented with 50 \(\mu\)M EGTA to sequester any possibly existing residual Ca\(^{2+}\) ions. Upon exposure of the cells to orfamide A, we observed only a very small increase in Ca\(^{2+}\) in this case, indicating that the orfamide-triggered rise in Ca\(^{2+}\) requires an influx of Ca\(^{2+}\) from outside the cell (Fig. 5d). We also investigated the link between the presence of Ca\(^{2+}\) in the medium,
the action of orfamide A, and delagellation. For this purpose, we examined the rate of orfamide-induced delagellation in *C. reinhardtii* in a medium with or without Ca**2+**. Clearly, orfamide A-based delagellation depends on the presence of Ca**2+** in the medium (Fig. 5e). These findings link the Ca**2+** uptake caused by the presence of orfamide A and consequently triggered delagellation, pointing to the cytosolic Ca**2+** elevation as the trigger for delagellation.

In the next step, we used a mutant known as *adf-1*, which encodes for a Ca**2+** channel shown to be responsible for delagellation via influx of Ca**2+** upon acid shock. The involved Ca**2+** channel was recently identified as TRP15**30**, **31**. The *adf-1* mutant was efficiently delagelled upon orfamide A treatment (Fig. 5f), suggesting that the TRP15 channel is not involved (or is at least not the only Ca**2+** channel) in the orfamide A signaling pathway. If the Ca**2+** channel blocked in the *adf-1* mutant was the sole channel mediating the Ca**2+** influx, orfamide A would not cause a significant increase in Ca**2+** in this mutant and the mutant would not delagger. In contrast, if another Ca**2+**-channel of *Chlamydomonas* is involved, delagellation would occur.

To test this possibility, we used lanthanum (La**3+**), a well-known inhibitor of Ca**2+** channels**29**, **35**. The addition of orfamide A in the presence of La**3+** resulted in a massively reduced level of Ca**2+** compared to the absence of La**3+** (Fig. 5g). Altogether, our results indicate that orfamide A from *P. protegens* induces the influx of extracellular Ca**2+** through an unidentified Ca**2+** channel (other than TRP15) in the algal plasma membrane.

**Discussion**

Ca**2+** signaling has been implicated in early stages of higher plant–microbe interactions of both symbiotic and antagonistic nature**36**. Likewise, in marine diatoms, environmental signals and intraspecies interactions can activate Ca**2+** signaling**37**, **38**. By means of an aequorin reporter line, we have demonstrated that bacteria substantially perturb the cytosolic Ca**2+** levels in *C. reinhardtii* at different time scales, within minutes (Fig. 4a) to hours (Supplementary Fig. 13). The changes in Ca**2+** do not necessarily result in delagellation of a *C. reinhardtii* cell, but are also involved in light-signaling pathways from the eyespot to the flagella, thus controlling algal movements**26**, **29**, **30**. Similarly, elevated salt concentrations evoke an increase in Ca**2+**, but not delagellation**30**. The strong rise in Ca**2+** in response to acidification and salt stress**26**, **29**, **30**, **26** was confirmed with the aequorin assay in this study (Fig. 3e, f), and a rapid increase in Ca**2+** is also evident upon orfamide A and B treatment (Fig. 4d).

Experiments with calcium-sensitive dyes have shown that localization of Ca**2+** primarily in the apical part of the algal cell and the timing of the Ca**2+** signals are important factors in acid-induced delagellation**26**. In agreement with this regulatory role of Ca**2+** signals in delagellation, we observed that both orfamide-induced Ca**2+** elevation (Fig. 5d) and delagellation (Fig. 5e) depend on the presence of extracellular Ca**2+** ions. Although stresses often elicit Ca**2+** responses in *C. reinhardtii* that can lead to delagellation**29**, **30**, our results point to a more specific role of orfamides. We show that orfamide A exposure results in cytosolic Ca**2+** increase, which requires a Ca**2+** influx from outside the cell without causing a major permeabilization of the plasma membrane (Figs. 4c and 5b, d). The fact that this Ca**2+** signal is inhibited by La**3+** (Fig. 5g), a Ca**2+** channel blocker**29**, **35**, suggests that orfamide A targets a Ca**2+** channel in the algal plasma membrane. In the light of these findings, an alternative mode of action of orfamides as Ca**2+** carriers or small Ca**2+**-selective pores that are not permeable to Evans blue seems to be less likely, but remains to be tested.

In this context, it is also of interest that *Chlamydomonas* has an unusually high number (>30 predicted by its genome) of Ca**2+** channels, especially of the TRP type**40**, most of which have not been characterized so far. It seems likely that one of them is part of the orfamide-signaling pathway. Since the well-characterized *Chlamydomonas* TRP15 Ca**2+** channel**34** is not involved in orfamide A-triggered Ca**2+** uptake (or is not the only Ca**2+** channel involved in this process) (Fig. 5f), the target of orfamide remains to be identified. Orfamide A may bind directly to its Ca**2+** channel target, or induce channel opening indirectly, for example, by binding to another protein or lipid component of the plasma membrane. The distinct response of orfamide B (Fig. 4d) that differs from orfamide A by only one methyl group (Fig. 2b) might indicate that orfamide B may even trigger a different pathway than orfamide A. The specificity of orfamide A in immobilizing Chlorophyceae algae, but not two species from the Pedinophyceae and Euglenophytes, respectively (Table 1), further supports a specific mechanism and a signaling role of these cyclic lipopeptides that involves specialized membrane components unique to Chlorophyceae.

The majority of characterized lipopeptides interacts with lipid components of membranes, such as daptomycin, which was recently found to interfere with fluid membrane microdomains**41**. In some cases, however, protein targets were also identified**42**. In addition to the delagellating and Ca**2+**-eliciting activities associated with orfamide A, which manifest themselves within minutes (Figs. 2c and 4c), this secondary metabolite also starts to disturb the morphology of *C. reinhardtii* over a time scale of 24 h (Fig. 5a). While staining with Evans blue indicates that orfamide A does not disrupt the algal cell membrane on the short term (Fig. 5b), membrane disruption may occur on the long term. Whether the different effects of orfamide A on *C. reinhardtii* are...
based on a single or multiple molecular interactions with components of the algal plasma membrane needs to be addressed in future work. In zoospores of the oomycete Phytophthora ramorum, a nonphotosynthetic member of the Heterokontophyta, orfamide A causes both immobilization and cell lysis within minutes. The structure of the cell wall, which is absent from P. ramorum zoospores, and also the composition of the plasma membrane may explain why orfamide A exerts different effects on P. ramorum and different photosynthetic algae (Table 1; Figs. 1d and 2c).

Mutation of ofaA in P. protegens results in the stop of orfamide production and defects in swimming (movement on soft agar) of the bacteria. Bacterial lipopeptides, specifically orfamides, have been known to play different roles, e.g., as biosurfactants, as antifungal biocontrol agents, or as insecticides. We have now revealed an interkingdom function of orfamides A and B as precise saboteurs of eukaryotic signaling pathways, resulting in the immobilization of flagellated algal cells by interference with intracellular Ca\(^{2+}\) homeostasis. These cyclic lipopeptides thus represent novel bioactive factors that cause Ca\(^{2+}\)-dependent deflagellation in microalgae. Experiments with the \(\Delta ofaA\) mutant (Fig. 4e, f, g) showed that orfamides are not essential for P. protegens to induce Ca\(^{2+}\) signaling and deflagellation in C. reinhardtii. The algal Ca\(^{2+}\) signal elicited by the \(\Delta ofaA\) mutant was slightly faster than the signal elicited by the wild type (Fig. 4e), whereas deflagellation was delayed when an extract from the \(\Delta ofaA\) mutant was used (Fig. 4f). Although the reason for these different effects is currently unclear, not every Ca\(^{2+}\) signal triggers deflagellation (as mentioned above), and different Ca\(^{2+}\) signatures may induce different downstream responses with different efficiencies. Our findings with the \(\Delta ofaA\) mutant further show that P. protegens also produces other chemical mediators in addition to orfamides that possess Ca\(^{2+}\)-eliciting and deflagelling activities, albeit rapid deflagellation (within 30 s) seems to be mainly associated with exposure to orfamides (Fig. 4f). Although these mediators may employ the same or alternative signaling pathways compared to orfamide A, the observed redundancy may indicate that Ca\(^{2+}\) elicitors and deflagelling compounds could aid to improve the fitness of P. protegens. In any case, the recovery of algal growth in the presence of the \(\Delta ofaA\) mutant after ~10 days (Fig. 4g) indicates that a few algae survive the encounter when orfamides are absent, highlighting that orfamides help P. protegens to compete with C. reinhardtii.

The strategy used by P. protegens (Fig. 6) differs markedly from previously reported tactics of bacteria that secrete algidical agents and results in high concentrations of orfamides around the algal cells simply by the accumulation of the bacteria around the algae, as visualized in Supplementary Movie 1. This behavior results in the stop of the algal growth and altered cell morphology (Fig. 1b, d). We hypothesize that immobilization of the algal cells helps the bacteria to acquire nutrients from the algae. Bacterial growth is enhanced in coculture when the surrounding medium lacks trace elements (Supplementary Fig. 2), which are most likely delivered by the algal cells. However, the evidence for a benefit for the bacteria is currently limited, and further experiments are necessary to identify the specific nutrients obtained from the compromised algal cells. In addition to one or more trace elements, phosphate or sources of carbon or nitrogen that are present in the used medium may also have a role in the algal–bacterial interplay.

Beyond the new molecular insights, our findings also seem to be relevant from an ecological perspective because they contribute to a better mechanistic understanding of algal blooms that are part of phytoplankton. Algal blooms can affect the environment in two ways; they are not only major contributors to global photosynthesis, but can also be highly toxic, poisoning fish and shellfish. Unicellular flagellated algae and colony-forming algae such as Gonium or Pleodorina, which are related to C. reinhardtii, can also form blooms. Our data show that closely related wet soil and freshwater, as well as marine Chlorophyceae are likely subject to a similar deflagellation mechanism as C. reinhardtii because they get immobilized by orfamide A (Table 1). P. protegens is found in all these environments and P. protegens and a closely related P. fluorescens strain have been reported to control algal blooms in freshwater and marine ecosystems, respectively. Therefore, the microbial interaction that we unraveled seems to be transferable to other flagellate Chlorophyceae algae, thus providing new tools to interfere with algal blooms of such species.

**Methods**

**Strains and culture conditions.** C. reinhardtii strain SAG 73.72 (int\(^{+}\)) was used as a wild type for most of the performed experiments. In some cases, mutant adf-1 (CC-2919) with a defect TRP1 \(\Delta^{+}\) channel was used together with its parental strain CC-620. SAG 73.72 was obtained from the algal culture collection in Göttingen and CC-620, as well as the mutant CC-2919 from the Chlamydomonas Center at the University of Minnesota, USA. C. reinhardtii cells were grown in Tris-acetate-phosphate (TAP) medium \(^{11}\) in a light dark regime of 12:12 (unless otherwise indicated) with a light intensity of 50 \(\mu\mathrm{mol}\) photons m\(^{-2}\) s\(^{-1}\) and stirring (250 rpm). The bacterial strains E. johnsoniae UW101\(^{18}\), X. campestris pv. campestris ATCC33913 \(^{19}\), and P. fluorescens Pf-5 \(^{20}\) renamed to P. protegens Pf-5\(^{21}\) were usually grown in LB medium at 28 °C with orbital shaking (200 rpm). The \(\Delta ofaA\) deletion mutant \(^{25}\) was kindly provided by H. Gross (University of Tübingen), which he had obtained in turn from J.E. Loper (Oregon State University, USA) and B.T. Shafer (OSU and UCSD-ARS). For the agar-based coculture assay, algal cells were grown under continuous light conditions (50 \(\mu\mathrm{mol}\) photons m\(^{-2}\) s\(^{-1}\)) and bacterial cells were grown in TAP medium for this coculture assay. Transgenic cell lines expressing the apo-aequorin reporter (AEQ, see below) were maintained on TAP agar plates (2% agar) with hygromycin B (20 \(\mu\mathrm{g}\) ml\(^{-1}\)). For Ca\(^{2+}\) measurement experiments, algal cells were grown in TAP medium without any antibiotics and the bacteria were grown in LB medium.

Other flagellate algae were obtained from the culture collection of algae and protozoa of the Scottish Marine Institute in United Kingdom (Haematococcus pluvialis, strain CCAP 34/8 and Gonium pectorale, strain CCAP 32/4), from the culture collection in Göttingen, Germany (Choanephora hindii, strain CCAP 25.89 that was identified as CCM P 235, see Supplementary Fig. 6 and Euglena gracilis, strain SAG 1224-5/25) or were kindly provided by C. Wilhelm (Pedinomonas minor, SAG 1965-3), University of Leipzig, Germany. These flagellate algae were
grown in media whose recipes are provided by the mentioned algae collections. Briefly, H. pluvialis, G. pectorale, and P. minor were grown in 3N-BBM + V medium, E. gracilis in 3N-BBM+V supplemented with 1 g l⁻¹ sodium acetate trihydrate, 1 g l⁻¹ meat extract, 2 g l⁻¹ trypone, and 2 g l⁻¹ yeast extract, and Ochlamydomonas sp. SAG 25.89 in a modified version of 3N-BBM+V where 32 g l⁻¹ NaCl had been added and the NaN₂O₃ had been replaced with 7 mM NH₄Cl.

Co-cultivation of C. reinhardtii and bacteria. For mixed cultivation of algae and bacteria on agar plates, a TAP plate (2% agar, 92 mm diameter) was overlaid with 3.3 ml 0.5% TAP agar (cover agar) containing 2 × 10⁸ C. reinhardtii cells. Overall, 15 µl of an overnight bacterial culture (containing between 2.1 and 2.5 × 10⁶ cells ml⁻¹) was then applied to the surface of the solidified plate. The plates were incubated under continuous light (50 µmol photons m⁻² s⁻¹) at 20 °C and documented by scanning. For the analysis of alginate production, 1 ml of each culture was transferred into a 2 ml Eppendorf tube, cooled down to 4 °C and centrifuged for 2.5 min at 200g. The supernatant containing the bacteria was transferred to a new Eppendorf tube and the pellet bearing the algal cells was resuspended in 1 ml of TAP medium. OD was then measured at 700 nm and the cell density was estimated using a calibration curve obtained from the cell suspensions in the asexual cultures of C. reinhardtii.

For mixed cultivation of C. reinhardtii and P. protegens in liquid TAP medium free of trace elements (TAP-TE), bacteria were washed three times in TAP-TE to prevent growth of bacteria. The washed cells were used for the inoculation of the TAP-TE medium and used to inoculate 50 ml TAP-TE in 100 ml conical flasks. The cells were visualized and recorded in the asexual cultures of C. reinhardtii. For mixed cultivation of C. reinhardtii and P. protegens in liquid TAP medium and used to inoculate 50 ml TAP-TE in 100 ml conical flasks to an initial density of 10⁶ cells ml⁻¹. Bacteria were grown under continuous light (50 µmol photons m⁻² s⁻¹) and stirring (200 rpm) at 20 °C. After 24 h, algae were added at a ratio of 1:100 algae to bacteria, and the growth of the bacteria was determined by plating different dilutions on LB agar plates and counting CFUs.

To record Supplementary Movie 1, wild-type cells of C. reinhardtii were grown to a cell density of 3–4 × 10⁸ cells ml⁻¹. 20 µl of algal cells in TAP medium were dripped onto a coverslip and the coverslip was precleaned over the surface of 10 µl of a bacterial culture grown overnight (in LB medium) and used to inoculate 50 ml TAP-TE with 1.5 × 10⁸ CFU ml⁻¹. The cells were visualized and recorded in the asexual cultures of C. reinhardtii.

Acridine orange staining and fluorescence microscopy. C. reinhardtii (4 × 10⁶ cells ml⁻¹) and P. protegens (1.7 × 10⁶ CFU ml⁻¹) were mixed in a ratio of 100:1 of algae to bacteria. For the monitoring of C. reinhardtii and other flagellate algae, 18 µl of algal culture (3 × 10⁶ ml⁻¹) were placed as drop on an 8 well diagnostic glass slide (Thermo scientific) except for E. gracilis, where a concentration of 6 × 10⁶ ml⁻¹ cells was used due to its large cell size. Overall, 2 µl of orfamide A in the corresponding medium were carefully added to the droplet to reach a final concentration of 1 µg ml⁻¹ orfamide A. The cells were visualized and recorded in movie using the software Zen (blue edition, Carl Zeiss). In case of P. minor and E. gracilis, movies were taken after 5 min to verify that orfamide A still has no effect after a longer period. All experiments for the Supplementary Movies were replicated twice.

Acridine orange staining and fluorescence microscopy. C. reinhardtii and P. protegens were grown in 5 ml LB broth in test tubes for 24 h at 28 °C and orbital shaking at 200 rpm. ~7 × 10⁸ cells from 0.5 ml of each culture were washed three times with TAP medium and used to inoculate 250 ml of TAP medium in 1 liter conical flask. The culture was grown at room temperature with shaking at 110 rpm. To prepare cocultures, C. reinhardtii cells were added after 24 h in a ratio of 1:100 (alga to bacteria) and incubated for further 24 h. The cells were removed by cell filtration (20 min, 16,000 × g, 4 °C). The supernatant (CHROMAFIL® CA-20/25 (S), pore size 0.2 µm), extracted three times with ethyl acetate (250 ml), and the extract was dried by rotary evaporation at 40 °C. The dried residue was dissolved in 250 µl of methanol (HPLC grade), and the solution passed through a filter with a pore size of 0.45 µm.

LC-high-resolution MS. The analyses were performed on a Q-Exactive Orbitrap mass spectrometer coupled to an Acela AS LC system (Thermo Fisher, San José, USA). Organic solvents (LC-MS grade) and reagents used for LC-MS analysis were purchased from Sigma Aldrich. The pHyg3 vector was used for transformation. Its complete sequence is shown in Supplementary Fig. 7. This vector carries a codon optimized apo-aequorin expression and transformation of C. reinhardtii. The pHD-AEQ2 vector was used for transformation. Its complete sequence is shown in Supplementary Fig. 7. This vector carries a codon optimized apo-aequorin gene that was synthesized by GeneArt Life Technologies. The apo-aequorin gene was put under control of the HSP70A/RBCS2 tandem promoter along with the first intron of RBCS2 and was set in front of the RACKY 3’ UTR representing a constitutive reference transcript. The vector also carries the hygromycin B resistance from the Hyg3 vector for selection in C. reinhardtii as well as an ampicillin resistance (selection marker for E. coli). C. reinhardtii wild-type cells (strain SAG 73.72) were transformed with the autolysin method as described earlier. SAG 73.72 cells were then selected for either Hyg3 or pHD-AEQ2 (AEQ) lines. Hygromycin-resistant colonies were further examined in immunoblots (see below) for expression of apo-aequorin.
Intracellular Ca²⁺ measurement using an aequorin reporter

Current study are available from the corresponding authors on reasonable request.

Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding authors on reasonable request. Plasmids and transgenic lines are available from MM on basis of a Material Transfer Agreement.

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Author contributions
C.H., S.S., and M.M. designed the experiments. P.A., D.S., M.G.-A., D.C.F., and H.D. conducted the experiments. P.A., D.S., M.G.-A., D.C.F., C.H., S.S., and M.M. wrote the article.

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