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SHORT COMMUNICATION

Bacterial chemotaxis towards the extracellular products of the toxic phytoplankton *Heterosigma akashiwo*

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Marine bacteria exhibit positive chemotactic responses to the extracellular exudates of the toxic phytoplankton Heterosigma akashiwo. In the environment, this will support bacteria–algae associations with potential implications for harmful algal bloom dynamics.

The occurrence of harmful algal blooms (HABs), caused by toxic phytoplankton, has detrimental environmental, economic and human health effects within many aquatic habitats (Horner *et al.*, 1997). Understanding the environmental conditions that control the development and decline of HABs has consequently become an important element of ecosystem management within heavily affected environments. Potentially significant links between HAB dynamics and heterotrophic bacteria have been reported, with various species of aquatic bacteria implicated in both the promotion and inhibition of HABs (Kim *et al.*, 1998; Skerratt *et al.*, 2002; Liu *et al.*, 2008a, b). This has revealed the unseen complexity of HAB ecology, as well as arousing interest in the potential application of algicidal bacteria as biocontrols to terminate HABs (Kim *et al.*, 2007).

The raphidophyte phytoplankton *Heterosigma akashiwo* is a toxic species that has been implicated in several major fish kills worldwide (Honjo, 1993). The mechanism behind *H. akashiwo* toxicity is still unresolved, but increases in the toxic effects of this species have been linked to ecological interactions with heterotrophic bacteria (Carrasquero-Verde, 1999). Furthermore, heterotrophic bacteria can both enhance (Liu *et al.*, 2008a, b) and inhibit the growth of *H. akashiwo* (Kim *et al.*, 1998; Lovejoy *et al.*, 1998; Yoshinaga *et al.*, 1998; Liu *et al.*, 2008a, b), indicating that bacterial-algal interactions play a fundamental role in the ecology and environmental impact of this toxic species. Behavioural responses, including localized bacterial clustering around *H. akashiwo* cells (Lovejoy *et al.*, 1998), forming specific algal–bacterial consortia (Carrasquero-Verde, 1999), have been predicted to be an important element of these interactions. In this study, we examined whether the chemical products of *H. akashiwo* act as chemoattractants for three environmentally relevant strains of marine bacteria, to elucidate how behavioural

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responses may catalyse the ecological coupling between this toxic phytoplankton species and associated bacteria.

Axenic cultures of *Heterosigma akashiwo* (CCMP452) were grown in O3 medium (McIntosh and Cattolico, 1978) to mid-exponential phase. The extracellular exudates of *H. akashiwo* were obtained using the method of Bell and Mitchell (Bell and Mitchell, 1972). Cells were centrifuged for 5 min at 500 *g* and then gently filtered through sterile 0.2 μm membrane filters (Millipore). The culture filtrate (exudates) was then employed as a chemoattractant substrate for three environmentally relevant marine bacterial isolates. These included (i) *Pseudoalteromonas haloplanktis* (ATCC700530), a species previously shown to increase the toxicity of *H. akashiwo* (Carrasquero-Verde, 1999); (ii) *Vibrio alginolyticus* (12G01), a species shown to be inhibited by *H. akashiwo* (Oda *et al.*, 1997); and (iii) *Silicibacter sp.* (TM1040), a species known to establish close spatial associations with other phytoplankton species (Miller *et al.*, 2004).

Prior to experiments, bacteria were grown and prepared for chemotaxis experiments according to previously established protocols (Malmcrona-Friberg *et al.*, 1990; Mitchell *et al.*, 1996; Miller *et al.*, 2004). *Pseudoalteromonas haloplanktis* was grown in 1% Tryptic Soy Broth supplemented with 400 mM NaCl. Cells were harvested at mid-exponential growth phase and diluted 1:20 in artificial seawater (ASW) solution, before being starved at room temperature for 72 h (modified from Mitchell *et al.*, 1996). *Vibrio alginolyticus* was grown in *Vibrio* Nine Salt Solution (VNSS) to mid-exponential phase, centrifuged at 1500 *g* for 5 min and washed in Nine Salt Solution (NSS) (Malmcrona-Friberg *et al.*, 1990). *Silicibacter sp.* (TM1040) was grown in half-strength 2216 marine broth (Miller *et al.*, 2004) to mid-exponential phase, centrifuged at 1500 *g* for 5 min and washed in ASW.

A microfluidic chemotaxis assay, described in detail elsewhere (Seymour *et al.*, 2008), was used to study the chemotactic response of bacteria to the extracellular products of *H. akashiwo*. The microfluidic device consisted of a 45 mm long, 3 mm wide and 50 μm deep microchannel (Fig. 1A). Two in-line inlet ports were used to simultaneously introduce bacteria (inlet *1*) and chemoattractants (inlet *2*) into the channel, at a constant flow rate using a syringe pump. Inlet *2* led to a 100 μm wide microinjector, which produced a 300 μm wide band of chemoattractants in the centre of the channel. Bacteria were advected along either side of the chemoattractant band, until flow in the channel was stopped by turning off the syringe pump. At this point, the chemoattractant band was "released", spreading laterally at a rate set by molecular diffusion. The chemotactic response of the bacteria was then measured.

Positions of bacteria across the microchannel were visualized using an inverted microscope (Nikon TE2000e) and "movies" (200 frames) were recorded at 32 frames s^{-1} at 2 min intervals using a CCD camera (PCO 1600, Cooke, Romulus, MI, USA). Image analysis software (IPlab, BD Biosciences Bioimaging, MD, USA) was used to determine the mean distribution of bacteria across the channel (*x* direction). To compare the strength of chemotactic accumulation between experiments, cell concentrations were normalized with respect to the mean concentration in the outermost right 600 μm of the microchannel. A chemotactic index I_C, defined as the mean of the normalized cell

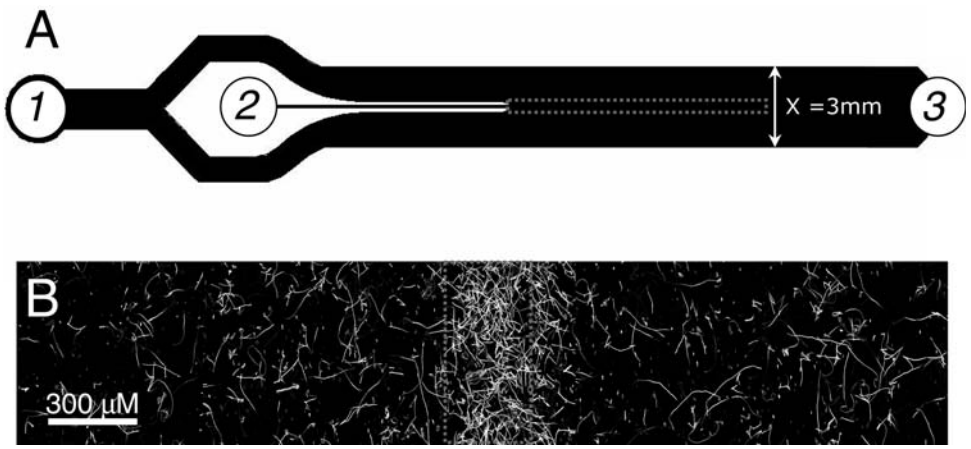

Fig. 1. (**A**) Schematic diagram of the microfluidic chemotaxis assay. Bacteria and chemoattractants were injected into the channel via inlets *1* and *2*, respectively. The channel outlet is denoted by *3*. (**B**) Swimming trajectories of *V. alginolyticus* bacteria within a band of *Heterosigma akashiwo* extracellular exudates, demonstrating accumulation of cells. Each white path is the trajectory of a single bacterium. Trajectories were obtained from a 6.2 s long movie recorded at 32.4 frames per second. Note, the orientation of (B) has been rotated 90° from (A). In both panels, the area bound by the grey dotted box corresponds to the approximate initial position of the band of injected *H. akashiwo* exudates.

Table I: Mean values of the chemotactic index I_C for heterotrophic bacteria responding to the extracellular exudates of Heterosigma akashiwo at different time points after flow in the microfluidic channel was stopped

Time (min)	*P. haloplanktis*	TM1040	*V. alginolyticus*
2	0.85 ± 0.1	3.6 ± 1.2	1.5 ± 0.4
4	0.95 ± 0.2	3.3 ± 0.9	2.0 ± 0.4
6	0.95 ± 0.2	2.4 ± 0.4	2.9 ± 0.5
8	1.04 ± 0.2	1.9 ± 0.2	3.3 ± 0.6
10	0.94 ± 0.01	1.5 ± 0.5	4.6 ± 1.3

Mean and standard deviation values were calculated from triplicate experiments.

concentration over the central 600 μm of the microchannel, corresponding to the position of the chemoattractant band, was also computed (Seymour *et al.*, 2008). $I_C = 1$ corresponds to a uniform cell distribution (no chemotaxis), while strong chemotaxis is characterized by larger values of I_C. Experiments were conducted in triplicate and the intensity, speed and duration of the chemotactic responses were compared between bacteria for each chemoattractant by comparing mean I_C values using *t*-tests.

Different degrees of chemotactic response were exhibited by the three strains of heterotrophic bacteria (Table I). On average, *V. alginolyticus* exhibited the strongest attraction to the *H. akashiwo* exudates, with cells accumulating within the centre of the microfluidic channel in correspondence with the position of the chemoattractant band (Fig. 1B). Within this central band, cell densities exceeded background concentrations by up to 6.5-fold (Fig. 2A). *Vibrio alginolyticus* attained a maximum mean I_C of 4.7 ± 1.3 SD, 10 min after the band of exudates was released. *Silicibacter sp.* TM1040 also exhibited a similar ($P > 0.05$) positive chemotactic response (Fig. 2B), reaching mean $I_C = 3.6 \pm 1.2$. While the strength of attraction was comparable, relative to *V. alginolyticus*, TM1040 reached maximum levels of chemotactic attraction much more rapidly, with peak cell concentrations within the chemoattractant band occurring after 2 min (Table I). In contrast, *P. haloplanktis* did not exhibit chemotactic attraction to the extracellular products of *H. akashiwo* (Fig. 2C), reaching a maximum I_C of only 1.0 ± 0.2 (Table I). This response by *P. haloplanktis* was significantly weaker ($P < 0.05$) than the responses of *V. alginolyticus* and *Silicibacter sp.* TM1040, and not distinguishable ($P > 0.05$) from the control experiment, where O3 growth medium was used as a chemoattractant. Alternatively, in the case of *V. alginolyticus* and *Silicibacter sp.* TM1040, no positive chemotactic response was exhibited in the control

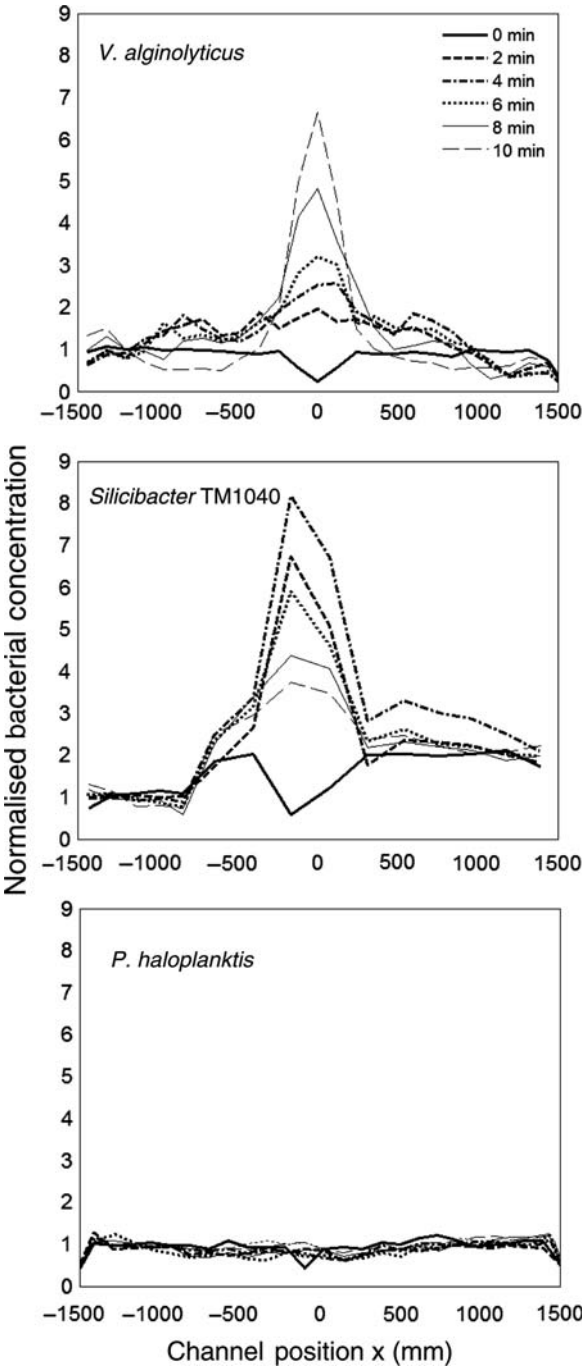

Fig. 2. Representative experimental distributions of bacteria across the width of the microfluidic channel at different times following the injection of a band of *Heterosigma akashiwo* exudates. The chemoattractant band was initially between $x = \pm 150$ μm, and $t = 0$ corresponds to the release of the chemoattractant band. Bacterial concentrations were normalized to the mean concentration measured in the 600 μm closest to the outer right wall of the microchannel.

experiments (mean $I_C = 1.1 \pm 0.2$), confirming that the significant ($P < 0.05$) chemotactic aggregation by *V. alginolyticus* and *Silicibacter sp.* TM1040 in the experimental

treatments occurred in response to the chemical products of *H. akashiwo*.

These experiments indicate that the chemical products of the toxic phytoplankton *H. akashiwo* act as chemoattractants for some species of marine bacteria. Bacterial chemotaxis to the chemical exudates of phytoplankton is widespread (Bell and Mitchell, 1972; Miller *et al*., 2004; Stocker *et al*., 2008) and plays an important role in aquatic microbial trophodynamics. Localized associations between heterotrophic bacteria and phytoplankton allow bacteria to gain enhanced exposure to the dissolved organic carbon (DOC) exuded by phytoplankton cells, while phytoplankton may receive increased remineralized inorganic nutrients from the bacteria (Azam and Ammerman, 1984). Alternatively, increased exposure to algicidal strains of bacteria can inhibit the growth of phytoplankton or kill them (Mayali and Azam, 2004).

Pseudoalteromonas strains are often algicidal (Skerratt *et al*., 2002; Mayali and Azam, 2004) and have been shown to kill *H. akashiwo* (Lovejoy *et al*., 1998). Furthermore, *P. haloplanktis* has been demonstrated to markedly increase the toxic effects of *H. akashiwo* on fish (Carrasquero-Verde, 1999). Hence, localized associations between *P. haloplanktis* and *H. akashiwo* may have implications at several levels. However, while we have previously found *P. haloplanktis* to exhibit strong chemotactic attraction to the exudates of diatoms, chlorophytes and cyanobacteria (Stocker *et al*., 2008; Seymour *et al*., 2008), no chemotactic response was observed here. While the reason for this lack of attraction is not clear, if *P. haloplanktis* has an algicidal effect on *H. akashiwo* like other *Pseudoalteromonas* strains, it is plausible that *H. akashiwo* may benefit from the production of chemicals that repel or inhibit the chemotaxis of this bacterium (Strom, 2008). Alternatively, the chemical constituents of *H. akashiwo* extracellular products may simply not match the chemoreceptive capabilities of *P. haloplanktis*.

Vibrio alginolyticus exhibited the strongest attraction to the *H. akashiwo* exudates. Interestingly, *H. akashiwo* has been demonstrated to inhibit the growth of *V. alginolyticus* through the production of reactive oxygen compounds (Oda *et al*., 1997). Our results indicate that the chemical products of *H. akashiwo* encourage attraction by *V. alginolyticus* despite this potential inhibitory effect.

Silicibacter TM1040, like other members of the *Roseobacter* clade, uses chemotaxis to form close spatial associations with dinoflagellates (Miller *et al*., 2004) and this behaviour significantly aids the growth of both the bacterium and the associated phytoplankton (Miller and Belas, 2006). We have also previously demonstrated that TM1040 exhibits chemotactic responses to the exudates of other phytoplankton species (Seymour *et al*., 2008). The positive chemotactic behaviour exhibited here is in line with these observations, but since no ecological links between members of the *Roseobacter* clade and *H. akashiwo* have previously been reported, the potential implications of the chemotactic attraction observed here remain open to further investigation.

The variability in the strength of chemotactic response exhibited between bacterial strains tested here could be driven by several factors. Differences in swimming speed and chemosensory capabilities will fundamentally influence how rapidly bacteria can exploit a diffusing nutrient source. Chemosensory sensitivities are also likely to be tuned to environmental parameters, with bacteria inhabiting more oligotrophic habitats, such as *Silicibacter*, potentially more sensitive to low concentrations of substrates. Finally, additional behavioural strategies, such as quorum sensing, may allow some strains of bacteria to enhance levels of accumulation following the initial detection of a diffusing nutrient source.

Rather than simply reflecting the growth response of bacteria to phytoplankton-derived DOC, it is becoming evident that associations between phytoplankton and heterotrophic bacteria are multi-faceted and complex (Mayali and Azam, 2004). This is particularly true within the context of HAB-forming toxic phytoplankton. The experiments presented here are the first to directly demonstrate that the chemical products of the toxic species *H. akashiwo* can provoke a strong behavioural response in some marine bacteria. This chemotactic behaviour may facilitate tight spatial coupling between bacteria and *H. akashiwo*, which in the environment will have potential implications for the growth and toxicity of this HAB forming species. These observations indicate the need for further *in situ* investigations into the composition of bacterial communities occurring in association with HAB forming phytoplankton and the subsequent influence of bacterial populations on the toxicity of species such as *H. akashiwo*.

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