Chitin in Diatoms and Its Association with the Cell Wall†

Colleen A. Durkin,1 Thomas Mock,2 and E. Virginia Armbrust1*

School of Oceanography, University of Washington, Box 357940, Seattle, Washington 98195,1 and School of Environmental Sciences, University of East Anglia, Norwich NR4 7TJ, United Kingdom2

Received 8 March 2009/Accepted 24 April 2009

Chitin is a globally abundant polymer widely distributed throughout eukaryotes that has been well characterized in only a few lineages. Diatoms are members of the eukaryotic lineage of stramenopiles. Of the hundreds of diatom genera, two produce long fibers of chitin that extrude through their cell walls of silica. We identify and describe here genes encoding putative chitin synthases in a variety of additional diatom genera, indicating that the ability to produce chitin is more widespread and likely plays a more central role in diatom biology than previously considered. Diatom chitin synthases fall into four phylogenetic clades. Protein domain predictions and differential gene expression patterns provide evidence that chitin synthases have multiple functions within a diatom cell. Thalassiosira pseudonana possesses six genes encoding three types of chitin synthases. Transcript abundance of the gene encoding one of these chitin synthase types increases when cells resume division after short-term silicic acid starvation and during short-term limitation by silicic acid or iron, two nutrient conditions connected in the environment and known to affect the cell wall. During long-term silicic acid starvation transcript abundance of this gene and one additional chitin synthase gene increased at the same time a chitin-binding lectin localized to the girdle band region of the cell wall. Together, these results suggest that the ability to produce chitin is more widespread in diatoms than previously thought and that a subset of the chitin produced by diatoms is associated with the cell wall.

Chitin is the most abundant polymer in the ocean and the second most abundant polymer on earth, exceeded only by cellulose (see, for example, references 1, 16, and 27). The pervasiveness of chitin is attributed to its use by diverse eukaryotic organisms, including fungi, insects, mollusks, crustaceans, algae, and protists. These diverged lineages commonly use chitin to strengthen their cell walls or skeletons. Chitin synthase is used by all chitin-producing organisms to generate the hundreds of diatom genera, two produce long fibers of chitin that extrude through their cell walls of silica. We identify and describe here genes encoding putative chitin synthases in a variety of additional diatom genera, indicating that the ability to produce chitin is more widespread and likely plays a more central role in diatom biology than previously considered. Diatom chitin synthases fall into four phylogenetic clades. Protein domain predictions and differential gene expression patterns provide evidence that chitin synthases have multiple functions within a diatom cell. Thalassiosira pseudonana possesses six genes encoding three types of chitin synthases. Transcript abundance of the gene encoding one of these chitin synthase types increases when cells resume division after short-term silicic acid starvation and during short-term limitation by silicic acid or iron, two nutrient conditions connected in the environment and known to affect the cell wall. During long-term silicic acid starvation transcript abundance of this gene and one additional chitin synthase gene increased at the same time a chitin-binding lectin localized to the girdle band region of the cell wall. Together, these results suggest that the ability to produce chitin is more widespread in diatoms than previously thought and that a subset of the chitin produced by diatoms is associated with the cell wall.

* Corresponding author. Mailing address: School of Oceanography, University of Washington, P.O. Box 357940, Seattle, WA 98195. Phone: (206) 616-1783. Fax: (206) 685-6651. E-mail: armbrust@u.washington.edu.
† Supplemental material for this article may be found at http://ec.asm.org.
‡ Published ahead of print on 8 May 2009.
other molecules (9, 35, 61). Specialized vesicles targeted to an invaginated membrane just below the fultoportulae have been identified and were proposed to carry the molecular and chemical machinery necessary for chitin synthesis, which likely includes the chitin synthases (20). In *T. weissflogii*, chitin synthases are estimated to generate 700,000 N-acetylglucosamine linkages s⁻¹μm⁻² of specialized membrane (20).

Analysis of the whole-genome sequences for *T. pseudonana* (2) and the pennate diatom *Phaeodactylum tricornutum* (3) suggests a previously unsuspected and complex role for chitin-related processes in diatoms. All genes necessary for chitin synthesis were found in *T. pseudonana*, as well as *P. tricornutum*, despite the fact that *P. tricornutum* does not produce chitin fibers. Both diatoms appear to encode multiple chitin synthases; moreover, *T. pseudonana* possesses more than 20 genes that encode putative chitin-degrading chitinases and more than 20 genes that encode putative proteins with chitin-binding domains. Two of the putative chitin-binding proteins in *T. pseudonana* localize to the girdle band region of the cell wall, a region not previously suspected to contain chitin (6). The genes encoding these chitin-binding proteins, named p150 and p150-like, are highly expressed when cells are grown in the presence of high concentrations of copper or when cells are starved of silicic acid or iron, conditions that all result in abnormally elongated theca (6, 40). We recently identified 84 genes upregulated when cells were deprived of iron or silicic acid; many of these coregulated genes encode proteins known to be involved in cell wall processes (40). Surprisingly, a gene encoding a chitin synthase was among this subset of upregulated genes, suggesting that chitin may play a role in diatom

![FIG. 1. Maximum-likelihood phylogenetic tree of translated diatom chitin synthase gene sequences. Sequences amplified by CODEHOP primers are followed by numerical identifiers 1 through 10, while sequences identified from genomes are followed by their protein ID number. The outgroup *S. cerevisiae* chitin synthase 3 (NCBI accession no. P29465) was used to root the tree. Bootstrap values are indicated at nodes. A scale bar indicates the number of amino acid changes. The inset shows a simplified phylogeny of diatom evolution adapted from data from Medlin and Kaczmarska (36), and genera listed in the inset correspond to those present in the gene tree.](image-url)
cell walls processes. Additional support for this possibility comes from a recent analysis of the organic composition of *T. pseudonana* cell walls using solid-state nuclear magnetic resonance (62). That study identified chitin as the main carbohydrate component embedded within the silica cell walls, although the authors attributed this presence to contaminating chitin fibers.

The genetic complexity of chitin-related genes in *T. pseudonana*, the identification of potential chitin synthases in *P. tricornutum*, and the unexpected expression pattern of one chitin synthase gene in *T. pseudonana* suggests a more complex role of chitin synthesis in diatoms than was previously thought. We describe here our characterization of diatom chitin synthase genes through comparative genetics and physiological experimentation in order to better understand the evolution, function, and ecological consequences of chitin synthesis in diatoms.

**MATERIALS AND METHODS**

**In silico analyses.** Putative chitin synthase genes were identified in the *T. pseudonana* and *P. tricornutum* genomes by combining automated gene predictions (www.doe.jgi.gov) and homology of predicted proteins to known chitin synthases in the NCBI nonredundant database based on nBLAST and pBLAST tools (ncbi.nlm.nih.gov/blast/blast.cgi). BLAST identification of chitin synthases was considered significant when the e values were ≤10\(^{-10}\) and when a majority of identified homologs were annotated as chitin synthases. Domain structures of identified homologs were annotated as chitin synthases. Domain predictions were considered significant when the e values were ≤10\(^{-5}\).

**Phytoplankton cultures and experimental setup.** An isolate of *T. punctigera* and *T. rotula* was provided by P. von Dassow (64) and an isolate of *Ditylum brightwelli* was provided by J. Koester; both isolates were collected from Puget Sound, WA. An isolate of *Pseudo-nitzschia multiseries* from the North Atlantic was provided by S. Bates and C. Leger. All other phytoplankton isolates (Table 1) were purchased from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP [ccmp.bigelow.org]). Cultures were maintained exponentially at 100 to 200 μmol of photons m\(^{-2}\) s\(^{-1}\) in 250 to 500 ml of f/2-amended seawater (17, 18) that had been filtered through a 0.45-μm-pore-size polycarbonate filter (Millipore).

Silicic acid starvation and recovery were monitored on both short and long time scales. Short-term recovery from silicic acid starvation was analyzed by first growing triplicate cultures in Aquil medium (50) under continuous light at 100 μmol of photons m\(^{-2}\) s\(^{-1}\) and constant bubbling with sterile air until the middle of the exponential growth phase. In a manner similar to those described by Hildebrandt et al. (22), cells were harvested by centrifugation at 4,500 × g for 15 min, washed once with, and inoculated into Aquil medium without silicic acid at a final concentration of 0.6 × 10\(^{-4}\) cells ml\(^{-1}\). At 48 h, silicic acid was added to the cultures at a final concentration of 200 μM, and the cultures were monitored for an additional 8 h as they recovered from silicic acid starvation and resumed cell division. Long-term responses to silicic acid starvation were monitored in triplicate cultures transferred from exponential growth in fully amended f medium (17) into f medium with low silicic acid (0.067 μM) at 175 to 275 μmol of photons m\(^{-2}\) s\(^{-1}\). Changes in chlorophyll a fluorescence over time were measured with a fluorometer (Turner), and cell concentrations were determined with an InFlux cell sorter flow cytometer (Cytopeia) equipped with a 488-nm laser. An aliquot of 2-μm fluorescein isothiocyanate bead stock (Polysciences) of known concentration was added to 1 ml of culture, and the particles were counted together. Photosynthetic capacity (Fv/Fm) was determined with a PhytoPAM fluorometer (Walz). To confirm that cell division had ceased due to silicic acid starvation, a final concentration of 214 μM silicic acid was added to a 50-ml aliquot of each triplicate culture, and the relative fluorescence was monitored for a day. Algaicots of the culture media on day 4 were also filtered through a 0.2-μm-pore-size filter and analyzed for dissolved silicic acid content by using the molybdate method (5). A pulse of silicic acid similar to the short-term experiment (214 μM) was added to the silicic acid-starved cultures after 6 days in stationary phase, and the response of the cells was monitored for 48 h. Samples were collected from both types of experiments for quantitative reverse transcriptase PCR (qRT-PCR) by filtering between 200 and 1,000 ml of culture onto two 2-μm-pore-size polycarbonate filters (Millipore) (see below). Filtered cells were also collected from previously described experiments (40) with nutrient-replete cultures and short-term (<1 day) silicic acid-, iron-, and nitrate-limited cultures.

**DNA and RNA extraction and cDNA synthesis.** DNA was extracted with a plant DNA extraction kit (Qiagen) from exponentially maintained cultures (Table 1) that had been concentrated and frozen onto 0.45-μm-pore-size polycarbonate filters (Millipore). RNA was extracted with the plant RNA isolation reagent (Invitrogen) from frozen cells on 2-μm-pore-size polycarbonate filters. Contaminating DNA was eliminated by incubating the isolated RNA with DNase (Ambion) at 37°C for 1 to 2 h; DNA-free RNA was purified with an RNasy MiniElute cleanup kit (Qiagen). cDNA was synthesized from 2 μg of purified RNA with a Superscript III first-strand synthesis system for RT-PCR (Invitrogen). The 20 μl of cDNA was subsequently diluted with water to 100 μl. One microliter of RNA was used in PCRs (see below) to ensure that all contaminating DNA had been eliminated.

**TABLE 1. Organisms tested by PCR for the presence of chitin synthase genes and identification of those in which chitin synthase gene fragments were successfully amplified.**

| Organism                      | Source      | Group                      | Known chitin producer | Chitin synthase identified |
|-------------------------------|-------------|----------------------------|-----------------------|---------------------------|
| Phaeocystis globosa           | CCMP 629    | Prymnesiophyte             | Yes                   | No                        |
| Gyrodinium sp.                | CCMP 1737   | Dinoflagellate             | No                    | No                        |
| Chlorella minutissima         | CCMP 1941   | Red algae                 | No                    | No                        |
| Thalassiosira pseudonana      | CCMP 1335   | Multicellular diatom       | Yes                   | Yes                       |
| Thalassiosira guillardii      | CCMP 988    | Multicellular diatom       | Yes                   | Yes                       |
| Thalassiosira minuta          | CCMP 1093   | Multicellular diatom       | Yes                   | Yes                       |
| Thalassiosira oceanica        | CCMP 999    | Multicellular diatom       | Yes                   | Yes                       |
| Thalassiosira punctigera      | P. von Dassow| Multicellular diatom       | Yes                   | Yes                       |
| Thalassiosira weissflottii    | CCMP 1336   | Multicellular diatom       | Yes                   | Yes                       |
| Skeletonema costatum          | CCMP 780    | Multicellular diatom       | No                    | Yes                       |
| Chaetoceros socialis          | CCMP 205    | Multicellular diatom       | No                    | Yes                       |
| Lithodesmium undulatatum      | CCMP 472    | Multicellular diatom       | No                    | Yes                       |
| Ditylum brightwelli           | J. Koester  | Multicellular diatom       | No                    | No                        |
| Stephanopysis palmeriana      | CCMP 814    | Radial centric diatom      | No                    | No                        |
| Phaeodactylus tricornutum     | CCMP 632    | Pennate diatom             | No                    | Yes (genome)              |
| Entomoneis alata              | CCMP 1522   | Pennate diatom             | No                    | No                        |
| Fragilaria pinnata            | CCMP 295    | Pennate diatom             | No                    | No                        |
| Pseudo-nitzschia multiseries  | C. Leger and S. Bates | Pennate diatom          | No                    | No                        |
| Toxarium undulatum            | CCMP 2277   | Pennate diatom             | No                    | No                        |
**Determination of full-length gene sequences.** Full-length sequence of the *T. pseudonana* chitin synthase cDNAs was determined by DNA sequencing of PCR-generated fragments using a total of 40 primers (not listed) designed across the entire length of the modeled gene sequences (www.doe.jgi.gov), and open reading frames that appeared to extend up or downstream of gene models. PCRs amplified the entire length of the modeled gene sequences (www.doe.jgi.gov), and open reading frames that appeared to extend up or downstream of gene models. PCRs were designed to amplify a 100- to 250-bp fragment from genes encoding three chitin synthases (protein IDs 7305, 6575, and 4368) and two genes encoding girdle band-associated chitin-binding proteins p150 and p150-like (protein IDs 12594 and 26041). Triplicate qRT-PCR amplifications consisted of an initial denaturation of 94°C for 2 min, followed by 30 cycles of 94°C for 10 s, 60°C for 30 s, and 72°C for 50 s; followed by a stepwise increase in primer set, and then 72°C for 90 s. Amplified fragments were separated on an agarose gel, and fragments of the appropriate size were cut out of the gel and extracted with the QiaQuick gel extraction kit (Qiagen). Between 50 and 100 ng of purified PCR product was cycle sequenced with DYEnamic ET dye terminator (GE Healthcare Biosciences Corp.) and analyzed on a MegaBACE 1000 (GE Healthcare Biosciences Corp.). Rapid amplification of cDNA ends (RACE) was performed with First Choice RLM-RACE kit (Amersham Pharmacia Biotech). RACE PCR primers designed to amplify a 100-bp fragment from genes encoding three chitin synthases (protein IDs 7305, 6575, and 4368) were identified by using BLAST.

**Gene expression.** Transcript abundances in *T. pseudonana* were determined by qRT-PCR. Primers (Table 2) were designed to amplify a 100- to 250-bp fragment for four reference genes encoding the actin-like protein (protein identification [protein ID] 269504), actin (protein ID 25772), 40S ribosomal (protein ID 31084), and beta tubulin (protein ID 31569). Primers were also designed to amplify an 100-bp fragment from genes encoding three chitin synthases (protein IDs 7305, 6575, and 4368) and two genes encoding girdle band-associated chitin-binding proteins p150 and p150-like (protein IDs 12594 and 26041). Triplicate qRT-PCRs included 2 μl of cDNA; 0.8 μM concentrations of forward and reverse primers; and 10, 20, or 25 μl of iQ Supermix (Bio-Rad) and consisted of an initial denaturation at 95°C for 3 min; followed by 30 cycles of 94°C for 10 s, 60°C for 30 s, and 72°C for 90 s. Amplified DNA fragments were separated and purified from an agarose gel as described above, ligated into the TOPO vector (Invitrogen), and used to transform *Escherichia coli* TOP 10 cells. Clones were sequenced and analyzed as described above. To determine the efficiency of each primerset, and then 72°C for 90 s. Amplified fragments were separated on an agarose gel, and fragments of the appropriate size were cut out of the gel and extracted with the QiaQuick gel extraction kit (Qiagen). Between 50 and 100 ng of purified PCR product was cycle sequenced with DYEnamic ET dye terminator (GE Healthcare Biosciences Corp.) and analyzed on a MegaBACE 1000 (GE Healthcare Biosciences Corp.). Rapid amplification of cDNA ends (RACE) was performed with First Choice RLM-RACE kit (Amersham Pharmacia Biotech). RACE PCR primers designed to amplify a 100-bp fragment from genes encoding three chitin synthases (protein IDs 7305, 6575, and 4368) were identified by using BLAST.

**Gene expression.** Transcript abundances in *T. pseudonana* were determined by qRT-PCR. Primers (Table 2) were designed to amplify a 100- to 250-bp fragment for four reference genes encoding the actin-like protein (protein identification [protein ID] 269504), actin (protein ID 25772), 40S ribosomal (protein ID 31084), and beta tubulin (protein ID 31569). Primers were also designed to amplify an 100-bp fragment from genes encoding three chitin synthases (protein IDs 7305, 6575, and 4368) and two genes encoding girdle band-associated chitin-binding proteins p150 and p150-like (protein IDs 12594 and 26041). Triplicate qRT-PCRs included 2 μl of cDNA; 0.8 μM concentrations of forward and reverse primers; and 10, 20, or 25 μl of iQ Supermix (Bio-Rad, Richmond, CA) in a 20-, 40-, or 50-μl reaction volume. Amplifications were carried out in an iCycler (Bio-Rad) and consisted of an initial denaturation at 95°C for 3 min; followed by 30 cycles of 94°C for 10 s, 60°C for 30 s, and 72°C for 90 s. Amplified DNA fragments were separated and purified from an agarose gel as described above, ligated into the TOPO vector (Invitrogen), and used to transform *Escherichia coli* TOP 10 cells. Plasmids from 3 to 10 positive transformants were amplified with TemPlPhi (GE Healthcare Biosciences Corp.) and sequenced with M13 forward and reverse primers as described above. Resulting DNA sequences were analyzed in Sequencer and aligned with CLUSTAL W. Positive identification of fragments corresponding to chitin synthase genes was based on the presence of the sequence that encoded EDR and QRRR motifs. Sequences that differed by three or more nucleotides were considered distinct. A maximum-likelihood tree was calculated in proml in the PHYLIP software package (12) based on the alignment of all translated sequence fragments amplified with CODEHOP primers.
ers, *T. pseudonana* and *P. tricornutum* sequences, with *Saccharomyces cerevisiae* chitin synthase 3 (P29465) as an outgroup. Sequence alignment was anchored by the EDR, QRRRW, and SWG motifs. Regions corresponding to amino acids 1007 to 1073 in *T. pseudonana* 705, amino acids 573 to 770 in *P. tricornutum* 37908, and amino acids 1012 to 1078 in *S. cerevisiae* P29465 were eliminated due to the uncertainty of alignment in this region. The resulting alignment was 111 amino acids long, and bootstrap values were calculated from the construction of 1,000 trees.

**Localizing and quantifying chitin and silica deposition.** Silica deposition was determined by incubating 50-ml aliquots of cultures with 0.5 μM 2-(4-pyridyl)-5-(4-2(dimethylaminomethyl)-aminobenzamido)-methylphenyl oxazole (PDMPO; Invitrogen) for 12 to 14 h in the same light and temperature conditions as the experimental conditions (56). Cells were analyzed with an Influx cell sorter flow cytometer (Cytopeia) equipped with a 355-nm UV laser. Fluorescent emission was detected at 460 nm (50-nm band-pass). Internal standards of 3-μm UV beads (Spherotech) were used for calibration. Epifluorescence was also visualized with an i80 microscope (Nikon) after excitation at 300 to 400 nm and emission detected with a 420-nm long-pass filter (Chroma). Potential chitin localization was determined by incubating cells with 4 μg of fluorescein isothiocyanate-labeled wheat germ agglutinin (FITC-WGA; Sigma) for 15 to 30 min. Cells were centrifuged at 15,000 *g* for 5 min and resuspended in 1 ml of 1/2 media or phosphate-buffered saline buffer (137 mM NaCl, 2.7 mM KCl, 10.4 mM Na2HPO4, 1.8 mM KH2PO4) to remove unbound FITC-WGA. The resulting fluorescence signal was quantified by flow cytometry after excitation with a 488-nm laser, and 2-μm yellow-green beads (Polysciences) were used as internal standards. Emission was detected at 530 nm (40 nm band-pass). Localization of FITC-WGA binding was visualized by fluorescence microscopy with a 500- to 570-nm wavelength filter (Chroma) after excitation at 455 to 500 nm. Competitive binding experiments with chitotriose and N-acetylglucosamine (41, 48) were used to determine whether the FITC-WGA bound specifically to chitin in *T. pseudonana*. First, 4 μg of FITC-WGA was incubated with 1 μg to 2.4 μg of chitotriose in 100 μl of water for 3 h, followed by a 30-min incubation with 100 μl of cell culture at >105 cells ml−1. Excess FITC-WGA was washed from the cells as described above, and the amount of FITC-WGA bound to cells was quantified by flow cytometry and microscopy. Afterward, similar incubations were conducted with 1 μg to 9.6 μg of N-acetylglucosamine as the competitive binding substrate. Flow cytometry signal comparisons were calculated in MatLab by using a two-sided Kolmogorov-Smirnov test with a 95% confidence interval. Modes were determined by creating a histogram of the 65,000 possible fluorescence intensities detected by the flow cytometer with a bin size of 200. If a distribution had two modes the average of the two numbers was used. Distributions were visualized by using FlowJo (Tree Star, Inc.).

Exponentially growing and silicic acid starved *T. pseudonana* cells were also incubated with FITC-labeled chitin-binding probe (FITC-chb) purified from *Bacillus circulans* (New England Biolabs) by pelleting the cells in a centrifuge at 15,000 × *g* for 10 min, resuspending them in 500 μl of TBS (0.05 M Tris base, 0.15 M NaCl), and then incubating them with 1 μl of FITC-chb for at least 3 h. The cells were washed, and FITC-chb localization was visualized as described above. Cells were also stained with 10 μg of calcofluor white (Sigma) for 30 min and visualized after excitation at 300 to 400 nm and emission detected with a 420-nm long-pass filter (Chroma). Additional diatom species *T. punctigera*, *T. rotula*, and *S. costatum* were also silicic acid starred and stained with FITC-WGA as described above.

**Genetic sequences.** Chitin synthase sequence fragments amplified from CODEHOP primers were deposited in the NCBI database under the following accession numbers: *C. socialis* (FJ544943 to FJ544945), *L. undulatum* (FJ544946 and FJ544947), *S. costatum* (FJ544948 to FJ544952), *T. guillardi* (FJ544953 to FJ544960), *T. minuta* (FJ544961 to FJ544965), *T. oceanica* (FJ544966 to FJ544974), *T. punctigera* (FJ544975 to FJ544977), and *W. iweissflogii* (FJ3500876 to FJ350085).

**RESULTS**

In silico and phylogenetic analyses of putative chitin synthases in diatoms. Automated annotation of the *T. pseudonana* whole genome sequence identified six genes that encoded putative chitin synthases (Table 3) based on detection of the chitin synthase active site domain with the conserved amino acid motifs EDR, QRRRW, and SWG. Full-length sequences and intron locations of the identified genes were confirmed through a combination of cDNA sequencing and RACE. The six *T. pseudonana* genes are located on three chromosomes. Chitin synthase genes occur as inverted repeats once on chromosome 4 and again on chromosome 7. Because the genes in both inverted repeats are identical to each other, it was not possible to determine whether retrieved cDNA sequences corresponded to both members of a repeat. Automated gene model predictions from version 3 of the *T. pseudonana* genome were consistent with transcribed regions confirmed by cDNA sequencing and RACE, except modeled protein ID 4368 incorrectly predicted a short exon at the 3′ end.

Automated annotation of *P. tricornutum* whole genome sequence identified two genes that encoded putative chitin synthases (Table 3) that also contained the chitin synthase active-site domain with conserved amino acid motifs, despite the fact that this diatom is not known to produce chitin. The gene model for one chitin synthase (ID 44759) displayed expressed sequence tag (EST) support without additional open reading frames detected either upstream or downstream of the modeled gene and was therefore assumed to be accurate. The second gene (ID 37908) lacked EST support but displayed 61% nucleotide sequence identity and 57% translated nucleotide identity to the gene encoding 44759.

PCR products from the active site region of chitin synthase genes were successfully amplified from a variety of multipolar species including the known chitin fiber producers *T. oceanica*, *T. punctigera*, *T. minuta*, *T. guillardi*, and *W. iweissflogii*, as well as species not known to produce fibers, including *Skeltonema costatum*, *Chaetoceros socialis*, and *Lithodesmium undulatum*. PCR products were not successfully amplified from the other examined phytoplankton (Table 1). Phylogenetic analyses grouped the diatom chitin synthases into three clades with bootstrap support of 96, 71, and 100; a fourth potential clade has weaker bootstrap support of 45 (Fig. 1). The two *P. tricornutum* sequences formed a clade, identified here as clade D. The multipolar diatom sequences were distributed among three clades, identified here as clades A, B, and C. Each of these three clades contained sequences from multiple species. Moreover, most examined multipolar species encoded proteins that fell into more than one clade. Although clone libraries were not sequenced to saturation, it is of interest to note that

| Genotype and protein ID | Nucleotides (bp) | Intron length (bp) | Chromosome location | Confirmation method |
|------------------------|------------------|--------------------|--------------------|--------------------|
| **T. pseudonana**       |                  |                    |                    |                    |
| 6575                   | 4,408            | 96,99              | 6                  | cDNA sequencing    |
| 7305                   | 3,887            | 143                | 7 (IR)             | cDNA sequencing    |
| 7306                   | 3,887            | 143                | 7 (IR)             | cDNA sequencing    |
| 4368                   | 4,374            | None               | 4                  | cDNA sequencing    |
| 4413                   | 3,012            | None               | 4 (IR)             | cDNA sequencing    |
| 4414                   | 3,012            | None               | 4 (IR)             | cDNA sequencing    |
| **P. tricornutum**      |                  |                    |                    |                    |
| 44759                  | 2,806            | 91                 | 5                  | EST coverage       |
| 37908                  | 2,875            | 73                 | 14                 | Similarity to 44759 |

* IR, inverted repeat.
only species possessing fultoportula encoded proteins associated with clade A sequences.

The large sequence divergence of clade D from clades A, B, and C is similar to the divergence between division 1 and division 2 fungal chitin synthases. The P. tricornutum chitin synthases do not have transmembrane domains N terminal to the QRRRW motif. This motif is also located relatively near the N-terminal end, which is typical of division 1 fungal and oomycete chitin synthases. In contrast, the T. pseudonana chitin synthases possess additional transmembrane domains and a cytochrome b5 domain toward the N-terminal end and their QRRRW motif is located closer toward the C-terminal end, features that characterize division 2 fungal chitin synthases (54) (Fig. 2). The chitin synthases from clades A and C contain a myosin motor head domain and have identical protein domain predictions overall. The clade B chitin synthase lacks the myosin motor head domain and has an additional transmembrane domain. The three different protein domain structures further support the identification of phylogenetically distinct clades A, B, and D.

**Differential expression of chitin synthase genes in T. pseudonana.** Relative transcript abundance for the chitin synthase genes from the three clades was not significantly different (P > 0.01) under nutrient-replete or nitrate-depleted conditions (Fig. 3A). Relative transcript abundance for the genes from clades A and C was also not significantly different when growth was slowed by iron or silicic acid depletion relative to nutrient-replete conditions (P > 0.01). In contrast, the gene from clade B was upregulated by 7.6- ± 0.5-fold and 4.9- ± 0.5-fold (P < 0.01) relative to the control after experiencing less than 24 h of silicic acid or iron depletion, respectively. This treatment is expected to stop growth of a portion of cells in the same phase of the cell cycle (22).

To assess transcript abundance in synchronized cells, cells were first starved of silicic acid for 48 h to block the majority of cells in the same phase of the cell cycle, and then replenished with sufficient silicic acid to resume cell division. This is similar to a treatment shown to synchronize T. pseudonana (22), modified in the present study with an additional day of silicic acid starvation. Transcript abundance of genes from clades A and C did not differ significantly (P > 0.01) among the 11 time points examined during the 8 h after replenishment of silicic acid. In contrast, transcript abundance of the clade B gene remained significantly lower (P < 0.01) during the early time points (5 min and 0.5, 1, and 2 h) after the addition of silicic acid than during the later time points (4, 6, and 8 h); transcript abundance also differed significantly between the 5-min and 7-h time points (Fig. 3B). This treatment appears to influence transcription of the clade B chitin synthase gene once cells reach a particular stage in cell division.

The effects of long-term silicic acid starvation and recovery were also examined. Asynchronous cells maintained in media with growth limiting concentrations of silicic acid increased exponentially (μ = 1.8 day⁻¹) for 3 days, after which point silicic acid was depleted to 0 μM in all bottles. Photosynthetic capacity decreased in these silicic acid starved cells, as measured by a drop in the Fv/Fm from 0.64 ± 0.02 to 0.5 ± 0.05 (Fig. 4A), the cell division was blocked, and cells began to display aberrant morphology and aggregation. The chlorophyll a relative fluorescence units (RFUs) doubled in 50-ml aliquots from each day-4 culture 1 day after the addition of 214 μM silicic acid. Together, these data indicate that growth of cells was stopped by silicic acid depletion. The tendency for aggregation persisted until the starved culture was replenished with silicic acid on day 9. By day 10, the Fv/Fm had increased to 0.62 ± 0.03 and cultures resumed asynchronous exponential growth (μ = 1.1 day⁻¹) (Fig. 4A).

During the 3 days of exponential growth, the relative transcript abundance was low for the chitin synthase genes from clades A, B, and C and the two genes encoding chitin-binding proteins, p150 and p150-like, that localize to the girdle bands (days 2 and 3, Fig. 4B and C). A slight yet significant increase in clade B and a decrease in clade A chitin synthases occurred on day 3, suggesting that cells were beginning to experience
silicic acid depletion. On day 4, when silicic acid was depleted and cell division stopped, the relative transcript abundance of both the clade B and C chitin synthases increased by 4.9- ± 0.6-fold and 2.3- ± 0.4-fold, respectively, relative to the previous day. This increased transcript abundance was significantly maintained through day 10 for the clade C chitin synthase and through day 11 for the clade B chitin synthase, although the levels did begin to drop as cells resumed unsynchronized division. Transcript abundance of the chitin binding gene p150 also increased 3.8- ± 0.8-fold on day 4. In contrast, transcript abundance of the clade A chitin synthase gene and p150-like gene did not increase during the period of silicic acid starvation. When related to different reference genes, the absolute value of transcript abundance, but not the direction of the daily changes, was affected by the choice of reference gene (see Fig. S1 in the supplemental material). Transcript abundance of the actin-like gene (ID 269504) appeared to maintain constant expression or increase during silicic acid starvation and decrease during exponential growth, depending on the reference gene used (see Fig. S1A in the supplemental material), and was thus identified as the most conservative reference gene for target gene expression. This gene was previously shown to have similar expression levels in different growth conditions (40).

Chitin localization in diatoms. Competitive binding experiments were conducted to determine whether FITC-WGA binds specifically to T. pseudonana chitin and thus can be used to determine chitin localization. As concentrations of chitotriose preincubated with FITC-WGA increased, reduced amounts bound to T. pseudonana cells, as detected with flow cytometry (Fig. 5A). Distributions of the FITC-WGA fluorescence of cells incubated with or without chitotriose were sig-
nificantly different ($P < 0.05$) at every concentration, and the mode consistently decreased as chitotriose concentrations increased, to a final mode 70% less than noncompetitively bound cells. In contrast, the fluorescence distributions of FITC-WGA-bound cells with or without N-acetylglucosamine were not significantly different ($P > 0.05$) until concentrations of 22 mM were reached and the mode values varied by no more than $\pm 5\%$ (Fig. 5B). These results suggested that the FITC-WGA bound specifically to *T. pseudonana* chitin.

The impact of silicic acid starvation and recovery on silica deposition and chitin localization were examined by staining cells with FITC-WGA and PDMPO, a stain that incorporates into newly deposited silica (56). During the transition of *T. pseudonana* from exponential growth to silicic acid depletion, silica deposition and chitin exposure on the cell wall were inversely correlated. Exponentially growing cells had a relatively narrow distribution of high intensity (mode $= 135 \pm 5$ RFUs) PDMPO staining as detected with flow cytometry (Fig. 6A). Epifluorescence microscopy confirmed that these exponentially growing cells were depositing silica at valves and girdle bands (Fig. 6B and C). In contrast, the FITC-WGA fluorescence of exponentially growing cells was slightly higher (mode $= 66 \pm 4$ RFUs) than unstained cells (mode $= 46 \pm 2$). FITC-WGA staining of cells was not detectable with epifluorescence microscopy. As cells entered silicic acid depletion on day 4 (Fig. 4A), the distribution of PDMPO fluorescence broadened, with more cells displaying lower intensity fluorescence, and the average mode decreased by 35% (Fig. 6A). Microscopic examination of cells indicated that many still deposited silica at the girdle bands but not the valves. Mode fluorescence of FITC-WGA per cell increased on day 4 by 35%. Microscopy revealed lectin localization around the girdle bands (Fig. 6C). The PDMPO fluorescent intensity per cell continued to decrease until the average mode was 1% of the original intensity on day 5, and no staining of cells was observed (Fig. 6C). The distribution of FITC-WGA fluorescence per cell continued to decrease until the average mode was 1% of the original intensity on day 5, and no staining of cells was observed (Fig. 6C). The distribution of FITC-WGA fluorescence shifted to a higher intensity, with the highest average mode on day 9 being about twice the original intensity. Most cells had FITC-WGA bound to the girdle-band region. These cells also had an elongated and bent phenotype. This was particularly evident in the highest-fluorescing cells, where this exposed bent joint between the two thecae appeared to be larger (Fig. 6C). No binding of FITC-WGA was seen at the chitin fibers. After silicic acid replenishment on day 9, the mode of PDMPO fluorescence increased significantly ($119 \pm 5$ RFUs) and the mode of FITC-WGA fluorescence decreased significantly ($72 \pm 1$ RFUs) ($P < 0.01$).

To confirm the localization of chitin at the girdle bands, additional fluorescent chitin binding probes were used to label both exponentially growing and silicic acid-starved *T. pseudonana* cultures. Cells were incubated with FITC-WGA, calcofluor white, and the commercially available chitin-binding protein derived from bacteria, FITC-chb. All three stains lo-
The localization of chitin at the girdle band region in additional species of diatoms was confirmed by labeling silicic acid-starved *T. punctigera*, *T. rotula*, and *S. costatum* with FITC-WGA (Fig. 8). All three species displayed an abnormal phenotype elongated at the theca. As in *T. pseudonana*, FITC-WGA localized to these elongated regions.

**DISCUSSION**

The discovery of chitin synthase genes in a variety of diatoms emphasizes the widespread distribution of chitin throughout eukaryotic lineages. Stramenopile chitin synthases have been sparsely characterized, and the present study provides a more complete picture of the evolutionary history of this polymer.
Chitin is often widely distributed within lineages due to its fundamental structural function in the organisms; however, it can also evolve divergent functions and structures. For example, in fungi and insects chitin is a primitive, indispensable character that plays a fundamental structural role in forming the cell wall and exoskeleton and thus is present throughout these two groups of organisms. However, within the fungi, chitin has different functions during cell wall formation, bud scar formation, and cell division (52). In mollusks, chitin is also widely distributed and is used for diverse structural functions, such as the formation of the shells in bivalves (33), the structural pens in squid (26), and the radula of snails (60). Identification of chitin synthase genes in diatom genera (52x), the radula of snails (60). Identification of chitin synthase genes in diatom genera (52), the formation of the shells in bivalves (33), the structural pens in squid (26), and the radula of snails (60).

The patterns of transmembrane domains and thus are expected to assume different folded structures across the membrane. In addition, only the T. pseudonana chitin synthase genes encode a cytochrome b5 domain, which can anchor proteins into lipid bilayers (15) and may have served as a template for the evolution of a lipid binding site (39). This difference in the presence of cytochrome b5 domains is also observed between the two divisions of fungal chitin synthases. The acquisition or loss of this N terminus from the ancestral chitin synthase is hypothesized to have driven the divergence of the two fungal chitin synthase divisions (54). Fungi often contain chitin synthases from both divisions. In contrast, the few stramenopile lineages that have been examined do not contain this level of diversity within a single organism. The other chitin synthases identified in stramenopiles are from oomycetes, whose sequences are phylogenetically similar to division 1 fungal chitin synthases (54). In diatoms, T. pseudonana chitin synthases appear to be similar to division 2 fungal chitin synthases, while P. tricornutum chitin synthases are more similar to those of division 1.

Three clades of chitin synthase genes were detected in multipolar diatoms; two were supported by high bootstrap values, and the distinctiveness of the third was supported by additional gene expression data. This suggests different evolutionary histories and possibly different functions for chitin within the cells. Each clade contains sequences from multiple species, and a single species commonly has chitin synthase genes in multiple clades. The lone exception to this is T. weissfloggi, with multiple sequences all assigned only to clade A; this could be due to insufficient sampling of clone libraries, and the large number of
copies may be related to the large genome size of this strain (64). Clades B and C contain gene fragments from all successfully tested genera, including the chitin-producing *Thalassiosira* species and the non-chitin-fiber-producing *C. socialis, L. undulatum, and S. costatum*. Representatives from these two gene clades were likely present in the common ancestor prior to the evolution of the chitin-fiber-producing species. Interestingly, the encoded proteins from these two clades in *T. pseudonana* appear to utilize different modes of intracellular transport: the clade C protein possesses a myosin motor head domain N-terminal to the cytochrome *b*2 domain, whereas the clade B protein lacks this domain. Clade A sequences are dominated by members of the genus *Thalassiosira*. The single exception is a gene fragment from *Skeletonema costatum*. This genus is derived from *Thalassiosira* (28) and also has fultoportula, the specialized pores in the silica wall through which chitin fibers emerge. However, rather than chitin fibers extending from the fultoportula, the *S. costatum* silica structures instead remain fused between divided cells and create distinctive chains of cells. Now, only species with fultoportula contain clade A chitin synthases. Therefore, clade A gene products are likely associated with the fultoportula structures, including the synthesis of the long chitin fibers. The clade A chitin synthase also possesses a myosin motor head domain in a similar location as the protein from clade C, suggesting a similar evolutionary history for these two gene families.

Support for functional differences between chitin synthases also comes from the differential transcription of genes in *T. pseudonana* cells exposed to different nutrient conditions. Relative transcript abundance of the clade B gene is particularly sensitive to short-term depletion of either silicic acid or iron. These conditions resulted in an increased transcript abundance of this clade B gene but not of the clade A or C genes. Coregulation of transcript abundance by silicic acid and iron depletion has been linked to cell wall processes (40). Transcript abundance of only the clade B gene varied depending on the amount of time elapsed since synchronized cells resumed division after short-term silicic acid starvation (22), suggesting that the clade B gene may have a role during a particular part of the cell cycle and particularly under silicic acid and iron stresses. When cells were submitted to longer-term silicic acid starvation, a response from both the clade B and clade C chitin synthases was detected, but the clade C response was of a much smaller magnitude. This gene may also be related to cell wall processes, although it appears to have less sensitivity to changing silicic acid conditions. Interestingly, similar differences in transcription level was found for the two genes encoding girdle-band associated chitin-binding proteins both in the present study and others (7). None of the tested conditions affected the transcript abundance of the clade A chitin synthase, suggesting that it is not directly related to these cell wall processes.

The chitin-specific lectin WGA was used to correlate clade B and C transcript abundance with chitin synthesis. The lectin bound to the girdle band region of cells. WGA can bind to multiple substrates, so its specificity for chitin on the cell was confirmed both by competitive binding experiments and by similar localization patterns using two additional chitin-binding probes with different binding mechanisms and access to the binding substrate. Interestingly, the lectin was not visibly bound to the chitin fibers extruded from the fultoportula. This might be due to the pure beta configuration of the fiber polymers, whose highly crystalline parallel arrangement may not be accessible to the lectin-binding sites. In contrast, both the chitin-binding probe and calcofluor white were visible on both the girdle-bands and the fultoportula chitin fibers, indicating a more general binding mechanism that does not exclude specific forms of chitin. When cells experienced silicic acid depletion and longer-term starvation, transcript abundance increased for the clade B and C chitin synthases and for a gene encoding a chitin-binding protein, p150, previously localized to cell wall girdle bands (6). The increase in transcripts corresponded to the increase in WGA binding at the girdle bands, which suggests that the clade B and C proteins are related to synthesis of chitin at the girdle bands in times of cell wall stress. The clade B gene also appears to be transcribed during normal progression through the cell cycle. The relative amount of transcript abundance of the clade B gene depended on length of exposure to silicic acid starvation and/or amount of time since recovery from different degrees of silicic acid starvation, events known to influence cell cycle progression (22). Further experiments are needed to determine the exact relation between chitin synthesis and cell cycle progression. However, both the chitin binding probe and calcofluor white localized to the girdle band region of exponentially growing cells preparing to separate after completing division. This supports a connection between chitin synthesis with the cell cycle and suggests that chitin synthesis is enhanced in stressed cells unable to precipitate silica and divide.

The identification of chitin synthase genes in a wide variety of diatoms and the localization of chitin at the girdle bands of *T. pseudonana* suggests the possibility that this use for chitin may be more widespread in diatoms. Silicic acid-starved *T. punctigera, T. rotula*, and *S. costatum* were labeled with WGA, and similar localization patterns were identified. The association of chitin with the cell wall should be considered in other diatom species; however, more detailed analysis within each lineage is needed.

Chitin appears to be intimately connected with silica processes because of its relationship to the cell wall, and this connection is illustrated in several ways. The distinctive fultoportula that serve as pores through the silica cell wall appear to have formed specifically to extrude chitin fibers (24), implying that this structure likely coevolved with the ability to produce chitin fibers at this cellular location, perhaps with the evolution of the clade A chitin synthase genes. Interestingly, chitin and silica have also evolved to form structures of similar functions. Diatoms can be found as solitary cells or in chains depending on the species. Either chitin fibers or silica spines can extend out from the cell to increase drag in the water and also form the connections between chains of cells. The evolution of either chitin or silica connections between cells may result from the different costs associated with production of rigid silica spines versus flexible chitin fibers; for example, rigid chains experience larger shear flow compared to flexible chains, which has the potential to increase their encounter rate with other particles and nutrients (29). The discovery of chitin at the girdle band region reveals another connection between chitin and silica. Our results suggest that chitin is localized to the girdle band region as a normal function of cell division and also under conditions when the cells are unable to precipitate ad-
ditional silica. This stress-induced localization of chitin around the girdle band region is associated with cells that are elongated and bent and that tend to aggregate and sink. The potential interactions between silica and chitin likely reflect the necessary coordination of the organic and inorganic components of a functional cell wall.

Chitin synthase is proposed to have been present in a primitive eukaryote because of its widespread distribution throughout the eukaryotic tree of life (54). Thus, it seems probable that the ability to synthesize chitin was present in diatom ancestors as they evolved the ability to generate cell walls of silica. However, further work is needed to investigate whether chitin synthases can be identified in the more primitive centric lineage or a sister group to diatoms. Perhaps chitin has been maintained as an essential component of the cell wall, and it is only during times of cell wall stress or certain stages of the cell cycle that it can be detected through lectin binding at specialized locations. The use of chitin as a matrix for the precipitation of inorganic structures is a common strategy in eukaryotes (10, 11, 65), and the potential for chitin-silica structures specifically has been demonstrated in vitro (46). It is intriguing to speculate whether direct interactions between silica and chitin occur in diatoms; however, the current understanding of diatom silica precipitation is that it occurs in an adhesive vesicle and does not rely on chitin (30–32). Interestingly, a recent microscopy-based study has shown that an uncharacterized organic matrix of fibers forms the core of the girdle bands onto which the silica structure is formed (23). Our data indicate a role for chitin in the cell wall, and future microscopy-based studies are needed to clarify that role. Chitin may be a component of the cell wall that is linked to silica but functionally separate. For example, chitin, rather than silica, may be used for more flexible components of the cell wall during the cell division cycle. Similarly, the apparently larger quantities of chitin detected at the girdle bands during silicic acid starvation may serve as a replacement cell wall material during conditions in which silica cannot be deposited. Notably, the increased synthesis of chitin at girdle bands in stressful conditions is reminiscent of chitinous cyst formation, a survival strategy used by other protists (42).

The relationship between silica and chitin has ecological implications for both diatoms and the global ocean. Chitin is the most abundant polymer in the ocean and serves as an enormous reservoir of organic carbon and nitrogen. Diatoms have traditionally been excluded from considerations of chitin production (27, 59). Our work suggests that diatoms are likely larger contributors to this organic reservoir than previously thought. Chitin is an attractive source of nutrients for microbes (37, 44), and the deposition of chitin at diatom girdle bands has the potential to facilitate diatom-microbe interactions (19, 25).

The morphology of cell wall-stressed, chitin-producing cells is also of ecological importance. These cells are elongated and bent with a tendency to aggregate, which causes them to sink. If these processes observed in a laboratory translate to post-bloom environmental conditions, it suggests that production of chitin on the cell wall is enhanced when growth is no longer possible, followed by sinking out of the surface layer. Sinking is proposed to be a survival strategy for cells in hostile surface environments and enables them to persist at depth until upwelling brings them back into favorable conditions (58). Thus, the deposition of chitin around the cell as it sinks to less hostile conditions may be a component of a survival strategy to persist through fluctuating ocean conditions. In addition, if cells sink below the mixing depth, sinking becomes a mechanism to pump carbon and other nutrients to the deep ocean (57).

ACKNOWLEDGMENTS

This study was supported by a Gordon and Betty Moore Foundation Marine Microbiology Investigator Award to E.V.A. We thank Rhonda Morales, Julie Koester, and Thomas Connolly for help with flow cytometry analysis; Ellen O. Lin and Audrey Djunaedi for help with RNA extraction and qRT-PCR. We also thank Julie Koester, Micaela Parker, and Nils Kröger for helpful comments and recommendations.

REFERENCES

1. Aluwihare, L. I., D. J. Repeta, S. Pantoja, and C. G. Johnson. 2005. Two chemically distinct pools of organic nitrogen accumulate in the ocean. Science 307:1097–1101.

2. Armbrust, E. V., J. A. Berges, C. Bowler, B. R. Green, D. Martinez, N. H. Putnam, S. G. Zhou, E. A. Allen, K. E. Apt, M. Bechner, M. A. Brzezinski, B. K. Chaal, A. Chiavotti, A. K. Davis, M. S. Demarest, J. C. Detter, T. Glavish, B. D. Goodstein, M. Z. Hadli, U. Heltstein, M. Hildebrand, B. D. Jenkins, J. Jurka, V. V. Kapitonov, N. Kröger, W. W. Y. Lau, T. W. Lane, F. W. Larimer, J. C. Lippmeier, S. Lucas, M. Medina, A. Montsant, M. Obernig, M. S. Parker, B. Palenik, G. J. Pauzour, P. M. Richardson, T. A. Rynearson, M. A. Saito, D. C. Schwartz, K. Thamatrakoln, K. Valentin, A. Vardi, F. P. Wilkerson, and D. S. Roksar. 2004. The genome of the diatom Thalassiosira pseudonana: ecology, evolution, and metabolism. Science 306:79–86.

3. Bowler, C., A. E. Allen, J. H. Badger, J. Grimwood, K. Jabbari, A. Kuo, U. Maheswarith, C. Martens, F. Maumus, R. P. Ottillar, E. Rayko, A. Salamov, K. Vandepoele, B. Beszteri, A. Gruber, M. Heijde, M. Katinka, T. Mock, K. Valentijn, F. Verrel, J. A. Berges, C. Brownlee, J.-P. Cadoret, A. Chiavotti, C. J. Choi, S. Coessel, A. De Martino, J. C. Detter, C. Durkin, A. Falcilatore, J. Fournet, M. Horuta, M. J. Huyseman, B. D. Jenkins, K. Jiroutova, R. E. Jorgensen, J. Joubert, A. Kaplan, N. Kröger, P. G. Kroth, J. La Roche, E. Lindquist, M. Lommer, V. Martin-Jezequel, J. P. Lopez, S. Lucas, M. Manggona, K. McGinnis, L. K. Medlin, A. Montsant, M.-P.-O. Secq, C. Napoli, M. Obernig, M. S. Parker, J.-L. Petit, B. R. Porcel, N. Poulten, M. Robison, L. Ryckebusch, T. A. Rynearson, J. Schmutz, H. Shapiro, M. Siaut, M. Stanley, M. R. Sussman, A. R. Taylor, A. Vardi, P. von Dassow, W. Wysmerman, A. Willis, L. S. Wyrzyk, D. S. Roksar, J. Weissbenz, E. V. Armbrust, B. R. Green, V. Van de Peer, and J. I. V. Grigoriev. 2008. The Phaeodactylum genome reveals the evolutionary history of diatom genomes. Nature 456:239–244.

4. Broit, L. L., R. R. Naik, D. J. Pikas, S. M. Kirkpatrick, D. W. Tomlin, P. W. Whitlock, S. J. Clarson, and M. O. Stone. 2001. Ultrafast holographic nanoscale-patterning of biocatalytically formed silica. Nature 413:291–293.

5. Brzezinski, M. A., and D. M. Nelson. 1986. A solvent-extraction method for the colorimetric determination of nanomolar concentrations of silicic acid in seawater. Mar. Chem. 271:139–151.

6. Davis, A. K., M. Hildebrand, and B. Palenik. 2005. A stress-induced protein associated with the girdle band region of the diatom Thalassiosira pseudonana (Bacillariophyta). J. Phycol. 41:577–589.

7. Davis, A. K., M. Hildebrand, and B. Palenik. 2006. Gene expression induced by copper stress in the diatom Thalassiosira pseudonana. Eukaryot. Cell 5:1157–1168.

8. Dugdale, R. C., F. P. Wilkerson, and H. J. Minas. 1995. The role of a silicate pump in driving new production. Deep-Sea Res. I Oceanogr. Res. Papers 42:697–719.

9. Dzwietz, N. E., J. R. Colvin, and A. G. McNelles. 1968. Studies on chitin (β-(1-4)-linked 2-acetamido-2-deoxy-d-glucan) fibers of the diatom Thalassiosira fluviatilis. J. Cell. Physiol. 69:777–779.

10. Ehrlich, H., M. Krautter, T. Hanke, M. Schiebel, S. Weis, H. Roden, and M. Worch. 2007. First evidence of the presence of chitin in skeletons of marine sponges II. Glass sponges (Hexactinellida: Porifera). J. Exp. Zool. B Mol. Dev. Evol. 308B:473–483.

11. Falini, G., S. Albeck, S. Weiner, and L. Addadi. 1996. Control of aragonite or calcite polymorphism by mollusk shell macromolecules. Science 271:67–69.

12. Felsenstein, J. 1989. PHYLIP: phylogeny inference package (version 3.2). Cladistics 5:164–166.

13. Field, C. B., M. J. Behrenfeld, J. T. Randerson, and P. Falkowsky. 1998. Primary production of the biosphere: integrating terrestrial and oceanic components. Science 281:237–240.

14. Frigeri, L. G., T. R. Radabaugh, P. A. Haynes, and M. Hildebrand. 2006. Identification of proteins from a cell wall fraction of the diatom Thalassiosira
