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ORIGINAL ARTICLE

# Ubiquitous marine bacterium inhibits diatom cell division

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**Intricate relationships between microorganisms structure the exchange of molecules between taxa, driving their physiology and evolution. On a global scale, this molecular trade is an integral component of biogeochemical cycling. As important microorganisms in the world's oceans, diatoms and bacteria have a large impact on marine biogeochemistry. Here, we describe antagonistic effects of the globally distributed flavobacterium *Croceibacter atlanticus* on a phylogenetically diverse group of diatoms. We used the model diatom *Thalassiosira pseudonana* to study the antagonistic impact in more detail. In co-culture, *C. atlanticus* attaches to *T. pseudonana* and inhibits cell division, inducing diatom cells to become larger and increase in chlorophyll *a* fluorescence. These changes could be explained by an absence of cytokinesis that causes individual *T. pseudonana* cells to elongate, accumulate more plastids and become polyploid. These morphological changes could benefit *C. atlanticus* by augmenting the colonizable surface area of the diatom, its photosynthetic capabilities and possibly its metabolic secretions.**
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## Introduction

The ocean is a dilute environment with a microbial density of ~$10^6$ bacteria per milliliter of seawater (Whitman *et al.*, 1998) and 10–1000 times fewer phytoplankton. To survive low nutrient concentrations, marine microbes commonly form close associations with one another to facilitate metabolic and nutrient exchanges. Consequently, interacting networks of phytoplankton and bacteria recycle most fresh organic carbon in the sunlit layers of the upper ocean, while a relatively small fraction of photosynthetic products remains dissolved in the water column or sinks to the sea floor (Azam *et al.*, 1994; Azam, 1998).

Marine microbes experience a complex and heterogeneous chemical milieu that is unlike the macro-environment described by bulk measurements (Stocker, 2012). A thin boundary layer of fluid termed 'the phycosphere' surrounds algal cells (Bell and Mitchell, 1972). In the phycosphere, molecular gradients are governed by diffusion (Lazier and Mann, 1989) and altered by the activities of bacteria that accumulate through chemotaxis (Stocker and Seymour, 2012; Smriga *et al.*, 2016) and reproduction. As the thickness of diffusive boundary layers scales with surface area (Karp-Boss *et al.*, 1996), larger phytoplankton may harbor more bacteria than smaller phytoplankton. Bacteria modify the phycosphere by excreting metabolites, consuming metabolites produced by the algae or other bacteria, consuming and regenerating nutrients or making nutrients more bioavailable. As a result, bacteria turn the phycosphere into a dynamic microniche by shaping the environmental signals perceived by algae. Over time, co-evolution between algae and bacteria is likely to have resulted in specific interactions between these two groups.

Diatoms are an ecologically important group of phytoplankton that have a major role in supporting fisheries and driving the marine carbon cycle (Armbrust, 2009; Agusti *et al.*, 2015). Specific bacterial phyla, mainly the Proteobacteria and Bacteroidetes (Amin *et al.*, 2012), are commonly associated with diatoms. Beneficial and detrimental bacteria can be isolated from phytoplankton cultures, but their roles in the ocean and their mechanisms of antagonistic activity remain under-studied (Mayali and Azam, 2004; Amin *et al.*, 2012). Beneficial bacteria typically provide a useful service to the diatom in exchange for organic nutrients; for example, *Ruegeria pomeroyi* provides vitamin $B_{12}$ to *Thalassiosira pseudonana* in exchange for the organic sulfur compound 2,3-dihydroxypropane-1-sulfonate (Durham *et al.*, 2015). Many detrimental bacteria are from the phylum Bacteroidetes or the class Gammaproteobacteria (Mayali and Azam, 2004) and are

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thought to harm phytoplankton by secreting proteases or other algaecides (Lee *et al*., 2000; Paul and Pohnert, 2011; Seyedsayamdost *et al*., 2011).

Previously, we isolated and identified bacteria belonging to the *Sulfitobacter*, *Hyphomonas*, *Limnobacter*, *Marinobacter* and *Croceibacter* genera from several cultures of the coastal diatom *Pseudo-nitzschia multiseries* originating from the Atlantic and Pacific Oceans. To probe interactions between the diatom and these bacteria, we made *P. multiseries* cultures axenic and performed a series of co-culture experiments with the different bacterial isolates. Some isolates increased the growth, whereas others inhibited the growth of *P. multiseries* (Amin *et al*., 2015). Here we report on the activities of *Croceibacter atlanticus*, an antagonistic flavobacterium from the *P. multiseries* consortium, which impacts diatom DNA content, cell division and cell morphology. This type of antagonistic effect has not, to our knowledge, been previously reported in the marine environment and could have important implications for understanding bloom dynamics and organic matter fluxes in the ocean.

## Materials and methods

*Culture acquisition and maintenance*

*Nitzschia* sp. RCC 80 was acquired from the Roscoff Culture Collection (Roscoff, France); *Thalassiosira weissfloggii* CCMP 1052, *T. oceanica* CCMP 1003 and *T. pseudonana* CCMP 1335 from the National Center for Marine Algae and Microbiota (NCMA, East Boothbay, ME, USA); *Pseudo-nitzschia multiseries* CLNN-17 and *P. multiseries* CLN-47 from Stephen Bates and Claude Léger of Fisheries and Oceans Canada (Moncton, NB, Canada). *P. multiseries* CLN-47 is the product of a cross performed *ca* 2001 between environmental strains CL-143 and CL-147 collected from the Bay of Fundy, Canada; *P. multiseries* CLNN-17 is the product of a cross between CLN-47's siblings CLN-35 and CLN-48 performed at the end of 2006. *P. multiseries* PC9 and GGA2, and *P. fraudulenta* OC1 were isolated from Penn Cove WA, in 2010, from Golden Gardens WA in 2010, and from Newport Beach CA in 2011 by Michael Carlson, and *P. multiseries* IOES-1 from East Sound WA in 2010 by Irina Oleinikov. *Pseudo-nitzschia* species were identified using PCR primers to amplify and sequence a polymorphic region of the internal transcribed spacer 1 (Hubbard *et al*., 2008).

All strains were maintained as semicontinuous batch cultures in f/2 medium (Guillard, 1975) at 13 °C in a 16-h light/8-h dark diurnal cycle (~120 $\mu E\,m^{-2}\,s^{-1}$) except RCC 80 and CCMP 1003, which were maintained at 20 °C in 24-h light conditions (~120 $\mu E\,m^{-2}\,s^{-1}$). For experimental work, *Pseudo-nitzschia* strains and CCMP 1335 were acclimated to the synthetic seawater medium Aquil (Price *et al*., 1989). To monitor diatom division rates, *in vivo* chlorophyll *a* fluorescence was measured daily with a 10-AU fluorometer (Turner Designs, Sunnyvale, CA, USA) 1–2 h before the end of the dark period.

The bacteria *Marinobacter* sp. SA14 and *C. atlanticus* SA60 were isolated from *P. multiseries* CLNN-17 in 2010; *C. atlanticus* HV2 was isolated from *P. multiseries* GGA2 in 2012. These strains were identified by their 16S ribosomal RNA gene sequence (Amin *et al*., 2015; GenBank accession no.: KM033246, KM033280, KM033233). We acquired the *C. atlanticus* type strain HTCC 2559 from the American Type Culture Collection (ATCC, Manassas, VA, USA). All bacterial isolates were stored in 15% glycerol stocks at − 80 °C.

*Pseudo-nitzschia* strains IOES-1, PC9, GGA2 and OC1 were 'cured' of associated bacteria by adapting a protocol from Shishlyannikov *et al.* (2011), treating cultures with the detergent Triton X-100 (20 $\mu g\,ml^{-1}$) and an antibiotic cocktail (per ml: 50 µg streptomycin, 67 µg gentamycin, 20 µg ciprofloxacin, 2.2 µg chloramphenicol and 100 µg ampicillin) as described in Amin *et al.* (2015). CCMP 1335 is maintained bacteria-free by the National Center for Marine Algae and Microbiota (NCMA). The absence of bacteria in these cultures was verified periodically by examining SYBR green-stained cultures with epifluorescence microscopy and by checking for bacterial growth in marine broth (MB; per l: 5 g peptone, 0.5 g yeast extract and 750 ml 0.2 µm filtered seawater; ZoBell, 1941).

*Co-culture experiments*

To assess the range of possible hosts for *C. atlanticus*, we performed a series of co-culturing experiments between different diatoms and the three *C. atlanticus* strains. Before each experiment, bacterial glycerol stocks were plated at room temperature on marine agar (MB with 1.5% w/v agar). Single colonies were inoculated into MB and grown overnight with shaking at 30 °C. Triplicate co-cultures were initiated by inoculating an exponentially growing diatom culture (initial relative chlorophyll *a* fluorescence units ~0.5) with $\sim 4 \times 10^5$ bacteria $ml^{-1}$ (initial bacterial concentration). This concentration of *C. atlanticus* was chosen because it is within the range of concentrations certain flavobacteria reach in the euphotic zone during a diatom bloom (Teeling *et al*., 2012). Control cultures were treated in each experiment with 0.01–0.20% (v/v) MB to match the initial concentrations of growth media added with bacteria.

More comprehensive experiments were conducted with *T. pseudonana* CCMP 1335 and *C. atlanticus* SA60 with the goal of honing in on the mechanism of antagonistic activity. *T. pseudonana* cells were grown exponentially in the organic carbon-free artificial medium Aquil (Price *et al*., 1989) to $\sim 10^5$ cells $ml^{-1}$ and diluted to an initial concentration of 2500 cells $ml^{-1}$ for the experiment. Overnight cultures of *C. atlanticus* were grown in MB to a

density of ~$10^9$ cells $ml^{-1}$ and either added directly to the diluted *T. pseudonana* cultures or added after washing 2× with either Aquil, fresh MB, or the spent MB from an overnight grown culture after the bacteria were removed by centrifugation and the media filtered through a 0.2 µm filter. Washing consisted of centrifuging 1 ml cell cultures at 3220 × *g* and 10 °C for 5 min and replacing the supernatant with 1 ml media. These experiments were designed to determine whether *C. atlanticus* secretes a growth inhibitor into MB in the absence of diatoms, and whether *C. atlanticus* can grow on diatom-produced metabolites. As *C. atlanticus* is non-motile except for surface gliding (Cho and Giovannoni, 2003; McBride and Zhu, 2013), we increased the initial concentration of *C. atlanticus* added to each co-culture to ~$2 \times 10^6$ cells $ml^{-1}$ for an initial ratio of about 800 bacteria per diatom cell, enhancing the encounter rate with diatoms and increasing the likelihood of early attachment. For diatom control treatments, we added 0.2% v/v of fresh MB, fresh Aquil media, or 0.2% v/v of the sterile-filtered spent overnight MB media to cultures of *T. pseudonana* growing in Aquil.

Samples (1–2 ml) for flow cytometry were collected each day 0.5–1.5 h after the start of the light period and preserved in 1% buffered paraformaldehyde and 0.05% glutaraldehyde, incubated 20–30 min, flash frozen in liquid nitrogen and stored at − 80 °C (Marie *et al.*, 1999). Samples (1–2 ml) for epifluorescence microscopy were taken from a single replicate of each sample and were preserved in 8–9 ml Aquil with 2% glutaraldehyde and stored in the dark at 4 °C.

Specific growth rates (µ, $day^{-1}$) were estimated from *in vivo* chlorophyll *a* fluorescence (relative fluorescence units) or cell counts (flow cytometry) measured during the exponential growth phase. Specific growth rate is defined as the natural log cell number or fluorescence at time $t_2$ over the cell number or fluorescence at time $t_1$ divided by the difference between $t_2$ and $t_1$. Percent growth inhibition for the co-cultures was defined as the difference between $\mu_{control}$ and $\mu_{co\text{-}culture}$ divided by $\mu_{control}$ multiplied by 100. Final cell concentrations (*C,* cells $ml^{-1}$) were defined as the maximum value for each replicate growth curve measured by flow cytometry cell counts. Means were calculated from triplicate cultures unless otherwise indicated.

In a separate experiment, we tested the hypothesis that *C. atlanticus* requires amino acids as a supplementary nutrient in co-culture. *T. pseudonana* was grown exponentially in Aquil to ~$3 \times 10^5$ cells $ml^{-1}$ and diluted to an initial concentration of ~2000 cells $ml^{-1}$. Overnight cultures of *C. atlanticus* were grown in MB to a density of ~$10^9$ cells $ml^{-1}$, washed 2× with Aquil and added to *T. pseudonana* at an initial concentration of ~$2 \times 10^6$ cells for an initial ratio of 1000 bacteria per diatom cell. Each co-culture was amended with MEM Amino Acids solution (1:200 dilution, product no.: M5550, Sigma-Aldrich Inc., St Louis, MO, USA), MEM non-essential amino-acid solution (1:400 dilution, product no.: M7145, Sigma-Aldrich Inc.), a mix of both solutions, or nothing (duplicate samples each). After a 5-day incubation period, flow cytometry samples were collected as described previously.

*Epifluorescence microscopy*

Microscopy was used to provide phenotypic evidence of the interaction and to interpret flow cytometry results. Polyvinyl alcohol basal solution (Mowiol 4-88, Sigma-Aldrich Inc.) was prepared as described by Lunau *et al.* (2005) and stored as 200 µl aliquots in amber microcentrifuge tubes at − 20 °C. Fresh staining solution was prepared by adding 2 µl of freshly prepared 1 M ascorbic acid and 4 µl SYBR Green I (Invitrogen, Waltham MA, USA) to 200 µl basal solution. Samples preserved in 2% glutaraldehyde were gently filtered onto black polycarbonate membrane filters (0.2 µm pore size, 25 mm diameter, Poretics, Osmonics Inc., Minnetonka, MN, USA). The filters were air dried on glass slides cleaned with 70% ethanol and then attached to the slide with 5 µl of staining solution. An additional 14 µl of staining solution was pipetted onto a cover slip that was flipped onto the filter. Air bubbles were squeezed out and the cover slip was sealed in place with clear nail polish. The samples were examined by epifluorescence microscopy with a 470/40 nm blue excitation 515 nm long-pass emission filter set (×100 magnification, Eclipse 80i, Nikon, Tokyo, Japan; 11001v2, Chroma Corp., Rockingham, VT, USA) on the day of slide preparation and sections of the slide were imaged (MicroPublisher 3.3 RTV, QImaging, Surrey, British Columbia, Canada). Slides were stored in the dark at − 20 °C. All microscopy images can be accessed from the figshare online digital repository (doi: 10.6084/m9.figshare.3471488).

ImageJ (Rasband, 2016), image analysis software, was used to measure diatom length and diameter (or just diameter depending on the cell orientation), plastid surface area, and to count nuclei from control and co-culture treatments at $t = 2$ days and $t = 6$ days. Each image was split into three channels Red Green Blue (RGB) and measurements were made on cells that were in focus. The wand tool was used in the red channel to measure the surface area covered by plastids for each diatom cell. The number of nuclei per cell was counted in the green channel. Length and diameter measurements (or minimum and maximum lengths) were also taken with the straight-line selection tool in the green channel, using nonspecific SYBR Green staining or attached bacteria as a guideline for the cell boundaries.

*Flow cytometry*

Flow cytometry was used both to count *T. pseudonana* and unattached *C. atlanticus* cells as well as to compare the light scatter and emission properties of

the *T. pseudonana* population in co-culture and mono-culture. Flow cytometry measurements were made on an influx flow cytometer (BD/Cytopeia Inc., Seattle, WA, USA) equipped with two 10% neutral density filters and a 488 nm laser; data collection was triggered by forward light scatter. Samples were stained with SYBR Green I (1:10 000 dilution) and incubated in the dark on ice for at least 20 min. To minimize coincidence and improve population resolution, flow rates and sample concentrations were adjusted to achieve event rates below 1000 $s^{-1}$. Two-micrometer beads were added to each sample as an internal standard (PolySciences Inc., Warrington, PA, USA). Samples were run for 5 min before data collection to allow the stain to equilibrate. Samples were run until at least 200 *T. pseudonana* cells were counted. Forward light scatter, side scatter and emission at 692 nm (40 nm band pass) (chlorophyll *a* fluorescence) were measured on a log scale. A flow meter was used to measure the volume sampled.

Flow cytometry standard files were processed with the flowCore and flowStats libraries in *R* (Ellis *et al*., 2015b; Hahne *et al*., 2015). Each population of cells was semiautomatically gated with the lymphGate function from the flowStats library after a rectangular pre-selection. *T. pseudonana* was gated in the forward scatter and 692/40 nm emission dimensions, whereas *C. atlanticus* and the 2 μm beads were gated in the forward scatter and 530/40 nm emission dimensions. These gating parameters were chosen to effectively separate the population of chlorophyll *a*-containing particles from the unattached bacteria. The accuracy of gated populations was visualized with scatter plots from the flowViz library (Ellis *et al*., 2015a). *T. pseudonana* distributions were normalized to the 2 μm beads before analysis. Beads were inadvertently not added to a *T. pseudonana* ($t = 3$ days) replicate and a *T. pseudonana*+fresh MB ($t = 5$ days) replicate before data collection; these samples were therefore removed from further analysis. Flow cytometry data can be accessed from the figshare online digital repository (doi: 10.6084/m9.figshare.3471455).

*Statistical analyses*

*R* was used to plot *in vivo* chlorophyll *a* fluorescence and flow cytometry data, and the *R* stats package (https://stat.ethz.ch/R-manual/R-devel/library/stats/DESCRIPTION) was used to perform all statistical analyses (v. 3.22.6). Two sample *t*-tests with a significance cut-off value of 0.05 were used to compare growth rates and final cell concentrations ($\mu$, $C$) of different treatments as well as microscopy measurements made with ImageJ. A chi-squared test with a significance cutoff value of 0.05 was used to compare the distributions of nuclei counted in different treatments.

When discussing the co-culture treatments, we sometimes refer to *T. pseudonana* cells with attached bacteria as 'colonized cells.' The flow cytometry data were not normally distributed according to Shapiro–Wilk test results. Q-Q plots showed that control populations tended to have a slightly positive skew while colonized populations skewed increasingly negative over the course of the experiment, making data transformations intractable. We therefore used one-tail Wilcoxon rank sum tests with a significance cutoff of 0.05 to test for significant differences between the forward scatter, side scatter, and red fluorescence per cell of the *T. pseudonana* populations over time. Given that nonparametric tests with large sample sizes can be sensitive to small statistical differences with little biological meaning, we designed the test to detect a significant shift for a given time point if the colonized *T. pseudonana* population mean was greater than the control population mean by as much as the upper quartile of all samples from the control population, a relatively consistent metric over time. Replicate samples were merged to compare control and colonized treatments over time.

*C. atlanticus genomes*

We used the sequenced genomes of *C. atlanticus* to look for evidence of metabolic deficiencies in the bacterium that could be supplemented by metabolites from diatoms. *C. atlanticus* strain Pm1 was sequenced by the Joint Genome Institute as part of the *P. multiseries* genome project. The *C. atlanticus* strain Pm1 genome corresponds to the first scaffold in the prokaryotic assembly file (http://genome.jgi-psf.org/Psemu1/download/Psemu1_prokaryotic_scaffolds.fasta.gz). The *C. atlanticus* type strain genome sequence (HTCC 2559) is publically available from GenBank (accession number CP002046). Strain SA60 was sequenced at the Center for Environmental Genomics (Seattle, WA, USA). DNA from SA60 was extracted using a DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA, USA), sheared into 1–2-kb fragments (Hydroshear, Applied Biosystems, Waltham, MA, USA), and a 50 bp × 50 bp mate-paired library was generated (Genomic Solutions Ltd, Cambridgeshire, UK). Emulsion PCR was used to attach the library to beads (Life Technologies, Research and Development Unit, Carlsbad, CA, USA); approximately 56 million beads were deposited and sequenced with a SOLiD 4 Next Generation Sequencer (Life Technologies). Sequencing generated 98 959 690 reads (or 49 479 845 mate pairs), which resulted in 74 025 907 reads (12 275 915 singlets and 30 874 996 mate pairs) after quality control. The short-read de Bruijn graph assembler Velvet was used for *de novo* contig assembly (Zerbino and Birney, 2008) and five scaffolds were generated with SEAStAR graph_ops (Iverson *et al*., 2012). Scaffold order was determined by alignment to the HTCC 2559 genome.

All three genomes were submitted to the Integrated Microbial Genomes Expert Review (IMG ER)

gene prediction and annotation pipeline (Markowitz *et al*., 2009). The genome sequences and their annotations are publically available through IMG (http://img.jgi.doe.gov submissions: 15010, 11665, 11666). LASTZ was used to align the Pm1 and SA60 genomes to the type strain (Harris, 2007); aligned segments were plotted (Guy *et al*., 2010) in *R* (v. 3.22.6). Overall genome percent identity was calculated from a bl2seq alignment as the number of matches $\times 100$ divided by the alignment length between each genome and HTCC 2559.

The BioCyc organism-specific Pathway/Genome Databases (PGDBs; Caspi *et al*., 2014) were used to compare the amino-acid biosynthesis, vitamin biosynthesis and inorganic nutrient utilization pathways of *T. pseudonana* CCMP 1335 and *C. atlanticus* HTCC 2559. The HTCC 2559 PGDB is not curated and the *T. pseudonana* PGDB is missing some genes, likely due to lack of homology. For this reason, the results were verified in the SA60 genome and partial pathways were sometimes considered to be evidence that the whole pathway is present in *T. pseudonana*.

## Results and discussion

*C. atlanticus is commonly associated with P. multiseries in culture*

We identified three strains of *C. atlanticus* from three different *P. multiseries* cultures based on 16S ribosomal DNA sequence identity with the *C. atlanticus* type strain HTCC 2559 isolated from the Sargasso Sea (Cho and Giovannoni, 2003). The *P. multiseries* isolates were collected from different locales and maintained in culture for varying lengths of time. *C. atlanticus* HV2 was isolated after 2 years in culture with *P. multiseries* GGA2. *C. atlanticus* strain SA60 was isolated from *P. multiseries* strain CLNN-17, although the bacterial consortium was likely derived from the original parental strains (CL-143, CL-147), both isolated from the Bay of Fundy ~10 years prior. The genome sequence of *C. atlanticus* strain Pm1 was identified in sequences derived from the *P. multiseries* strain CLN-47 genome project. CLN-47 is an F1 generation cross produced *ca* 2001 from parental strains CL-143 and CL-147 and sequenced after 5 years in culture (Supplementary Table S1); CLN-47 has since been lost and no available isolate is associated with the *C. atlanticus* Pm1 sequence. The detection of *C. atlanticus* in several diatom cultures with different origins and culturing time in the laboratory indicates that it is a common, long-term member of the bacterial consortia associated with *P. multiseries* in culture.

*Antagonistic impact of C. atlanticus on diatoms*

When co-cultured with *P. multiseries* IOES-1, *C. atlanticus* strains SA60, HV2 and HTCC 2559 all displayed a similarly negative impact on the growth of the diatom. Within hours of inoculating *P. multiseries* with the different strains of *C. atlanticus*, *in vivo* chlorophyll *a* fluorescence of the co-culture began to decline, leveling off within about 6 days (Figure 1). Similar results were obtained with the closely related diatom *P. fraudulenta* (Table 1). Epifluorescence microscopy revealed that *C. atlanticus* strain HV2 proliferated on the cell surface of both *P. multiseries* and *P. fraudulenta* (Supplementary Figure S1). These results differ significantly from co-culturing with other bacteria from the consortium. For example, in previous work *Sulfitobacter* sp. SA11 enhanced the growth of *P. multiseries* (Amin, *et al*., 2015), whereas *Marinobacter* sp. SA14 did not influence diatom growth (Figure 1). The antagonistic effect of *C. atlanticus* was unexpected given the observed long-term co-habitation of this bacterium with various *P. multiseries* strains in culture.

All three *C. atlanticus* strains negatively impacted a variety of diatoms to varying degrees of severity (Table 1). Growth inhibition by *C. atlanticus* was not restricted to diatoms nor did it correlate with diatom phylogeny. Strong inhibition was observed with the closely related *P. fraudulenta* but not with *Nitzschia* sp. RCC 80; moderate inhibition was observed with two distantly related diatoms *T. pseudonana* and *T. oceanica* and no inhibition was observed with *T. weissflogii* (Table 1). Frazier *et al.* (2007) found that the pelagophyte *Aureococcus anophagefferens* was also inhibited by an isolate of *C. atlanticus* from

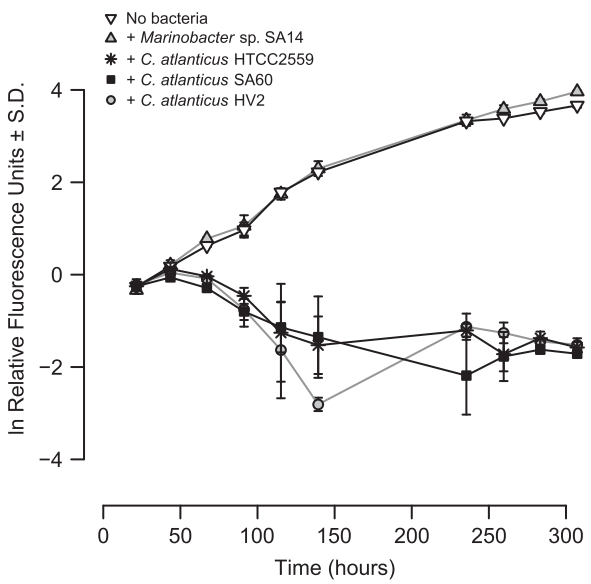


**Figure 1** Effect of three different *C. atlanticus* isolates on the growth rates of *P. multiseries* IOES-1. The growth rates of *P. multiseries* are indistinguishable from each other when 0.05% v/v of either fresh MB media ($n=2$) or an overnight grown culture of *Marinobacter* sp. (strain SA14) at $\sim 4\times10^5$ bacteria $ml^{-1}$ ($n=3$) is added to diatom cultures at $t=0$ h. Growth rates and cell yield are significantly inhibited when overnight grown *C. atlanticus* isolates ($n=3$ each; Table 1, Bonferonni-adjusted $P\leqslant0.05$) are added to the diatom culture at a final concentration of $\sim 4\times10^5$ bacteria $ml^{-1}$. Error bars indicate ± 1 s.d. or the range (for $n=3$ or 2, respectively) and are shown when larger than symbol size.

**Table 1** *C. atlanticus* impacts on selected heterokonts

| *Heterokont ID* | *Axenic* | *Croceibacter strains* | *Marine broth concentration (% v/v)* | $\mu_{control}$[a] ± *s.d.* (*day*$^{-1}$) | $\mu_{co\text{-}culture}$ ± *s.d.* (*day* $^{-1}$) | *% Growth inhibition* |
|---|---|---|---|---|---|---|
| *Pseudo-nitzschia multiseries* | Yes | HTCC 2559 | 0.10 | 0.68 ± 0.02 | 0.18 ± 0.10 | 73 |
| PC9 | | SA60 | 0.10 | | 0.32 ± 0.04 | 53 |
| *Pseudo-nitzschia multiseries* | Yes | HTCC 2559 | 0.04 | 0.50 ± 0.02 | − 0.26 ± 0.14 | 151 |
| IOES-1 | | SA60 | 0.05 | | − 0.22 ± 0.18 | 144 |
| | | HV2 | 0.03 | | − 0.53 ± 0.03 | 204 |
| *Pseudo-nitzschia fraudulenta* | Yes | HTCC 2559 | 0.01 | 0.63 ± 0.03 | 0.44 ± 0.02 | 30 |
| OC1 | | SA60 | 0.01 | | 0.41 ± 0.06 | 34 |
| | | HV2 | 0.01 | | 0.45 ± 0.02 | 29 |
| | | | 0.02 | 0.82 ± 0.04 | 0.00 ± 0.03 | 100 |
| *Nitzschia* sp. | No | SA60 | 0.02 | 1.37 ± 0.04 | 1.38 ± 0.04 | 0 |
| RCC 80 | | | | | | |
| *Thalassiosira weissfloggii* | No | SA60 | 0.02 | 1.57 ± 0.03 | 1.58 ± 0.03 | 0 |
| CCMP 1052 | | | | | | |
| *Thalassiosira oceanica* | No | SA60 | 0.02 | 0.92 ± 0.01 | 0.78 ± 0.00 | 15 |
| CCMP 1003 | | | | | | |
| *Thalassiosira pseudonana* | Yes | SA60 | 0.01 | 0.93 ± 0.02 | 0.63 ± 0.02 | 33 |
| CCMP 1335 | | | | | | |
| *Aureococcus anophagefferens* | No | Isolate from Great South Bay, New York | 0.20 | ND | ND | >0 |
| CCMP 1784[b] | | | | | | |

Abbreviation: ND, no data.
[a]$\mu_{control}$ is the diatom growth rate in the absence of additional *C. atlanticus.*
[b]From Frazier *et al.*, 2007.

Great South Bay, New York, although the extent of the inhibitory effect was not reported. Two of the seven tested diatoms were not impacted by the addition of *C. atlanticus*; neither of these diatom strains was axenic, raising the possibility that members of the pre-existing bacterial consortia from those diatoms mitigated the impacts of *C. atlanticus*. This possibility may explain how *C. atlanticus* remained a consistent member of the *P. multiseries* consortium without completely arresting diatom growth. Complex host-independent patterns of interaction have been observed in many host-dependent microbiomes (for example, Trosvik *et al.*, 2010), and likely exist in the diatom microenvironment as well.

### *Mechanism of antagonistic effect of C. atlanticus on T. pseudonana*

We chose to focus on the diatom-associated strain SA60 given the similarity in antagonistic phenotypes of the three *C. atlanticus* strains and their high genome sequence identities—the SA60 genome is 98% identical and the Pm1 genome is 99% identical to HTCC 2559 at the nucleotide level and both are missing a genome segment involved in alginate metabolism (Supplementary Figure S2). We examined in more detail, the interaction between strain SA60, and the model diatom *T. pseudonana* CCMP 1335 because the annotated whole genome of this diatom is available (Armbrust *et al.*, 2004), with complementary metabolomic data (Longnecker *et al.*, 2015), and because the cells are small enough to analyze with flow cytometry.

Epifluorescence microscopy revealed that in co-culture, *C. atlanticus* cells colonized the cell surface of *T. pseudonana* while a number of *C. atlanticus* cells remained unattached to the diatoms. As *C. atlanticus* is non-motile except for surface gliding (Cho and Giovannoni, 2003; McBride and Zhu, 2013), association with the diatom likely proceeds by random encounter followed by attachment and replication on the cell surface of the diatom. In acclimated non-axenic cultures and in nature, it may be that vertical transmission from parent to daughter cells is a more important mechanism of maintaining associations over multiple generations. The *C. atlanticus* population counted by flow cytometry consists of the unattached portion of the bacterial community—those cells that did not encounter *T. pseudonana* and any cells that detached from the diatom either naturally or during sample processing.

In the previous co-culture experiments (Figure 1, Table 1), an aliquot of overnight grown cultures of *C. atlanticus* cells in spent MB was added directly to the different diatom cultures. Although in each instance the added MB media was diluted 500–10 000-fold (Table 1), variable amounts of undefined organic matter, as well as any growth inhibitor potentially released by *C. atlanticus* into the MB, were also added to the co-cultures. We therefore carried out a series of experiments to determine potential sources of the antagonistic effects on diatoms.

First, we tested whether *C. atlanticus* constitutively released a diatom growth inhibitor into the MB during overnight growth in the absence of diatoms.

An aliquot of filter-sterilized MB media from an overnight grown *C. atlanticus* culture was added to a *T. pseudonana* culture and did not significantly decrease the growth rate ($\mu_{1335+fresh\ MB} = 1.01 \pm 0.02\ day^{-1}$, $\mu_{1335+spent\ \ MB} = 1.08 \pm 0.05\ day^{-1}$, $P = 0.13$) or final cell concentration ($C_{1335+fresh\ \ MB} = 7.76 \pm 0.57$ ($\times 10^5$) cells $ml^{-1}$, $C_{1335+spent\ \ MB} = 7.71 \pm 0.80$ ($\times 10^5$) cells $ml^{-1}$, $P = 0.94$) of the diatom (Figure 2a). These results suggest that *C. atlanticus* does not constitutively release a growth inhibitor into MB under these conditions, at least at an effective concentration. By contrast, another study showed that the flavobacterium *Kordia algicida* constitutively secretes a protease that negatively impacts several diatoms (Paul and Pohnert, 2011). One diatom, *Chaetoceros didymus*, appears to deactivate the algicidal activity of *K. algicida* through production of its own counter-acting protease-degrading enzymes (Paul and Pohnert, 2013). It is not clear whether *T. pseudonana* can similarly degrade low concentrations of the *C. atlanticus* growth inhibitor.

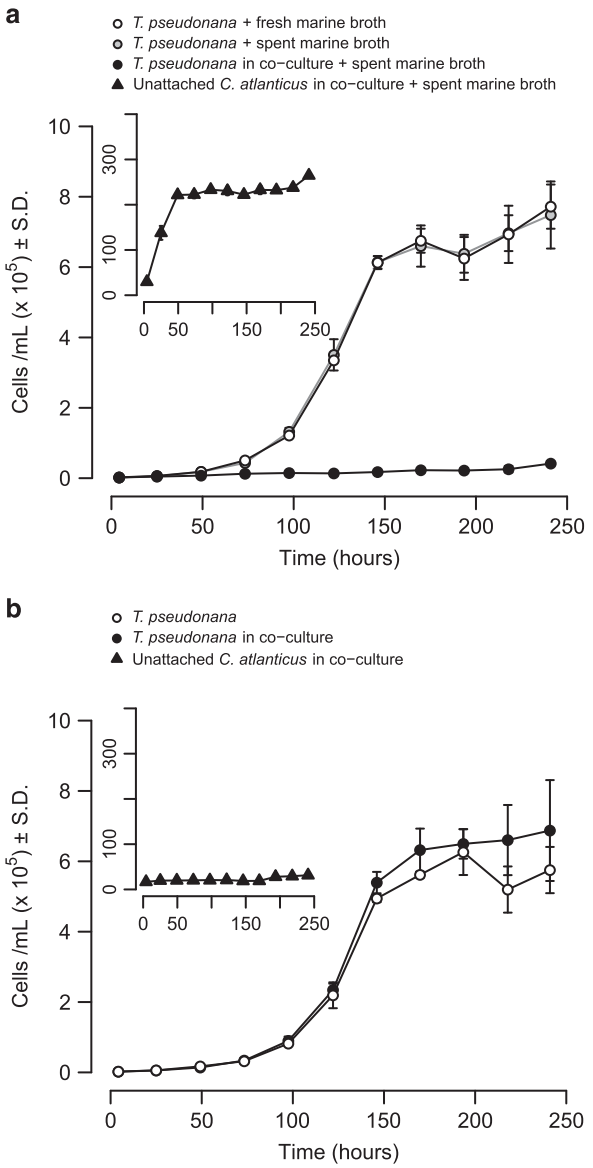


**Figure 2** Effects of *C. atlanticus* SA60 and its growth media on the growth of *T. pseudonana* CCMP 1335. (**a**) Filter-sterilized spent MB from an overnight culture of *C. atlanticus* has an indistinguishable effect relative to fresh MB on the growth of *T. pseudonana*, whereas a direct inoculate of overnight grown *C. atlanticus* drastically inhibits the growth of *T. pseudonana*; unattached *C. atlanticus* cells in co-culture display growth (inset). (**b**) *C. atlanticus* cells that were washed with Aquil do not grow in co-culture with *T. pseudonana* (inset) and do not impact the growth of *T. pseudonana* in co-culture. Symbols mark the mean of diatom and unattached bacterial cell concentration (circles, triangles) when grown alone, in the presence of *C. atlanticus* waste products, or in co-culture (white, gray, black; $n = 3$ biological replicates ± 1 s.d. and are shown when larger than symbol size).

Second, we asked whether *C. atlanticus* required a component of the organic matter carried over in the MB inoculum to elicit the negative effect on the diatom. When *C. atlanticus* cells were washed with the organic carbon-free diatom medium Aquil before addition to the *T. pseudonana* culture, the bacteria did not divide in co-culture with *T. pseudonana*, did not attach to the *T. pseudonana* cells nor did they impact the growth rate or final cell concentration of the diatom ($\mu_{1335} = 0.93 \pm 0.05\ day^{-1}$, $\mu_{1335+SA60} = 0.97 \pm 0.04\ day^{-1}$, $P = 0.40$; $C_{1335} = 6.26 \pm 0.65$ ($\times 10^5$) cells $ml^{-1}$, $C_{1335+SA60} = 7.70 \pm 0.03$ ($\times 10^5$) cells $ml^{-1}$, $P = 0.06$; Table 2, Figure 2b). In contrast, a *C. atlanticus* overnight culture washed with spent MB before addition to the co-culture remained antagonistic indicating that the washing step itself did not impact the *C. atlanticus* phenotype ($\mu_{1335+SA60} = 0.39 \pm 0.07\ day^{-1}$, $\mu_{1335+SA60\ \ centrifuged} = 0.44 \pm 0.03\ day^{-1}$, $P = 0.29$; $C_{1335+SA60} = 4.15 \pm 0.1$ ($\times 10^4$) cells $ml^{-1}$, $C_{1335+SA60\ \ centrifuged} = 4.07 \pm 1.42$ ($\times 10^4$) cells $ml^{-1}$, $P = 0.9$; Supplementary Figure S3). This result suggests that specific nutrients present in MB and required in co-culture were not supplied by the diatom, at least at the start of the experiment.

To rule out that washing the cells in Aquil was toxic to the bacterium and inhibited its antagonistic activity, amino acids were added to Aquil-washed *C. atlanticus* in co-culture with *T. pseudonana* and bacterial growth was rescued (Supplementary Figure S4). The amino-acid formulations used in this experiment include two mixtures of amino acids (named essential and non-essential based on cultured mammalian cell nutrient requirements). *C. atlanticus* requires both mixtures for growth,

**Table 2** Impact of *C. atlanticus* on the growth rate of *T. pseudonana*

| *Media supplement* | $\mu_{control}$[a] ± *s.d.* (*day* $^{-1}$) | $\mu_{co\text{-}culture}$ ± *s.d.* (*day* $^{-1}$) | *% Growth inhibition* |
|---|---|---|---|
| None (Aquil) | 0.93 ± 0.05 | 0.97 ± 0.04 | 0 |
| 0.2% v/v fresh marine broth | 1.01 ± 0.02 | 0.29 ± 0.03 | 71 |
| 0.2% v/v spent marine broth | 1.08 ± 0.05 | 0.44 ± 0.03 | 59 |

[a]$\mu_{control}$ is the diatom growth rate in the absence of bacteria.

implying that there are amino acids in both solutions that are required by *C. atlanticus*.

The extent of the negative impact when supplemented with MB appeared to be related to *C. atlanticus* growth rate in co-culture. When *C. atlanticus* cells were washed with spent MB rather than fresh MB, the unattached *C. atlanticus* cells displayed a lower growth rate in co-culture ($\mu_{spent} = 1.81 \pm 0.08$ day$^{-1}$, $\mu_{fresh} = 2.26 \pm 0.11$ day$^{-1}$, $P = 0.01$) and reached a lower final cell concentration ($C_{spent} = 2.57 \pm 0.06$ ($\times 10^7$) cells ml$^{-1}$, $C_{fresh} = 3.01 \pm 0.01$ ($\times 10^7$) cells ml$^{-1}$, $P < 0.01$). This lower *C. atlanticus* growth rate translated into a significantly reduced impact on the *T. pseudonana* growth rate (59% vs 71% inhibition, Table 2). These results reinforce the idea that the observed detrimental effect is driven by proliferation of *C. atlanticus* whose growth is supported by nutrients present in MB.

These co-culturing experiments show that the Aquil-washing step eliminates nutrients required for the growth of *C. atlanticus* in co-culture, and that early exponential phase cultures of *T. pseudonana* do not provide sufficient organic matter to support *C. atlanticus* cell division. *C. atlanticus* growth can, however, be rescued by the addition of amino acids or MB to co-culture. In the absence of nutrients, *C. atlanticus* does not attach to the diatom cells and the antagonistic phenotype is not induced. In contrast, when *C. atlanticus* cells are added to the diatom cultures along with sufficient organic nutrients, the unattached cells continue to divide, presumably attaining a cell density that provides a sufficiently high encounter rate with the diatom cells. *C. atlanticus* likely has a mechanism to recognize and attach to diatom cells; perhaps algal exudates serve as infochemicals that induce colonization after an encounter. Once *C. atlanticus* cells attach to a diatom, they appear to proliferate on the cell surface, likely due to the enhanced flux of diatom-released nutrients at the cell surface not seen in the bulk media.

*Effects of C. atlanticus on the cell size, morphology and DNA content of T. pseudonana*

Microscopy and flow cytometry measurements of diatoms in mono-culture and co-culture indicated that over the course of a few days, *T. pseudonana* cells with attached bacteria elongate (Figures 3a and b, Supplementary Figure S5), are filled with more plastids (Figure 3c), and typically possess more nuclei (Figure 3d) than cells without attached bacteria (Figure 4). In treatments where both *C. atlanticus* and MB were added, the vast majority of *T. pseudonana* cells hosted attached bacteria by the second day of co-culture (Figure 4a).

In examining both the means and distributions of flow cytometry parameters and microscopy measurements, a time course of the impact of *C. atlanticus* on *T. pseudonana* was apparent. Within 2 days of the addition of *C. atlanticus* cells, the growth rate of *T. pseudonana* in co-culture decreased significantly relative to the control (Figure 2a), whereas the chlorophyll *a* fluorescence (692/50 nm emission) per cell in colonized populations increased significantly ($P < 0.001$) (Figure 4b, Supplementary Figure S6). Microscopy measurements of the cellular surface area covered by plastids suggest that elevated chlorophyll *a* fluorescence is correlated with an increase in the number of plastids per cell (Figures 3c and 4b). By day 3, the forward scatter of colonized *T. pseudonana* populations increased ($P < 0.001$) (Figure 4b, Supplementary Figure S6). Increased forward scatter is often correlated to increased cell size (Olson *et al*., 1989), although this relationship is sometimes affected by other refractive properties of the cell (Shapiro, 2003). However, microscopy measurements of *T. pseudonana* diameter and length (Figures 3a and b) lead us to conclude that increased forward scatter of colonized cells can be attributed to their increased cell size. By day 4, side scatter values also increase significantly ($P < 0.001$) (Figure 4b, Supplementary Figure S6). The reason for these shifts are not known, but changes in side scatter distributions may be related to cell shape, cytoplasmic granularity and differences in internal cell structures (Shapiro, 2003). We also observed that *T. pseudonana* sank to the bottom of the culture tubes in treatments where the cells were colonized, possibly as a result of morphological changes. The similar pattern between cell length measurements and cell area covered by plastids suggests that the number of plastids scales with cell size.

Epifluorescence microscopy indicated that by day 6 the larger, SYBR Green-stained colonized *T. pseudonana* cells commonly possessed two or more nuclei rather than a single nucleus as observed in control cultures (Figure 3d). A significant proportion of cells without a nucleus were also observed on day 6 using microscopy (Figure 3d). This is suggestive of nuclear disintegration and DNA fragmentation, which accompanies apoptosis (Huang *et al*., 2005). *C. atlanticus* thus appears to manipulate the cell cycle of the diatom, stimulating both polyploidy and nuclear disintegration.

Increased DNA content is often correlated with enhanced cell size (Von Dassow *et al*., 2008; Koester *et al*., 2010) as well as increased metabolic activity (Wildermuth, 2010; Edgar *et al*., 2014). We hypothesize that manipulation of the diatom cell cycle could therefore be an effective strategy for *C. atlanticus* to induce morphological and physiological changes in its host that enhance the release of required metabolites. For example, *C. atlanticus* is unable to synthesize the amino acids isoleucine, valine, leucine and arginine, all of which *T. pseudonana* produces within its plastids and mitochondria, which may be more abundant now that the diatom is larger (Supplementary Table S2, Armbrust *et al*., 2004). In addition, the *C. atlanticus* genome encodes a variety of peptidases and carbohydrate active enzymes (CAZYmes) and *C. atlanticus* can grow in defined media with amino acids as its sole source

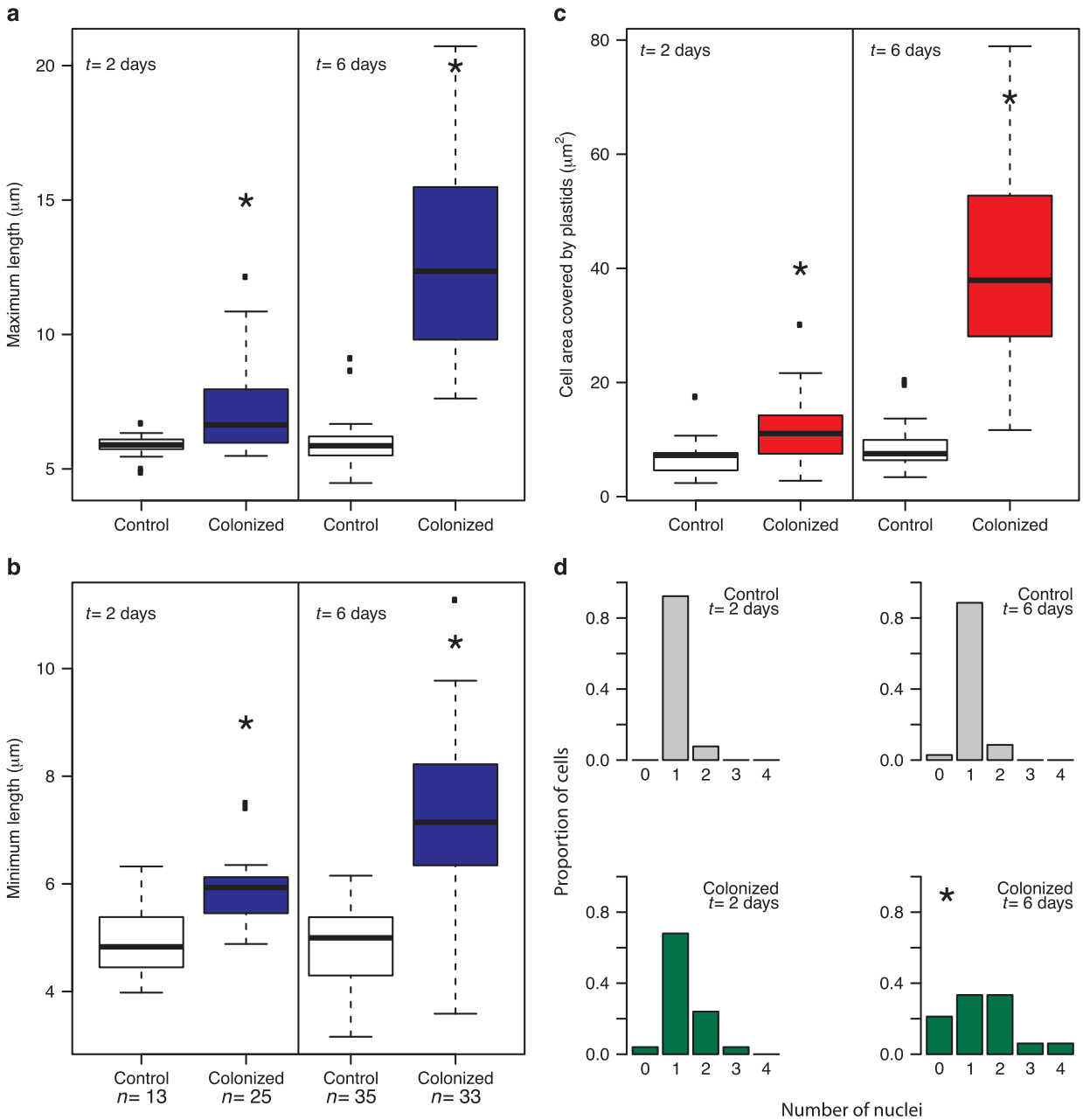


**Figure 3** Microscopy measurements comparing colonized and control diatom cells after 2 and 6 days in culture. Box-and-whiskers plots of the (**a**) maximum and (**b**) minimum lengths and (**c**) the surface area per cell covered by plastids show the distribution of data including the median, quartiles, and range. *N* represents the number of cell measurements made. (**d**) Histograms of nucleus count per cell for colonized and control diatoms after 2 and 6 days in culture. Asterisks represent treatments that are statistically different from the control for the same incubation period ($P<0.05$).

of organic carbon and nitrogen (data not shown). Secretion of carbohydrates and peptides by *T. pseudonana* (Longnecker *et al.*, 2015) could also fulfill *C. atlanticus* nutritional requirements.

Polyploidy occurs naturally in a variety of plant, mammalian and insect cell types (Edgar *et al.*, 2014). It has also been observed at nutrient exchange points between fungal or bacterial pathogens or symbionts and their host cells (Wildermuth, 2010). As ploidy correlates linearly with gene expression and metabolic flux, it has been hypothesized that increased DNA content may be a common biological mechanism to cope with increased metabolic demands (Wildermuth, 2010). In particular, primary metabolism (glycolysis, respiration and fermentation), transport, and plastid-localized production of metabolites are sensitive to increases in ploidy (Wildermuth, 2010). In mammals, pathogenic bacteria are known to target cell cycle progression of their host cells through production of effectors known as cyclomodulins (Nougayrède *et al.*, 2005). These effectors regulate the expression of cyclins and cyclin-

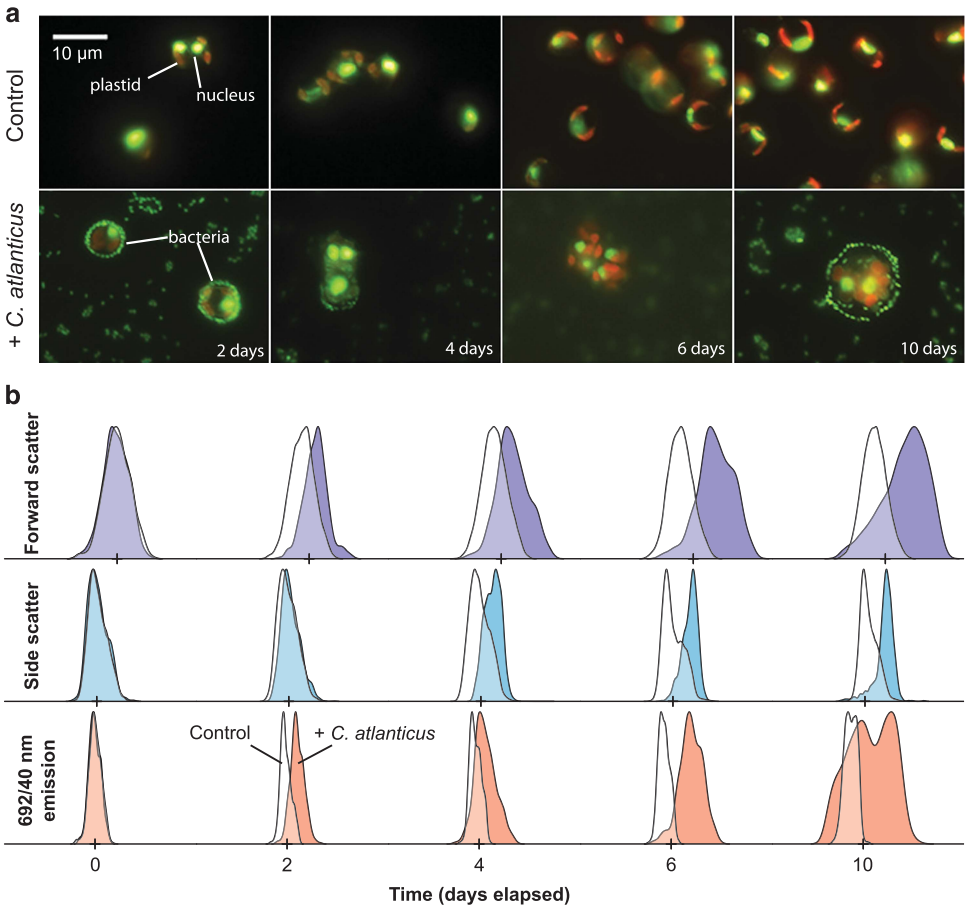


**Figure 4** Attachment of *C. atlanticus* to *T. pseudonana* and impacts on diatom morphology. (**a**) Representative epifluorescence micrographs from the experiment portrayed in Figure 2 show *C. atlanticus* attachment to *T. pseudonana* and morphological changes over the course of the experiment. Diatom plastids autofluoresce red, SYBR Green-stained DNA from the diatom nucleus and the bacteria fluoresce green. The co-culture micrographs for day 4 and 6 focus on the diatoms rather than the attached bacteria to show examples of polyploidy. Scale bar = 10 µm. (**b**) Increased forward and side scatter values (blue, turquoise) over time show how *C. atlanticus* impacts the size and shape of *T. pseudonana*. Shifts in the 692/40 nm emission distribution (red) over time indicate that *C. atlanticus* impacts the amount of chlorophyll per cell. Colored polygons are representative distributions of *T. pseudonana* population scatter and fluorescence characteristics in co-culture; white polygons show the same for a control population. Distributions represent the fractions of total cells in a population.

dependent kinases, required for cell cycle progression. Analogously, growth-promoting and pathogenic bacteria can interfere with the plant cell cycle hormonal balance to modulate host cyclin and cyclin-dependent kinase activity (Stes *et al.*, 2011). Whether *C. atlanticus* manipulates *T. pseudonana* cell division through a similar disruption of cyclin-mediated cell cycle progression requires further study.

## Conclusion

Flavobacteria such as *C. atlanticus* have a reputation as fast-growing r-strategists that specialize on initiating organic matter degradation near the end of algal blooms (Teeling, *et al.*, 2012). Our results raise the possibility that some flavobacteria could contribute to diatom bloom decline if they, like *C. atlanticus*, are capable of halting cell division. The colonized diatom population appears to survive until the late stationary phase when there is some indication of nuclear disintegration. As *C. atlanticus* is only capable of gliding motility, it cannot actively seek out a new host and must continue to colonize an arrested diatom cell. By causing *T. pseudonana* to elongate, *C. atlanticus* increases the surface area of its niche. In addition, *C. atlanticus* benefits from colonizing a live diatom that continues to synthesize photosynthetic products. However, once nutrients are depleted in solution and the diatom stops its metabolic activities, it would be more beneficial for *C. atlanticus* to consume all available organics from the failing diatom and detach from the cell before it sinks.

The mechanisms of diatom–bacteria interactions can give us new insights into the dynamics of marine microbial ecology. Although we do not yet know whether bacteria affect the cell cycle of diatoms in nature, we have evidence that *C. atlanticus* can affect the DNA content of *T. pseudonana* and prior evidence that *Sulfitobacter* sp. can upregulate G1-S

phase cyclins of *P. multiseries* in culture (Amin *et al*., 2015). It may be that the potential for cell cycle manipulations is widespread in the marine environment and is a common ecological strategy. *C. atlanticus* sequences are prevalent across the global ocean (Yooseph *et al*., 2010), and the interaction appears to lack specificity. If *C. atlanticus* is capable of antagonizing a variety of phytoplankton in nature, then this microbe could have an important role in the remineralization of organic matter in the ocean. Interactions between *C. atlanticus* and other algal-associated bacteria may also be involved in mediating these antagonistic interactions.

## Conflict of Interest

The authors declare no conflict of interest.

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