Environmental Microbiology (2006) **8**(9), 1648–1659 doi:10.1111/j.1462-2920.2006.01071.x

Motility is involved in *Silicibacter* sp. TM1040 interaction with dinoflagellates

Todd R. Miller[†] and Robert Belas*
Center of Marine Biotechnology, University of Maryland Biotechnology Institute, 701 East Pratt Street, Baltimore, MD 21202, USA.

Summary

***Silicibacter* sp. TM1040, originally isolated from a culture of the dinoflagellate *Pfiesteria piscicida*, senses and responds to the dinoflagellate secondary metabolite dimethylsulfoniopropionate (DMSP) by flagella-mediated chemotaxis behaviour. In this report we show that swimming motility is important for initiating the interaction between the bacterium and dinoflagellate. Following transposon mutagenesis, three mutants defective in wild-type swimming motility (Mot$^-$) were identified. The defects in motility were found to be in homologues of *cckA* and *ctrA*, encoding a two-component regulatory circuit, and in a novel gene, *flaA*, likely to function in flagellar export or biogenesis. Mutation of *flaA* or *cckA* results in the loss of flagella and non-motile cells (Fla$^-$), while CtrA$^-$ cells possess flagella, but have reduced motility due to increased cell length. All three Mot$^-$ mutants were defective in attaching to the dinoflagellate, particularly to regions that colocalized with intracellular organelles. The growth rate of the dinoflagellates was reduced in the presence of the Fla$^-$ mutants compared with Fla$^+$ cells. These results indicate that bacterial motility is important for the *Silicibacter* sp. TM1040–*P. piscicida* interaction.**

Introduction

In marine environments, algae interact with a diverse community of heterotrophic bacteria that compete for carbon or other nutrients produced by algal populations and regenerate essential inorganic nutrients for algal growth. These processes are essential for the cycling of organic matter in the ocean and drive global nutrient cycles. Some bacteria may utilize specialized behaviours to compete for algal-derived nutrients including chemotaxis towards specific algal compounds, production of lytic substances, and attachment or invasion of algal cells. It is clear that not all bacteria possess these traits and some are more likely to survive in an algal niche than others.

Bacteria phylogenetically related to the *Roseobacter* clade are an abundant group of marine bacteria that are associated with algae in both laboratory and field studies (Lafay *et al*., 1995; Prokic *et al*., 1998; Alavi *et al*., 2001; Hold *et al*., 2001; Zubkov *et al*., 2001a). Although many of the bacterial taxa abundant in the marine environment have never been cultured, the *Roseobacter* clade is an exception and can be readily cultured in the laboratory. Most roseobacters are motile via one or more flagella (Gonzalez *et al*., 2003; Miller *et al*., 2004). These bacteria are the major consumers of the algal osmolyte, dimethylsulfoniopropionate (DMSP), which may serve as a sole source of carbon and sulfur for some *Roseobacter* species (Gonzalez *et al*., 1999). In addition, the production and activity of *Roseobacter* species in the environment is significantly correlated with DMSP-producing algal blooms, especially those of dinoflagellates and prymnesiophytes (Gonzalez and Moran, 1997; Zubkov *et al*., 2001b). Furthermore, some *Roseobacter* species exhibit close physical (attached and intracellular) or physiological (growth-enhancing) relationships with DMSP-producing dinoflagellates, including *Prorocentrum* (Lafay *et al*., 1995), *Alexandrium* (Dantzer and Levin, 1997; Gallacher *et al*., 1997) and *Pfiesteria* species (Alavi *et al*., 2001; Miller and Belas, 2004).

Silicibacter sp. strain TM1040 is a member of the *Roseobacter* clade and was isolated from a culture of the dinoflagellate *Pfiesteria piscicida* (Alavi *et al*., 2001; Miller and Belas, 2003). This bacterium degrades DMSP produced by *P. piscicida* via a demethylation pathway to produce 3-methylmercaptopropionate (MMPA) as a major breakdown product (Miller and Belas, 2004). *Silicibacter* sp. TM1040 is actively motile by means of two to three lophotrichous flagella and is chemotactic towards DMSP, MMPA, and amino acids produced by *P. piscicida* as well as towards other unidentified products of dinoflagellate metabolism (Miller and Belas, 2004).

In this report we show that *Silicibacter* sp. TM1040 is an attached and/or intracellular bacterium that promotes

Received 1 February, 2006; accepted 11 May, 2006. *For correspondence. E-mail belas@umbi.umd.edu; Tel. (+1) 410 234 8876; Fax (+1) 410 234 8896.[†]Present address: Johns Hopkins University, 615 N., Wolfe Street, Baltimore, MD 21205, USA.

growth of *P. piscicida* in laboratory cultures. To strengthen our understanding of this interaction, a library of random transposon insertional mutants was screened, for non-motile mutants, and three of the mutants were tested for their ability to physically interact with the dinoflagellate and to promote dinoflagellate growth. The results indicate that swimming motility is important for the development of the *Silicibacter* sp. TM1040–*P. piscicida* interaction.

Results and discussion

Visualization and localization of Silicibacter *sp. TM1040 on dinoflagellate cells*

Previous reports (Alavi *et al*., 2001) have shown that roseobacters in the community associated with *P. piscicida* physically interact with and attach to the dinoflagellate cell. The interaction of *Silicibacter* sp. TM1040 with *P. piscicida* was therefore measured by microscopic imaging of *Silicibacter* sp. TM1040 bacteria that had been pre-stained with a fluorescent dye (CFDA/SE). Pairs of fluorescent and phase-contrast images of the identical microscope field containing dinoflagellate zoospores and bacteria were superimposed and analysed by computer image analysis to determine the number and location of *Silicibacter* sp. TM1040 cells on each dinoflagellate. Figure 1 shows a single zoospore with several attached bacteria (Fig. 1B), and is representative of the dataset. When Fig. 1A and B are superimposed the location of the bacteria relative to the dinoflagellate cell is revealed (Fig. 1C). An analysis of these data indicates that the interaction of *Silicibacter* sp. TM1040 with *P. piscicida* results in a close physical association between the bacteria and the dinoflagellate. In this study, bacteria were often observed on the dinoflagellate periphery (Fig. 1C), and surprisingly were also found at sites that colocalized within the zoospore. These hint that the bacteria may also find their way to locations beneath the surface of the dinoflagellate, either in deep grooves on the outer surface of the host cell, or in intracellular spaces internal to the dinoflagellate.

To obtain a more precise location of *Silicibacter* sp. TM1040 cells when they interact with the dinoflagellate, a series of Z-section optical images using confocal scanning laser microscopy (CSLM) were obtained. The optical sections were combined into a composite image in which the objects were colour-coded based on their Z-axis distance (depth). Figure 2 shows a representative image from this analysis. In these images, green pixels represent objects that are close to the proximal side of the dinoflagellate cell surface (0–2 μm), blue tints denote objects on the distal side of the dinoflagellate (10–12 μm), and a pink or red colour is used for objects in the centre of the zoospore (2–8 μm). As previously shown in Fig. 1, bacteria were found on the periphery of the dinoflagellate, but were also frequently observed at sites that colocalize to areas between the proximal and distal surfaces of the dinoflagellate. For example, in Fig. 2, at least six bacteria colocalize with the Z-axis centre (5–10 μm below the surface) of the epitheca (upper hemisphere) or the hypotheca (lower hemisphere) of the zoospore.

The CSLM image analysis suggests that *Silicibacter* sp. TM1040 may be within the cytoplasm of the dinoflagellate. However, there are other interpretations of these data. One remote possibility is that the bacteria have been ingested by the dinoflagellates and are in the food vacuole. This is unlikely, as we have not seen bacterial cells within food vacuoles using other methods, a result that

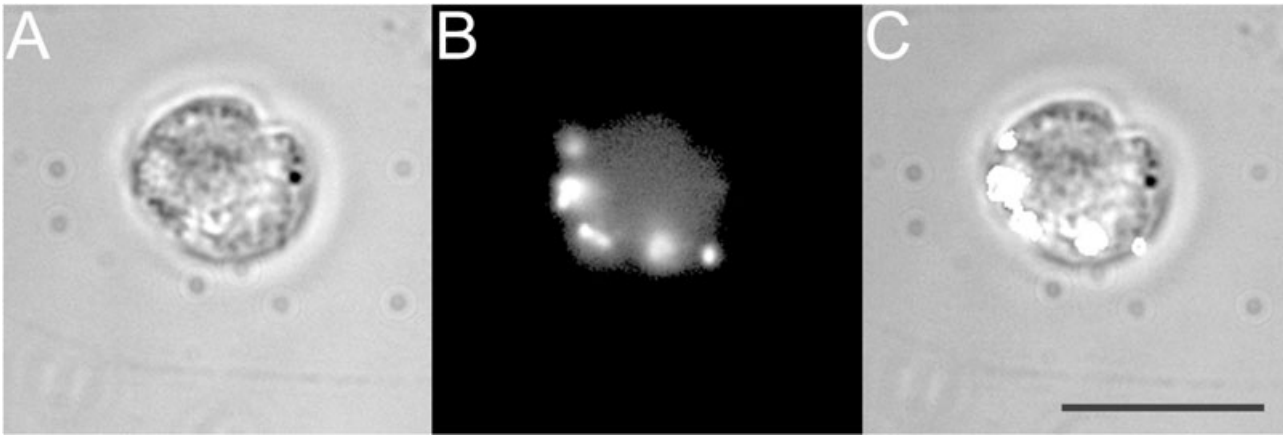

Fig. 1. Visualization and colocalization of *Silicibacter* sp. TM1040 cells interacting with *P. piscicida*. Bacteria were pre-stained with a fluorescent tracer dye and added to washed *P. piscicida* zoospores. After 2 h, samples were removed, chemically fixed and viewed by phase contrast (A) and fluorescence microscopy (B). (C) The phase-contrast and fluorescent images of the same specimen were overlaid, and the bacteria colocalized with the dinoflagellate cells as described in *Experimental procedures*. Numerous clusters of fluorescent bacteria can be seen colocalized to a crescent-shaped area within the periphery of a settled zoospore. The bar represents 10 μm.

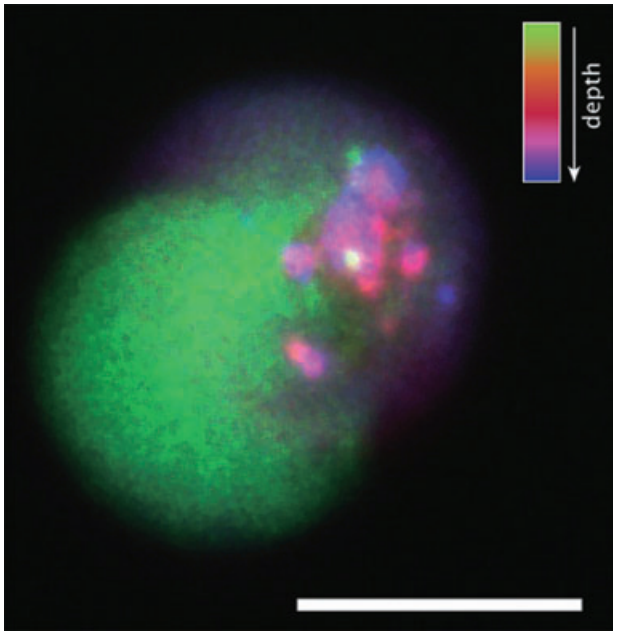

Fig. 2. Serial Z-section composite image of the interaction between *Silicibacter* sp. TM1040 and *P. piscicida*. The bacteria were fluorescently labelled as described in *Experimental procedures*, and the samples were chemically fixed and visualized using confocal microscopy. Optical Z-section slices through individual dinoflagellates were captured in 0.5 μm increments to create a series of images through the Z-axis of the cell (proximal to distal surface of the zoospore). The resulting series was then colour coded according to depth (Z-axis length) and merged into a single image, where objects tinted in green are nearer the viewer, those in shades of red and pink are in the midsection of the dinoflagellate cell, and objects near the distal surface are represented in shades of blue. Many of the bacterial cells are observed to colocalize to the middle depths of the image, suggestive of being in or near the cytoplasm of the dinoflagellate. The bar represents 10 μm.

agrees with the reports of others (Silva, 1978; Biegala *et al.*, 2002). Alternatively, the bacteria may be on the exterior of the dinoflagellate, but lodged in one of several deep grooves or indentations on the dinoflagellate cell surface, i.e. the sulcus or cingulum. When viewed in CSLM composite Z-sections, a bacterial cell located at the bottom of one of these grooves could colocalize with objects in the dinoflagellate cytoplasm. This possibility is currently under investigation. For the current study, we simply distinguish between bacteria that colocalize to the dinoflagellate surface from those that colocalize beneath the surface of the zoospore (see *Experimental procedures* for further description).

Random mutagenesis of Silicibacter *sp. TM1040*

We hypothesize that bacteria first swim within close proximity of the dinoflagellate and then attach. Therefore, mutants of *Silicibacter* sp. TM1040 defective in swimming motility were constructed and tested for their ability to interact with the dinoflagellate. A technique was developed that permits random mutations to be constructed using a Tn*5* derivative (the EZ::TN <R6Kγ*ori*/KAN-2> transposome). The method is highly effective and results in 1×10^3–1×10^4 kanamycin-resistant colonies per 1 μl of the transposome. Using this method, a bank of 3724 kanamycin-resistant mutants was constructed and screened for loss of motility and auxotrophy. By comparison, six independent auxotrophs were obtained from the bank. These auxotrophs were not further characterized in this study. Nine non-motile Mot$^-$ mutants were identified and three, TM2014, TM2017 and TM2038, were chosen for further analysis.

Phenotypic analysis of Silicibacter *sp. TM1040 motility mutants*

Two of the three strains, TM2014 and TM2017, are non-motile (Mot$^-$) in semisolid marine motility agar (Fig. 3) and do not swim in liquid media. Neither of the two mutants produces flagella (Fla$^-$), as measured by a flagellum silver stain (Fig. 4). Both are capable of forming rosettes, star-shaped clusters of cells typical of this species, and their growth rates and cell morphology are indistinguishable from wild type (Fig. 5 and Table 1).

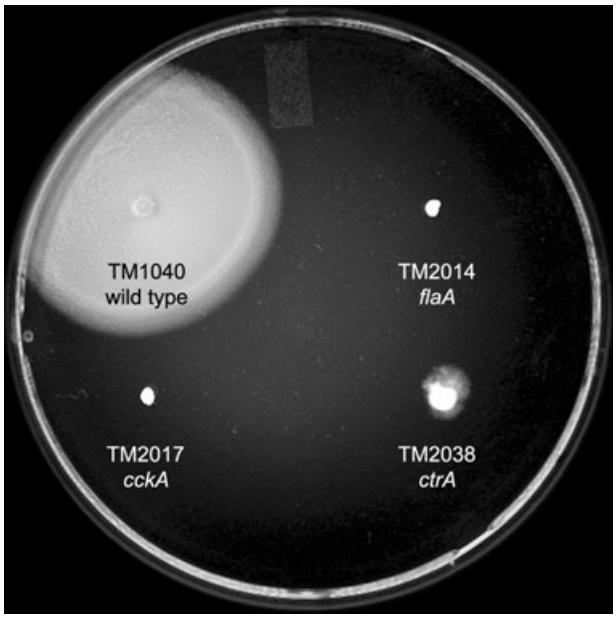

Fig. 3. Motility screening of transposon-insertion mutants. Mutant strains obtained from random transposon insertional mutagenesis were screened for their ability to swim through semisolid Mot agar (*Experimental procedures*). This assay identifies mutations in genes encoding structural components of the flagellum, hook basal body, and motor, chemotaxis signal transduction proteins, as well as global regulators of flagellar gene transcription. Clockwise from upper left are *Silicibacter* sp. TM1040 (wild-type motility), plus three strains with motility defects (TM2014, TM2017 and TM2038). Strains TM2014 and TM2017 are non-motile by this assay, while TM2038 produces flares of motile cells.

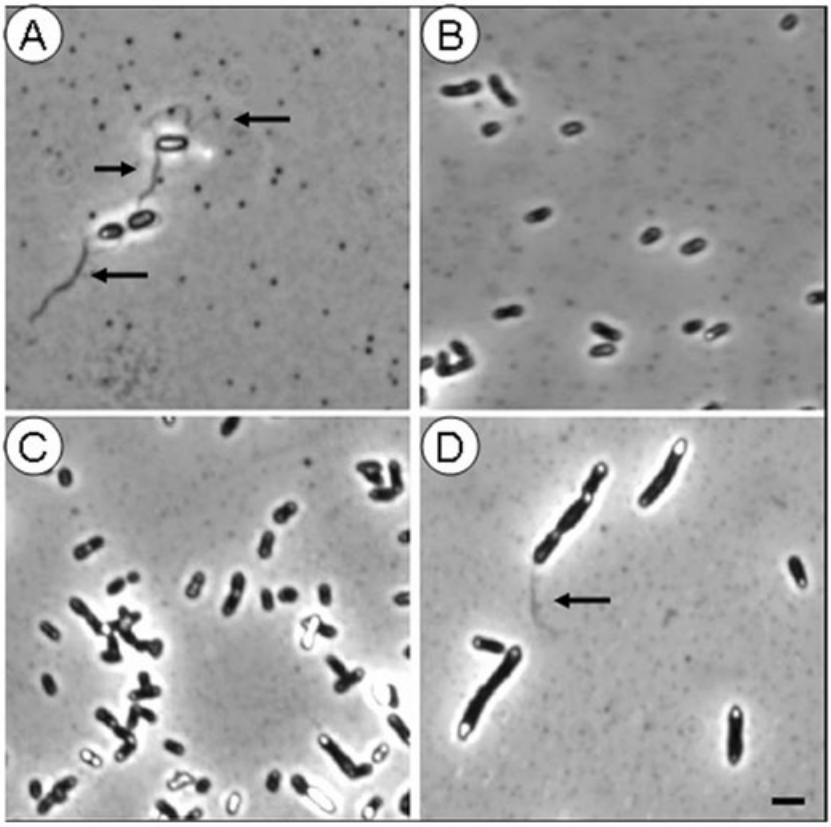

Fig. 4. Non-motile mutants lack flagella. Flagellar filaments were observed by phase-contrast microscopy after staining with silver nitrate according to the method of West and colleagues (1977). (A) *Silicibacter* sp. TM1040 (wild type); (B) TM2014 (*flaA*); (C) TM2017 (*cckA*), and (D) TM2038 (*ctrA*). The arrows point to flagellar filaments observed in both the wild-type and TM2038 cells. The bar represents 1 μm.

Conversely, strain TM2038 is non-motile in semisolid media when examined within 24–48 h post inoculation, but produces small flares of motile cells (Fig. 3). In liquid media, the majority (> 99.9%) of the cells were non-motile. Silver staining of the flagella confirmed that a few TM2038 cells produced flagella (Fig. 4). These data indicate that the mutation in TM2038 leads to a severe downregulation of flagellar synthesis. Interestingly, the mutation also resulted in an increase in the length of TM2038 cells (Fig. 5). The mean length of a TM2038 cell is 6.9 μm or nearly four times the length (1.5 μm) of the wild type (Table 1). Taken together, these results suggest that the mutation in TM2038 has likely affected a regulatory circuit that controls flagellar synthesis and cell elongation.

Genomic analysis of the mutations causing motility defects

The transposon and flanking DNA sequences from strains TM2014, TM2017 and TM2038 were obtained by rescue-cloning (*Experimental procedures*). Following trimming of transposon nucleotide sequence, the DNA flanking the site of each insertion was used to search for DNA : DNA homology to the draft annotation of the genome of

Table 1. Phenotypic analysis of *Silicibacter* sp. TM1040 and motility mutants.

Test	TM1040	TM2014	TM2017	TM2038
Motile in broth[a]	++	–	–	+
Motile in agar[b]	++	–	–	+
Flagella	Yes	No	No	Yes
Growth[c]	00.58 h^{-1}	Same as wild type	Same as wild type	Same as wild type
Cell length (μm)	1.8 ± 0.1	1.6 ± 0.1	1.5 ± 0.1	6.9 ± 0.3
Rosette formation	Yes	Yes	Yes	Yes

a. The percentage of motile cells per microscope field was scored on a plus/minus scale: –, no motile cells; +, at 10%–50% motile cells; and ++, > 50% motile cells.
b. Motility in agar was assessed using marine motility agar. The distance cells travelled outwards in motility agar was scored on a plus/minus scale: –, no outward movement; +, one or more flares of motile cells; and ++, wild-type motility.
c. Growth rate of wild type and mot$^-$ mutants was determined by measuring increases in the OD_{600} of cultures over a 2 day period.

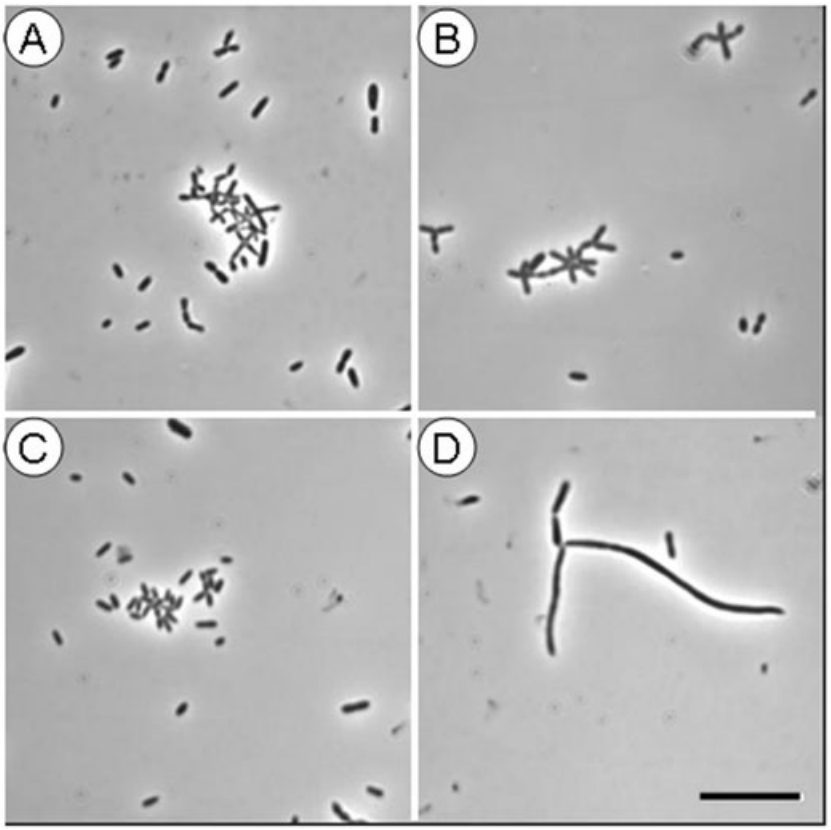

Fig. 5. The mutation in strain TM1038 leads to elongated cells. The cellular morphology of *Silicibacter* sp. TM1040 and the three Mot$^-$ mutant strains was observed by phase-contrast microscopy after incubation of the cells to the mid-exponential phase of growth. The cells of the (A) parent (strain TM1040), (B) FlaA$^-$, TM2014 and (C), CckA$^-$, TM2017 possess wild-type cell morphology, with a mean cell length of *c.* 1.6 μm, while cells of (D) CtrA$^-$, TM2038 are elongated (mean of 6.9 μm). The bar represents 5 μm.

Silicibacter sp. TM1040 (http://genome.ornl.gov/microbial/rose_tm1040). Open reading frames (ORFs) were identified and the deduced amino acid sequence of these proteins was used in further searches with BLAST (Altschul *et al*., 1990) of sequences contained in GenBank and the draft annotation of another roseobacter species, *Jannaschia* sp. CCS1 (http://genome.ornl.gov/microbial/jann/). These data were used to establish the degree of homology between the mutated ORFs and known proteins in the databases.

The results of this analysis are shown in Fig. 6. The Mot$^-$ mutant TM2014 has a mutation in a gene (GenBank accession number ZP_003388108.1; JGI contig 52, ORF1857, and gene ID #402609350) that hereafter will be referred to as *flaA*. The *flaA* ORF has negligible homology to protein sequences from non-roseobacter genera, but reasonably good homology to ORFs of unknown function from both *Silicibacter pomeroyi* DSS-3 (GenBank accession number AAV93530.1; E = 3.0 e-76 and 27% identities) and *Jannaschia* sp. CCS1 (JGI contig 27, ORF2948; E = 8e-25 and 24% identities). This suggests that *flaA* may have a roseobacter-specific function involving flagellar motility.

While FlaA protein : protein searches failed to suggest a function for this ORF, an analysis of the ORFs adjacent to *flaA* was enlightening. *flaA* is the last gene in a group of five genes (*fliL*, hypothetical protein, hypothetical protein, *motA*, *flaA*) each aligned in the same transcriptional orientation, suggesting that *flaA* may be part of an operon. Two of the five genes in this operon have homology to genes encoding proteins known to be involved in flagellar biosynthesis or energetics. For example, *flaA* is downstream of the predicted stop codon for a gene encoding a protein that is homologous to *Caulobacter crescentus motA* (E = 2.0 e-87, 55% identities). In addition, downstream of *flaA* and separate from it by a 335 bp gap is the start codon of a gene with nearly perfect homology to *flhA* from *Rhodobacter sphaeroides* (E = 0.0, 65% identities) whose function is involved in flagellar protein export. Homologues to *fliR* and *flhB*, other genes encoding proteins required for flagellar export, are located downstream to the *flhA* homologue; further strengthening the belief that *flaA* encodes a protein involved in flagellar biosynthesis or export.

The mutation in strain TM2017 (Fig. 6), the second Fla$^-$ mutant, is in an ORF (GenBank accession ZP_00339586.1; JGI contig 56, ORF3662; gene ID #402627660) that has homology to many sensor histidine kinases, with significant homology to *R. capsulatus* CckA (E = 1 e-162, 58% identities). The *Silicibacter* sp. TM1040 CckA homologue is the last gene in a group of four ORFs each transcribed in the same direction (right to left, as

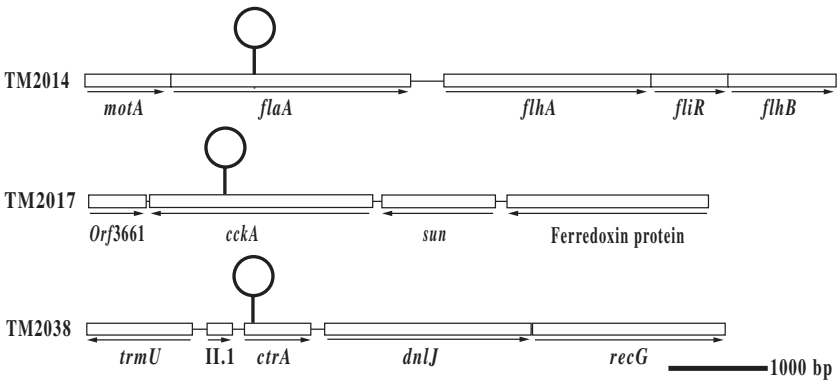

Fig. 6. Genomic analysis of the mutations leading to Mot⁻ phenotype. Mot⁻ strain TM2014 has a transposon insertion in a gene of unknown function (henceforth referred to as *flaA*) that resides in a group of genes with the same transcriptional orientation, and that appear to encode proteins required for flagellar export and/or rotation. The mutation in TM2017 affects a gene that encodes a protein with strong homology to CckA, while the mutation in TM2038 lies in a gene whose deduced amino acid sequence has homology to CtrA. In other genera, CckA, a histidine kinase, and CtrA, a DNA-binding response regulator, compose a two-component regulatory circuit that regulates flagellar synthesis and other metabolic functions (Lang and Beatty, 2002; Jacobs *et al*., 2003).

shown in Fig. 6). The other genes in this locus encode proteins with homology to *R. sphaeroides* Fmu (Sun family of 16S rRNA cytosine methyltransferase), *R. sphaeroides* ferrodoxin, and L-carnitine dehydratase from *Cupriavidus necator* (formerly *Ralstonia eutropha* JMP134).

The mutation that produced strain TM2038 is in an ORF (GenBank accession ZP_00339421.1; JGI contig 56, ORF 3516; gene ID 402626200) with homology to the response regulator protein, CtrA (Fig. 6) of *R. capsulatus* (E = 1.0 e-116, 89% identity). CtrA, a DNA-binding protein, acts in concert with CckA as a two-component regulatory circuit to regulate motility and genetic exchange in *R. capsulatus* (Lang and Beatty, 2002) and the cell cycle of *C. crescentus* (Jacobs *et al*., 2003), perhaps offering clues as to the homologous gene functions in *Silicibacter* sp. TM1040.

The transposon insertions adversely affect the physical interaction

The Mot⁻ mutants were compared with the wild-type parent for their ability to physically interact with *P. piscicida* zoospores by analysing CSLM composite images of fluorescently tagged bacteria. Figure 7 shows a representative set of these composite images. When compared with the wild-type bacteria (Fig. 7A), micrographs of the three Mot⁻ strains (Fig. 7B–D) showed fewer bacteria colocalized to interior regions of the zoospore. For example, images of the *flaA* (TM2014, Fla⁻) and *cckA* (TM2017, Fla⁻) mutant, Fig. 7B and C respectively, lack fluorescently stained bacterial cells that colocalize with interior regions of the zoospore (red or pink colours), yet bacteria attached to the periphery (green or blue colour) of the dinoflagellate are readily apparent. The lack of bacteria that colocalized to interior regions of the dinoflagellate was also evident with the *ctrA* (TM2038, Mot⁻) mutant, which also appeared to have reduced ability to attach to the surface of the zoospore (Fig. 7D).

A quantitative measurement of the number of bacterial cells attached to the surface or colocalized to interior regions of the zoospore was performed, and its results (Fig. 8) support the CSLM image analysis. Between one and six wild-type bacteria (mean 1.52 ± 0.14) were attached to the periphery and from zero to six bacteria (mean 0.6 ± 0.08) colocalized to interior regions. The mean of these values was set at 100% and used to compare the effect of the three mutations on the physical interaction with *P. piscicida*. The results, shown in Fig. 8, are consistent with the CSLM data: the Mot⁻ strains are defective in their physical interaction with the zoospore. Both the *flaA* (TM2014 Fla⁻) and *cckA* (TM2017 Fla⁻) mutations have little effect on the attachment of these bacteria to the surface of the dinoflagellates ($79.75 \pm 12.53\%$ and $111.39 \pm 28.64\%$ of wild type values respectively), while the number of bacteria colocalized to the interior of the dinoflagellate was significantly reduced ($16.13 \pm 4.56\%$ and $38.71 \pm 9.12\%$ of the wild-type value respectively) in these two mutants. The *ctrA* mutant (TM2038, Mot⁻) failed to localize to either the surface ($31.65 \pm 1.0\%$ of wild type) or the interior ($38.71 \pm 1.79\%$ of wild type) of the zoospore compared with wild-type cells. The attachment of heat-killed wild-type cells was reduced to $54.43 \pm 34.01\%$, a value that is not statistically different from living wild-type cells. However, no heat-killed bacteria were found colocalized with intracellular regions of the dinoflagellate suggesting that attachment to the dinoflagellate surface does not require a living cell, but attachment to areas beneath the surface does.

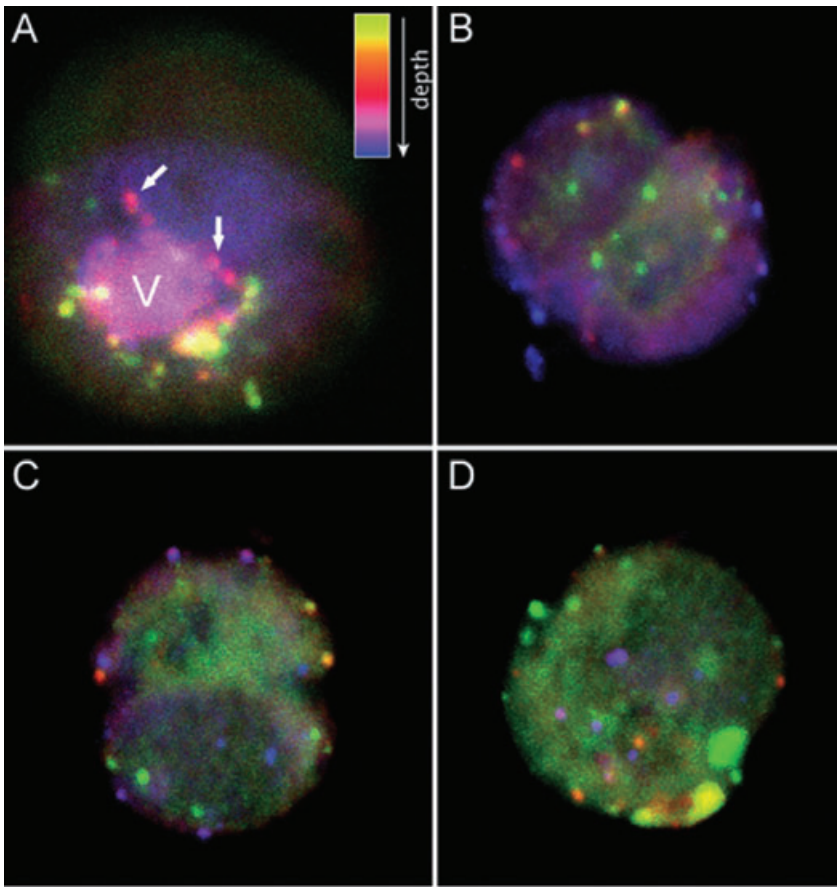

Fig. 7. Serial Z-section composite images of the interaction between Mot⁻ mutants of *Silicibacter* sp. TM1040 and *P. piscicida*. The labelling of the bacteria and preparation of the composite Z-section images for confocal microscopy is as described in *Experimental procedures*. The depth of an object is colour-coded, such that green objects are nearer the viewer (on the proximal facing surface), reds and pinks are in the midsection of the zoospore, and blue objects are distal. The panels show composite Z-section images of (A) *Silicibacter* sp. TM1040, (B) TM2014 (*flaA*), (C) TM2017 (*cckA*), and (D) TM2038 (*ctrA*) associated with a dinoflagellate zoospore. The two arrows in panel A denote TM1040 cells at the same depth as a food vacuole (V). All three mutations reduce the number of bacteria found to colocalize with the midsection of the zoospore.

Not all zoospores harbour bacteria therefore the per cent of dinoflagellates with bacteria attached to their surface or bacteria colocalized beneath the surface was measured (Fig. 8B). The percentage of zoospores in the population that harboured wild-type bacteria attached to their surface was 56%, while 21% of the dinoflagellates had wild-type cells colocalized to areas beneath the surface (Fig. 8B, first pair of bars). One of the mutations affected the attachment of the mutant cells to the dinoflagellate surface, yet all three mutations had a statistically significant negative effect on the interaction and reduced the number of bacteria colocalized with the interior of the zoospore (Fig. 8B). Taken as a whole, these results underscore the requirement of bacterial motility or functions related to the CckA/CtrA signal transduction pathway in the interaction of *Silicibacter* sp. TM1040 with *P. piscicida*, and suggest that these functions may be important in allowing the bacteria to gain access to subsurface regions of the zoospore.

Enhanced dinoflagellate growth in the presence of the wild type is altered by the Mot⁻ mutations

Our previous results have shown that *P. piscicida* grows poorly without bacteria, a condition that may be overcome by the addition of a roseobacter (Alavi *et al*., 2001). In keeping with this, wild-type *Silicibacter* sp. TM1040 also aids the growth of *P. piscicida* in an axenic culture (Fig. 9). As shown in Fig. 9, in the presence of wild-type cells, dinoflagellate density reached 9.5×10^3 cells per ml on day 9, compared with a zoospore density of 2.9×10^3 cells per ml on day 9 in the absence of bacteria, a reduction of over 70%. The *ctrA* (TM2038, Mot⁻) mutant promoted growth of the dinoflagellate equal to wild type. In contrast, both the Fla⁻ mutants [TM2014 (*flaA*) and TM2017 (*cckA*)] did not promote dinoflagellate growth equal to wild-type TM1040, reducing the rate by *c*. 30%. However, this reduction was not statistically significant at the $P < 0.05$ level using the Wilcoxon rank sum test.

The data suggest that TM1040 aids in the growth of the dinoflagellates within the axenic culture and that motility is involved. While not statistically significant, the slight reduction in dinoflagellate growth yield in the presence of TM2017 and TM2014 Mot⁻ mutants is reproducible and was consistently observed in multiple experiments suggesting that bacterial motility is involved in promoting dinoflagellate growth, but that other bacterial processes are also required. While the CtrA⁻ (TM2038) mutant did not show deficiencies in promoting dinoflagellate growth, this protein is known to be involved in a large number of

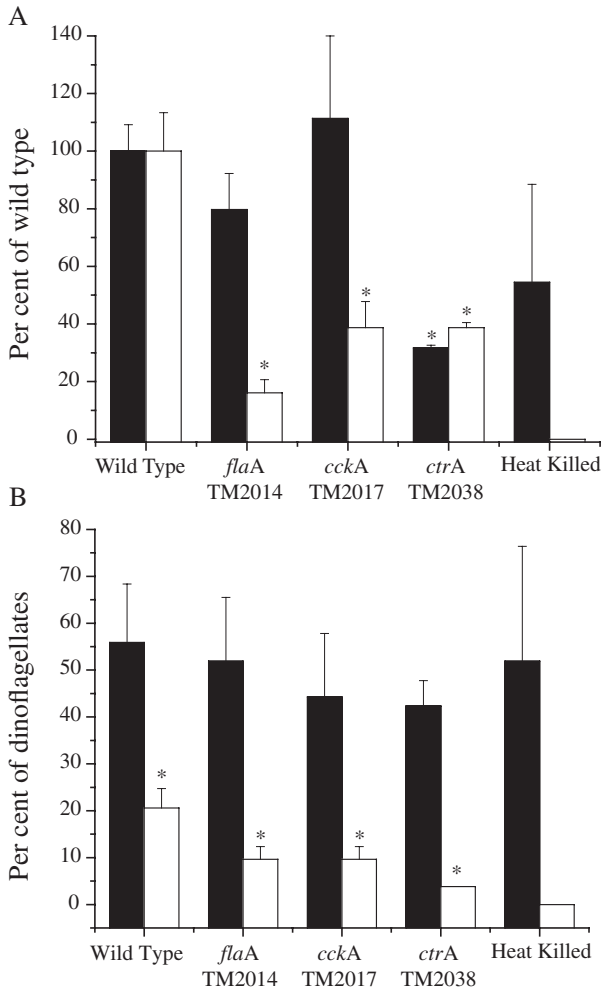

Fig. 8. Quantitative measurement of the effect of Mot⁻ mutations on bacterial attachment to the dinoflagellate cell. Wild-type and Mot⁻ strains of *Silicibacter* sp. TM1040 were fluorescently labelled and incubated with washed zoospores. Following fixation, the samples were examined by confocal microscopy, and the depth of each bacterial cell determined relative to the proximal and distal surfaces of the dinoflagellate. The mean number of bacteria on the surface (black bars) or colocalized to the interior of the dinoflagellate (white bars) for each strain was determined as a percentage relative to wild-type cells.
A. The per cent of wild-type colocalization to either the surface or interior of the dinoflagellate for TM1040 (wild type) and each Mot⁻ mutant (TM2014 FlaA⁻, TM2017 CckA⁻ and TM2038 CtrA⁻).
B. The per cent of dinoflagellates in the population observed to possess bacteria on their surface or colocalized to their interior. Heat-killed *Silicibacter* sp. TM1040 cells served as a negative control in these experiments. Asterisks indicate that the mean of that sample is significantly different from the mean of the wild-type cells ($P < 0.05$; $n = 180$).

cellular processes in addition to motility and flagellar synthesis. Data from an analysis of the *Silicibacter* sp. TM1040 genome sequence show that CtrA, as in other α-Proteobacteria, controls the expression of multiple processes including cell division, flagellar synthesis, motility, chemotaxis and genetic exchange (R. Suvanasuthi, J. Ness and R. Belas, unpubl. results). CtrA is also likely involved in a large number of other more cryptic functions, based on the presence of CtrA-binding sites in the upstream regulatory sequences of several genes whose products may affect the interaction of *Silicibacter* sp. TM1040 with the dinoflagellate. As CtrA controls many pathways, a mutation in *ctrA* could mask a slight deficiency caused by the lack of motility. This would happen if CtrA represses TM1040 genes or pathways whose products enhance dinoflagellate growth. Thus, a CtrA⁻ mutant would upregulate those genes, enhancing dinoflagellate growth, even though those mutant cells were deficient in attachment to the dinoflagellate. It is possible that disturbance of this complex signalling network masks any deficiencies in dinoflagellate growth promotion caused by a lack of motility in the CtrA⁻ mutant.

Conclusions

Members of the *Roseobacter* clade interact with dinoflagellates both in the environment and the laboratory (Alavi *et al.*, 2001; Zubkov *et al.*, 2001a; Adachi *et al.*, 2004; Miller *et al.*, 2004). However, little is known about the molecular mechanisms used by the bacteria to establish the interaction with the zoospore. Previous studies have shown that *Silicibacter* sp. TM1040 positively responds via chemotaxis to *P. piscicida* homogenates, specifically sensing DMSP and amino acids produced by the dinoflagellate (Miller and Belas, 2004). The current report extends this knowledge by demonstrating that the

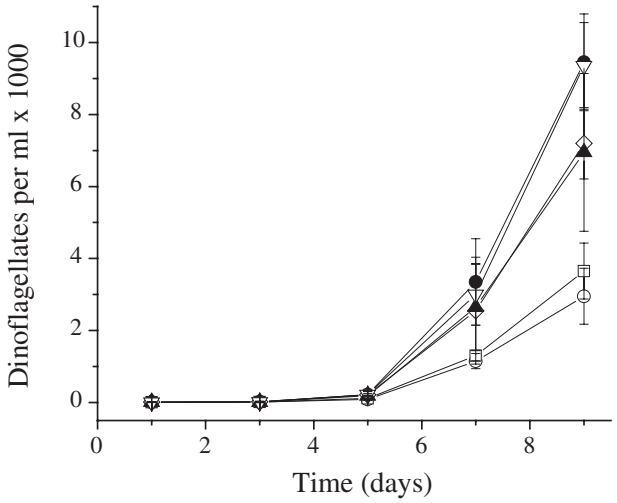

Fig. 9. Growth of axenic *P. piscicida* zoospores in the presence and absence of *Silicibacter* sp. TM1040 and three Mot⁻ mutant strains. Add-back experiments were performed to analyse the contribution of wild-type and Mot⁻ cells on the growth of *P. piscicida*. The mean (± SEM) *P. piscicida* cell density is plotted over the period of the experiment (9 days). The symbols represent: (●) *Silicibacter* sp. TM1040; (○) no bacteria control; (□) heat-killed TM1040 cells; (◇) TM2014 (*flaA*); (▲) TM2017 (*cckA*); and (▽) TM2038 (*ctrA*).

interaction has both a physical component, involving attachment of the bacteria to the dinoflagellate cell periphery, as well as areas beneath the surface, and a physiological component whereby the presence of bacteria enhances the growth of the dinoflagellates. These findings are consistent with reports showing *Roseobacter* clade species in associations with dinoflagellates in the marine environment (Zubkov *et al*., 2001a; 2002; Adachi *et al*., 2003). Therefore, in a broader context, the *Silicibacter–Pfiesteria* interaction described in laboratory culture serves as a model system for studying algal bacterial interactions in the ocean.

Three mutants with defects in wild-type swimming motility were measured for their ability to interact with the zoospores. All three mutants, FlaA$^-$, CckA$^-$ and CtrA$^-$, are defective in the physical component of the interaction. Specifically, they are all less likely to be found colocalized beneath the surface of the zoospore. As the mutants are either devoid of swimming motility (TM2014 and TM2017), or poorly motile, in the case of TM2038, one explanation is that motility is required for colocalization of the bacteria to subsurface regions. The CckA/CtrA pathway controls many functions, one of which is motility. Therefore, it is possible that other, as yet unknown mechanisms, have been affected in TM2017 (*cckA*) and TM2038 (*ctrA*). Further analysis of this regulatory circuit as it affects the interaction of the bacteria and zoospores will be enlighting.

The interaction of *Silicibacter* sp. TM1040 with *P. piscicida* is best described as a mutualistic or symbiotic relationship, where both organisms benefit from the interaction. For the bacteria one significant benefit is the acquisition of nutrients from the dinoflagellate in the form of DMSP and amino acids. The bacteria use swimming motility and chemotaxis to move towards concentration gradients of nutrients produced by the swimming dinoflagellate. As the bacteria close in on their host, they are confronted with several options, including swimming along with the moving dinoflagellate, finding dinoflagellates that have settled and orbit around these non-motile hosts, or coming in close proximity to the zoospore whereupon the bacteria attach. In this attached niche, the bacteria not only are bathed in the nutrients they require, but also may be in a 'safe-zone', free from ciliates and flagellate bacterivores. Moreover, bacteria attached to areas beneath the dinoflagellate surface are certain to have an even greater level of protection and sustenance.

While the data show that zoospore growth is improved by the presence of *Silicibacter* sp. TM1040, the mechanism by which this occurs is not so obvious. Bacteria may enhance the growth of algal species by several different mechanisms. Bacteria may remove toxic waste products, regenerate essential nutrients, produce compounds necessary for dinoflagellate growth, or produce compounds that deter predation (reviewed in Doucette, 1995). *Pfiesteria piscicida* is a heterotroph, consuming whole algal cells as a food source. The consumption and digestion of these algal cells could result in the build-up of excess metabolites in the culture that limit dinoflagellate growth. The bacteria may function to degrade those compounds as they accumulate. Alternatively, the bacteria may partially digest the algal prey making their capture by *P. piscicida* less energetically expensive. This is currently under investigation.

On the other hand, *Silicibacter* sp. TM1040 may be required by *P. piscicida* to achieve balanced growth. This is the basis for a number of symbiotic and/or syntrophic relationships among organisms in nature. For example, in another α-Proteobacterium genus, *Rhizobium*, nitrogen is supplied to the plant host in return for carbon (Ausubel, 1982). We propose that *Silicibacter* sp. TM1040 provides growth factors, cofactors, or nutrients to the zoospores that are otherwise in limiting concentrations. The molecular nature of these growth-enhancing substances is not known, but is under investigation.

Experimental procedures

Bacteria and media

Silicibacter sp. TM1040 was grown in HIASW broth consisting of 25 g Difco Heart Infusion broth (Becton-Dickinson, Franklin Lakes, NJ) supplemented with 10 g of artificial sea salts per litre (Instant Ocean, Aquarium Systems, Mentor, OH) (Miller and Belas, 2004) or in half-strength 2216 marine broth (Becton-Dickinson) as described by Alavi and colleagues (2001). Marine motility agar was made by supplementing half-strength 2216 marine broth with 3.0 g of Bacto-agar per litre. Basal minimal (BM) broth containing glycerol as the sole carbon source was made according to Miller and Belas (2004). *Escherichia coli* DH5α λ *pir* was grown in Luria–Bertani (LB) broth (Sambrook *et al*., 1989). Bacto-agar at 1.5% (w/v) was added to broths as required. As appropriate, kanamycin was used at 120 μg per ml for *Silicibacter* strains and 50 μg per ml for *E. coli* DH5α λ *pir*.

Dinoflagellates and cultivation

Pfiesteria piscicida CCMP1830 was grown as previously described (Alavi *et al*., 2001). Dinoflagellates were fed a diet of axenic *Rhodomonas* sp. CCMP768, using the method described by Alavi and colleagues (2001). All dinoflagellate culture manipulations were done in a laminar flow hood.

Transposon mutagenesis and phenotypic analyses

Electrocompetent *Silicibacter* sp. TM1040 was prepared following the procedures of Garg and colleagues (1999) with minor changes. Strain TM1040 was incubated in HIASW broth at 30°C with shaking to an optical density at 600 nm (OD_{600}) of 0.5. The cells were harvested by centrifugation at

8000 *g* for 10 min at 4°C, the supernatant was discarded, and the cell pellet washed four times in ice-cold distilled water. Following the final wash, the cell pellet was suspended in 10% glycerol and held on ice. A 50 μl sample of cells was mixed with 1 μl (25 ng) of the EZ::TN <R6Kγ*ori*/KAN-2> transposome (Epicentre, Madison, WI), and the mixture was electroporated at 16 kV per cm, yielding a time constant of *c*. 9.2 ms. The cells were suspended in 2 ml of prewarmed HIASW broth and incubated 14–16 h at 30°C with shaking. Samples of this culture were then spread onto HIASW agar containing kanamycin and incubated for 1–2 days at 30°C. The resulting kanamycin-resistant colonies were transferred to HIASW agar plus kanamycin and arranged in a 7-by-7 array to facilitate future analysis. Following incubation, the colonies were replicated on three media: fresh HIASW agar, marine motility agar to screen for motility defects, and BM plus glycerol to screen for auxotrophs. Motility (Mot$^-$) mutants and auxotrophs were identified from the initial bank, picked to fresh media, and re-tested two more times to confirm the phenotype.

Phenotypic characterization of Mot$^-$ mutants

Mot$^-$ mutants were further analysed for additional linked phenotypes. As motility agar screening does not discriminate between defects in flagella or chemotaxis, each Mot$^-$ mutant was examined by phase-contrast microscopy for its ability to swim in HIASW, BM plus glycerol, and half-strength marine broths. The synthesis of flagella was determined using the flagellar silver staining protocol of West and colleagues (1977). The length of individual cells was measured by analysis of phase-contrast images captured using a Quantix CCD camera (Photometrics, Tucson, AZ) and IPLab computer software (Scanalytics, Fairfax, VA), with 5 μm beads (Ted Pella, Redding, CA) serving as a size reference. The ability of the mutants to form rosettes [groups of cells bound to each other at their cell poles, a characteristic of *Silicibacter* sp. TM1040 and other roseobacters (Yoshida *et al*., 1997)] was determined by light microscopic examination of cells grown in BM plus glycerol broth with shaking at 30°C for 2 days. The growth rate of the Mot$^-$ mutants was determined by measuring the OD_{600} over a 2 day period while incubating in HIASW broth at 30°C with shaking.

Cloning and sequencing of EZ:TN flanking DNA

The EZ:TN transposon and flanking DNA were rescue-cloned by using the *ori*R6K origin of replication on the EZ:TN transposon. Genomic DNA from each Mot$^-$ mutant was extracted using phenol-chloroform extraction plus CTAB (cetyltrimethyl ammonium bromide) following standard protocols (Ausubel, 2001). Genomic DNA was digested with NcoI restriction endonuclease and treated with T4 DNA ligase to circularize the molecules. This was then transformed into *E. coli* DH5α λ*pir* by electroporation (Ausubel, 2001). The transformed cells were spread onto LB containing kanamycin and incubated at 37°C overnight. Plasmid DNA was extracted from the resulting kanamycin-resistant colonies using a Qiagen Midi kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The nucleotide sequence of the DNA flanking the transposon of a representative plasmid from each rescue-cloning was obtained using two oligonucleotide primers, KAN-2 FP-1 and R6KAN-2 RP-1, as described by the manufacturer (Epicentre).

Analysis of transposon insertion site

The site of transposon insertion in the genome of *Silicibacter* sp. TM1040 was determined through DNA : DNA homology searches using the DNA sequence flanking each transposon and the draft annotation of the *Silicibacter* sp. TM1040 genome provided by the Joint Genome Institute (JGI), US Department of Energy (Walnut Creek, CA; http://genome.ornl.gov/microbial/rose_tm1040/). The genome sequence is deposited in GenBank (Bethesda, MD) under accession number NZ_AAFG00000000. Nucleotide sequences flanking the transposon were aligned with the draft TM1040 genome sequence using BLASTN to identify the mutated gene (Altschul *et al*., 1990). Open reading frames were identified using the GENEMARK program (Lukashin and Borodovsky, 1998) and a BLASTP alignment of the deduced amino acid sequence. Further characterization of the putative gene products was made through a search for protein family domains using the Simple Modular Architecture Research Tool (SMART 4.0) (Letunic *et al*., 2004).

Fluorescent staining of living bacteria

Silicibacter strains were stained with 5-(and 6-)-carboxyfluorescein diacetate succinimidyl ester (CFDA/SE, Molecular Probes, Eugene, OR) fluorescent tracer dye according to the method of Fuller *et al*. (2000). A 5 ml aliquot of half-strength marine broth was inoculated with either one of three Mot$^-$ mutants or the wild-type cells, taking as an inoculum cells growing at the periphery of a 2-day-old colony grown in marine motility agar. After overnight incubation at 30°C, the cells were pelleted by centrifugation at 4000 *g* for 10 min at room temperature, and suspended in 10 parts per thousand (ppt) artificial seawater (ASW; Instant Ocean) containing 1 mM succinate to an OD_{600} of 0.6. A 10 μl aliquot of 4 mM CFDA/SE tracer dye dissolved in DMSO was added to 1 ml of cells and the tubes inverted several times to mix. After 4 h staining in the dark, the cells were washed three times with 10 ppt ASW at 8000 *g* for 10 min to remove unbound stain. After staining and prior to the final washing step a sample of stained wild-type cells was killed by heating at 65°C for 10 min.

Measurement of attachment of Silicibacter *sp. TM1040 to* P. piscicida

Dinoflagellates were prepared by behavioural washing using the method described by Alavi and colleagues (2001) with the following modifications. A 10 ml aliquot of *P. piscicida* culture at ~10^5 cells per ml was added to a 14 ml polypropylene test tube (Falcon #352059) and allowed to remain undisturbed for 30 min. During this time dinoflagellates actively swim to the bottom of the container. One millilitre of the turbid bottom layer containing the dinoflagellate zoospores was carefully removed from the tube and added to 10 ml of sterile 10 ppt ASW in a separate tube. This process was repeated

three more times, removing the majority of the unattached or free-living bacteria from the dinoflagellates (Alavi *et al*., 2001).

The washed dinoflagellates (1 ml sample) were added to 1 ml of 10 ppt ASW contained in a well of a tissue culture plate (Falcon 353046, Becton Dickson). Samples of mutant, wild-type and heat-killed TM1040 cells stained with CFDA/SE were diluted in 10 ppt ASW to *c.* 10^5 cells per ml and 20 μl of each dilution was added to 2 ml of the washed zoospores. After 2 h, 1 ml of each suspension was fixed with 2.5% paraformaldehyde (w/v final concentration) on ice for 15 min. The fixed cells were pelleted by centrifugation at 8000 *g* for 10 min at 4°C and suspended in 100 μl of 10 ppt ASW. Fifty microlitres of the cells was placed on a clean glass slide and the cells allowed to adhere to the glass. After 10 min, excess fluid was removed and the cells were covered with 10 μl of Prolong antifade solution (Molecular Probes, Eugene, OR) and a glass coverslip. The number of CFDA/SE-stained bacteria associated with individual *P. piscicida* zoospores was determine using CSLM and epifluorescence.

Confocal scanning laser microscopy

Dinoflagellates and physically associated CFDA/SE-stained *Silicibacter* strains were visualized with a Bio-Rad Radiance 2100 AGR-3Q laser scanning confocal microscope with argon, green He/Ne and red diode lasers (Bio-Rad, Hercules, CA), with an excitation wavelength of 488 nm and detected using a 500–535 nm band pass filter. Optical Z-sections at 0.5 μm increments were obtained and composite images assembled as previously described (Alavi *et al*., 2001). Sections were colour-coded according to depth and merged into a single image. Bacteria were considered on the bacterial surface if they either occurred (i) at the periphery of the zoospore cells or (ii) in optical Z-sections between 0 and 2 μm (proximal) or between 10 and 12 μm (distal). Bacteria were considered to be colocalized beneath the dinoflagellate surface if they met the following three criteria: (i) the bacteria occur within Z-sections at a depth of 2–8 μm; (ii) the bacteria are near the centre of one of the two hemispheres (epitheca or hypotheca), and not at the periphery; and (iii) the bacteria are at the same Z-axis depth as cytoplasmic organelles such as the food vacuole when such organelles are clearly evident. The presence of a food vacuole was confirmed by scanning the specimen with the 488 nm laser and detection of prey algal chlorophyll fluorescence using a Rhodomine filter.

Epifluorescence microscopy

CFDA/SE-stained samples were also visualized using an Olympus BX60 upright microscope equipped with filter cubes giving excitation and emission maxima of 480 and 525 nm respectively (Olympus, Melville, NY). Phase contrast and fluorescent images were captured and phase-contrast and epifluorescent micrographs of the same field merged using ImageJ software (Rasbad, NIH, MD) with the colocalization plug-in (Bourdoncle, Institut Jacques Monod, Paris).

Bacterial add-back experiments

Axenic zoospores were produced using the method of Alavi and colleagues (2001), which typically produced between 1000 and 2000 axenic zoospores per 10 ml culture. Axenic zoospore cultures were fed *Rhodomonas* sp. algae at a ratio of dinoflagellate to alga of 1:800, a ratio empirically determined to provide maximal growth of *P. piscicida* under these conditions. *Silicibacter* sp. TM1040 and Mot^- mutant strains were incubated in HIASW broth overnight at 30°C with shaking and washed twice in 10 ppt ASW. Each culture was normalized to an OD_{600} of 0.2 and placed on ice prior to use. A sample of wild-type strain TM1040 culture was heat-killed at 65°C for 10 min to serve as a negative control. A 50 μl sample of each bacterial suspension was added to 10 ml of the axenic zoospores with gentle mixing to give *c.* 10^4 bacteria per ml. Cultures were incubated at 20°C on a 14:10 light : dark cycle for 9 days. A sample of each culture was taken on days 1, 3, 5, 7 and 9 for enumeration of dinoflagellate and prey algal cell densities. Denaturing gradient gel electrophoresis (DGGE) was used to ensure the absence of unwanted, contaminating bacteria. Only the data from experiments shown to be free of contaminants at day 9 (end of experiment) were used.

Cell counts

Pfiesteria piscicida and *Rhodomonas* sp. were fixed in 10% (v/v) Bouin's fixative (Sigma, St Louis, MO), diluted in 10 ppt ASW (as required), and counted using a hemacytometer (Corning).

Denaturing gradient gel electrophoresis

Denaturing gradient gel electrophoresis was used to determine the composition of the bacterial species during add-back experiments. PCR amplification of 16S rDNA was done according to the method of Ferris and colleagues (1996), and the products analysed by DGGE according to Wang and colleagues (2004). The oligonucleotide primers used to amplify the 16S rDNA gene specific for most eubacteria were 1055f (ATGGCTGTCGTCAGCT) and 1392rGC (CGCCCGCCGCGCCCCGCGCCCGGCCC-GCCGCCCCCGCCCCACGGGCGGTGTGTAC) containing a GC-clamp (underlined portion) (Ferris *et al*., 1996).

Acknowledgements

The authors wish to thank Mary Ann Moran (University of Georgia) and Allison Buchan (Yale University) for their advice on roseobacter microbiology and transposon mutagenesis, Rooge Suvanasuthi and Jeremy Ness for sharing unpublished data on CtrA-binding sites, and the COMB Confocal Microscopy Facility for use of the microscope. We gratefully acknowledge the comments, advice and encouragement given by the members of the Belas laboratory and Miller dissertation committee. This work was supported by a grant from the National Science Foundation (MCB0446001) and a COMB grant-in-aid.

References

Adachi, M., Kanno, T., Okamoto, R., Itakura, S., Yamaguchi, M., and Nishijima, T. (2003) Population structure of *Alexandrium* (Dinophyceae) cyst formation-promoting bacteria

in Hiroshima Bay, Japan. *Appl Environ Microbiol* **69:** 6560–6568.

Adachi, M., Kanno, T., Okamoto, R., Shinozaki, A., Fujikawa-Adachi, K., and Nishijima, T. (2004) *Jannaschia cystaugens* sp. nov., an *Alexandrium* (Dinophyceae) cyst formation-promoting bacterium from Hiroshima Bay, Japan. *Intl J Evol Syst Microbiol* **54:** 1687–1692.

Alavi, M., Miller, T., Erlandson, K., Schneider, R., and Belas, R. (2001) Bacterial community associated with *Pfiesteria*-like dinoflagellate cultures. *Environ Microbiol* **3:** 380–396.

Altschul, S., Gish, W., Miller, W., Myers, E., and Lipman, D. (1990) Basic local alignment search tool. *J Mol Biol* **215:** 403–410.

Ausubel, F.M. (1982) Molecular genetics of symbiotic nitrogen fixation. *Cell* **29:** 1–2.

Ausubel, F.M. (2001) *Current Protocols in Molecular Biology*. New York, USA: J. Wiley.

Biegala, I.C., Kennaway, G., Alverca, E., Lennon, J., Vaulot, D., and Simon, N. (2002) Identification of bacteria associated with dinoflagellates (Dinophyceae) *Alexandrium* spp. using tyramide signal amplification-fluorescent in situ hybridization and confocal microscopy. *J Phycol* **38:** 404–411.

Dantzer, W.R., and Levin, R.E. (1997) Bacterial influence on the production of paralytic shellfish toxins by dinoflagellate algae. *J Appl Microbiol* **83:** 464–469.

Doucette, G. (1995) Interactions between bacteria and harmful algae: a review. *Nat Toxins* **3:** 65–74.

Ferris, M.J., Muyzer, G., and Ward, D.M. (1996) Denaturing gradient gel electrophoresis profiles of 16S rRNA-defined populations inhabiting a hot spring microbial mat community. *Appl Environ Microbiol* **62:** 340–346.

Fuller, M.E., Streger, S.H., Rothmel, R.K., Mailloux, B.J., Hall, J.A., Onstott, T.C., *et al.* (2000) Development of a vital fluorescent staining method for monitoring bacterial transport in subsurface environments. *Appl Environ Microbiol* **66:** 4486–4496.

Gallacher, S., Flynn, K.J., Franco, J.M., Brueggemann, E.E., and Hines, H.B. (1997) Evidence for production of paralytic shellfish toxins by bacteria associated with *Alexandrium* spp. (Dinophyta) in culture. *Appl Environ Microbiol* **63:** 239–245.

Garg, B., Dogra, R.C., and Sharma, P.K. (1999) High-efficiency transformation of *Rhizobium leguminosarum* by electroporation. *Appl Environ Microbiol* **65:** 2802–2804.

Gonzalez, J.M., and Moran, M.A. (1997) Numerical dominance of a group of marine bacteria in the alpha-subclass of the class *Proteobacteria* in coastal seawater. *Appl Environ Microbiol* **63:** 4237–4242.

Gonzalez, J.M., Kiene, R.P., and Moran, M.A. (1999) Transformation of sulfur compounds by an abundant lineage of marine bacteria in the alpha-subclass of the class *Proteobacteria*. *Appl Environ Microbiol* **65:** 3810–3819.

Gonzalez, J.M., Covert, J.S., Whitman, W.B., Henriksen, J.R., Mayer, F., Scharf, B., *et al.* (2003) *Silicibacter pomeroyi* sp. nov. and *Roseovarius nubinhibens* sp. nov., dimethylsulfoniopropionate-demethylating bacteria from marine environments. *Int J Sys Evol Microbiol* **53:** 1261–1269.

Hold, G.L., Smith, E.A., Rappe, M.S., Maas, E.W., Moore, E.R.B., Stroempl, C., *et al.* (2001) Characterisation of bacterial communities associated with toxic and non-toxic dinoflagellates: *Alexandrium* spp. and *Scrippsiella trochoidea*. *FEMS Microbiol Ecol* **37:** 161–173.

Jacobs, C., Ausmees, N., Cordwell, S.J., Shapiro, L., and Laub, M.T. (2003) Functions of the CckA histidine kinase in *Caulobacter* cell cycle control. *Mol Microbiol* **47:** 1279–1290.

Lafay, B., Ruimy, R., de Traubenberg, C.R., Breittmayer, V., Gauthier, M.J., and Christen, R. (1995) *Roseobacter algicola sp*. nov., a new marine bacterium isolated from the phycosphere of the toxin-producing dinoflagellate *Prorocentrum lima*. *Int J Syst Bacteriol* **45:** 290–296.

Lang, A.S., and Beatty, J.T. (2002) A bacterial signal transduction system controls genetic exchange and motility. *J Bacteriol* **184:** 913–918.

Letunic, I., Copley, R.R., Schmidt, S., Ciccarelli, F.D., Doerks, T., Schultz, J., *et al.* (2004) SMART 4.0: towards genomic data integration. *Nucleic Acids Res* **32:** 142–144.

Lukashin, A.V., and Borodovsky, M. (1998) GeneMark.hmm: new solutions for gene finding. *Nucleic Acids Res* **26:** 1107–1115.

Miller, T.R., and Belas, R. (2003) *Pfiesteria piscicida, P. shumwayae*, and other *Pfiesteria*-like dinoflagellates. *Res Microbiol* **154:** 85–90.

Miller, T.R., and Belas, R. (2004) Dimethylsulfoniopropionate (DMSP) metabolism by *Pfiesteria*-associated *Roseobacter*. *Appl Environ Microbiol* **70:** 3383–3391.

Miller, T.R., Hnilicka, K., Dziedzic, A., Desplats, P., and Belas, R. (2004) Chemotaxis of *Silicibacter* sp. TM1040 toward dinoflagellate products. *Appl Environ Microbiol* **70:** 4692–4701.

Prokic, I., Bruemmer, F., Brigge, T., Goertz, H.D., Gerdts, G., Schuett, C., *et al.* (1998) Bacteria of the genus *Roseobacter* associated with the toxic dinoflagellate *Prorocentrum lima*. *Protist* **149:** 347–357.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor, NY, USA: Cold Spring Harbor Laboratory Press.

Silva, E.S.E. (1978) Endonuclear bacteria in two species of dinoflagellates. *Protistologica* **14:** 113–119.

Wang, Q., Deeds, J.R., Place, A.R., and Belas, R. (2004) Dinoflagellate community analysis of a fish kill using denaturing gradient gel electrophoresis. *Harm Algae* **4:** 151–162.

West, M., Burdash, N.M., and Freimuth, F. (1977) Simplified silver-plating stain for flagella. *J Clin Microbiol* **6:** 414–419.

Yoshida, T., Inglis, V., Misawa, N., Kruger, R., and Sakai, M. (1997) *In vitro* adhesion of *Pasteurella piscicida* to cultured fish cells. *J Fish Dis* **20:** 77–80.

Zubkov, M.V., Fuchs, B.M., Archer, S.D., Kiene, R.P., Amann, R., and Burkill, P.H. (2001a) Linking the composition of bacterioplankton to rapid turnover of dissolved dimethylsulphoniopropionate in an algal bloom in the North Sea. *Environ Microbiol* **3:** 304–311.

Zubkov, M.V., Fuchs, B.M., Burkill, P.H., and Amann, R. (2001b) Comparison of cellular and biomass specific activities of dominant bacterioplankton groups in stratified waters of the Celtic Sea. *Appl Environ Microbiol* **67:** 5210–5218.

Zubkov, M.V., Fuchs, B.M., Archer, S.D., Kiene, R.P., Amann, R., and Burkill, P.H. (2002) Rapid turnover of dissolved DMS and DMSP by defined bacterioplankton communities in the stratified euphotic zone of the North Sea. *Deep-Sea Res* **II:** 3017–3038.

