

Chemotactic response of marine bacteria to the extracellular products of *Synechococcus* and *Prochlorococcus*

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ABSTRACT: The cyanobacterial genera *Prochlorococcus* and *Synechococcus* are key phototrophic organisms in the open ocean, and ecological interactions between these groups and heterotrophic bacteria have fundamental importance for marine carbon and nutrient cycling. We applied a microfluidic chemotaxis assay to study the chemotactic response of 3 marine bacterial isolates (*Pseudoalteromonas haloplanktis*, *Vibrio alginolyticus* and *Silicibacter* sp. TM1040) to the chemical products of *Synechococcus elongatus* and *Prochlorococcus marinus* MED 4Ax. In the ocean, chemical products from these cyanobacteria may be released into the water column via extracellular exudation or cell lysis. Strong and rapid chemotactic responses by all 3 bacterial strains occurred in reaction to the *Synechococcus* products. *P. haloplanktis* cells exhibited the strongest attraction, accumulating within the chemoattractant band in concentrations of up to 9-fold above background levels. Positive chemotaxis to *Prochlorococcus* chemical products also occurred, with *P. haloplanktis* and *Silicibacter* sp. TM1040 exhibiting the strongest responses. These observations indicate that marine bacteria can exhibit behavioural responses to the chemical products of *Synechococcus* and *Prochlorococcus*, which may support ecological associations between these important populations of marine prokaryotes in the environment.

KEY WORDS: Chemotaxis · Cyanobacteria · Heterotrophic bacteria · DOC

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INTRODUCTION

Marine bacteria are predicted to cluster around living phytoplankton cells, to take advantage of microzones of exuded dissolved organic carbon (DOC; Azam & Ammerman 1984), and to aggregate within patches of DOC released from lysed phytoplankton cells (Blackburn et al. 1998). This active clustering requires chemotaxis, i.e. the ability to direct movement in response to chemical stimuli. Chemotaxis is a common phenotype among marine bacteria (Malmcrona-Friberg et al. 1990, Barbara & Mitchell 2003a), and the chemical products of phytoplankton are potent chemoattractants for natural communities (Bell & Mitchell

1972) and isolated species (Miller et al. 2004) of bacteria. As a consequence of chemotactic behaviour, heterotrophic bacteria often occur in close spatial associations with phytoplankton cells (Alavi et al. 2001, Barbara & Mitchell 2003b). Chemotaxis can therefore play an important role in bacteria-phytoplankton interactions, which in turn shape carbon and nutrient cycling in the upper ocean.

While tight ecological associations between bacteria and eukaryotic phytoplankton are well established, prokaryotic phytoplankton are dominant across much of the open ocean. In particular, the 2 cyanobacterial genera *Prochlorococcus* and *Synechococcus* often constitute the bulk of photosynthetic biomass and are

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responsible for a significant, but variable, proportion of primary production in oligotrophic waters (Waterbury et al. 1979, Chisholm et al. 1988, Vaultot et al. 1995, Fernández et al. 2003). Cyanobacteria exude DOC (Paerl & Pinckney 1996, Bertilsson et al. 2005) and as a consequence of bacterial chemotaxis, spatial associations between heterotrophic bacteria and other species of cyanobacteria occur in aquatic habitats (Paerl & Galucci 1985, Stevenson & Waterbury 2006). However, no quantitative evidence for chemotaxis by heterotrophic bacteria towards the chemical products of *Prochlorococcus* and *Synechococcus* populations has yet been reported. An assessment of the behavioural relationships between these important functional units of the microbial food web will aid in obtaining an understanding of how they co-exist, cooperate, or compete in the ocean.

MATERIALS AND METHODS

Heterotrophic bacteria. Three marine bacterial species, including 2 gammaproteobacteria, *Pseudoalteromonas haloplanktis* (ATCC700530) and *Vibrio alginolyticus* (12G01), and 1 alphaproteobacterium, *Silicibacter* sp. (TM1040), were grown to mid-exponential phase and prepared for chemotaxis studies using previously established protocols. *P. haloplanktis* was grown in 1% tryptic soy broth (TSB; Difco) supplemented with 400 mM NaCl. Cells were then diluted 1:20 in artificial seawater (ASW), before being starved for 72 h (Mitchell et al. 1996). *Silicibacter* sp. (TM1040) was grown in half-strength 2216 marine broth (Miller et al. 2004), centrifuged at $1500 \times g$ for 5 min and washed in ASW. *V. alginolyticus* was grown in *Vibrio* nine salt solution (VNSS), centrifuged at $1500 \times g$ for 5 min and washed in NSS (Malmcrona-Friberg et al. 1990).

Cultivation of *Synechococcus* and *Prochlorococcus* extracellular products. Axenic cultures of *Synechococcus elongatus* (CCMP 1630) and *Prochlorococcus marinus* strain MED 4Ax were grown in f/2 medium and Pro99 medium, respectively, at 20°C, under constant light conditions ($36 \mu\text{E m}^{-2} \text{s}^{-1}$) to mid-exponential growth phase. The extracellular products (culture filtrates) of each strain were obtained using the methods of Bell & Mitchell (1972). Cells were centrifuged for 5 min at $500 \times g$, and 5 ml of a 10 ml total volume were then gently filtered through a syringe-mounted 0.2 μm filter (Pall). To determine the concentration of DOC in each culture filtrate, the filtrate was acidified with phosphoric acid to pH 2, sparged with compressed air to remove inorganic carbon, and measured with a Shimadzu TOC-5000 total carbon analyser. The filtrate, containing the extracellular pro-

ducts, was then employed as a chemoattractant for bacteria. While it is possible that during the filtration process some cell lysis may have occurred, cyanobacterial lysis products, occurring as a result of viral infection (Suttle & Chan 1993, Sullivan et al. 2003), are also likely to represent a significant source of DOC in the environment. For each bacterial species, single control experiments were performed using f/2 and Pro99 media, pre-filtered through 0.2 μm membrane filters, to ensure that the cyanobacterial growth media, or extracts from membrane filters, did not provoke chemotactic responses by any of the bacteria.

Chemotaxis assay and image analysis. A microfluidic chemotaxis assay, described in detail elsewhere (Seymour et al. 2008), was applied to study the chemotactic response of the 3 bacterial species to the extracellular chemical products of *Synechococcus* and *Prochlorococcus*. The assay employed a 45 mm long, 3 mm wide and 50 μm deep microchannel (Fig. 1). Two inline inlet ports simultaneously introduced bacteria (Inlet A) and chemoattractants (Inlet B) into the channel, at $240 \mu\text{m s}^{-1}$ using a syringe pump. Inlet B was joined to a 100 μm wide microinjector, which generated 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

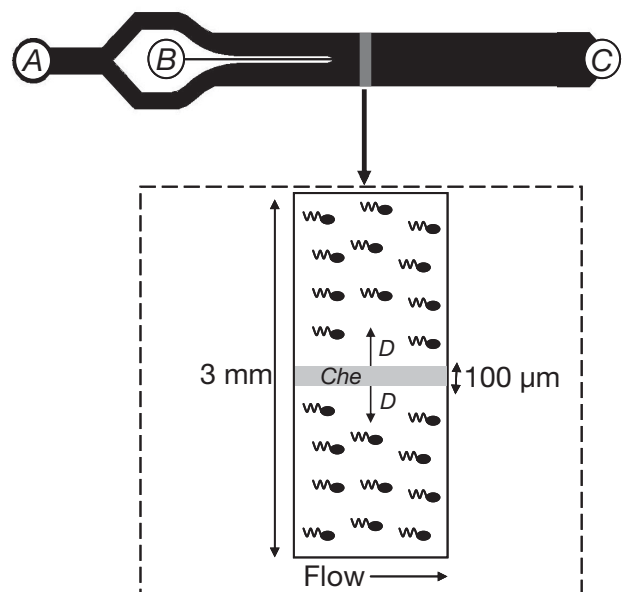


Fig. 1. Microfluidic chemotaxis assay. Bacteria and chemoattractants were injected into the channel via Inlets A and B, respectively. C denotes the channel's outlet. The grey box represents the position 3 mm downstream of the microinjector's tip where data were acquired. Inset: Magnified schematic of the channel region denoted by the grey box, showing the initial conditions within the channel, including the initial position and width of the chemoattractant band (Che), the direction of flow, and the direction of diffusion of the chemoattractant band (D) at the onset of the experiment

the channel was stopped when the syringe pump was disengaged. At this point, fluid flow stopped instantaneously and the chemoattractant band spread laterally at a rate set by molecular diffusion, allowing for the chemotactic response of the bacteria to be measured in flow-free conditions.

An inverted microscope (Nikon TE2000e) was used to visualise the positions of bacteria across the channel, and 'movies' (sequences of 200 frames recorded at 32.4 frames s^{-1}) were captured at 2 min intervals using a 1600 × 1200 pixel CCD camera (PCO 1600, Cooke). Experiments were run for 10 to 12 min, which is consistent with the time period within which maximum chemotactic accumulations of bacteria within the channel return to uniformity as a consequence of the diffusion and uptake of the chemoattractant band (Stocker et al. 2008, Seymour et al. 2009). With the aid of image analysis software (IPLab, BD Biosciences Bioimaging), the mean distribution of bacteria across the channel (x direction) was recorded at each time point.

Data analysis. To measure the strength of the chemotactic response and compare treatments using a single quantitative parameter, we computed a chemotactic index $I_C = C_C/C_O$, where C_C is the mean cell concentration in the central 600 μm of the microchannel, corresponding to the position of the chemoattractant band, and C_O is the mean cell concentration in the outermost 600 μm of the channel (corresponding to the upper 600 μm in Fig. 1) (Seymour et al. 2008). A uniform cell distribution (no chemotaxis) yields $I_C = 1$, whereas strong chemotaxis is characterised by large values of I_C . Experiments were conducted in triplicate, and the intensity of the chemotactic responses was compared between bacteria for each chemoattractant by comparing I_C values.

Data were tested for normality using the Kolmogorov-Smirnov test, and where normality was satisfied, I_C values for different bacteria were compared using analysis of variance (ANOVA), coupled with Tukey test post hoc analysis. In cases where data were not normally distributed, or the sample size was small, the non-parametric Mann-Whitney test was employed.

RESULTS AND DISCUSSION

Occurrence of experimental organisms in the environment

To determine whether the bacterial species used here are representative of those occurring in the oligotrophic ocean, where *Prochlorococcus* and *Synechococcus* dominate, we performed an examination of 15 randomly selected open ocean sites, spanning the Atlantic, Indian and Pacific Oceans, from the Global

Ocean Sampling (GOS) data set (Rusch et al. 2007). The Metagenome Rapid Annotation Subsystem Technology (MG-Rast) system was used to Blast protein encoding genes using the SEED framework (Overbeek et al. 2005, Meyer et al. 2008), using a maximum e value of 10^{-5} and a minimum alignment length of 50 bp. Hits for *Silicibacter* TM1040 and *Pseudoalteromonas haloplanktis* occurred within 100% of the open ocean metagenomes examined, while hits for *Vibrio alginolyticus* occurred in 93% of metagenomes. This indicates that the bacterial strains used here widely occur in the oligotrophic open ocean and are likely to co-occur with *Synechococcus* and *Prochlorococcus* in the environment.

Chemotactic responses to *Synechococcus* extracellular exudates

Synechococcus elongatus is a comparatively large (1 to 3 μm) species of *Synechococcus*, which occurs widely in both open ocean and coastal environments, typically accounting for ~10% of total *Synechococcus* hits in our analysis of GOS samples. In the chemotactic experiments, the extracellular products of *S. elongatus* had a mean DOC concentration of 279 ± 91 (SD) μM and elicited a strong and rapid chemotactic response by each of the 3 bacterial isolates (Figs. 2b & 3, Table 1). Dense accumulations of each of the heterotrophic bacterial species occurred within the centre of the microfluidic channel, corresponding to the position of the chemoattractant band, within 2 to 4 min (Fig. 2b). A strong and rapid response was exhibited by *Pseudoalteromonas haloplanktis*, which has previously been shown to exhibit chemotactic responses to the extracellular products of eukaryotic phytoplankton (Seymour et al. 2009). *P. haloplanktis* cells accumulated within the band of *S. elongatus* exudates in concentrations of up to 9-fold above background levels (Fig. 3a), reaching a mean I_C value of 6.7. This high level of attraction was not significantly different ($p > 0.05$) from that exhibited by *Silicibacter* TM1040, which also strongly responded to the *S. elongatus* filtrates, reaching a maximum I_C value of 5.9. *Vibrio alginolyticus* reached a maximum I_C value of 3.4, which was significantly different ($p < 0.05$) and weaker than the responses of both other bacteria. No evidence for positive chemotaxis was exhibited by any of the bacterial strains to the control case of f/2 media ($I_C = 0.7$ to 1.3; e.g. Figs. 2a & 3d), and in each case the chemotactic response to the *Synechococcus* products was significantly different ($p < 0.05$) from the control.

The rapid positive responses exhibited by all 3 bacterial strains here indicate that the extracellular products of *Synechococcus elongatus* contain chemical con-

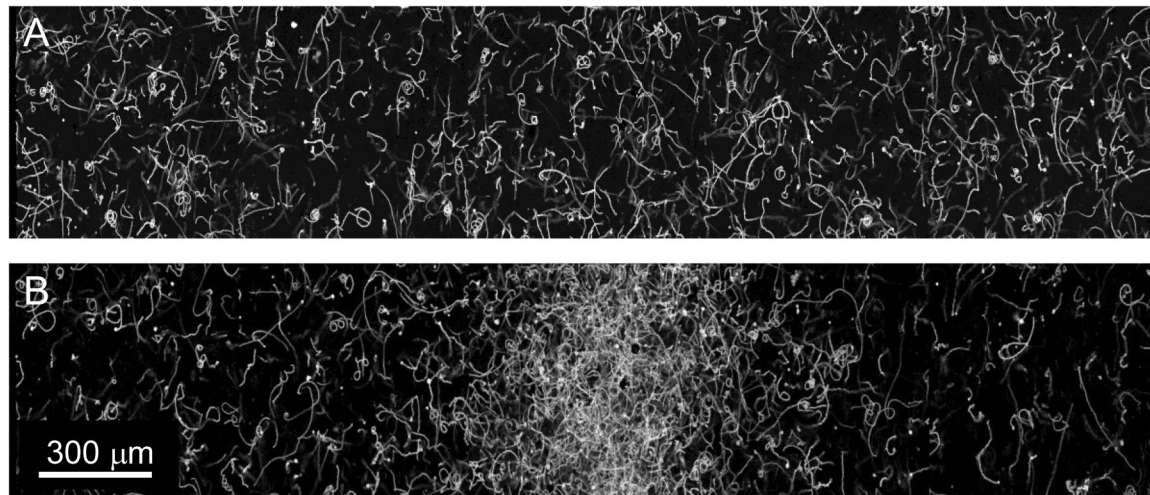


Fig. 2. *Silicibacter* sp. TM1040. (A) Swimming trajectories of TM1040 cells across the microfluidic channel following injection of *f/2* growth medium as a chemoattractant (control). Each white path is the trajectory of a single bacterium. (B) Swimming trajectories of TM1040 cells within a band of *Synechococcus elongatus* extracellular products, demonstrating strong accumulation in the band of chemoattractant. Note that the orientation has been rotated 90° from Fig. 1

stituents that act as strong chemoattractants for these marine bacteria. However, we observed marked differences in the speed of the response, indicating differences in the chemotactic velocity of the 3 bacterial species (see below), with *Pseudoalteromonas haloplanktis* reaching maximum accumulation within the patch within 2 min, compared to 4 and 8 min for TM1040 and *Vibrio alginolyticus*, respectively (Table 1).

Chemotactic responses to *Prochlorococcus* extracellular exudates

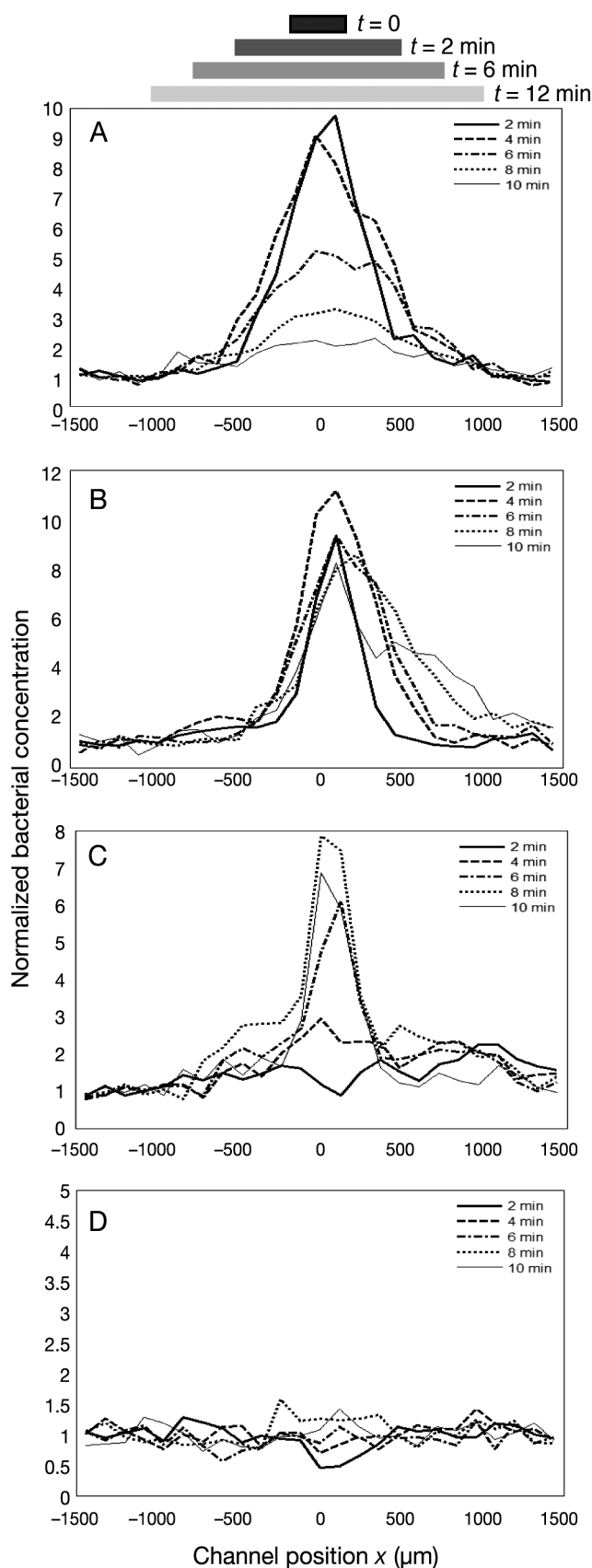
Two of the 3 strains of heterotrophic bacteria also exhibited clear chemotactic responses towards the extracellular products of *Prochlorococcus marinus* (MED 4Ax; Fig. 4). *Silicibacter* TM1040, which has previously been shown to use chemotaxis to establish close ecological associations with phytoplankton

(Miller et al. 2004), reached a mean maximum I_C value of 3.1. *Pseudoalteromonas haloplanktis* also exhibited a positive chemotactic response ($I_C = 2.4$), which again did not differ significantly from *Silicibacter* TM1040 ($p > 0.05$). In contrast, significantly different ($p < 0.05$) and weaker responses were exhibited by *Vibrio alginolyticus*, which in some cases showed a short-lived minor response (Fig. 4c), but typically displayed I_C values only marginally above 1 (Table 1) and not significantly different ($p > 0.05$) from the control case, where Pro99 medium was used as a chemoattractant ($I_C = 0.9$ to 1.4).

In line with the *Synechococcus* filtrate experiments, *Pseudoalteromonas haloplanktis*, once again reached maximum accumulation levels faster than the other 2 bacterial strains (Table 1). Variability in response time is driven by differences in the chemotactic velocity of the bacterial populations (Rivero et al. 1989), which is a fraction (typically 5 to 35%, Ahmed & Stocker 2008) of

Table 1. Mean values of the chemotactic index I_C for heterotrophic bacteria responding to the extracellular chemical products of *Synechococcus* and *Prochlorococcus*. Mean \pm SD were calculated from triplicate experiments. Time: time elapsed since release of the chemoattractant

Time (min)	<i>Synechococcus</i>			<i>Prochlorococcus</i>		
	<i>Pseudoalteromonas haloplanktis</i>	<i>Silicibacter</i> sp. TM1040	<i>Vibrio alginolyticus</i>	<i>Pseudoalteromonas haloplanktis</i>	<i>Silicibacter</i> sp. TM1040	<i>Vibrio alginolyticus</i>
2	6.7 \pm 1.8	4.2 \pm 1.4	1.2 \pm 0.2	2.4 \pm 0.1	2.7 \pm 0.5	1.2 \pm 0.3
4	6.6 \pm 1.2	5.9 \pm 2.4	1.9 \pm 0.4	2.0 \pm 0.3	3.1 \pm 0.9	1.2 \pm 0.7
6	4.2 \pm 1.0	5.6 \pm 2.9	2.7 \pm 0.9	1.8 \pm 0.2	2.2 \pm 0.4	0.9 \pm 0.4
8	2.7 \pm 0.3	4.1 \pm 1.8	3.4 \pm 2.4	1.5 \pm 0.3	2.1 \pm 0.5	0.6 \pm 0.1
10	2.0 \pm 0.2	3.6 \pm 1.5	3.3 \pm 1.1	1.3 \pm 0.1	1.7 \pm 0.3	0.7 \pm 0.2



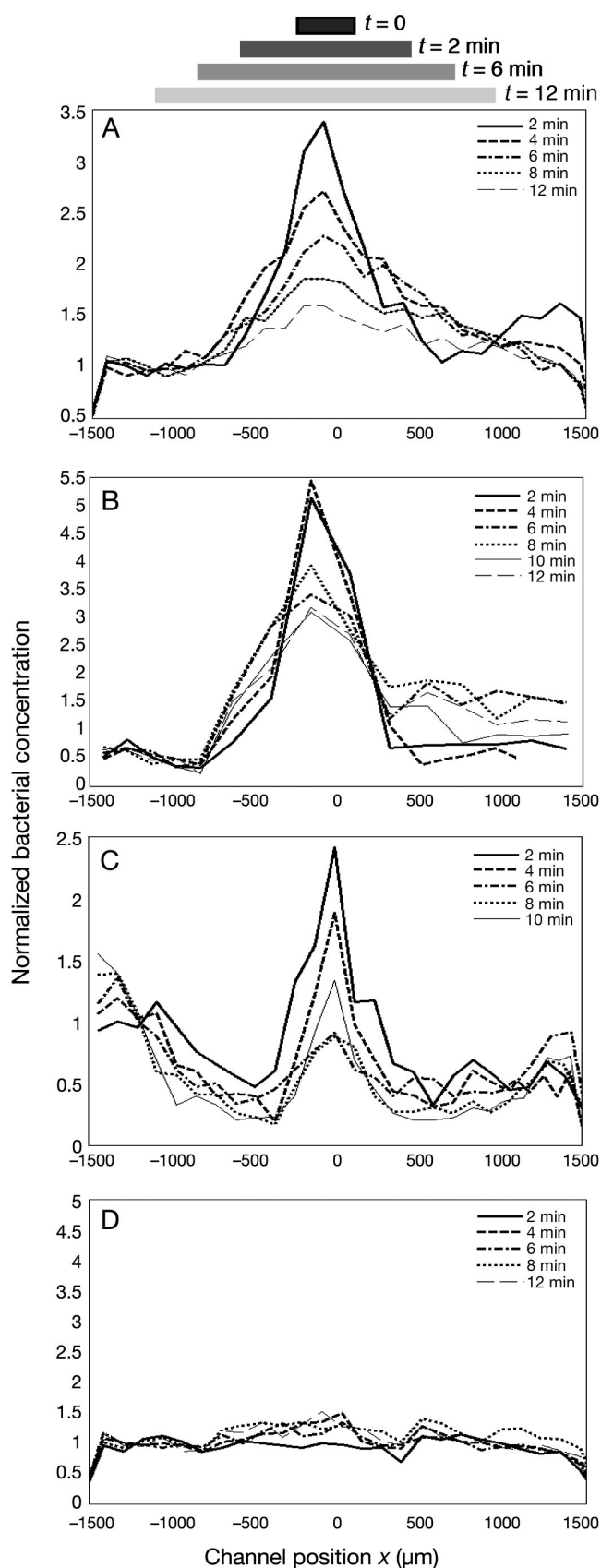
the bacterial swimming speed, but also depends on chemoreceptor sensitivity. The mean swimming speed of *P. haloplanktis* ($85 \mu\text{m s}^{-1}$) was substantially higher than both *Silicibacter* TM1040 ($52 \mu\text{m s}^{-1}$) and *Vibrio alginolyticus* ($54 \mu\text{m s}^{-1}$), indicating that swimming speeds indeed had a bearing on the rate of the chemotactic response. This supports the hypothesis that where nutrient pulses are rapidly dissipated by physical processes including diffusion and turbulence (e.g. the ocean), fast swimming speeds will be advantageous for rapid exploitation by bacteria (Stocker et al. 2008).

Ecological implications

The experiments presented here provide the first quantitative evidence for bacterial chemotaxis in response to the chemical products of the important marine genera *Synechococcus* and *Prochlorococcus*. The extracellular products of *Synechococcus* and *Prochlorococcus* are primarily composed of low molecular weight DOC compounds, which are highly labile substrates for heterotrophic bacteria (Chang 1981, Bertilsson et al. 2005). The release of these chemical products into the water column may occur as a consequence of both extracellular exudation and cell lysis. Particularly in oligotrophic waters, the chemical products from these cyanobacterial groups are expected to represent an essential pool of DOC for heterotrophic bacterial growth (Bertilsson et al. 2005). Therefore, chemotactic responses to the DOC released from *Synechococcus* and *Prochlorococcus* cells may provide a strong competitive advantage for some heterotrophic bacteria, which will gain exposure to elevated nutrient concentrations before their competitors (Stocker et al. 2008).

In the dynamic ocean environment, however, physical constraints must be considered when extrapolating chemotaxis to potential localised associations of cells.

Fig. 3. Representative distributions of bacteria across the width of the microfluidic channel at different times following the injection of a band of *Synechococcus elongatus* filtrates. The chemoattractant band was initially between $x = \pm 150 \mu\text{m}$, and $t = 0$ corresponds to the release of the chemoattractant band when flow in the channel was stopped. Bacterial concentrations were normalised to the mean concentration measured in the $600 \mu\text{m}$ closest to the right wall of the microchannel. (A) *Pseudoalteromonas haloplanktis*, (B) *Silicibacter* sp. TM1040, (C) *Vibrio alginolyticus*, (D) an example of control experiment, where TM1040 bacteria were presented with a band of f/2 growth medium, demonstrating the lack of chemotactic response. Bars above panel (A) represent the width of the diffusing chemoattractant band at different times during the experiment



It has previously been calculated that the smallest nutrient microzone that a swimming bacterium could detect in the ocean would correspond to a 2 μm radius phytoplankton cell (Jackson 1987). *Prochlorococcus* and *Synechococcus* cells are typically only 0.6 to 1.5 μm . However, these previous calculations considered a bacterium with the swimming characteristics of *Escherichia coli*. It is now recognised that marine bacteria, including the strains studied here, exhibit markedly different chemotactic behaviours than *E. coli* (Barbara & Mitchell 2003a, Stocker et al. 2008). Mitchell et al. (1996) suggested that the rapid reaction times exhibited by marine bacteria allow them to sense and cluster within nutrient patches associated with cells down to the size of *Prochlorococcus* and *Synechococcus*.

Using atomic force microscopy, Malfatti & Azam (2009) recently demonstrated that localised associations between individual *Synechococcus* cells and heterotrophic bacteria can indeed commonly occur in the ocean. Such microscale associations may have significant implications for nutrient cycling rates and microbial competition. The chemotactic behaviour exhibited here provides a potential mechanism for the development of these associations.

The strength of bacterial chemotactic responses in the environment will ultimately depend upon the concentration of labile organic material within the cyanobacterial chemical products. In our experiments, the mean DOC concentration in the *Synechococcus* and *Prochlorococcus* culture filtrates was 279 and 133 μM , respectively. While these DOC values are about 3- to 5-fold higher than open ocean bulk concentrations (Wiebinga & de Baar 1998, Doval et al. 2001), they are well within the range of DOC concentrations observed during phytoplankton blooms (Gobler et al. 2004, Packard et al. 2000). In the environment, associations between heterotrophs and cyanobacteria occur in a microenvironmental scenario, where the concentrations of exudates near the surfaces of cells will be substantially higher than in the bulk phase. Furthermore, there is both *in situ* (Seymour et al. 2005) and experimental (Koblíček et al. 2000) evidence for the existence of localised aggregations of *Synechococcus* cells, which will generate larger and more concentrated clouds of DOC than are produced by individual cells (Rothschild et al. 1999). The DOC concentrations

Fig. 4. As in Fig. 3, but following the injection of a band of *Prochlorococcus* MED 4Ax filtrates. (A) *Pseudoalteromonas haloplanktis*, (B) *Silicibacter* sp. TM1040, (C) *Vibrio alginolyticus*, (D) an example of control experiment, where TM1040 bacteria were presented with a band of Pro99 growth medium, demonstrating the lack of chemotactic response

in these scenarios are likely comparable to, or higher than, the DOC concentrations applied in these experiments, and we propose that the strong behavioural responses observed here are also likely to occur in the environment. These observations, along with recent demonstrations of microscale associations (Seymour et al. 2005, Malfatti & Azam 2009) and evidence of mutualistic relationships (Morris et al. 2008), suggest the need for a closer examination of the ecological and biogeochemical ties between heterotrophic bacteria and these 2 important groups of marine cyanobacteria.

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