APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Oct. 2003, p. 6243–6249 Vol. 69, No. 10
0099-2240/03/$08.00+0 DOI: 10.1128/AEM.69.10.6243–6249.2003

Detection of *Prochlorothrix* in Brackish Waters by Specific Amplification of *pcb* Genes

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Received 21 April 2003/Accepted 21 July 2003

***Prochlorothrix hollandica* is the only filamentous chlorophyll *b* (Chl*b*)-containing oxyphotobacterium that has been found in freshwater habitats to date. Chl*b* serves as a light-harvesting pigment which is bound to special binding proteins (Pcb). Even though *Prochlorothrix* was initially characterized as a highly salt-sensitive species, we detected it in a brackish water environment that is characterized by salinities of up to 12 practical salinity units. Using PCR and reverse transcription, we amplified *pcb* gene fragments of phytoplankton samples taken along a salinity gradient in the eutrophic Darss-Zingst estuary (southern Baltic Sea). After sequencing, high levels of homology to the *pcbB* and *pcbC* genes of *P. hollandica* were found. Furthermore, autofluorescence of *Prochlorothrix*-like filaments that indicated that Chl*b* was present was detected in enrichment cultures prepared from the estuarine phytoplankton. The detection of Chl*b*-containing filaments, as well as the *pcb* and 16S ribosomal DNA sequences, suggests that *Prochlorothrix* is an indigenous genus in the Darss-Zingst estuary and may also inhabit many other brackish water environments. The potential of using *pcb* gene detection to differentiate *Prochlorothrix* from morphologically indistinguishable species belonging to the genera *Pseudanabaena* and *Planktothrix* (*Oscillatoria*) in phytoplankton analyses is discussed.**

The Prochlorophyta is an unusual group among the oxyphotobacteria. Like the chloroplasts in higher plants, the members of this group contain chlorophyll *b* (Chl*b*) as an accessory pigment. For many years, the symbiont *Prochloron didemni* (19) was the only known representative of the Prochlorophyta. The first filamentous strain (5) in the group of Chl*b*-synthesizing prokaryotes, the free-living species *Prochlorothrix hollandica*, was isolated in 1984 from a lake in The Netherlands (4). *Prochlorothrix* sp. strain NIVA-8/90, tentatively named *Prochlorothrix scandica*, has been proposed as a second species (28). In contrast to the salt-sensitive filamentous oxyphotobacteria, coccoid Chl*b*-containing species of the genus *Prochlorococcus* are very abundant in the central oceans (7, 26). However, phylogenetic analyses of 16S ribosomal DNA (rDNA) sequences have clearly indicated that the oxyphotobacteria do not form a separate bacterial lineage but are specially pigmented members of the old cyanobacterial evolutionary radiation (39).

Many investigations have concentrated on the evolutionary importance of the Chl*b*-containing oxyphotobacteria and the specificities of their photosynthetic machinery (3, 12, 19, 23, 37, 38). However, since most attention has been paid to the biology of the globally important genus *Prochlorococcus* in marine ecosystems (8, 11, 21, 22, 26, 33), our understanding of *Prochlorothrix* ecology is limited. The isolation of *Prochlorothrix* strains from the Loosdrecht lakes (6) and Lake Malaren (28) and the results of laboratory studies (5) imply that *Prochlorothrix* spp. are freshwater organisms with a preference for shallow eutrophic water. The highest levels of *Prochlorothrix* were found during the summer in shallow, phosphate-limited regions of the Loosdrecht lakes (5). The oxygenic photosynthetic activity was found to be highly resistant to inhibition by sulfide (30). Experiments focusing on phosphorus nutrition resulted in characterization of *P. hollandica* as a high-affinity storage strategist (9).

While *Prochlorococcus* cells dominate phytoplankton communities in wide areas of the oceans (27), *Prochlorothrix* occurs at most times of the year at rather low levels, while cyanobacteria that are very similar morphologically seem to dominate. It is particularly difficult to distinguish *Prochlorothrix* from members of the cyanobacterial genera *Pseudanabaena* and *Planktothrix* (*Oscillatoria*). In the past, epifluorescence microscopy was used to investigate phytoplankton from the Loosdrecht lakes (43), the original source of *P. hollandica* (5). This technique is based on the phycoblisome autofluorescence that is characteristic of filamentous cyanobacteria but is missing in *Prochlorothrix* because of its different pigmentation. Detecting *Prochlorothrix* based on the absence of fluorescent trichomes is not a very reliable method (5, 43). Modern molecular techniques may be much more suitable for this purpose. The currently used methods, such as 16S rDNA denaturing gradient gel electrophoresis and sequence analysis, are restricted to analysis of species that occur at rather high levels in the environment (42). Methods based on genes which are restricted to specific bacterial groups could provide greater sensitivity. Genes encoding Chl*a*/*b* binding proteins (*pcb*) may be good candidates for specific detection of Chl*b*-containing oxyphotobacteria. These genes exhibit only low levels of similarity to members of the extended gene family encoding eukaryotic Chl*a*/*b* and Chl*a*/*c* light-harvesting proteins (18). The antenna polypeptides are encoded by three genes in *P. hollandica*

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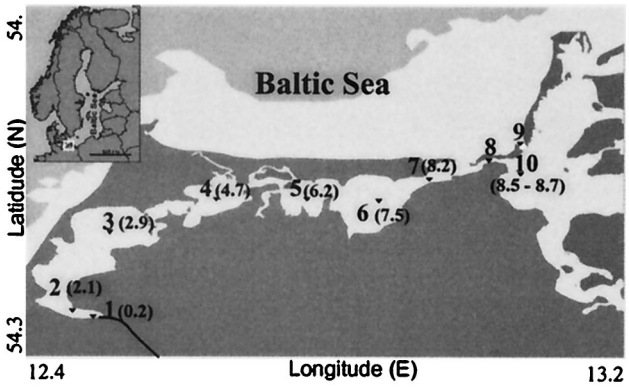

FIG. 1. Map of sampling sites in the Darss-Zingst estuary. Sampling sites 1 to 10 correspond to buoys Recknitz, R2, R84, R37/44, R1/B65, B53, B37/46, B12/13, B18/19, and B35, respectively. The salinity at each sampling site (in PSU) is indicated in parentheses. The inset is a map of the Baltic Sea.

(*pcbABC*). The *pcbC* gene is significantly different from *pcbA* and *pcbB*, which exhibit high levels of similarity to iron stress-induced *isiA* genes of cyanobacteria (41). The genes of the multicistronic *pcbABC* operon are transcribed largely independent of the light intensity applied (24). Seven different *pcb*-like genes were found to be expressed in a *Prochlorococcus* strain (12). In general, the known sequences of *pcb* genes from *P. hollandica* allow the design of PCR primers which are highly specific for this genus.

In this paper we present evidence that the genus *Prochlorothrix* is much more widespread than the previous occasional observations indicated. During our investigation of cyanobacterial diversity in an estuary in the Baltic Sea (unpublished data), DNA fragments with high levels of sequence similarity to *pcb* genes of *P. hollandica* were obtained. The search for *Prochlorothrix*-like filaments and their characteristic autofluorescence signatures in enrichment cultures was augmented with tests for the salt tolerance of *P. hollandica* cells to verify the genetic results.

MATERIALS AND METHODS

Sampling sites and samples. Plankton samples were taken in July 2001 from various locations (Fig. 1) in the Darss-Zingst estuary. The samples originated from 10 stations, which comprise a 55-km line along a salinity gradient from 0.2 to 8.7 practical salinity units (PSU). All salinity values were derived from measurements made with a conductivity-measuring cell (LT 197; WTW Weilheim) according to the definition of the conductivity ratio of standard seawater (salinity, 35 PSU) and a solution containing 32.4356 g of KCl per kg of solution at 15°C (International Association for the Physical Sciences of the Ocean). The water temperature ranged from 22 to 25°C. The sampling sites were designated by using a series of increasing numbers that followed the west-east axis of the estuary and increasing salinity (Fig. 1). Samples for RNA extraction were quickly frozen on board.

Cells of the isolate from Lake Malaren, *Prochlorothrix* sp. strain NIVA-8/90 (the organism proposed as *P. scandica*), were obtained as a frozen pellet.

Phytoplankton counting and differential epifluorescence microscopy. Phytoplankton cells were counted by using Lugol-fixed samples and an inverted microscope (20, 40) at a magnification of ×256. For the dominant cyanobacteria and members of the Chlorophyceae, which form large spherical colonies, numbers of cells were determined by examining 10 randomly chosen colonies per sample with green light excitation at a magnification of ×1,250. From the mean cell number, the total number of cells per species was calculated. Cell diameters were measured to calculate cell volumes (10), and the amount of carbon biomass was estimated by using 11.25% carbon per fresh weight (15) at a specific density of 1.04 g cm^{-3}. Additionally, unicellular rod-shaped cyanobacteria in the pico size class (diameter, <2 μm) were quantified by using glutaraldehyde-fixed samples (which were filtered through a 0.2-μm-pore-size Millipore filter prior to analysis) and epifluorescence illumination (green light; BP 545 nm; magnification, ×1,250), and a mean cell volume of 0.86 μm^3 (unpublished data) was used for biomass calculations. Differential epifluorescence microscopy and microphotography were performed with the equipment described by Schubert and Schumann (36), which allowed detection of Chl*a* (photosystem II) autofluorescence of living cells after highly selective excitation in the blue absorption bands of either Chl*a* or Chl*b*.

DNA and RNA techniques. For extraction of nucleic acids, 70 mg (fresh weight) of phytoplankton cells was used. The cells were disrupted by bead beating (5,000 rpm; 10 s; 0.1-mm-diameter glass beads). For extraction of chromosomal DNA, a Qiagen plant extraction kit (Qiagen) was used. An additional preextraction step with hot phenol (Aqua-Roti-Phenol; Roth) for 10 min (pH 4.5 to 5.0; 65°C) was used for RNA extraction. Total RNA was isolated by using a High Pure RNA isolation kit (Roche Biochemicals). All PCR were performed by using *Taq* PCR Master Mix (Qiagen), and reverse transcription (RT) reactions were carried out with SuperScriptII Rnase H reverse transcriptase (Gibco BRL Life Technologies).

Cyanobacterial 16S rDNA fragments (424 bp) were amplified with degenerate standard primers CYA359F and CYA781R (25). The 16S rDNA primers that specifically targeted *P. hollandica* (16S-Pholl-fw [5′-ACA CAG CTT AAC TGT GGG AGA-3′] and 16S-Pholl-rev [5′-AGT TGG CTG CTC TTT GTC CCT-3′]) were based on the alignment obtained with the software BIOEDIT (14). Besides the exact match with *P. hollandica*, the primer sequences are identical to sequences of the unidentified cyanobacterial clones LD16 and LD22 (accession no. AJ007866 and AJ006285) from the Loosdrecht lakes. These clones exhibit more than 99% identity to *P. hollandica* (44). All primers were used at an annealing temperature of 55°C. The *pcbB* gene was obtained by first using primers optisi-fw (5′-AAD TAY GAH TGG TGG GC-3′) and hlwha-l-rev (5′-GCG TGC CAS AGR TGA CC-3′) and then using reverse primer pyfadt-rev (5′-CGT TTC GGC AAA RTA RGG-3′) in a second seminested PCR. Specific amplification of the *pcbC* gene was performed with primers pcbC-fw (5′-GTA ATA TCC GCC TCG TAG AC-3′) and pcbC-rev (5′-CTA ACC GTC AGA CCT TAA CC-3′). The PCR program and all other procedures have been described previously (13). Sequencing of cloned fragments was done at least in triplicate by using a capillary sequencer (Beckman-Coulter).

Computer analysis. We searched for sequence similarities in databases with the assistance of the BLAST software (1). Sequence alignment was performed with the software BIOEDIT (14). A consensus tree was constructed by using the multiple-sequence alignment and the software program PAUP (Phylogenetic Analysis Using Parsimony, beta version 4.0; David Swofford, Laboratory of Molecular Systematics, Smithsonian Institution). Rooted cladograms were constructed after phylogenetic analyses of the 16S rDNA sequences (maximum-parsimony method) and of the *pcbB* and *pcbC* sequences (neighbor-joining method) with 1,000 replications. The cladograms were constructed with TreeView (version 1.5; R. D. M. Page, 1998).

Cultivation and high-performance liquid chromatography (HPLC) analysis. Estuarine phytoplankton were enriched by cultivating 1 ml of the natural phytoplankton assemblage in 3 ml of BG11 medium (32) at 23°C. The enrichment cultures were exposed to daylight.

P. hollandica SAG 10.89 cells were grown at 23°C in plates containing 15 ml of BG11 medium (32) supplemented with different concentrations of NaCl with constant illumination of 9 μmol of photons m^{-2} s^{-1} (photosynthetically active radiation; fluorescent tubes; color code 25; Philips). The NaCl concentrations used were 34.2 mM (2 PSU), 68.4 mM (4 PSU), and 102.6 mM (6 PSU). Cells were harvested after 10 days by filtration onto membrane filters (pore size, 2 μm) for determination of the net dry weight and compatible solutes. For analysis of compatible solutes by HPLC, the cells on filters were extracted in 2 ml of 80% ethanol for 3 h at 65°C. After an internal standard (50 μg of sorbitol) was added, the particulate material was removed by centrifugation, and the supernatant was dried in a Speed-Vac. Prepurification of low-molecular-weight carbohydrates was performed by sequentially dissolving the dried supernatants in different solvents (0.5 ml of deionized water, 0.5 ml of 100% ethanol, 100 μl of HPLC grade water). The concentrations of low-molecular-weight carbohydrates were analyzed with an HPLC setup as described by Schoor et al. (35). All chromatographic experiments were performed with a chromatograph consisting of LC-9A pumps, SIL-9A autoinjector (1- to 50-μl sample loop), a CTO-6A column oven, a DGU-4A solvent degasser, and a RID-6A refractive index detector (Shimadzu Corp., Kyoto, Japan). A reverse-phase column filled with Hypersil 120 ODS and a sugar-alcohol column (Aminex HPX-87C; Bio-Rad) were the columns used.

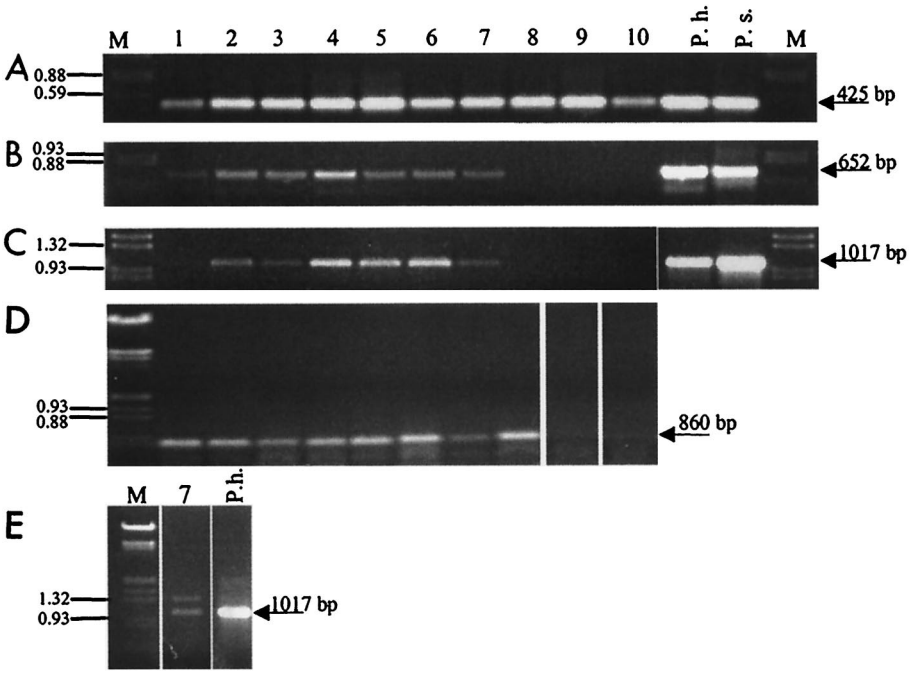

FIG. 2. Separation of PCR fragments obtained with DNA (A to C) or cDNA (D and E) from 10 sampling sites. The lane numbers indicate the sampling sites (Fig. 1). The following different primer pairs were used on the DNA level: degenerate cyanobacterial 16S rDNA primers CYA359F and CYA781R (A), *Prochlorothrix*-specific 16S rDNA primers 16S-Pholl-fw and 16S-Pholl-rev (B), and *Prochlorothrix*-specific *pcbC* primers pcbCfw and pcbCrev (C). On the cDNA level, degenerate primers optisi-fw, hlwha-l-rev, and pyfadt-rev for *pcbB* amplification in nested PCR (D) and *Prochlorothrix*-specific *pcbC* primers pcbCfw and pcbCrev (E) were used. DNA from cultures of *P. hollandica* (P.h.) and *P. scandica* (P.s.) served as positive controls. Lanes M contained a marker (*Eco*RI/*Hin*dIII-digested λ DNA).

Nucleotide sequence accession numbers. The nucleotide sequences of the 16S rDNA of *P. hollandica* (accession no. AJ007907), *Leptolyngbya* sp. strain PCC 7104 (AB039012), *Thermosynechococcus elongatus* BP-1 (AP005376), *Prochlorococcus marinus* SSW5 (X63140), *Prochloron* sp. (X63141), uncultured cyanobacterial clones LD7 (AJ007864) and LD16 (AJ007866), *Escherichia coli* PK3 (X80731), *Gloeobacter violaceus* PCC 8105 (AF132791), *Prochlorococcus marinus* subsp. *pastoris* NATL2 (AF311219), *Prochlorococcus marinus* MIT 9303 (AF001469), *Planktothrix agardhii* NIVA CYA59 (AB045939), *Synechocystis* sp. strain PCC 6803 (AB041938), *Synechococcus* sp. strain PCC 7002 (AJ000716), *Prochlorococcus marinus* (X63140), *Nostoc* sp. strain PCC 7120 (AP003595), *Synechococcus* sp. strain WH 7805 (AF001478), and *Trichodesmium erythreum* IMS 101 (NZ_AAAU01000054) and the nucleotide sequences of the *pcbBC* genes of *P. hollandica* (X97043) and *Prochlorococcus marinus* CCMP 1375 (AF198526 and AF198528) were obtained from databases. Partial 16S rDNA and *pcbC* gene sequences of *Prochlorothrix* sp. strain NIVA-8/90 (accession no. AJ534944 and AJ534947) and of the uncultured estuarine *Prochlorothrix* (AJ534945 and AJ534946) were obtained in this study and were deposited in the databases.

RESULTS

Detection of *pcb*-like and 16S rDNA sequences in samples from the Darss-Zingst estuary. Gene fragments that exhibited high levels of sequence similarity (99.9%) to *pcbB* from *P. hollandica* were amplified by RT-PCR from sampling sites 1 to 9 (Fig. 2D). Until now, this gene has been described only for *P. hollandica* and *Prochlorococcus marinus.* In order to verify that *Prochlorothrix*-like organisms are present in the Darss-Zingst estuary, specific primers for amplification of the *pcbC* gene of *P. hollandica* were used in RT-PCR, as well as in PCR. Use of these *pcbC*-specific primers resulted in amplification of the expected 1-kb fragment with DNA from sampling sites 2 to 7 (Fig. 2C). Fragments of the same size were also obtained with cDNA, which were synthesized from total RNA from sampling sites 1, 3, 6, 7, and 9 (Fig. 2E). The similarities of the DNA fragments obtained to *pcbC* of *P. hollandica* were verified by Southern hybridization experiments, in which the fragments were recognized by a specific *pcbC* probe obtained from *P. hollandica* (data not shown). Restriction analyses with *Nco*I resulted in a fragment size pattern which was identical to that expected based on the *P. hollandica pcbC* sequence (data not shown). Several PCR fragments from all sampling sites were cloned and sequenced. All of the sequences were 99% identical to *pcbC* from *P. hollandica* regardless of the sampling site; i.e., just one genotype was found.

Finally, we searched for *P. hollandica*-like 16S rDNA sequences in environmental samples (Fig. 2A). This was done initially with cyanobacterium-specific primers (25), which amplify a 425-bp internal 16S rDNA fragment. Restriction analyses and sequencing of at least 10 randomly obtained 16S rDNA clones never resulted in a sequence similar to that of *P. hollandica*. In all cases, the sequences were similar to those of phycobilisome-containing cyanobacteria (data not shown). *Prochlorothrix*-type organisms could not be detected by this approach. However, the use of *P. hollandica*-specific 16S rDNA primers allowed amplification of the expected 650-bp fragments. The sequences of these fragments exhibited about 99% identity to sequences of *P. hollandica*. In addition, significant amounts of 16S rDNA fragments characteristic of *P. hollandica* were detected in *pcbC*-positive samples (Fig. 2B). Significant amounts of *P. hollandica*-like fragments were not detected in DNA from sampling sites 9 and 10.

The partial sequences of the *pcbC*, *pcbB*, and 16S rDNA

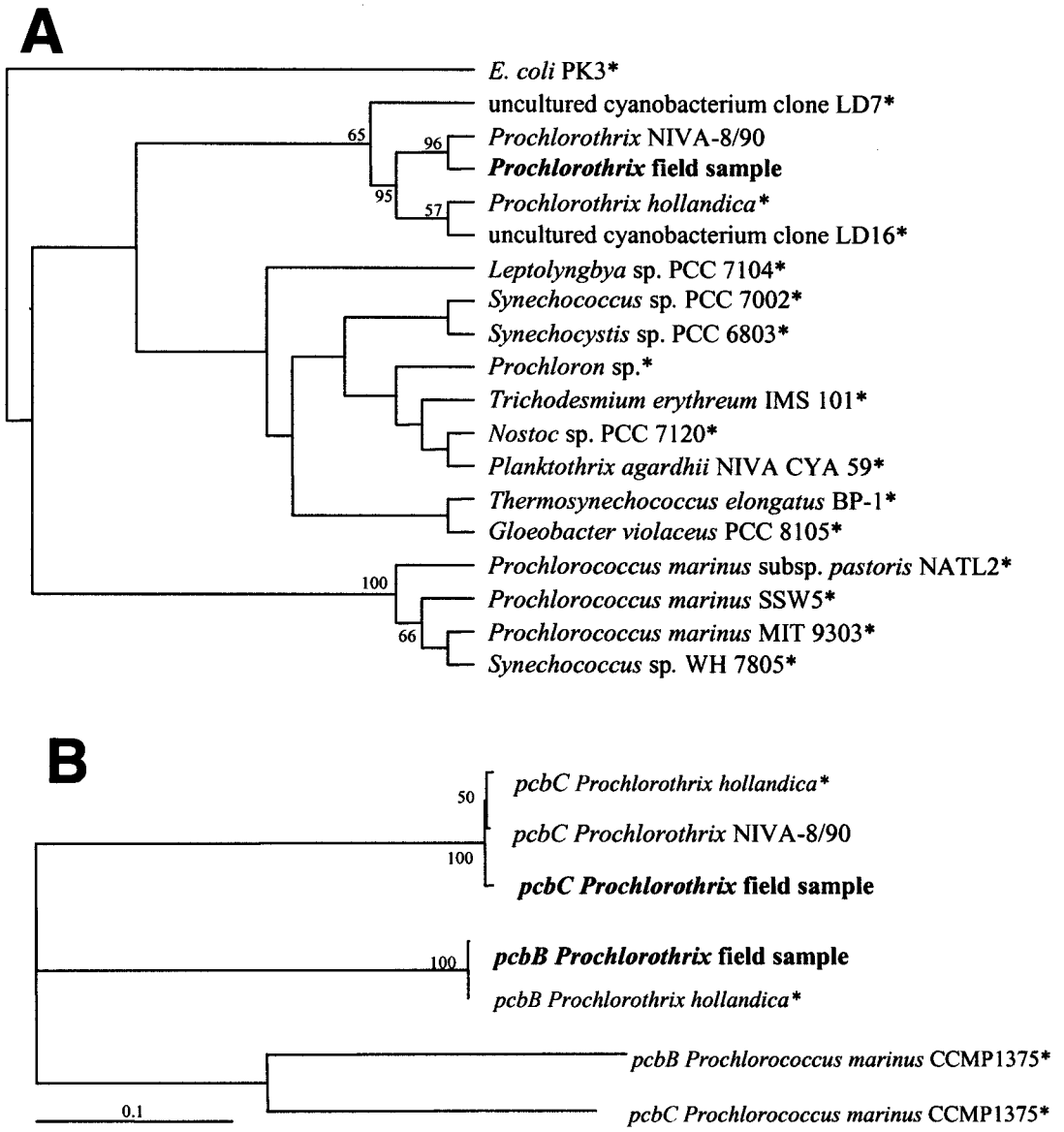

FIG. 3. Rooted cladograms obtained after phylogenetic analyses by the maximum-parsimony method (performed with PAUP, beta version 4.0; Laboratory of Molecular Systematics, Smithsonian Institution) of a 527-bp fragment of the 16S rDNA sequences (maximum-parsimony method) (A) and of a 828-bp fragment of the *pcbB* and *pcbC* sequences (neighbor-joining method) (B). Organisms whose sequences were obtained from databases are indicated by asterisks. Selected bootstrap values based on 1,000 replications are shown at the nodes; only values greater than 50% are shown. The cladograms were constructed with TreeView (version 1.5; R. D. M. Page).

genes were used for phylogenetic comparisons with similar sequences from the databases (Fig. 3A). Corresponding sequences were also obtained from the proposed new *Prochlorothrix* species, *P. scandica* (strain NIVA-9/80) (29). The *pcb* gene sequences from the estuarine phytoplankton samples clustered closely with those of *P. hollandica*, while the sequences of *Prochlorococcus marinus* were clearly not closely related (Fig. 3B). The *pcbC* sequence of *P. scandica* NIVA-8/90 was similar to the sequence of *P. hollandica*. In spite of minor differences, the resulting amino acid sequences were 99% identical (Table 1). Thus, the *pcbC* sequences of the Darss-Zingst estuary clones, *P. scandica*, and *P. hollandica* are almost identical. Analyzing the partial 16S rDNA sequences again led to close grouping of the 16S rDNA sequences in the genus *Prochlorothrix* (Fig. 3A). According to this alignment, the 16S rDNA sequence of the environmental samples exhibited a slightly higher level of similarity to the sequence of *P. scandica* NIVA-8/90 than to the sequence of *P. hollandica*.

Detection of *Prochlorothrix*-like trichomes in estuarine phytoplankton. Phytoplankton counts from the 10 sampling sites (Fig. 4) revealed that about one-half of the members of the Oscillatoriales could be morphologically attributed to the microscopically indistinguishable taxa *P. hollandica* and *Pseudoanabaena*. This morphotype represented an important fraction of the phytoplankton biomass (mean, 12%) at stations 1 to 7, but for stations 8 to 10 the percentage was less than 1% (mean, 0.4%), which basically corresponds to the results of the PCR analyses.

TABLE 1. Levels of similarity of the DNA sequences from *Prochlorothrix* field samples to sequences obtained from databases and from analyses in this study[a]

Organism	% Similarity		
	16S rDNA	*pcbB*	*pcbC*
Prochlorothrix sp. strain NIVA-8/90	99.6		99.0
Prochlorothrix hollandica[b]	98.9	99.9	99.3
Prochloron sp.[b]	90.5	ND[c]	ND
Prochlorococcus marinus SSW5[b]	91.3	ND	ND
Prochlorococcus marinus CCMP 1375[b]	ND	45.3	44.0
Cyanobacterium clone LD7[b]	94.9	ND	ND
Cyanobacterium clone LD16[b]	98.7	ND	ND
Planktothrix agardhii NIVA CYA53[b]	90.5	ND	ND

[a] 16S rDNA sequences (527 bp) and sequences of chlorophyll binding protein genes (*pcbB* and *pcbC*; 826 bp) were compared.
[b] Sequence obtained from database.
[c] ND, not determined.

The molecular data strongly suggest that *P. hollandica*-like organisms are present in the Darss-Zingst estuary. This encouraged us to search for *P. hollandica*-like trichomes. Since the Darss-Zingst estuary is a highly eutrophic aquatic system, phytoplankton are abundant, and many filamentous cyanobacteria of the *Planktothrix-Pseudoanabaena* type are present (Fig. 5A). This fact made it impossible to detect *P. hollandica* trichomes in water samples reliably. Culture experiments to enrich mixtures of filamentous cyanobacteria were started. The cultures were examined after 2 weeks by light microscopy and differential epifluorescence microscopy (Fig. 5B and C). Some trichomes produced clear autofluorescence signals after selective Chl*b*-exciting illumination, in contrast to other trichomes that had the same morphology and to colony-forming chroococcal cyanobacteria. Some green algae proved that the Chl*b* excitation was specific. This finding supported the assumption that parts of the filamentous cyanoplankton contain Chl*b*, like *Prochlorothrix* filaments. Furthermore, PCR fragments from DNA extracted from the enrichment culture contained the same *Prochlorothrix*-like sequences as the environmental samples contained.

Our data suggest that *P. hollandica*-like organisms are able to live throughout the estuary under low-salinity conditions, although such organisms have been isolated previously only from freshwater. *P. hollandica* SAG 10.89 was cultivated in NaCl-supplemented BG11 medium to mimic estuarine conditions. Growth was observed in media containing NaCl at salinities up to 6 PSU. In salt-treated cells, accumulation of sucrose was detected, while this sugar did not accumulate in *P. hollandica* cells under the low-salt conditions of standard BG11 medium (data not shown). These data indicate that sucrose might be used for osmoregulation, which can support acclimation to the salinities present in the Darss-Zingst estuary.

DISCUSSION

We corroborated initial indications that *Prochlorothrix*-like organisms are present in the natural phytoplankton community of coastal waters in the southern Baltic Sea. DNA fragments exhibiting high levels of sequence identity to *pcbB*, *pcbC*, or 16S rDNA of *P. hollandica* were amplified from DNA isolated from different sampling sites along a gradient of salinities ranging from 0.2 to 8.2 PSU. In particular, the *pcbC* sequence can be used as a specific marker for *P. hollandica*-like strains, since this gene is different from other *pcb* genes in oxyphotobacteria and from similar genes in eukaryotic algae (41). Furthermore, *P. hollandica*-like trichomes were enriched from the estuary and produced autofluorescence signatures that indicated that Chl*b* was present. Assuming that mRNA is a useful marker of cell viability because of its short half-life compared to that of DNA, *Prochlorothrix*-like cells are able to propagate at least in the enclosed parts of the Darss-Zingst estuary. Horizontal drifting of buoyant phytoplankton is caused by net horizontal water currents with velocities that are usually in the range from 0.1 to 0.5 m s^{-1} (34) (broad and narrow parts). Therefore, 30 to 150 h is the minimum drifting time from sampling site 1 to site 10 in the estuary. These time estimates were obtained by excluding mixing in the larger basins and periods of salt water inflow with inverted drift direction. Addition of *Prochlorothrix* originating from freshwater input is unlikely, since *pcb* mRNA half-lives were shown to be in the range from minutes (in light) to hours (in darkness) (24).

The occurrence of viable *Prochlorothrix* at salinities up to 8 PSU was initially surprising, because Burger-Wiersma et al. (5) characterized *P. hollandica* as a freshwater organism with a very low salt tolerance. Growth of *P. hollandica* ceased completely in the presence of 100 mM NaCl (17% of the seawater concentration or 6 PSU) and was inhibited in the presence of >25 mM NaCl (ca. 1.5 PSU). In contrast, our growth experiments with *P. hollandica* SAG 10.89 did not show growth inhibition at such salinities, and optimum growth was detected in the presence of about 85 mM NaCl (14% of the seawater concentration or 5 PSU) under the conditions used in this study. We assume that our cultivation method, which decreased shearing forces and other parameters that are not known, led to these differences in salt tolerance. Mechanical destruction of cell surfaces in particular might be critical for regulation of osmotic pressure by accumulation of compatible solutes and ion export. Cells of *P. hollandica* SAG 10.89 accumulated sucrose upon exposure to increased NaCl concentrations. Sucrose accumulation was found to be characteristic of the cyanobacterial strains with the lowest halotolerance (31). The optimal growth conditions for *P. hollandica* in cultures at

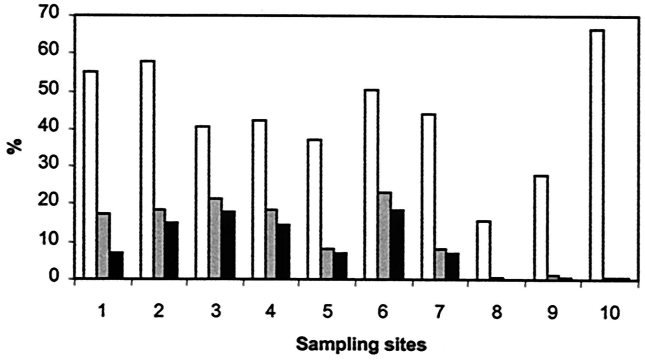

FIG. 4. Relative biomasses of *Planktothrix*-like filaments in different fractions of the phytoplankton in samples from the Darss-Zingst estuary. The percentages of biomass were calculated for Oscillatoriales (open bars), total cyanobacteria (grey bars), and total phytoplankton (solid bars).

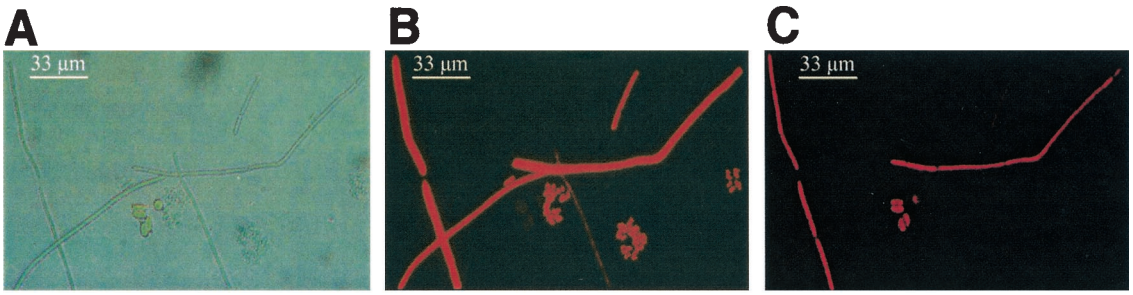

FIG. 5. Micrographs of cyanobacteria and eukaryotic algae in enrichment cultures from the Darss-Zingst estuary after 14 days of incubation. (A) Bright-field microscopy. (B and C) Epifluorescence microscopy of autofluorescence with illumination that preferentially excited Chl*a* (B) and Chl*b* (C).

salinities of about 4 PSU parallel those expected for *Prochlorothrix*-like cells in the estuary. Even though *pcbB* and *pcbC* DNA fragments were found in the western to central part of the Darss-Zingst estuary at all sampling sites, undetectable amounts of these fragments in the northeastern transition zone to the open Baltic Sea (station 10) support the suggestion that the organisms are restricted to salinities below 10 PSU.

The high levels of similarity of the 16S rDNA and functional *pcb* sequences (more than 99%) put the *Prochlorothrix* field sample sequences into the *Prochlorothrix* cluster with the type strain of *P. hollandica*. Only a few additional isolates, like *Prochlorothrix* strain NIVA-8/90, provisionally named *P. scandica* (28), and some uncultured clones from Lake Loosdrecht, exhibited high levels of similarity to *P. hollandica* (44). Based on the *pcbB* and *pcbC* sequence comparisons, the levels of similarity are even higher. Therefore, we believe that the *Prochlorothrix*-like gene fragments found in the Darss-Zingst estuary in fact indicate that *Prochlorothrix* species are present. On the 16S rDNA level the *Prochlorothrix* representative from the Darss-Zingst estuary showed a slightly higher level similarity to *P. scandica* NIVA-8/90 from Lake Malaren. The *pcbB-pcbC* sequence comparison implied that there is a closer relationship to *P. hollandica*. Nevertheless, it should be considered how significant differences are when levels of 16S rDNA sequence identity in the range from 98.8 to 99.6% are used to distinguish different species. At this time we are reluctant to assign the sequences to different species, and generally we refer to *P. hollandica*. In the case of *Prochlorococcus* species, all comparisons of 16S rDNA sequence homologies showed high degrees of identity, but there are stable ecotypes with completely different genome sizes (16). Therefore, further investigations of the genus *Prochlorothrix* could reveal more detailed information.

Monthly phytoplankton monitoring in the estuary failed to detect *Prochlorothrix* because of its inconspicuous and somewhat unclear morphological features. It is necessary to distinguish this taxon from the frequently occurring, morphologically similar cyanobacteria (i.e., *Pseudanabaena limnetica* [*Oscillatoria limnetica*]). These organisms can be distinguished by some ultrastructural features (29) or by different pigment-dependent autofluorescence signatures (42). However, electron microscopy is too costly to be used as a tool to search for phytoplankton species. In addition, acclimation to nutrient depletion and irradiance could lead to a significantly changed pigment content of *Pseudoanabaena* or *Planktothrix* (*Oscillatoria*) species (26). Phycobilisome fluorescence is not a reliable tool for distinguishing filamentous cyanobacterium-like organisms in the phytoplankton (5, 43). We used differential Chl*a*/*b* excitation of long-wavelength autofluorescence to differentiate *Prochlorothrix* from Chl*b*-free cyanobacteria. This positive indication allowed us to prove that *Prochlorothrix* was present. However, a heavily reduced Chl*b* antenna per cell also could lead to incorrect identification. Therefore, prechecking phytoplankton samples for the presence of *Prochlorothrix*-like DNA sequences with PCR can be a useful technique for reducing the risk of misinterpreting microscopic investigations of natural phytoplankton samples. Even in samples in which *Prochlorothrix*-like trichomes accounted for less than 1% of the total phytoplankton biomass (Fig. 4), *Prochlorothrix* could be detected by PCR. In addition, potential errors of microscopic investigation can be excluded completely when a set of *Prochlorothrix*-like filaments can be verified to be *Prochlorothrix* by using a PCR approach at the level of single trichomes. The latter method has already been successfully applied to cyanobacterial filaments (2, 17). Possibly, *Prochlorothrix* is much more widely distributed than currently expected. In fact, *Prochlorothrix*-like filaments were suspected to be components of cyanobacterial blooms in the Baltic Sea (Gulf of Finland, Baltic Proper) during monitoring cruises (http://meri.fimr.fi/Algaline/eng/EnAlgaline.nsf). Methods for the unambiguous identification of these organisms are necessary. Further investigations should lead to estimates of the ecological importance of *Prochlorothrix* in brackish phytoplankton.

ACKNOWLEDGMENTS

We thank H. Schubert (Department of Aquatic Ecology, University of Rostock) for the setup used for differential epifluorescence microscopy. B. Brzezinka is acknowledged for her technical support. We are grateful to the crew of the Zingst Biological Station (University of Rostock) for their kind support of the field work. Cells of *P. scandica* were kindly provided by H. C. P. Matthijs (Department of Microbiology, University of Amsterdam). Cells of *P. hollandica* were kindly provided by M. Lorenz (Sammlung von Algenkulturen, University of Göttingen).

This work was supported by a grant from the Deutsche Forschungsgemeinschaft (DFG).

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