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BACTERIA-INDUCED MOTILITY REDUCTION IN *LINGULODINIUM POLYEDRUM* (DINOPHYCEAE)[1]

Xavier Mayali,[2] *Peter J. S. Franks*

Scripps Institution of Oceanography, University of California San Diego, 9500 Gilman Drive, La Jolla, California 92093-0202, USA

Yuji Tanaka

Tokyo University of Marine Science and Technology, 4-5-7, Konan, Minato-ku, Tokyo, 108-8477, Japan

and Farooq Azam

Scripps Institution of Oceanography, University of California San Diego, 9500 Gilman Drive, La Jolla, California 92093-0202, USA

Biotic factors that affect phytoplankton physiology and behavior are not well characterized but probably play a crucial role in regulating their population dynamics in nature. We document evidence that some marine bacteria can decrease the swimming speed of motile phytoplankton through the release of putative protease(s). Using the dinoflagellate *Lingulodinium polyedrum* (F. Stein) J. D. Dodge as a model system, we showed that the motility-reducing components of bacterial-algal cocultures were mostly heat labile, were of high molecular weight (>50 kDa), and could be partially neutralized by incubations with protease inhibitors. We further showed that additions of the purified protease pronase E decreased dinoflagellate swimming speed in a concentration-dependent manner. We propose that motility can be used as a marker for dinoflagellate stress or general unhealthy status due to proteolytic bacteria, among other factors.

Key index words: **algicidal; bacteria; dinoflagellate; motility; protease**

Abbreviations: **AMC, 7-amido-4-methylcoumarin; DVM, diel vertical migration; PMSF, phenylmethanesulphonylfluoride**

Blooms of dinoflagellates and other members of the phytoplankton, many of which produce potent toxins that adversely affect wildlife and human health, appear to be increasing in incidence worldwide (Van Dolah 2000). Motility is a key element that allows dinoflagellates to outcompete other phytoplankton and form blooms, since they can perform diel vertical migration (DVM) to take up nutrients below the thermocline at night and photosynthesize at the surface during the day (Smayda 1997). One factor critical for dinoflagellates to successfully carry out DVM is the ability to swim quickly. Instantaneous speeds of *L. polyedrum* have been measured between 250 and 400 $\mu m \cdot s^{-1}$ (reviewed by Lewis and Hallett 1997), which is $\sim$10 body lengths $\cdot s^{-1}$. Various studies have described biologically mediated motility reduction of flagellates, although the actual swimming speeds of the organisms were not directly quantified. For example, toxins from *Anabaena flos-aquae* inhibited the motility of *Chlamydomonas reinhardtii* (Kearns and Hunter 2001), potentially explaining their negatively correlated abundances in nature. Both viral infection (Lawrence and Suttle 2004) and programmed cell death mediated by inorganic carbon limitation (Vardi et al. 1999) increased sinking rates and decreased flagellate motility, respectively. No studies to date have examined the effect of heterotrophic bacteria on the motility of eukaryotic phytoplankton.

Interactions between bacteria and phytoplankton play major roles in structuring marine pelagic ecosystem dynamics (Cole 1982, Azam 1998), yet it is probable that we have not yet identified many of these associations. There exist many examples of bacteria that directly affect phytoplankton physiology, leading to changes in growth (Ferrier et al. 2002), sexual reproduction (Adachi et al. 2001), and even cell death, the latter often induced by algicidal bacteria from the Flavobacteriaceae family of the Bacteroidetes division (see review by Mayali and Azam 2004). We have recently isolated three bacterial strains from this group, related to previously isolated algicidal bacteria, capable of decreasing net algal growth rates and inducing temporary cyst formation of the bloom-forming dinoflagellate *L. polyedrum* (Mayali et al. 2007). These bacteria were initially enriched and subsequently cultured from seawater samples added to axenic *L. polyedrum* cultures (16S rRNA GenBank accession numbers of the bacteria are EF527870, EF527871, and EF527872). We microscopically observed that before cyst formation, the algal cells appeared to be ''sluggish'' and swam more slowly than axenic (bacteria-free) control cultures. Since several studies (Lee et al.

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[2]Author for correspondence: e-mail xmayali@ucsd.edu.

2000a,b) have implicated excreted enzymes as putative algicidal agent(s), we examined whether excreted enzymes were implicated in this motility-reducing phenomenon. The hypotheses tested in this paper were the following: (i) algicidal bacteria isolates excrete proteases that reduce *L. polyedrum* motility, and (ii) additions of purified protease similarly reduce *L. polyedrum* motility.

MATERIALS AND METHODS

Laboratory incubations. Axenic *L. polyedrum* strain CCMP 1932 batch cultures were grown in 25 mL *f*/4 medium with 4X vitamin stock (Guillard 1975) at 18°C under 16:8 light:dark (L:D) cycled cool-white fluorescent tubes at 160 μmol photon $\cdot m^{-2} \cdot s^{-1}$. Bacterial isolates ALC1, LPK5, and LPK13 (Mayali et al. 2007) were grown in ZoBell 2216 medium (5 g peptone, 0.5 g yeast extract in 1 L autoclaved seawater), washed in *f*/4, and added to algal cultures at final concentrations of 10^6 bacterial cells $\cdot mL^{-1}$. Algal culture growth was monitored by in vivo fluorescence with a TD700 fluorometer (Turner Designs, Sunnyvale, CA, USA). When fluorescence in bacterial-algal coincubations decreased (compared to no addition controls) due to algal cells settling, both motility-inhibited and control cultures were filtered through a 0.22 μm acrodisc HT Tuffryn® filter (Pall Life Sciences, East Hills, NY, USA) to remove both algal and bacterial cells. Other types of filters were tested but were subsequently found to remove motility-inhibiting activity (data not shown). Filtrates were then size fractionated using Centricon® filters (Millipore, Billerica, MA, USA) and/or incubated at 80°C for 10 min to denature proteins. Three irreversible protease inhibitors were tested for their ability to suppress motility reduction. These inhibitors included the cysteine-protease inhibitor E-64 (Sigma cat. # E3132; working stock 2.8 mM in distilled water) and the serine-protease inhibitors phenylmethanesulphonylfluoride (PMSF; Sigma cat. # P7626; working stock 100 mM in isopropanol) and Pefabloc®SC (Sigma cat. # 76307; working stock 210 mM in distilled water, pH = 5.2). Filtrates were incubated in 10, 100, and 100 μM (final concentration) E-64, PMSF, and Pefabloc®, respectively, for 30 min at room temperature in the dark. Subsequently, these filtrates were washed through a Centricon®-50 to remove the unbound inhibitor. This step was crucial because the presence of unbound protease inhibitors decreased dinoflagellate motility (data not shown). For the same reason, reversible protease inhibitors such as amastatin or leupeptin were not tested. Treated filtrates were resuspended in *f*/4 medium (4.5 mL final volume), and *L. polyedrum* cells from late log phase cultures (500 μL) were added and incubated for 16 h in the dark. All treatments were performed in triplicate. Two sets of two experiments were carried out, each set using the same batch of stock *L. polyedrum* cultures and motility-reducing filtrates. The first two experiments were completed within 24 h of each other, and the second set 1 month later, also within 24 h of each other.

Analysis of motility patterns. After incubations, algal cells in treated filtrates were mixed gently and placed in a plastic cuvette. Samples were illuminated from the side with a metal-halide cold spot light source PCS-UMX250 (Nippon-PI, Tokyo, Japan). Cells were video recorded (also from the side) for 15 s using a FASTCAM-PCI R2 camera (512 × 480 pixel resolution; Photron Co. Ltd., Tokyo, Japan) with a BSL-Z50 lens (Sonic Co. Ltd., Osaka, Japan) and an additional 0.5× front converter. The data were saved as .avi files at 30 fps. Each video file was compressed into one image, and brightness and contrast adjusted with ImageJ (Abramoff et al. 2004). Cell tracks were qualified as fast, slow, or sinking (Fig. 1). Fast cells showed the characteristic helical swimming pattern of dinoflagellates. Sinking cells did not show any lateral movement and were unidirectional (usually vertical, although convection inside the cuvette sometimes altered the direction of sinking cells slightly). Slow cells exhibited an intermediate wave-like motility pattern, neither fully unidirectional nor helical. The percentages of fast and/or slow cells among treatments were compared with nonparametric Kruskal–Wallis tests.

Protease activity measurements. To determine if motility-reducing filtrates possessed higher protease activities than

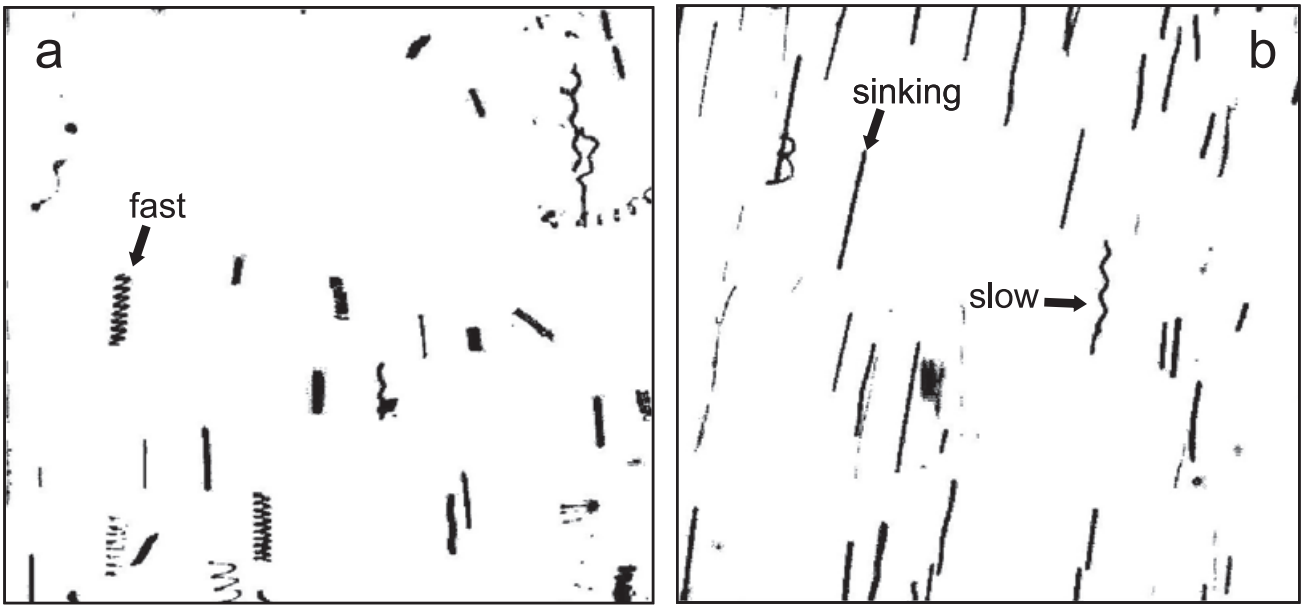

FIG. 1. Images (5.0 × 5.4 mm) of two-dimensional cell tracks (total time = 15 s) of axenic *Lingulodinium polyedrum* (a) and *L. polyedrum* incubated in the filtrate of bacterial strain ALC1 (b) recorded while the dinoflagellates swam in a cuvette. Tracks were counted as ''fast'' if they were of a corkscrew shape, ''slow'' if they were almost (not too fully) unidirectional, and ''sinking'' if they were fully unidirectional.

control filtrates, we compared cell-free axenic *L. polyedrum* filtrate to the filtrate from *L. polyedrum* cocultured with bacterial strain ALC1. We used four different 7-amido-4-methylcoumarin (AMC) labeled substrates (Hoppe 1983) conjugated to the amino acids leucine, serine, threonine, and glycine (Sigma). Change in fluorescence over time was monitored with a TD700 fluorometer with a Hoechst dye filter set (Turner Designs). Control incubations in autoclaved seawater were used as negative controls. Data were fitted to a standard curve of AMC and are reported as nmol substrate hydrolyzed $\cdot$ L^{-1} $\cdot$ min^{-1}.

Pronase experiments. Additions of relatively high (1 U $\cdot$ mL^{-1}) concentrations of pronase E to *L. polyedrum* cultures quickly led to motility reduction (within minutes), whereas additions of equivalent amounts of BSA, cellulase, phosphatase, lipase, or glucosidase had no effects (data not shown). Duplicate log phase *L. polyedrum* batch cultures (all from the same mother culture) were incubated in lower concentrations of pronase E (Sigma cat. # P6911) over time at final concentrations of 36, 9, and 1.8 $\mu g \cdot mL^{-1}$ (equivalent to 0.18, 0.045, and 0.009 U $\cdot$ mL^{-1}, respectively). Samples were taken 5 min, 30 min, 8 h, and 23 h after pronase addition and filmed as above. Motility quantification was performed as below.

Motility quantification. Videos from pronase E addition experiments were analyzed as follows (with Matlab; code available from P. J. S. F.). Fifteen frames, corresponding to 0.5 s of movie time, were combined to give streaks that represented a two-dimensional view of a dinoflagellate's swimming path. These images were made into binary (black and white) images by setting a threshold pixel intensity, and then the properties of the particle paths were analyzed using the *regionprops* function in Matlab. This step gave the length of the path (in pixels) for each organism in the field of view and was repeated for the entire 15 s (450 frame) movie. Scratches on the cuvette (pixels that did not move over the course of the entire 15 s period) were removed from analysis. Small objects (<10 pixels in area) were omitted, and each pixel represented ~10.63 μm (1 mm scale = 94 pixels). The resulting data set (for each movie) was a frequency distribution of path lengths (in pixels). Path lengths >35 pixels were not included in the analysis since they represent swimming speeds of 600 $\mu m \cdot s^{-1}$ (see calculation below), much greater than reported in literature data for *L. polyedrum* motility. Such paths usually originated from the overlap of two or more swimming trajectories; these could not be distinguished with our analysis software. To translate pixels to instantaneous swimming speeds, we removed the diameter of the cell from the path length: a nonmotile cell would have a path length equal to its cell diameter. We used an average cell diameter of 45 μm (average diameter of cells from one frame = 4.26 pixels):

$$\text{speed } (\mu m \cdot s^{-1}) = [(\#\text{ pixels} \times 10.63\ \mu m \cdot \text{pixels}^{-1}) - 45\ \mu m \cdot \text{cells}^{-1}] \times 2\ \text{frames} \cdot s^{-1}$$

These estimates of swimming speed are biased toward an underestimate of the true speed for two reasons: (i) the swimming path is helical, and we measure only the net displacement; and (ii) we are measuring a two-dimensional projection of a three-dimensional swimming path. Paths oriented at an angle to the imaging plane will appear shorter than they actually are. We have not corrected the data for these biases, because we expect them to be similar for all experiments. For the pronase E experiment sampled in duplicate, frequency distributions were combined before statistical analyses comparing treatments. Data were not normally distributed and were analyzed with nonparametric Kruskal–Wallis tests. Post hoc tests of significance (if necessary) were performed with a Wilcoxon test after *P*-value modification with the Dunn–Sidak method.

RESULTS

Motility effects. In most cases, a comparison of the fraction of fast cells was sufficient to compare treatments, although the fraction of slow cells was sometimes informative (both are reported on the figures). The results from the four separate experiments are summarized in Table S1 (in the supplementary material). In the first experiment, the motility-reducing component of the ALC1 filtrate originated in the >50 kDa fraction and was not detectable in the <50 kDa fraction (Fig. 2a; $P = 0.028$). The motility-reducing component from LPK13 was determined in both size fractions ($P = 0.024$). Fractionated axenic *L. polyedrum* filtrates (<50 and >50 kDa) were similar to the unfractionated control and are not reported.

In the second experiment, E-64-treated axenic control filtrates had no effect on algal motility, while Pefabloc®SC-treated axenic control filtrates showed minor motility reduction, though not statistically significant (Fig. 2b). Comparing bacterial filtrates to the controls, Pefabloc®SC neutralized the motility reduction in the ALC1 filtrate but not in the LPK13 filtrate ($P = 0.037$). The cysteine-protease inhibitor E-64 did not have a neutralizing effect on either ALC1 or LPK13 filtrate (both were statistically slower than the E-64-treated control filtrate; $P = 0.049$).

In the third experiment, the motility-reducing component of the LPK5 filtrate, as in strain ALC1, originated in the >50 kDa fraction and was not detectable in the <50 kDa fraction (Fig. 2c; $P = 0.026$). Treatment of the filtrates from the three bacterial strains with the serine-protease inhibitor PMSF did not fully neutralize motility reduction, although the treated LPK13 filtrate was significantly more motile than the untreated LPK13 filtrate ($P = 0.037$).

In the fourth experiment, the motility-reducing >50 kDa fraction from LPK13 could be denatured at 80°C and thus was likely a protein, while the motility-reducing <50 kDa fraction retained most of its activity after 80°C incubation (Fig. 2d; $P = 0.039$). The motility-reducing activity from LPK5 was almost fully neutralized upon treatment with the serine-protease inhibitor Pefabloc®SC, while the cysteine-protease inhibitor E-64 did not neutralize it ($P = 0.049$). Also included in this experiment were control PMSF incubations with axenic *L. polyedrum* filtrate, showing no effect of PMSF treatment on motility.

Protease activity. Comparison of protease activities from the ALC1 and axenic *L. polyedrum* filtrates did not show large differences in any of the four substrates tested (Table 1). Leucine-AMC and threonine-AMC hydrolysis rates were similar between the two

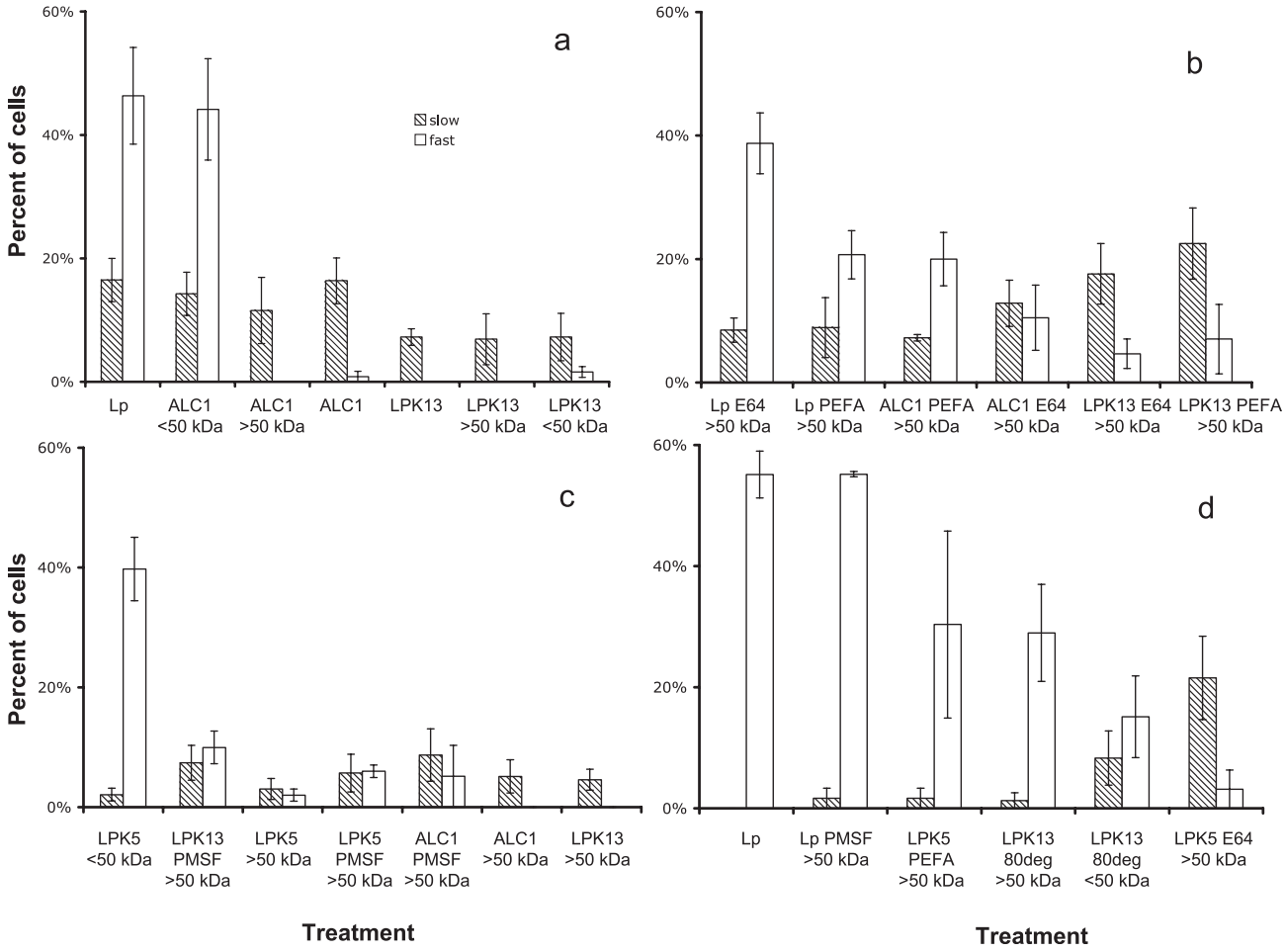

FIG. 2. Motility (percent of fast and slow cells) quantified after image analysis of video-recorded *Lingulodinium polyedrum* in cuvettes. Algal cells were incubated in filtrates from motility-inhibited cocultures, which were, in some cases, subsequently treated with the protease inhibitors Pefabloc®SC (PEFA), E-64, and PMSF. Additional treatments included 80° denaturation (80deg) and size fractionation with a 50 kDa filter. Bars indicate means and standard deviations of triplicate incubations. Statistically different treatments are pointed out in the text. ALC1, LPK5, and LPK13 refer to the bacterial strains with which *L. polyedrum* cells were initially incubated (Lp, axenic *L. polyedrum* control)

treatments. Serine-AMC and glycine-AMC were consistently higher in the ALC1 filtrate compared to the axenic *L. polyedrum* filtrates, but the magnitude of increase was minor (16% for both serine-AMC and glycine-AMC).

Pronase experiments. Before analyzing the motility data from algal cultures incubated with pronase E, we tested whether our analysis method could easily differentiate swimming and sinking cells. Frequency distributions of live, log phase *L. polyedrum* cells and formalin-fixed cells from the same culture were significantly different ($P < 0.0001$); thus, we were confident that we could statistically detect noticeable differences between treatments. The first motility evaluation was performed 5 min after addition of pronase E, and the cultures with the highest concentration added (36 $\mu g \cdot mL^{-1}$) were already swimming significantly slower than the controls (Fig. 3; $P < 0.0001$). This decrease was apparent throughout the 23 h experiment. Additions of 4-fold less pronase E led to decreased swimming speeds after 30 min ($P < 0.0001$), lasting throughout the experiment. Interestingly, additions of the lowest concentrations of pronase (1.8 $\mu g \cdot mL^{-1}$) led to an initial increase in motility, which lasted through the 30 min sampling ($P < 0.0001$). At the 8 and 23 h sampling times, the algal cells in those incubations

TABLE 1. Results of fluorescent protease activity assays (duplicates) from 0.22 μm filtrates of axenic *Lingulodinium polyedrum* and *L. polyedrum* incubated with motility-inhibiting bacterial strain ALC1.

Substrate	Axenic		ALC1	
Leucine-AMC	54.3	45.4	52.4	35.9
Serine-AMC	194.9	195.1	211.7	243.1
Glycine-AMC	9.42	10.04	11.27	11.26
Threonine-AMC	62.4	79.0	72.9	72.2

Values are reported in nmol substrate $\cdot L^{-1} \cdot min^{-1}$.
AMC, 7-amido-4-methylcoumarin.

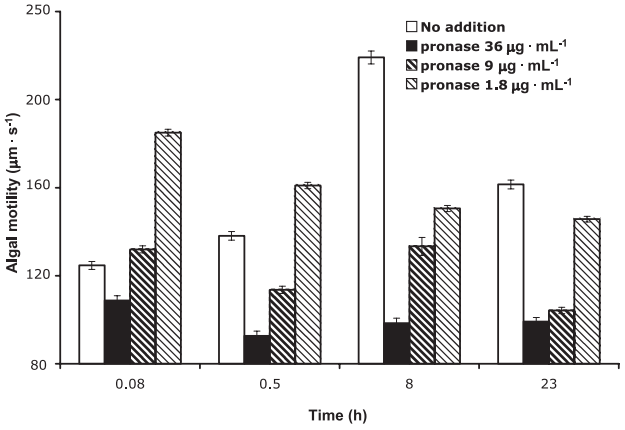

FIG. 3. Average speed of duplicate treatments of *Lingulodinium polyedrum* cells incubated with three different concentrations of pronase E, sampled at four different times after inoculation. Means and standard errors are reported (average N for each treatment, from two duplicate incubations, was ~4,000 measurements). Statistically different treatments are described in the text.

were swimming significantly slower than the controls, but significantly faster than the cells incubated with the higher pronase E concentrations ($P < 0.0001$).

DISCUSSION

Previous studies of bacterial interactions with phytoplankton have focused on easily observable effects, such as morphological change (e.g., cyst formation) or changes in growth rates (both positive and negative). Here we have revealed that some marine bacteria can excrete dissolved substances of high molecular weight that significantly decrease the swimming speed of the dinoflagellate *L. polyedrum*. Cell counts or fluorescence could not detect such changes in motility, and microscopic observation could only detect differences if they were extreme, such as when the algal cells were barely motile. Subtle differences in motility could be detected only after careful quantitative analysis of video files. A decrease of 10%–20% in instantaneous motility may not seem of great consequence; however, this change would either decrease the depth that a population of cells can migrate to or increase the time it takes to reach a particular depth. Either outcome will have important implications for the dinoflagellates' ability to compete with other phytoplankton since DVM is so critical to connect spatially dispersed nutrients and light under stratified conditions. Our results illustrate the idea that many potentially important interactions between heterotrophic bacteria and phytoplankton remain unobserved.

Our finding that the protease inhibitors Pefabloc®SC, PMSF, and E-64 differentially affected the motility-reducing filtrates suggests that the three bacterial strains tested did not excrete the same molecule(s). Strain LPK5 most likely excreted a serine protease, as Pefabloc®SC neutralized its motility-reducing component. Results of experiments with the other two bacterial strains were more ambiguous. ALC1 likely excreted one or more large proteins, possibly proteases as two protease inhibitors slightly counteracted motility reduction. LPK13 excreted both proteinaceous (>50 kDa, heat labile) and nonproteinaceous (<50 kDa, heat resistant) components, the former probably including a protease as PMSF significantly (but not fully) neutralized the motility reduction. Previous studies of algicidal bacteria have uncovered numerous types of algicidal agents, both proteinaceous (Baker and Herson 1978, Lee et al. 2000b) and nonproteinaceous (Dakhama et al. 1993, Skerratt et al. 2002), in accordance with our findings. It remains unknown whether our protease-mediated motility-reducing phenomenon is related to general algicidal activity, although this is likely. We do not believe that bacterial proteases specifically target motility in the dinoflagellates, but rather that motility reduction is a sign of an unhealthy status caused by protease activity on the cell as a whole.

Unexpectedly, we could not detect any difference in the protease activities (using four different exopeptidase substrates) between control and motility-reducing filtrates. We conclude that (i) axenic *L. polyedrum* cultures exhibited dissolved protease activity, and (ii) the motility-reducing proteases did not break down the exopeptidase substrates tested. The first conclusion is in accordance with previous work demonstrating that dinoflagellates produce protease activity (Stoecker and Gustafson 2003), although that study found the protease activity associated only with the cellular surface and not in the medium. Our second conclusion has some precedence since Obayashi and Suzuki (2005) determined that endopeptidase activity can be similar or higher than exopeptidase activity in surface seawater. Other protease substrates, particularly those specific for endopeptidases, might reveal strong differences between control and motility-reducing filtrates.

Additions of the purified serine-protease pronase E showed a concentration-dependent effect on *L. polyedrum* motility, and greater additions decreased motility faster. Previous work has shown that additions of pronase E increased diatom frustule dissolution (Bidle and Azam 1999) and decreased diatom cell aggregation (Smith et al. 1995) at concentrations of 1 and 0.1 $U \cdot mL^{-1}$, respectively. Here we have observed another effect of pronase E on phytoplankton with similar concentrations of added protease ($9\ \mu g \cdot mL^{-1}$ is equivalent to $0.045\ U \cdot mL^{-1}$). Since proteolytic activity is measurable in seawater (Obayashi and Suzuki 2005) and marine bacteria can have very high proteolytic rates (Martinez et al. 1996), it is likely that motility reduction by bacteria occurs in nature. These effects would be more significant during algal blooms, because bacterial abundances

(Gasol et al. 2005) and enzymatic activities of bacteria (Fandino et al. 2001) are elevated during such events. To corroborate the hypothesis that dissolved enzymes actively decrease algal motility in nature, future investigations might collect filtrate from field samples and test motility inhibition on laboratory cultures. In addition, a mechanistic understanding of proteolytic attack on dinoflagellates would help elucidate whether this phenomenon occurs in the environment. Regardless of the mechanism causing motility reduction, we suggest that decreased motility in dinoflagellates may be a useful marker for general unhealthy status, although more research on both internal and external factors that reduce flagellate motility will be necessary to establish this generalization.

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Supplementary Material

The following supplementary material is available for this article:

Table S1. Summary of Figure 2 data, indicating whether incubation of algal cells in treated filtrates strongly inhibited motility (−), had no effect (+), or weakly inhibited motility (±) compared to the control.

This material is available as part of the online article from: http://www.blackwell-synergy.com/doi/abs/10.1111/j.1529-8817.2008.00549.x.

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