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# Bacterial induction of temporary cyst formation by the dinoflagellate *Lingulodinium polyedrum*

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ABSTRACT: We report the isolation of 3 novel bacterial strains from the *Bacteroidetes* group capable of inducing temporary cyst formation by ecdysis in the bloom-forming dinoflagellate *Lingulodinium polyedrum*. Phylogenetic analysis of 16S rRNA revealed that 2 of these strains are most closely related to previously identified algicidal bacteria, indicating potentially similar mechanisms of interaction. Long-term (2 wk) co-incubations of algae and bacteria under a 12:12 h light:dark cycle resulted in decreased algal cell abundances (compared to bacteria-free controls) followed by temporary cyst formation. Short-term incubations in continuous light resulted in no apparent effects of the bacteria over 2 d, but incubations in continuous darkness resulted in algal ecdysis after 24 h followed by significant decreases in total algal cell abundances after 52 h compared to controls without algicidal bacteria. We also showed that ecdysis resulted in the removal of bacteria attached to the surface of the algal cells, demonstrating a potentially direct benefit to the algae if the bacteria are harmful. We further suggest that negative interactions of bacteria on phytoplankton may be enhanced in the absence of light.

KEY WORDS: Ecdysis · Cyst · Bacteria · Dinoflagellate · Bloom · Algicidal



## INTRODUCTION

The flow of energy from phytoplankton to bacteria is a major but variable flux in pelagic marine ecosystems, and its mechanisms are not fully characterized (reviewed by Azam & Smith 1991). One critical factor controlling this interaction is whether bacteria are free-living or attached to particles, as particles (both living and dead) can provide microenvironments rich in organic matter and nutrients where bacterial activity and growth can be enhanced (Smith et al. 1992). While dead phytoplankton cells (algal detritus) are quickly and intensely colonized by bacteria (Bidle & Azam 1999), it appears that live phytoplankton cells remain relatively free of bacterial colonization (Kogure et al. 1982). The mechanisms that prevent bacterial colonization of phytoplankton cells have not been clearly identified but may include the production of antibiotics (Cembella 2003), mucus (Rosowski 1992), or simply a low encounter rate due to low bacterial abundance (Vaqué et al. 1989). Clearly, there is a need to describe specific mechanisms by which phytoplankton cells remain uncolonized by bacteria.

Bacterial attachment may also play a role in the population dynamics (growth and death) of phytoplankton in the sea. Bacteria are sometimes considered to be passive utilizers of phytoplankton organic matter, but many previous studies have discussed (Azam & Ammerman 1984, Azam & Smith 1991) and modeled (Jackson 1987, Bowen et al. 1993) the concept that bacteria can 'attack' phytoplankton organic matter. Experimental evidence has also shown that bacteria can compete with phytoplankton for resources such as nitrogen (Caron et al. 1988) and phosphorus (Brussaard & Riegman 1998). There are many examples of bacteria that directly affect phytoplankton physiology, leading to increases in growth (Ferrier et al. 2002), inhibition of sexual reproduction (Adachi et al. 2001), and cell death (see review by Mayali & Azam 2004). The role of bacterial attachment in these various interactions has not been fully evaluated. In the relatively dilute world of seawater, attachment of bacteria to

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phytoplankton is crucial in the mediation of these interactions. For example, if bacteria release a chemical toxic to phytoplankton, the concentration of the chemical decreases dramatically away from the bacterium due to diffusion and advection. However, if the bacteria are attached to the algal cells, advection and diffusion are less significant, allowing persistent high concentrations of the molecules which can contact the algal cell.

Research on negative bacterial effects on phytoplankton has focused on the mechanisms of algicidal bacteria attack, but surprisingly little is known about algal defense strategies against bacteria. Three previous studies noted that some dinoflagellates produced temporary cysts in response to incubations with algicidal bacteria (Lovejoy et al. 1998, Nagasaki et al. 2000, Kitaguchi et al. 2001). Many species of armored dinoflagellates, in addition to having a complex sexual life cycle, have the ability to form such temporary (asexual) cysts through ecdysis, a process that involves shedding the theca and the outer plasma membrane (Morrill 1984). Temporary cysts are more resistant than vegetative cells to a variety of chemical treatments (Fensome et al. 1996), which has led to the hypothesis that cyst formation is a defense mechanism against unfavorable conditions. In the laboratory, temporary cyst formation has been induced by lowering temperature (Schmitter 1979), nutrient stress (Doucette et al. 1989), and incubation in the presence of an allelopathic competitor (Fistarol et al. 2004). Temporary cysts are found in natural populations of dinoflagellates, but their ecological role remains unclear (Olli 2004). Confusing matters further, some dinoflagellate species undergo asexual (Garcés et al. 1998) and sexual (Figueroa & Bravo 2005) division by ecdysis, forming cysts that closely resemble the temporary cysts caused by environmental disturbances. The function of temporary cysts as a short-term survival stage is still a hypothesis, based on laboratory findings that ecdysis is reversible: when the stress factor (nutrient, temperature, algicidal bacteria, etc.) is removed, excystment into vegetative cells occurs.

In our search for bacteria that negatively influence the growth of the bloom-forming dinoflagellate *Lingulodinium polyedrum*, we have isolated several bacteria that induced ecdysis in *L. polyedrum* unialgal and previously axenic (bacteria-free) cultures. In order to understand why dinoflagellates would form temporary cysts in the presence of algicidal bacteria, we investigated several aspects of this interaction. Since ecdysis results in the removal of the outer components of the algal cell, we examined bacterial attachment dynamics in the context of ecdysis formation. Ecdysis may be an adaptation to remove colonized bacteria. In addition, a previous study of a related dinoflagellate (*Alexandrium taylorii*) which undergoes ecdysis as its normal cell division mechanism when nitrogen starved (Giacobbe & Yang 1999) found that temporary cysts increased in abundance during the night (Garcés et al. 1998). We tested whether bacteria-induced ecdysis also increases during periods of darkness. The major hypotheses tested here were that (1) ecdysis-inducing bacteria are related to algicidal bacteria, (2) algicidal bacteria induce ecdysis faster in the dark, and (3) ecdysis results in the removal of bacteria from the surface of the algal cells.

## MATERIALS AND METHODS

**Bacterial strain isolation and phylogenetic analysis.** All incubations were performed at 18°C under 12:12 h light:dark cycled cool white fluorescent tubes at 160 µE $m^{-2}$ $s^{-1}$ unless specified otherwise. Surface whole seawater was collected from Scripps pier (La Jolla, CA) on various dates, filtered through 0.6 µm polycarbonate filters to remove protists, and added (10 µl) to an axenic strain of *Lingulodinium polyedrum* (CCMP 1932) grown in 25 ml *f*/4 medium with 4× vitamin stock (Guillard 1975). This medium has been shown to be optimal for axenic growth of this algal strain (P. Von Dassow pers. comm.). Such enrichments (mixed bacteria and viruses with *L. polyedrum*) as well as control (no addition) *L. polyedrum* cultures were monitored by *in vivo* fluorescence with a TD700 fluorometer (Turner Designs). Enrichments that led to decreased fluorescence compared to controls were considered to contain putative algicidal agents, and this activity could be transferred to new axenic *L. polyedrum* cultures by additions of 1 µl of the 'killed' culture. To isolate bacteria, these enrichments were spread on ZoBell agar (1.5%) plates (Oppenheimer & ZoBell 1952), and colonies picked with a sterile loop into new axenic *L. polyedrum* cultures. Three strains (ALC1, LPK13, LPK5) from 3 separate incubations exhibited putative algicidal activity. These isolates were PCR-amplified (MasterTaq) using 16S rRNA primers 27F and 1492R (Invitrogen) and sequenced bidirectionally using internal primers (Giovannoni 1991). The full-length (1488 bp) 16S sequences (deposited in GenBank under acc. nos. EF527870, EF527871, EF527872) were inserted into ARB (Ludwig et al. 2004), aligned using the internal aligner, and added to the global phylogenetic tree (January 2004 ARB database) using parsimony. Closely related aligned sequences were exported into PAUP* v. 4.0b10 (Swofford 2002) and further analyzed using maximum likelihood with 100 bootstrap replicates, using a model of molecular evolution chosen with modeltest (Posada & Crandall 1998).

**Effect of bacteria on algal growth.** Once the bacteria causing a decrease in fluorescence of axenic *Lingulodinium polyedrum* strain CCMP 1932 were isolated, the next step was to determine the specificity of this negative interaction. We again used *in vivo* fluorescence to quickly ascertain whether the bacteria had any effect on the algal strains tested (for a full list of tested algal cultures, see Table 1). These included 7 other *L. polyedrum* cultures (all xenic), 3 other xenic dinoflagellates species previously isolated from Scripps pier, and 2 other flagellates. Since ALC1 was isolated 2 yr before the other 2 bacterial strains, some of these growth experiments were performed at different times and are therefore plotted on separate diagrams. Bacteria were grown in ZoBell broth to log phase, washed in sterile *f*/4 medium, and added to triplicate exponentially growing algal cultures at $10^6$ bacterial cells $ml^{-1}$. Controls included the same algal cultures with no added bacteria (also in triplicate). Subsequently, a more detailed population dynamics experiment was performed with ALC1 and the axenic *L. polyedrum* culture. We performed cell counts of both *L. polyedrum* and ALC1 bacteria to determine if algal mortality occurred. Bacteria were inoculated at lower concentrations ($10^5$ cells $ml^{-1}$) than previous experiments to determine free-living bacterial growth dynamics. Triplicate 100 ml incubations in 250 ml Erlenmeyer flasks (both ALC1 additions and controls) were incubated as before and 1 ml samples were taken daily and fixed with 5% formalin. Samples were stained with DAPI, and filtered onto 0.22 µm polycarbonate filters (Millipore). Bacteria were counted under 1000× magnification and *L. polyedrum* cells under 10× magnification on an Olympus BX-51 microscope.

**Effect of darkness on ecdysis.** To determine if bacteria-induced ecdysis was affected by light, the 3 algicidal strains were inoculated separately as above but with higher abundances ($10^7$ cells $ml^{-1}$) into triplicate axenic *Lingulodinium polyedrum* cultures in continuous light or continuous darkness. We used higher bacterial numbers in order to induce an algal response faster, as we wanted to examine shorter time scales than previous experiments. As a control, we used a non-algicidal bacterium (*Flavobacterium* strain BBFL7) instead of no bacterial addition (also in triplicate). Samples were taken after 10, 24, and 52 h for quantification of total algal cells (which included vegetative cells, cysts, and protoplasts), temporary cysts, and bacterial attachment. Culture vessels were gently but thoroughly mixed before sampling to collect both swimming and non-motile cells/cysts. We counted total algal cells (vegetative + cysts) using epifluorescence microscopy after post-fixation DNA staining with SYBR Green II (Molecular Probes). We counted temporary cysts using light microscopy and considered rounded cells without thecae as cysts. If any protoplasts (vegetative cells that lost their thecae) were present, they would have been counted as 'cysts'. In dark incubations with algicidal bacteria after 52 h incubation, cysts and other non-motile cells were embedded in a matrix of empty thecae and bacterial biofilm, making them difficult to quantify, and so they are not reported. Statistical comparisons for total algal cells and cysts were performed with a Student's *t*-test and comparisons of bacterial attachment with a non-parametric Wilcoxon test.

**CARD-FISH.** In order to quantify the number of bacteria attached to dinoflagellate cells in the light:dark incubations, catalyzed reporter deposition fluorescent *in situ* hybridization (CARD-FISH) was used with a protocol slightly modified from Pernthaler et al. (2002). Samples were fixed with 5% unbuffered formalin (=1.8% formaldehyde) and gravity-settled overnight (or gently centrifuged at 200 × *g* for 10 min). This procedure allowed separation of algal cells from free-living bacteria. The algal cell pellet was then washed in 1× phosphate-buffered saline (PBS), and resuspended in 50% ethanol/PBS. Samples were spotted on Teflon-coated well slides and air-dried. The slides were dipped in 0.1% low-melting-point agarose, air-dried, and incubated in 1 mg $ml^{-1}$ lysozyme (Sigma) in Tris-ethylenediaminetetraacetic acid (TE) buffer at 37°C for 1 h. Slides were then washed 3× in Milli-Q water, incubated for 10 min in 0.1 N HCl, washed again 3× in 1× PBS (3 min), and finally dehydrated in an ethanol series (50%, 80%, 95% for 3 min each). Slides were incubated for 2 h at 35°C in a hydrated chamber with hybridization buffer (35% formamide, 900 mM NaCl, 20 mM Tris, 0.01% sodium dodecyl sulphate [SDS], 20% Roche Diagnostic Boehringer blocking reagent) containing 1 µl probe for every 25 µl buffer (final concentration = 2 ng $\mu l^{-1}$). We used eubacterial probe eub338 (Amann et al. 1995) conjugated with horseradish peroxidase (Eurogentec). Slides were subsequently washed for 20 min at 37°C in wash buffer (70 mM NaCl, 5 mM EDTA, 20 mM Tris, 0.01% SDS), rinsed in Milli-Q, and overlaid with TNT buffer (0.1 M Tris-HCl, pH 7.6, 0.15 M NaCl, 0.05% Tween-20) for 15 min. Wells were then incubated with 1 µl tyramide-Alexafluor488 (Molecular Probes) in 100 µl 1× PBS with 0.01% Boehringer blocking reagent for 30 min at room temperature in the dark. They were then washed with TNT buffer at 55°C, rinsed in water, air-dried, stained with DAPI, and mounted with Vectashield (Vectorlabs). Bacteria were visualized on an Olympus BX51 epifluorescence microscope with a standard DAPI filter set and a fluorescein isothiocyanate (FITC) Texas Red dual-band filter set (Chroma Technology). Up to 20 individual bacteria on an algal cell could be counted with minimal error, while higher numbers were cate-

gorized as >20 for statistical analyses. Our counts were most probably underestimates due to the inability to detect all probe-positive bacterial cells located behind the autofluorescent algal cells.

**Anoxia and excystment experiments.** Three additional experiments were performed to further constrain hypotheses about algicidal bacteria-induced temporary cyst formation in darkness. First, to determine the effect of continuous darkness on algal cell numbers and whether the presence of bacteria was a factor, we incubated axenic *Lingulodinium polyedrum* cultures (5 ml) with the <0.6 and <0.22 µm fractions from a freshly collected seawater sample. The former contained most free-living bacteria and viruses and the latter only viruses. Triplicate incubations were performed in continuous darkness and samples were taken daily for 3 d. Total algal cell numbers were counted after staining with SYBR Green II as above. We used a 2-way ANOVA to determine statistical significance.

To exclude the hypothesis that ecdysis in darkness was caused by anoxia due to enhanced bacterial activity, axenic *Lingulodinium polyedrum* cultures (triplicate 4 ml incubations in 16 mm plastic tubes) were incubated in the dark under both oxic and anoxic conditions for 24 h. Anoxic conditions were achieved by placing the tubes in a BBL™ GasPak Plus anaerobic system (Becton Dickinson). Temporary cysts were counted from samples collected after 24 h.

To determine the rate of excystment of *Lingulodinium polyedrum* temporary cysts incubated with the algicidal strains, we co-incubated log phase *L. polyedrum* with the 3 bacterial strains (separately) until temporary cyst formation was observed by microscopy. The algal cells were washed in sterile *f*/4 medium by centrifugation (1000 × *g*) to remove the free-living bacteria and resuspended into 3 ml *f*/4 (triplicate for each bacterial strain). After 24 h (including 12 h light), motile and non-motile cells were separately collected from the culture tubes that were not physically disturbed by mixing or centrifugation: the former were in the supernatant, the latter in the pellet. Samples were fixed and counted as above. The percent excystment was calculated as the number of swimming cells divided by the total number of cells (swimming + pellet). We assumed no additional cell division in 24 h from the swimming cells.

## RESULTS

### Bacterial strain isolation and phylogenetic analyses

Most 0.6 µm seawater filtrate additions to axenic *Lingulodinium polyedrum* cultures did not lead to premature culture crashes (data not shown), but on 3 occasions, such enrichments led to putative algicidal activities that could be transferred indefinitely to new axenic algal cultures. From each of these algicidal enrichments, bacterial isolates were found to be responsible for the activity: strains ALC1, LPK5, and LPK13. Strain LPK5 has been subsequently isolated 2 more times from 2 xenic *L. polyedrum* isolates that crashed earlier than other *L. polyedrum* cultures. As with the other 3 bacterial strains, this activity could be transferred to the axenic *L. polyedrum* culture upon re-addition of the bacterial isolates (data not shown).

The bacterial isolates ALC1, LPK5, and LPK13, as expected from their yellow colony coloration and ability to grow on kanamycin-containing agar plates, were members of the *Bacteroidetes* as shown by their 16S sequence. Sequence comparisons of strain ALC1 revealed no close relatives among cultured isolates, with the closest cultured strain sharing 97% similarity. A maximum likelihood phylogenetic analysis with full-length 16S sequences placed ALC1, with moderate (72%) bootstrap support, in a group that included many bacteria associated with algae (Fig. 1a), including Chlorophyta and Phaeophyta.

Sequence analysis of strain LPK13 revealed that its closest relative, with 100% bootstrap support (Fig. 1b), is an algicidal bacterium active against a dinoflagellate (Doucette et al. 1999). A group of other algicidal bacteria (*Kordia* and relatives) appears to be a sister taxon, although 16S sequence similarity to this group is low (90.5%). The phylogenetic analysis of strain LPK5 revealed, with 100% bootstrap support (Fig. 1c), that its closest relative is another algicidal bacterium active against a dinoflagellate (Kondo et al. 1999).

### Effect of bacteria on algal growth

Addition of bacterial strains ALC1, LPK5, and LPK13 to cultures of axenic *Lingulodinium polyedrum* (CCMP 1932) caused noticeable decreases in fluorescence compared to no-addition cultures (Fig. 2a,b). The other *L. polyedrum* cultures, all xenic, responded differently to the addition of the algicidal bacteria. For example, some were affected by strains ALC1, LPK5, and LPK13 (Fig. 2c,d) similarly to the axenic strain. Others were not affected (Fig. 2e), and still others were affected by one strain but not the other(s) (Fig. 2f). We also tested 3 other species of dinoflagellates (all isolates from Scripps pier) and 2 other flagellates and found that *Akashiwo sanguinea* was affected by strains LPK5 and LPK13 (Table 1). Moderate biomass decrease of *Isochrysis galbana* by strain LPK5 was also detected. For brevity, these experiments are not shown and the results are summarized in Table 1.

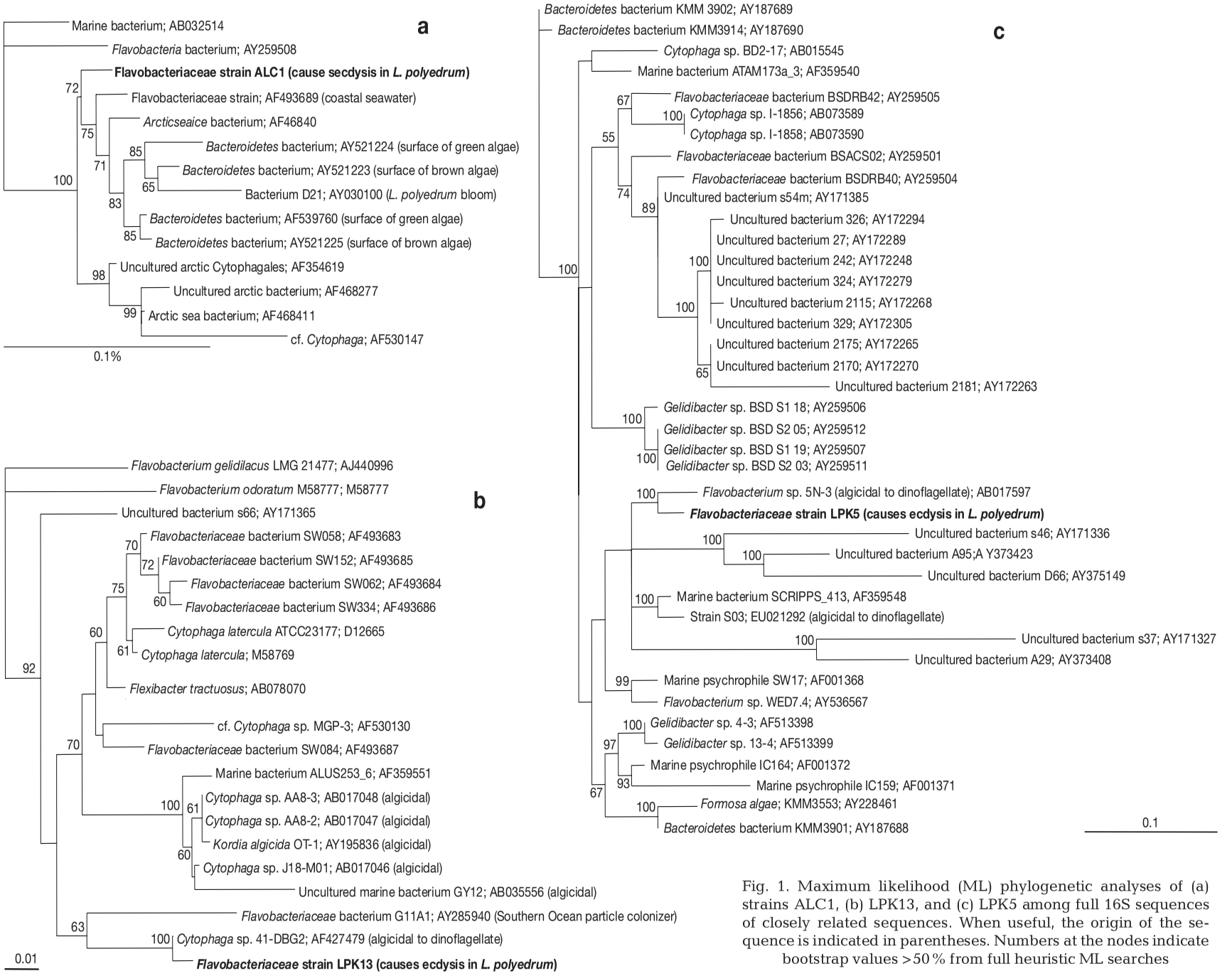


Fig. 1. Maximum likelihood (ML) phylogenetic analyses of (a) strains ALC1, (b) LPK13, and (c) LPK5 among full 16S sequences of closely related sequences. When useful, the origin of the sequence is indicated in parentheses. Numbers at the nodes indicate bootstrap values >50% from full heuristic ML searches

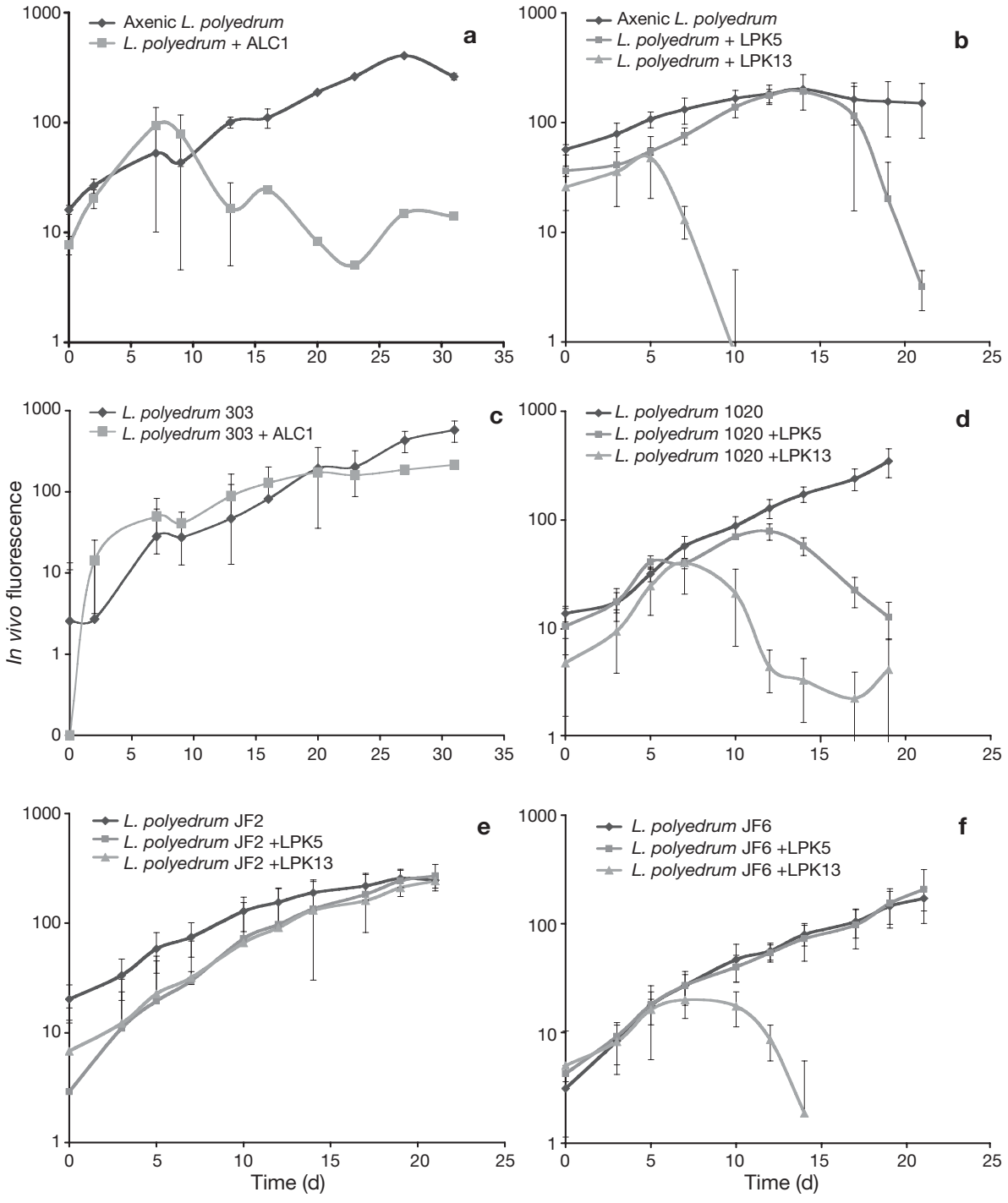


Fig. 2. *Lingulodinium polyedrum*. Growth of various cultures incubated with algicidal bacteria strains versus no-addition controls (a,b: axenic; c: 303; d: 1020; e: JF2; f: JF6) as monitored by *in vivo* fluorescence. Error bars indicate ± SD of triplicate cultures

When putative algicidy was detected by a decrease in fluorescence, *Lingulodinium polyedrum* cells formed a pellet at the bottom of the culture tubes. These pellets initially comprised vegetative cells that eventually formed temporary cysts if left undisturbed for ~1 wk. To determine if algal mortality occurred, we quantified algal and bacterial numbers in *L. polyedrum* cultures incubated with strain ALC1 (Fig. 3). Following inoculation at $10^5$ cells $ml^{-1}$, strain ALC1 grew over 1 order of magnitude over 24 h ($\mu$ = 0.17 $h^{-1}$) but thereafter never increased above $2 \times 10^7$ cells $ml^{-1}$. While total algal cell numbers (including cysts) were significantly

Table 1. Susceptibility of various *Lingulodinium polyedrum* cultures and other phytoplankton species to incubations with 3 algicidal bacteria strains as monitored by *in vivo* fluorescence compared to no-addition control cultures. Decrease in fluorescence is denoted as +, no difference to control as –, and blank as not tested. All cultures were xenic unless otherwise mentioned. Cultures without CCMP number were isolated from Scripps pier by various researchers

| Algal strain | ALC1 | LPK5 | LPK13 |
|---|---|---|---|
| Axenic *L. polyedrum* CCMP 1932 | + | + | + |
| *L. polyedrum* CCMP 1738 | – | + | + |
| *L. polyedrum* CCMP 1933 | + | – | – |
| *L. polyedrum* CCMP 1935 | + | | |
| *L. polyedrum* XM1020 | | + | + |
| *L. polyedrum* JF6 | | – | + |
| *L. polyedrum* JF2 | | – | – |
| *L. polyedrum* JF1 | | – | – |
| Axenic *Emiliania huxleyi* CCMP 374 | – | – | – |
| *Prorocentrum micans* AS623 | – | – | – |
| *Scrippsiella trochoidea* SIO strain | – | – | – |
| *Akashiwo sanguinea* SIO strain | – | – | – |
| *Isochrysis galbana* CCMP 462 | | + | – |

lower in the ALC1 incubations than the control after 6 d, they did not decrease below levels present at inoculation, demonstrating no net population mortality, but rather population growth reduction under the conditions tested. Incubations of *L. polyedrum* with strains LPK5 and LPK13 revealed similar dynamics and are not shown.

### Effect of darkness on ecdysis

Incubation with non-algicidal bacterium BBFL7 that served as a control did not induce ecdysis either in the light or dark (Fig. 4a). Bacterial attachment was significantly higher in the dark than in the light except at the first time point sampled (10 h), and showed temporal variability (in both light and dark), with increased colonization after 24 h compared to 10 and 52 h (Fig. 4b; $p < 0.0001$). Total algal cell concentrations remained constant over 52 h in the light. Darkness increased net algal cell concentration at 24 h (Fig. 4c; $p = 0.0009$), although the increased cell numbers were no longer significant at 52 h, possibly due to increased variance of the 52 h dark samples. No decrease in total algal cell numbers was detected, as measured by the difference between cell concentrations at 52 and 10 h within a treatment.

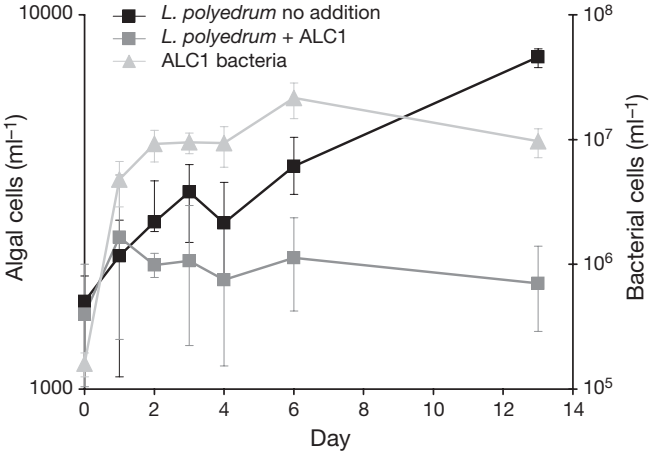


Fig. 3. *Lingulodinium polyedrum*. Growth of axenic culture incubated with strain ALC1 compared to a no-addition control, as monitored by cell counts. Bacterial numbers are also shown. Error bars indicate ±SD of triplicate cultures

Algicidal bacterium ALC1 induced ecdysis in the dark after 24 h incubation (Fig. 4d; $p = 0.0097$), while incubations in continuous light did not result in the formation of temporary cysts. Bacterial attachment was low compared to the other bacterial strains but increased with time, and there was no significant difference between light and dark incubations (Fig. 4e). For this strain, ecdysis did not result in a detectable decrease in bacterial colonization on all cells. Total algal cell concentration in the light exhibited a small but significant decrease after 52 h while the decrease in the dark was more pronounced (Fig. 4f; $p = 0.001$), with a loss of 80% of the cells after 52 h.

Algicidal bacterium LPK5 also induced ecdysis in the dark after 24 h incubation (Fig. 4g; $p = 0.0008$). Bacterial attachment in the light was very similar to the light incubations with non-algicidal strain BBFL7, showing an increase at 24 h. Bacterial attachment in the dark was initially higher than in the light (Fig. 4h; $p < 0.0001$) but exhibited a gradual and eventually significant decrease. For this strain, ecdysis in the dark was correlated with a decrease in bacterial colonization. Total algal cell concentration in darkness was significantly lower than in the light after 52 h (Fig. 4i; $p = 0.0011$), with dark-incubated treatments exhibiting a net population decrease of 84%.

Algicidal bacterium LPK13 caused a slight increase in the concentration of temporary cysts in the light after 24 h but a significant increase in the dark (Fig. 4j; $p = 0.0024$). No further temporary cyst formation occurred in the light after 52 h (data not shown). Bacterial attachment decreased over time, in both the light and dark (Fig. 4k; $p < 0.0001$). Total algal cell concentrations significantly decreased only in darkness, with a net population reduction of 66% (Fig. 4l; $p = 0.0012$).

### Anoxia and excystment experiments

One hypothesis to explain ecdysis in the presence of certain types of bacteria in the dark is anoxia due to the

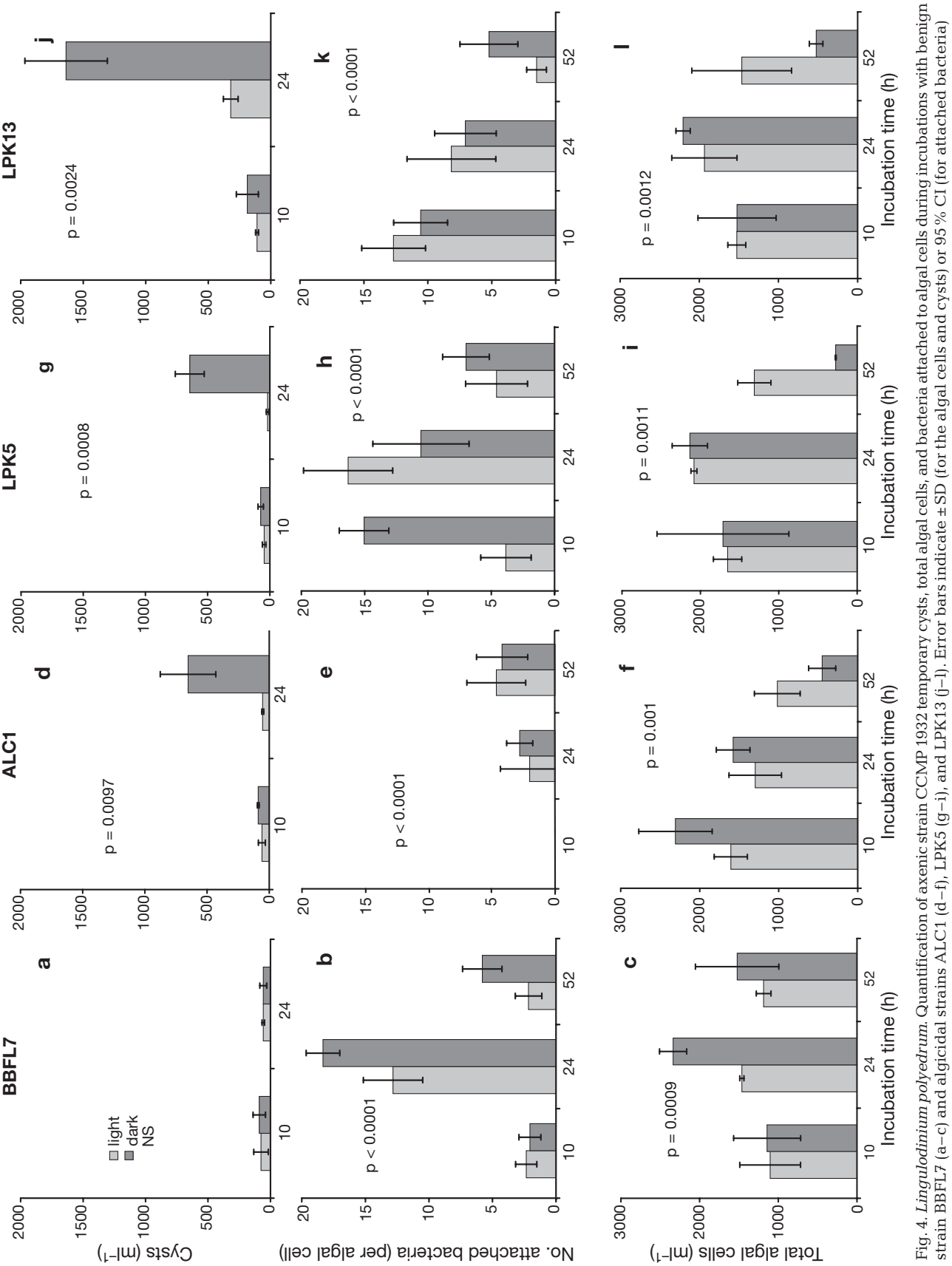


Fig. 4. *Lingulodinium polyedrum*. Quantification of axenic strain CCMP 1932 temporary cysts, total algal cells, and bacteria attached to algal cells during incubations with benign strain BBFL7 (a–c) and algicidal strains ALC1 (d–f), LPK5 (g–i), and LPK13 (j–l). Error bars indicate ±SD (for the algal cells and cysts) or 95% CI (for attached bacteria)

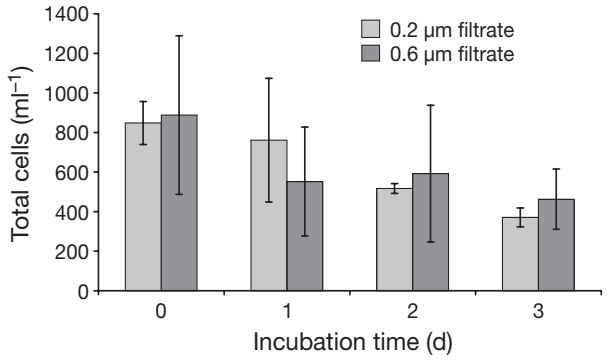


Fig. 5. Changes in algal cell numbers over the course of 3 d in continuous darkness, both with no bacteria (0.22 µm filtrate) and with the natural bacterial community from seawater (0.6 µm filtrate). Error bars indicate ±SD of triplicate cultures

lack of photosynthesis by the algal cells and high respiration rates by the phytoplankton and bacteria. We tested this by incubating *Lingulodinium polyedrum* cultures in anoxic and normal (oxic) conditions for 24 h in darkness. Incubations were not performed in the light because we did not expect ecdysis to occur under those conditions. We did not detect a significant difference ($p > 0.05$, ANOVA) in the number of temporary cysts in anoxic versus oxic conditions, rejecting the idea that anoxia alone caused ecdysis in the previous experiments.

To determine whether dark-induced algal mortality by bacteria was a general phenomenon, we incubated axenic *Lingulodinium polyedrum* cells in continuous darkness with and without a mixed bacterial community and monitored cell abundances over 3 d. There was no significant effect of the presence of bacteria, while time in darkness was a significant factor (Fig. 5; $p = 0.0265$). Population decrease over 2 d was on the order of 25%, much lower than dark incubations with algicidal bacteria from the previous experiments.

Following the removal of free-living algicidal bacteria and re-inoculation into fresh algal medium, *Lingulodinium polyedrum* temporary cysts formed swimming vegetative cells within 24 h. Excystment rates were 15.4 ± 1.6% (mean ± SD) for ALC1-, 16.1 ± 1.4% for LPK5-, and 2.4 ± 2.1% for LPK13-incubated dinoflagellates.

We did not always detect a strong decrease in the attachment of algicidal bacteria concurrent with ecdysis as hypothesized, and we often observed cysts colonized by bacteria. However, recolonization after ecdysis was possible in our laboratory incubations where new ecdysed cysts and colonized thecae were in physical contact. To directly determine if ecdysis physically removed bacteria attached to the dinoflagellate cells, we examined algal cells in the process of ecdysis sampled from the laboratory incubations. Vegetative cells without intact thecae (Fig. 6a) could be easily distinguished from the cysts (Fig. 6c) that exhibited the characteristic temporary cyst morphology (round with thick pellicle membrane). Cells in the process of ecdysis still had thecae attached (Fig. 6b). A number of

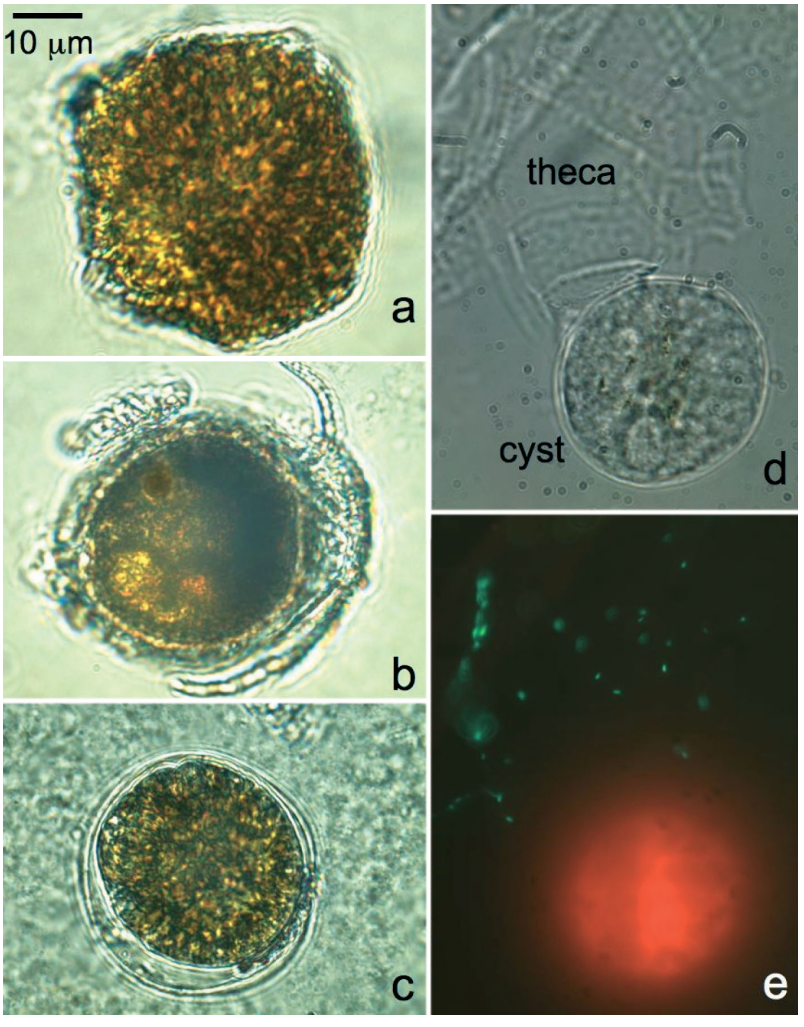


Fig. 6. *Lingulodinium polyedrum*. (a–d) Light and (e) epifluorescent micrographs of CCMP 1932 cells incubated with algicidal bacteria. (a) Motile vegetative cell; (b) vegetative cell undergoing ecdysis; (c) ecdysed temporary cyst with characteristically thick pellicle membrane; (d) temporary cyst (bottom) and cellulose theca (top); (e) same field of view as (d) under dual red and green fluorescence filter after CARD-FISH, showing chlorophyll-containing cyst (red) and bacteria-specific probe-stained cells (green)

these cells incubated with strain ALC1 (Fig. 6d,e), and the other 2 strains (not shown) revealed thecae heavily colonized with bacteria while the newly formed cysts were uncolonized.

## DISCUSSION

Three previous studies have reported dinoflagellate ecdysis in response to incubations with algicidal bacteria (Lovejoy et al. 1998, Nagasaki et al. 2000, Kitaguchi et al. 2001), but no knowledge exists on factors that influence bacteria-induced ecdysis and the potential benefits to the algal cells. We demonstrated that periods of darkness enhance bacterial induction of ecdysis in the dinoflagellate *Lingulodinium polyedrum* and further result in decreases in total algal cell abundances. In addition, we provided direct evidence that ecdysis removed colonized bacteria from the surface of algal cells, potentially providing an immediate benefit to the dinoflagellates.

According to the 16S phylogenetic analysis, strains LPK5 and LPK13 are the closest known relatives to algicidal bacteria strains 5N-3 (Kondo et al. 1999) and 41-DBG2 (Doucette et al. 1999), respectively. The implication of this finding is that the strain pairs share recent common ancestors and are likely to possess similar genomic architectures that enable them to display algicidal phenotypes. Algicidy and ecdysis induction are most likely related and may be regulated by the same biochemical processes. Indeed, under continuous darkness for 2 d, ecdysis-causing bacteria ALC1, LPK5, and LPK13 appeared to become algicidal, as total algal cell numbers (including cysts) decreased significantly compared to incubations with the non-algicidal bacterium BBFL7. This reinforces the notion that the definition of algicidy is ambiguous and interactions between potentially algicidal bacteria and their phytoplankton hosts are likely to vary with environmental conditions (Mayali & Azam 2004), in this case the presence of light. A further use of the phylogenetic analyses is the ability to identify environmental 16S sequences from uncultivated organisms as potentially algicidal if they are found to cluster in the groups discussed above. To date, no environmental sequences identical to those of strains LPK5 or LPK13 have been found. This may be a function of under-sampling, as only a handful of dinoflagellate blooms have been sampled for microbial community structure (reviewed by Garcés et al. 2007). In addition, these bacteria may be rare and found only attached to dinoflagellate cells, which would make them more difficult to detect in such studies. One study did find a sequence almost identical (1 bp difference, band ATT5, 99.3% similar, GenBank acc. no. AF125332) to that of ALC1 at the end of a 1997 *Lingulodinium polyedrum* bloom at Scripps pier (Fandino et al. 2001), implying it was present in relatively high abundances in nature. While few colony-forming bacteria are found to be numerically dominant in the environment (Bernard et al. 2000), we were successful in isolating strain ALC1 through enrichment with a culture of *L. polyedrum*. It remains unknown whether ALC1 exhibits algicidal or ecdysis-causing activity in the environment.

Incubations of *Lingulodinium polyedrum* cultures with the 3 algicidal strains in continuous light and continuous darkness convincingly revealed that darkness enhanced the ability of the bacteria to induce ecdysis. Non-algicidal strain BBFL7, another *Bacteroidetes* isolate, did not cause ecdysis under similar conditions. We used this isolate (16S GenBank acc. no. AY028207) because an identical sequence was found during a 1997 *L. polyedrum* bloom (Band ATT8, GenBank acc. no. AF125335), suggesting that it is present in abundant numbers during blooms of this dinoflagellate. Further incubation with algicidal bacteria in complete darkness (over 2 d) led to significant decreases in total algal cells compared to control incubations with BBFL7, as measured by counting DNA-containing cells. This is most likely due to the algal cells that did not form temporary cysts (vegetative cells and protoplasts) becoming non-viable. Another possibility is that some of the temporary cysts were lysed by the algicidal bacteria, as found in another study (Kitaguchi et al. 2001).

An unusual finding from our experiments is that darkness was crucial for algicidal bacteria to induce *Lingulodinium polyedrum* formation of temporary cysts. Our initial hypothesis of anoxia alone being responsible for this phenomenon was rejected because of the results of our experiments in the anoxic chambers. Darkness-enhanced ecdysis/algicidy may occur due to a combination of low oxygen and the production of algicidal agents. We offer 3 non-mutually exclusive hypotheses that may explain our findings. First, the algicidal agents may be light sensitive: several such compounds have been found to be proteins (Lee et al. 2000, Mitsutani et al. 2001) and would be denatured by UV radiation. Second, light may directly inhibit the production of algicidal compounds by bacteria, or indirectly through inhibition of bacterial growth. There is evidence that marine heterotrophic bacterial metabolism is enhanced in the dark (Morán et al. 2001), and secondary metabolite production would also be affected. Third, the algal cells may produce antibiotic compounds only during active photosynthesis in the light. For example, toxin production of *Alexandrium fundyense* is triggered by light (Taroncher-Oldenburg et al. 1997), and the production of reactive oxygen species (ROS) only occurs during photosynthesis. *L. poly-*

*edrum* produces ROS during the day and possesses a suite of enzymes designed to alleviate oxidative stress (Okamoto & Colepicolo 2001). The finding that ROS production by a marine flagellate inhibits the growth of a marine bacterium (Kim et al. 1999) further suggests a link between ROS production, light, and algicidal bacteria. A mechanistic understanding of algicidal bacteria killing phytoplankton is needed to clarify this issue. However, our findings suggest that light is a factor in mediating the interactions between algicidal bacteria and their phytoplankton host. Light may also be critical to other types of bacterial interactions such as commensalism and mutualism and merits further study in this context.

The last major aspect of our work examined whether ecdysis resulted in a noticeable benefit to the algal cells. Temporary cysts with their still-attached thecae revealed that newly formed cysts were not readily colonized, unlike the thecae. In terms of bacterial attachment in the culture incubations, the average number of bacteria per algal cell decreased significantly over time in incubations that were undergoing ecdysis (except for strain ALC1; Fig. 4). The case of strain ALC1 is a bit different because bacterial attachment did not start until after the 10 h time point, and attachment intensity was low compared to the other strains. Sampling of ALC1 incubations further in time might have revealed increased bacterial attachment but lower bacterial attachment in the dark compared to the light.

Temporary cysts are found in nature and there is evidence that some dinoflagellate blooms can end through ecdysis (Marasovic 1989, Wang et al. 2007). The potential significance of bacteria-induced ecdysis compared to ecdysis caused by other environmental factors remains unknown. It is likely that several factors work in combination. For example, nutrient limitation may make the dinoflagellates more susceptible to attack by ecdysis-causing bacteria. Programmed cell death (PCD) also appears to be a factor in bloom decline (Franklin et al. 2006), and the interaction between PCD and bacteria remains unstudied. Whether bacteria-induced ecdysis is significant in nature or is a laboratory artifact is a difficult question to answer. One strategy to corroborate these hypotheses is to quantify the abundance of algicidal (or ecdysis-causing) bacteria in nature in the context of bloom dynamics. Algicidal bacteria are phylogenetically diverse and different algicidal bacteria may occur in different blooms, making this a difficult task. Another strategy is to study the molecular mechanism(s) of ecdysis in the laboratory (from the point of view of the algae) and find markers specific for bacteria-induced ecdysis. Searching for these markers in algal cells during natural blooms would further provide evidence that bacteria induce ecdysis in the ocean.

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