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Detection of Stable Pre-rRNA in Toxigenic *Pseudo-nitzschia* Species

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Nucleotide sequence analysis of ribosomal DNA (rDNA) spacer regions is useful for taxonomic comparisons of closely related microorganisms. These regions have been less useful for routine microbial identification and detection, partly because rRNA precursors (pre-rRNAs) in microbial cells are assumed to be too labile to be detectable by high-throughput probe hybridization methods. We characterized the sequence diversity and physiological stability of pre-rRNA in the toxigenic marine diatoms *Pseudo-nitzschia australis*, *P. multiseries*, and *P. pungens*. As with nucleotide sequences of the first internal transcribed spacer (ITS1) reported previously, sequences of ITS2 and the 5′ external transcribed spacer (ETS1) exhibited considerable divergence among these species, including large insertions-deletions detectable by PCR-based spacer length analysis. In slot blot hybridization assays on RNA extracted from lysates of *Pseudo-nitzschia* cells, oligonucleotide probes directed to pre-rRNA spacers generated much stronger signals than did complementary probes directed to the coding strands of the rDNAs, indicating that the pre-rRNA-targeted probes detected multicopy transcripts. A group of probes directed to a discrete 90-base region within the ITS1 pre-rRNA gave no detectable signal, suggesting that this region is degraded early in the rRNA maturation pathway. Other pre-rRNA regions were always detectable and, in marked contrast to prokaryotic systems analyzed in this manner, were stable and abundant in both actively dividing and nondividing cells. Long, multilabeled RNA probes, which would exhibit considerable cross-reactivity if directed to mature rRNA sequences, detected species-specific pre-rRNA sequences from as few as 1,000 cells. Pre-rRNA is a potentially useful molecular target for detecting and identifying *Pseudo-nitzschia* species and possibly other unicellular eukaryotes as well.

Harmful algal blooms (HABs) occur in coastal regions worldwide, causing human illness and mortality as well as millions of dollars in annual economic losses. Studies of HABs involve identifying and counting phytoplankton collected in field samples, mapping their distribution with respect to space and time, and correlating those observations with properties of surrounding waters. Traditionally, researchers have relied on microscopy to identify harmful algae species, but such methods are problematic when applied to large numbers of samples. Therefore, we are developing DNA probe hybridization methods for detection and characterization of HAB events (26, 34, 35). We have chosen pennate diatoms of the genus *Pseudo-nitzschia* as the principal models for these efforts.

Certain *Pseudo-nitzschia* species produce the neurotoxin domoic acid (DA), which causes amnesic shellfish poisoning in humans and wildlife (31). Awareness of DA poisoning hazards has increased in recent years from localized events in eastern Canada and outbreaks along both North American coasts and in other regions of the world (4, 15, 16, 20, 23, 39–41). Over the same period, the number of *Pseudo-nitzschia* species linked to DA production has grown (7, 12, 23, 25, 32, 36). The toxicity of some species may vary geographically. For example, *P. pseudodelicatissima* has been associated with DA poisoning in eastern Canada but not in Denmark or California. Conversely, *P. pungens* blooms in eastern Canada have been considered nontoxic, but some isolates of this species from the western United States and New Zealand have produced DA in culture, and natural blooms in these areas have been linked to DA production (19a, 32). Clarification of these issues is hindered by the challenge of identifying *Pseudo-nitzschia* species by scanning electron microscopy of fine-scale morphological features (17–19). DNA probe methods could greatly simplify these efforts.

rRNA has long been used as a target for DNA probes because of its predictable phylogenetic specificity and high cellular copy numbers. Three of the four eukaryotic rRNA subunits are synthesized on a long transcript which is subsequently processed to form mature rRNA (10, 28, 37). The maturation pathway removes a 5′ leader (external transcribed spacer 1, or ETS1), an internal transcribed spacer (ITS1) between the small subunit (SSU) rRNA and the 5.8S rRNA, a second ITS (ITS2) between the 5.8S rRNA and the large subunit (LSU) rRNA, and a 3′ tail (ETS-2). Intermediates in this pathway are termed pre-rRNAs. Pre-rRNA spacer regions tolerate greater genetic drift than does mature rRNA, making the former useful for phylogenetic comparisons of closely related species and subspecies (1, 11, 14, 21, 28). Pre-rRNA spacer sequences of some harmful alga species have been reported. Manhart et al. (24) observed 27% divergence between the ITS1 sequences of *P. pungens* and the morphologically similar strain *P. multiseries*, supporting the recent taxonomic separation of these species. By comparison, the corresponding SSU rRNA sequences differed by less than 1% (24). ITS1 sequences have been used to characterize phylogenetic relationships among other diatoms (42) as well as those among toxigenic dinoflagellates of the genus *Alexandrium* (1).

Analysis of pre-rRNA spacers is normally conducted by PCR amplification and sequence or restriction fragment length polymorphism analysis of the chromosomal regions encoding rRNA transcripts (rDNA). For routine species identification

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and screening, direct DNA probe detection of pre-rRNA would be preferable to PCR and sequencing of rDNA, because of the relative economy and simplicity of DNA probe analysis and its compatibility with automated and high-throughput formats (27, 34). However, this approach is rarely attempted because of the expectedly short half-life of pre-rRNA in microbial cells. As intermediates in the growth-activated rRNA biosynthetic pathway, pre-rRNA molecules are expected to be particularly scarce in nongrowing cells. This expectation was confirmed for *Escherichia coli*, in which the cellular abundance of pre-16S rRNA declined by up to 1,000-fold under starvation conditions which did not measurably deplete the mature rRNA pool (5). Such observations raise the possibility of using species-specific, pre-rRNA-targeted probes to assess prokaryotic growth activity in mixed samples (5, 6).

In contrast to prokaryotes, measurements of rRNA transcription and processing activities in eukaryotic cells have suggested that some eukaryotes might retain stable pools of pre-rRNA upon cessation of cell division (8, 9, 22). However, measurements of individual steps in highly complex rRNA biosynthetic pathways cannot by themselves predict the abundance and stability of intermediates in these pathways. Moreover, no such measurements have been made on diatoms. Therefore, we determined the nucleotide sequences of ETS1 and ITS2 from three *Pseudo-nitzschia* species and characterized the stability of pre-rRNA pools in cultured *P. multiseries* cells by using the same probe hybridization methods used to assess these criteria in bacteria (5, 6). In contrast to that in bacteria, pre-rRNA in both growing and nongrowing *P. multiseries* cultures was stable and readily detectable. These data suggest that the use of pre-rRNA for direct identification and screening of *Pseudo-nitzschia* isolates, and perhaps of other eukaryotic microorganisms as well, may be more feasible than previously believed.

MATERIALS AND METHODS

Phytoplankton culture, harvest, and lysate preparation. Unialgal cultures of *Pseudo-nitzschia* species were isolated from Monterey Bay, Calif., identified by microscopy, and maintained as described previously (34, 35). Individual clones used in this investigation were *P. multiseries* mu1 and mu2; *P. pungens* pu12, pu14, pu15, and pu16; and *P. australis* au22, au23, and au27. All clones were held and cultured at the Monterey Bay Aquarium Research Institute (MBARI) and the University of California at Santa Cruz. Cells were cultured in f/2-enriched seawater (Fritz Chemical Co., Dallas, Tex.) at 15°C under a 10 h:14 h light:dark cycle, as described previously (13, 26). Cell densities were estimated by averaging the numbers of cells counted by light microscopy in three to five separate 5-μl samples in Lugol's iodine solution. For growth experiments, exponential-phase cultures were diluted 10-fold to approximately 5,000 cells/ml, and cell densities were measured daily throughout the subsequent incubation. A brief exponential growth phase was typically observed 1 to 3 days after dilution, and the cultures entered stationary phase 4 to 5 days after dilution at densities of approximately 90,000 cells/ml. Actively growing samples were taken 1 or 2 days after dilution (doubling time, 24 to 30 h), and stationary-phase samples were taken after growth had completely ceased for at least 1 day. Cells were harvested by collection of samples onto 25-mm-diameter hydrophilic Durapore membranes (0.65-μm pore size; Millipore) by using vacuum filtration. Filters were transferred to tubes containing 400 μl of lysis solution (50 mM glycine, 10 mM EDTA, 5% [vol/vol] *N*-lauroylsarcosine, 0.5% [vol/vol] ProClin 150 [Rohm and Haas, Philadelphia, Pa.], pH 11), vortexed gently, and then heated to 85°C for 5 min. Afterward, 600 μl of sample buffer (100 mM Tris, 17 mM EDTA, 8.35% formamide, 5 M guanidine thiocyanate, pH 7.5) was added to each tube, the tubes were capped with filter tips (Saigene Corporation, Bothell, Wash.), the contents were mixed, and the samples were pushed through the filters into clean tubes to remove particulates. Guanidine lysates were shipped frozen from MBARI to the Seattle Biomedical Research Institute and stored at −70°C until use.

DNA sequencing. DNA was extracted from guanidine lysates by using an IsoQuick Kit (Orca Research, Bothell, Wash.). The ITS2 region was amplified by using Perkin-Elmer Gene-Amp PCR reagents and primers 5.8S-R and LR3 (Table 1). An annealing temperature of 50°C yielded PCR products which migrated as a single band upon agarose gel electrophoresis. PCR products for direct sequencing were purified by standard protocols (33). Sequence determination was carried out at the Seattle Biomedical Research Institute's automated facility with Applied Biosystems hardware, software, and methods. Both strands were sequenced with primers 5.8S-R and AH1 (Table 1).

Because sequences upstream of the SSU rRNA region were not known, the ETS1 region was amplified from *Pseudo-nitzschia* DNA by an inverse PCR strategy (29). DNA was digested with restriction endonuclease *Hha*I, purified, diluted to a concentration of 2 ng/μl, and circularized with T4 DNA ligase. Fragments containing ETS1 and the 5′ portion of the SSU rRNA were amplified from the circularized DNA by PCR with the diverging 5′ SSU rDNA primers PMNVPCR1 and PMNVPCR2 (Table 1). Preliminary sequences of these fragments were used to design primer PAPCR2, which recognizes a conserved region approximately 345 bp upstream of the SSU rDNA 5′ terminus. This primer was used along with PMNVPCR1 to amplify ETS1 rDNA by conventional PCR. Both strands of the conventional PCR fragments were sequenced with PAPCR2 and PMR4 as primers (Table 1).

Nucleic acid extraction, preparation of slot blot filters, and probe labeling. RNA was extracted from guanidine lysates for slot blot hybridization as follows. One hundred microliters of lysate was added to a 2-ml microcentrifuge tube containing 350 μl of extraction buffer (50 mM Tris-HCl, 10 mM EDTA, 100 mM NaCl, and 5% [wt/vol] sodium dodecyl sulfate, pH 7.6), 100 μl of 1-methyl-2-pyrrolidinone (Aldrich Chemical Co., Milwaukee, Wis.), and 550 μl of phenol-water-chloroform (Applied Biosystems). The mixture was vortexed, heated to 85°C for 12 min, vortexed again, and centrifuged at 13,000 × *g* for 8 min. The aqueous layer (about 400 μl) was transferred to a new tube, to which was added 600 μl of phenol-water-chloroform. The tube was vortexed and centrifuged for 5 min as described above. The aqueous layer (about 300 μl) was transferred to a sterile 1.7-ml microcentrifuge tube, to which was added 0.1 volume of 3 M sodium acetate and 1 ml of ethanol. After incubation at −20°C for 1 h, the tube was vortexed and centrifuged as described above for 15 min. After the supernatant was decanted, the pellet was air dried for 5 to 10 min and then resuspended in 200 μl of TE buffer (33). The solution was diluted appropriately and applied to Nytran Plus membrane filters (Schleicher & Schuell, Keene, N.H.) by using a slot blot apparatus (27) and then fixed by UV cross-linking (6). Filters were cut in half for hybridization to two different probes. The final samples contained nucleic acid from 1×10^3 to 5×10^4 cells per half-slot, as specified in the text and figure legends.

Oligonucleotide probes (Table 1) were end labeled with [γ-^{32}P]ATP by using T4 polynucleotide kinase and purified by standard methods (27, 33). Oligonucleotide probes were used only if their specific activities after purification were within the range of 4×10^6 to 2×10^7 cpm/μg of probe. RNA probes were generated as described previously (6). Briefly, 90- to 345-bp fragments of rDNA were amplified using primer pairs listed in Table 1. The reverse primer in each pair had a linker containing the T7 RNA polymerase promoter and a spacer sequence. Transcripts complementary to pre-rRNAs were generated from the amplification products with the incorporation of multiple [α-^{32}P]uridine residues by using T7 RNA polymerase and purified as described previously (6). Amplification products were stored indefinitely at −20°C, and labeled transcripts were stored in a guanidine solution (6) for up to 2 weeks before use. Since it was not practical to control or measure RNA probe specific activity, all quantitative experiments using these probes (as well as those using oligonucleotides) were structured to prevent comparisons between results obtained with probes from different labeling reactions.

Hybridization. Appropriate conditions for RNA probe hybridization were determined empirically against nucleic acids from target and nontarget organisms. Oligonucleotide probes were designed to function under uniform conditions determined empirically for a subset of the probes. Slot blot filter hybridizations and washes were carried out in 15-ml screw-cap polypropylene tubes. Filter strips were prehybridized in up to 10 ml of formamide-Denhardt's hybridization solution (27) for 2 h at 42°C with orbital shaking. Probes were added to a final concentration of 10^6 cpm/ml, and hybridization proceeded overnight at 42°C with shaking. Membranes were washed in successive changes of wash solution (27) for 1 to 2 min at room temperature, 1 h at 42°C, and finally 30 min at 52°C (oligonucleotide probes) or 45 min at 80°C (RNA probes). The still-moist membranes were placed on Whatman filter paper and sealed in plastic wrap for exposure to Kodak X-Omat autoradiography film (1 to 7 days, depending on probe type and target abundance) or a Molecular Dynamics standard phosphor screen (2 to 48 h, depending on the same two variables). Exposed phosphor screens were scanned in a Storm 860 PhosporImager using ImageQuaNT software (both from Molecular Dynamics). Numerical results were generated by using the ImageQuaNT Peak Finder program. For graphic presentation, images were uniformly enhanced and annotated by using Microsoft PowerPoint.

Nucleotide sequence accession numbers. The nucleotide sequences of ETS1 and ITS2 from the following *Pseudo-nitzschia* species have been submitted to GenBank and given the following accession numbers: *P. multiseries*, AF001455 and AF001456, respectively; *P. pungens*, AF001457 and AF001458, respectively; and *P. australis*, AF001459 and AF001460, respectively.

RESULTS

Nucleotide sequences of ETS1 and ITS2. Nucleotide sequences of ETS1 and ITS2 from *P. multiseries*, *P. pungens*, and *P. australis* were determined by direct sequencing of PCR

TABLE 1. Primers and probes used in these studies

Probe or primer	Sequence[a]	Targeted region,[b] comments, and sequence reference
Oligonucleotide probes		
PMPR9	GAAGACTGATCTTGGATCGGTGTGGTAATG	ITS1, positions 1772–1801 (24)
PMPR9-C	CATTACCACACCGATCCAAGATCAGTCTTC	ITS1 DNA, complementary to PMPR9
PMPR10	GTCTAGATTAAAAACCTAAGCCACTGCAATCAG	ITS1, positions 1811–1843 (24)
PMPR11	GAGGGTTGCCGGTTAAGTTTGACTACAGTAG	ITS1, positions 1843–1873 (24)
PMPR12	GTGGCGGGCAACCCGAGATTAC	ITS1, positions 1878–1899 (24)
PMPR13	GGTCATTTGGTTCTTTCCAGTTACCAATCG	ITS1, positions 1906–1935 (24)
PMPR13-C	CGATTGGTAACTGGAAAGAACCAAATGACC	ITS1 DNA, complementary to PMPR13
PMPR14	GGTATAATCTGCAGGGCCACACTGACAG	ITS1, positions 1973–2000 (24)
PMPR16	CAGTGGTGATAAACCAAGTTGAACAAGTTC	ETS1, positions 167–196 (Fig. 1)
PMPR16-C	GAACTTGTTCAACTTGGTTTATCACCACTG	ETS1 DNA, complementary to PMPR16
PMPR17	GCTTCGGTGAGGGAAAGTCAAAATC	ETS1, positions 243–267 (Fig. 1)
PAPR1	GCTAAAGTGAAACAAACCAACACTGGCA	*P. australis* ETS1, corresponds to positions 274–300 (Fig. 1)
PMR3	TGATCCTTCCGCAGGTTCACCTACG	SSU, positions 1749–1773 (24)
PMR4	ATACTTAGACATGCATGGC	SSU, positions 31–49 (24)
PCR and sequencing primers		
5.8S-R	TCGATGAAGAACGCAGC	5.8S rDNA (24)
AH1	AGTTAGTTTCTTTTCCTC	LSU rDNA, positions 30–48 (35)
LR3	GGTCCGTGTTTCAAGAC	LSU rDNA, positions 556–572 (34)
PAPCR2	ATCTTTGTTGATATACTGTTCT	Upstream ETS1; see Materials and Methods
PAPCR3	*T7*CTAAAGTGAAACAAACCAACACTGGC	*P. australis* ETS1; overlaps PAPR1
PMNVPCR1	CTGATATAATGAGCCGTT	SSU rDNA, positions 73–90 (24)
PMNVPCR2	CTACTTGGATACCCGTAG	SSU rDNA, positions 116–133 (24)
PMPCR1	*T7*GGTATAATCTGCAGGGCCACACTGACAG	ITS1 rDNA, positions 1973–2000 (24)
PMPCR2	*T7*GTGGCGGGCAACCCGAGATTAC	ITS1 rDNA, positions 1878–1899 (24)
PMPCR3	*T7*GAGGGTTGCCGGTTAAGTTTGACTACAGTAG	ITS1 rDNA, positions 1843–1873 (24)
PMPR10-C	CTGATTGCAGTGGCTTAGGTTTTTAATCTAGAC	Complementary to PMPR10
PMPR12-C	GTAATCTCGGGTTGCCCGCCAC	Complementary to PMPR12
PMPCR4	AGCGCTGGCCTC	ITS2 rDNA, positions 71–82 (Fig. 1)
PMPCR5	*T7*TTAACTAGACAGGTCAAGC	ITS2 rDNA, positions 172–191 (Fig. 1)
PMPCR6	AGACGTGGAAGGCTT	ITS2 rDNA, positions 162–176 (Fig. 1)
PMPCR7	*T7*ACAAACTCATAGTAAACTCCA	ITS2 rDNA, positions 250–270 (Fig. 1)
PMPCR8	GTATGAAATATCTGGAGTTTAC	ITS2 rDNA, positions 238–259 (Fig. 1)
PMPCR9	*T7*AATGTATAACAGACTGTAAACAG	ITS2 rDNA, positions 320–342 (Fig. 1)
RNA probes		
PMT7-1		ITS1, positions 1878–2000 (primers PMPR12-C to PMPCR1)
PMT7-2		ITS1, positions 1811–1899 (primers PMPR10-C to PMPCR2)
PMT7-3		ITS1, positions 1773–1873 (primers PMPR9-C to PMPCR3)
PMT7-5		ITS2, positions 238–341 (primers PMPCR8 to PMPCR9)
PMT7-6		ITS2, positions 162–270 (primers PMPCR6 to PMPCR7)
PMT7-7		ITS2, positions 71–191 (primers PMPCR4 to PMPCR5)
PAT7-1		*P. australis* ETS1, positions −45 (approximate) to 300 (primers PAPCR2 to PAPCR3)

[a] *T7*, linker containing T7 RNA polymerase recognition site (AATTTAATACGACTCACTATAGGGA); used for generation of RNA probes.
[b] Designed against *P. multiseries* sequences except where indicated.

products. There were few ambiguities in these sequences, indicating that the templates were nearly homogeneous despite the presence of multiple copies of the rRNA operon. The sequenced regions may be strongly conserved between copies, or, alternatively, amplification primers or conditions may have favored the production of homogeneous subsets of copies.

ETS1 and ITS2 sequences are aligned in Fig. 1. As with previously reported ITS1 sequences from *P. multiseries* and *P. pungens* (24), ETS1 and ITS2 exhibited greater interspecies sequence diversity than does mature rRNA. The 290-bp region of *P. multiseries* rDNA containing the ETS1 coding region exhibited 19 and 22% divergence from the corresponding regions of *P. pungens* and *P. australis*, respectively, counting insertions-deletions (indels) as single events. ITS2 of *P. multiseries* (approximately 343 bases) exhibited 17 and 21% divergence, respectively, from those of *P. pungens* and *P. australis*. In contrast, comparison of mature SSU rDNAs from *P. multiseries* and *P. pungens* revealed only 14 point mutations and one indel

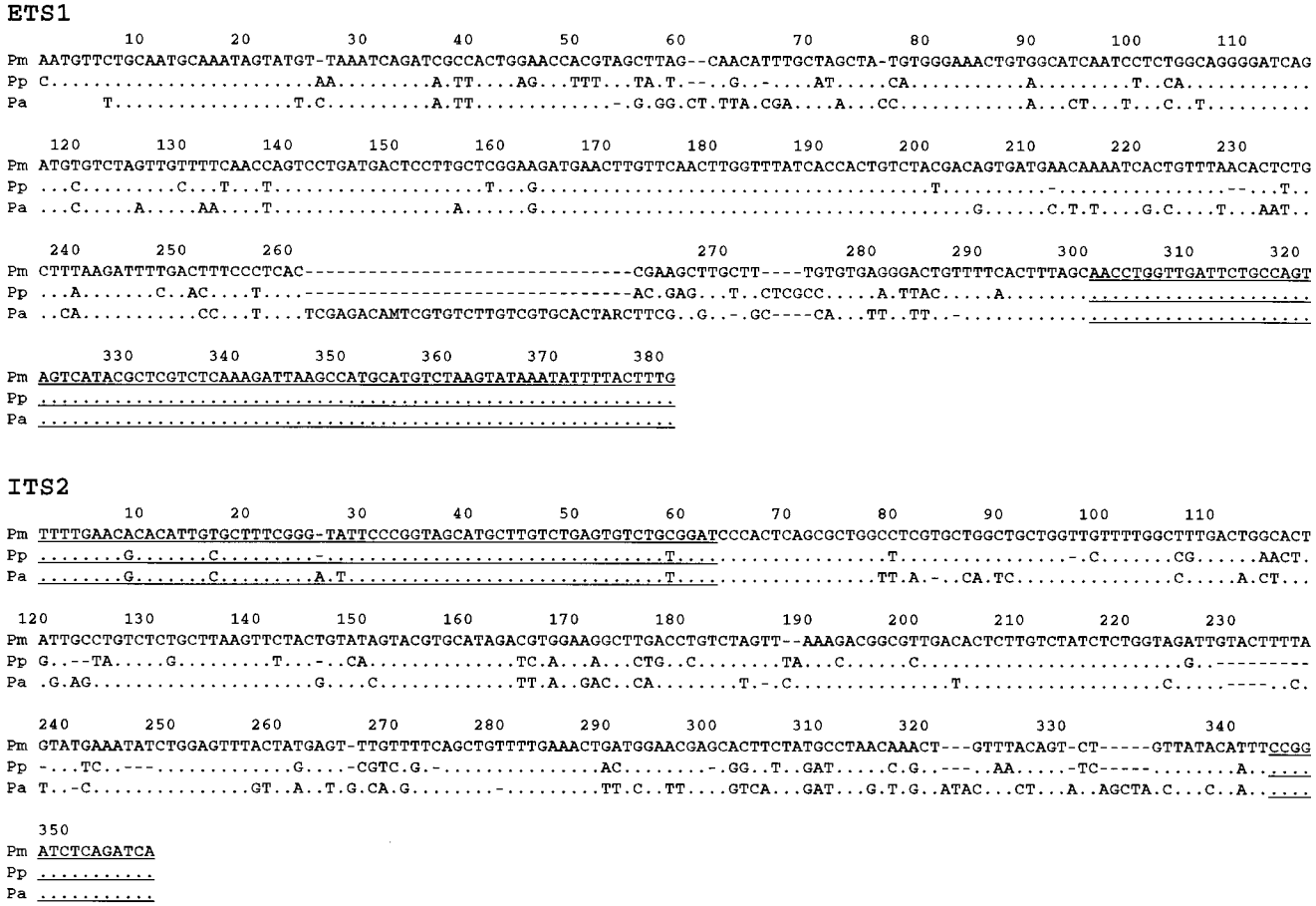

FIG. 1. Nucleotide sequences of *P. multiseries* mu1 (Pm), *P. pungens* pu14 and pu16 (Pp), and *P. australis* au22 (Pa), aligned with each other by FASTA analysis (30). ETS1 sequences end with mature SSU rRNA coding regions (underlined) inferred from the 100% homology between these regions and the SSU rRNA sequences of *P. pungens*, *P. multiseries* (24), and *Nitzschia apiculata* (GenBank accession no. M87334). ITS2 sequences begin with approximate positions of 5.8S rRNA-encoding rDNA (underlined) inferred from the 75% homology with the 5.8S rRNA sequence of *Stephanodiscus hantzschii* (GenBank accession no. U03078). ITS2 sequences end with approximate positions of LSU rRNA-encoding rDNA (underlined). These positions were inferred by homology of downstream regions (see GenBank accession no. AF001458 and AF001460) to published *Pseudo-nitzschia* LSU rRNA sequences (35) and to the complete LSU rRNA sequences of other unicellular protists (3).

in 1,770 bp, for less than 1% divergence (24). The ETS1 and ITS2 sequences provide further support for designating *P. pungens* (formerly *P. pungens* f. *pungens*) and *P. multiseries* (formerly *P. pungens* f. *multiseries*) as separate species.

Among the most pronounced variations in the spacer sequences was a 31-base indel situated about 39 bases upstream of the 5′ terminus of the mature SSU rRNA in the *P. multiseries* ETS1 (Fig. 1). Similar variations have been observed in ITS1 of *P. multiseries* and *P. pungens* (24) and in spacer regions of other groups of closely related taxa (1, 14, 21, 28). To gain an idea of the intraspecific stability of the 31-base indel in ETS1, we used primers PAPCR2 and PMR4 (Table 1) to amplify 400- to 440-bp fragments containing the indels from two isolates of *P. multiseries*, four isolates of *P. pungens*, and three isolates of *P. australis*. In all cases, amplification products were of the sizes expected if the 31-bp fragment was present in *P. australis* and absent in the other two species (Fig. 2), suggesting that this feature is stable in Monterey Bay isolates. Isolates of *P. pseudodelicatissima* and *P. fraudulenta* did not yield abundant amplification products of any size, possibly because of sequence divergence within the PAPCR2 recognition site.

Detection of multicopy pre-rRNA. We reported that pre-rRNA molecules in bacterial cells are readily detectable by direct probe hybridization if they are extracted and probed under appropriate conditions (5, 6). To determine whether the same is true of *Pseudo-nitzschia* pre-rRNA, we designed probes specific for most portions of ETS1, ITS1, and ITS2 of *P. multiseries* and/or *P. australis* (Fig. 3). Synthetic oligonucleotide probes (15 to 30 bases) were end labeled with ^{32}P, while longer RNA probes (90 to 345 bases) were labeled by incorporating multiple [α-^{32}P]uridine residues during synthesis by T7 RNA polymerase. Given the variability in the primary and secondary

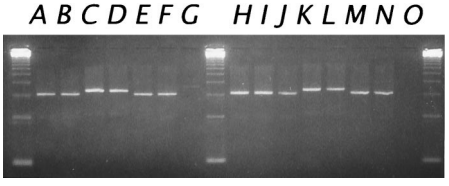

FIG. 2. PCR amplification of a 400- to 440-bp region of rDNA that spans the 5′ terminus of SSU rRNA and includes the 31-base indel in ETS1. The region was amplified from *P. australis* isolates au23 (C), au27 (D and K), and au22 (L); *P. pungens* isolates pu12 (E), pu14 (F), pu15 (H), and pu16 (I and M); and *P. multiseries* isolates mu1 (A and J) and mu2 (B and N). Little or no amplification product was obtained from *P. pseudodelicatissima* (G) or *P. fraudulenta* (O). Unmarked lanes contained DNA fragment length standards.

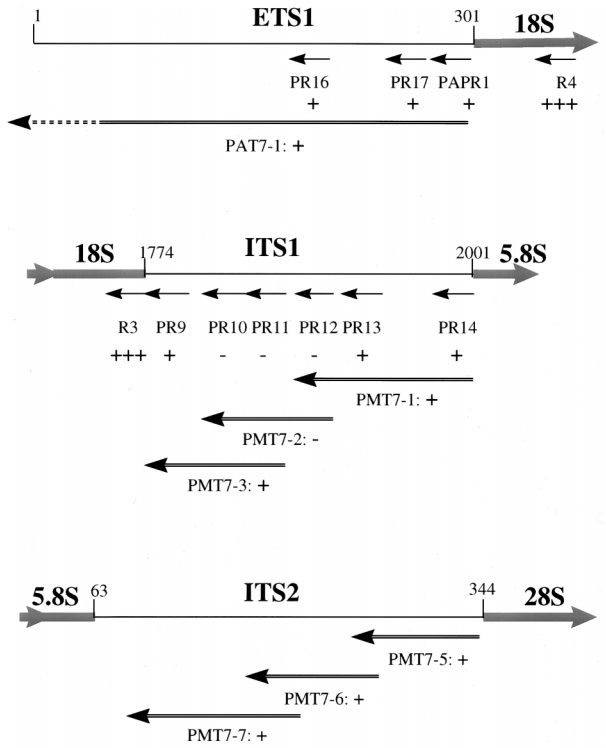

FIG. 3. Positions of probes targeting spacer and mature rRNAs. At the top of each of the three maps, spacer regions are shown as thin lines and inferred mature rRNA-coding regions are shown as thicker lines. The numbers on the ETS1 and ITS2 maps correspond to the *P. multiseries* sequences shown in Fig. 1. The numbers on the ITS1 map correspond to the published *P. multiseries* ITS1 sequence (24). The positions of oligonucleotide probes are shown as single-lined arrows below each map. Below the name of each oligonucleotide probe is a symbol indicating whether the probe generated a strong signal characteristic of mature rRNA when hybridized to RNA from *P. multiseries* (+++), a weaker signal characteristic of pre-rRNA (+), or no detectable signal (−). Each such classification is based on at least two independent experiments. Examples of each level of signal strength are illustrated in subsequent figures. Positions of RNA probes are shown as longer, double-lined arrows above probe names and indications of signal (+ or −, as described above). Probes PAPR1 and PAT7-1 targeted *P. australis* sequences, while the remaining probes targeted *P. multiseries*. To conserve space, names of *P. multiseries*-targeted oligonucleotide probes are abbreviated by omission of the "PM" prefix (e.g., PMPR16 is termed PR16 in this figure).

structures of target sites, as well as in the specific activities of individual probes, it is difficult to compare the relative abundances of targets recognized by different probes. While signals generated by pre-rRNA-targeted probes were always weaker than those generated by mature-rRNA-targeted probes, almost all pre-rRNA-targeted probes generated robust signals when hybridized to RNA extracted from as few as 10^4 cells (for oligonucleotide probes) or 10^3 cells (for RNA probes). The exceptions were oligonucleotide probes PMPR10, PMPR11, and PMPR12 and RNA probe PMT7-2, all of which targeted a discrete 90-base region of ITS1 (Fig. 3). This region may be degraded more quickly in the rRNA maturation pathway than other regions, or it is otherwise not accessible to hybridization under these conditions.

To determine whether pre-rRNA-targeted probes detected RNA transcripts or the rDNA sequences encoding such transcripts, we compared the signals generated by three of the oligonucleotide probes to those generated by the exact complements of these probes hybridized to the same samples. PMPR16 (ETS1), PMPR9 (5′ flank of ITS1), and PMPR13 (3′ flank of ITS1) gave much stronger signals than did their complements, indicating that they detected single-stranded, multiple-copy molecules which were presumably pre-rRNA (Fig. 4). ITS2 was not analyzed in this way because we did not target this region with oligonucleotide probes. However, RNA probes targeted to this region were as sensitive as RNA probes targeted to ITS1, suggesting that they detected similarly abundant molecules.

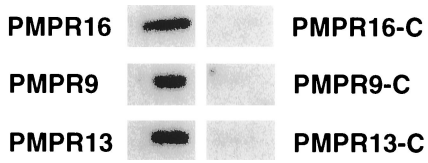

FIG. 4. Relative signal intensities generated by pre-rRNA-targeted probes (PMPR16, PMPR9, and PMPR13) and exactly complementary probes targeted to the coding strands of rDNA (PMPR16-C, PMPR9-C, and PMPR13-C, respectively). Each slot contained nucleic acid extracted from approximately 5×10^4 *P. multiseries* cells.

Specificity of RNA probes for pre-rRNA regions. In considering the use of direct probe hybridization to detect pre-rRNA, the low copy number of pre-rRNA molecules relative to mature rRNAs must be addressed. One approach to the problem is the use of ≥90-base-long, multiply labeled RNA transcripts rather than singly labeled oligodeoxynucleotide probes. Such probes would exhibit considerable cross-reactivity if targeted to mature rRNA, but they have exhibited excellent specificity when targeted to pre-rRNA, due to the variability of the latter (6). The probes in Fig. 3 were designed with emphasis on complete coverage of pre-rRNA regions rather than on species specificity; however, all exhibited at least partial specificity under our hybridization conditions, and PMT7-5 exhibited perfect specificity when hybridized to RNA from the three species used in this study (Fig. 5). This demonstrates that assay sensitivity sacrificed by targeting pre-rRNA rather than mature rRNA can be partially regained by using long RNA probes, without sacrificing assay specificity.

Physiological stability of pre-rRNA. We reported previously that the abundance of ETS1 and ITS1 pre-rRNA molecules per *E. coli* cell varies between growing and nongrowing cultures over a >10-fold to >1,000-fold range, depending on the conditions limiting growth (5). To determine whether such fluctuations also occur in *P. multiseries*, probes targeted to ETS1, ITS1, ITS2, and mature rRNA were hybridized to RNA extracted from lysates of two actively growing cultures, two stationary-phase cultures, and a culture which was allowed to enter stationary phase and then incubated in complete darkness for two additional days (all were grown in f/2-enriched seawater). Differences in pre-rRNA signal intensities per cell did not exceed a factor of five between these conditions, which is not much greater than the observed sample-to-sample error (Fig. 6). Over the course of this study, we examined 10 cultures of *P. multiseries* and 5 cultures of *P. australis* harvested in both logarithmic and stationary growth phases and never observed variations in cellular pre-rRNA abundance which appeared to be outside of this range. Therefore, *Pseudo-nitzschia* cells cultured in f/2-enriched seawater maintain constant or nearly constant pre-rRNA pools, despite significant changes in growth activity.

DISCUSSION

Our interest in pre-rRNA as a diagnostic target stems from two of its characteristics. First, it offers greater specificity for routine identification of microbial species and subspecies than

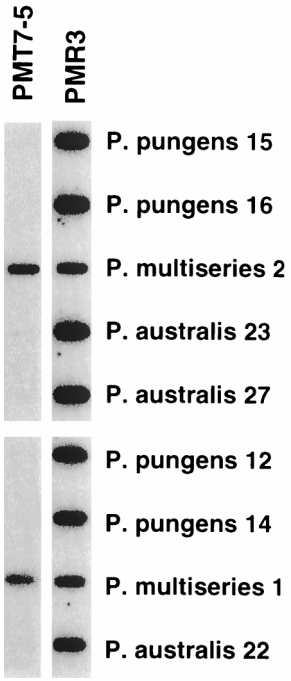

FIG. 5. Specificity of RNA probe PMT7-5 for *P. multiseries* pre-rRNA. The total nucleic acid extracted from approximately 5×10^3 cells of each *Pseudo-nitzschia* isolate was applied to a filter, which was cut in half and probed with PMT7-5 and the mature SSU rRNA-targeted probe PMR3. Positive signals generated by PMR3 showed that the *P. pungens* and *P. australis* samples contained at least as much RNA as the *P. multiseries* samples. A replicate experiment yielded the same results.

mature rRNA. Second, its abundance in bacterial cells fluctuates markedly with growth physiology, making it potentially useful as a species-specific indicator of growth in mixed or open systems (5, 6). To determine whether such fluctuations also occur in *Pseudo-nitzschia* cells, we conducted a detailed analysis employing the same methods we used on bacterial systems. We found that, in contrast to bacterial systems, pre-rRNA abundance in *P. multiseries* cells did not fluctuate strongly with growth physiology in f/2-enriched seawater. Therefore, pre-rRNA may not be useful as an indicator of *Pseudo-nitzschia* growth activity, at least under the conditions used in this study. However, it could be much more useful for routine identification of these and, perhaps, similar microorganisms than is generally assumed.

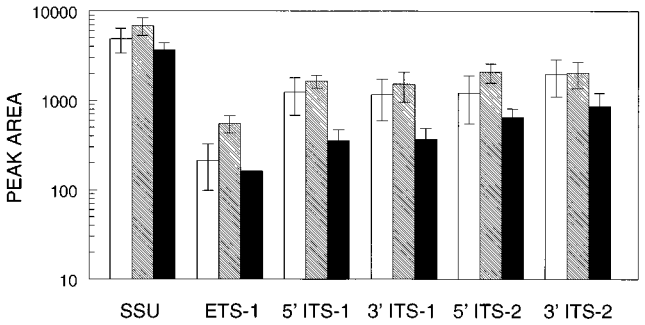

FIG. 6. Signal intensities generated by mature SSU rRNA-targeted probes and pre-rRNA-targeted probes hybridized to *P. multiseries* RNA from actively growing cultures (white bars), stationary-phase cultures (striped bars), and a stationary-phase culture incubated in total darkness for 2 days (light starved; black bars). Peak area values were generated by PhosphorImager analysis of slot blot signals as described in Materials and Methods, with a value of 10 considered background. Means and standard deviations for two actively growing harvests and two stationary-phase harvests are shown. Only one light-starved culture was tested, so means and standard deviations for two separate experiments on this culture are shown (error bars). Probes used in this analysis were as follows: SSU, oligonucleotide probe PMR3; ETS1, oligonucleotide probes PMPR16 and PMPR17 (labeled and applied together to maximize sensitivity); 5′ ITS1, PMT7-3; 3′ ITS1, PMT7-1; 5′ ITS2, PMT7-7; and 3′ ITS2, PMT7-5.

Pre-rRNA molecules were present in both dividing and nondividing *P. multiseries* cells in amounts which exceeded the rDNA sequences encoding them. Pre-rRNA pools were smaller than mature-rRNA pools, as is evident when mature SSU rRNA detected by a single labeled oligonucleotide is compared to ETS1 pre-rRNA detected by a pair of labeled oligonucleotides (Fig. 6). However, the extensive variability of pre-rRNA sequences permitted us to use long, multilabeled, T7 RNA polymerase-generated RNA probes to detect pre-rRNA with greater sensitivity than is possible with end-labeled oligonucleotides (note the relatively strong signals generated by RNA probes directed to ITS-1 and ITS-2 in Fig. 6). Pre-rRNA-targeted probes may never match the sensitivity of probes targeted to mature rRNA, but they are nonetheless sufficiently sensitive for use on small samples (10^3 to 10^4 cells) of *Pseudo-nitzschia* cultures. In contrast to our results, pre-rRNA was not detected in toxigenic dinoflagellates of the genus *Alexandrium* by a hybridization assay with sufficient sensitivity to detect rDNA (2). The single oligonucleotide probe used in the *Alexandrium* study may have targeted a region which is rapidly processed, as we believe to be the case for the undetectable 90-base region in the center of ITS1 in *P. multiseries* (Fig. 3). The existence of such regions must be taken into consideration when developing pre-rRNA-targeted probes.

Pre-rRNA sequences include relatively conserved regions involved in secondary structures required for rRNA maturation, as well as regions which are more variable than even the most hypervariable mature rRNA regions (1, 10, 11, 14, 21, 28, 38). Therefore, as with sequence analysis of rDNA spacers, probe analysis of pre-rRNA can provide information not obtainable by analysis of mature rRNA. In situations in which mature rRNA offers adequate specificity and greater resolving power is not needed, pre-rRNA-targeted probes can provide independent (and rigorous) confirmation of taxonomic identification based on mature rRNA.

The extent of pre-rRNA depletion in starved *E. coli* cells varies with conditions but always exceeds a factor of 10 (5). Pre-rRNA pools in *E. coli* are presumably depleted by inhibition of *rrn* operon transcription combined with ongoing pre-rRNA processing and/or degradation. The present study of *Pseudo-nitzschia* species used two sets of growth limitation conditions: illumination and lack of illumination of stationary-phase cells in f/2-enriched seawater. These conditions almost certainly depressed pre-rRNA synthesis relative to that of actively growing cultures, but they caused little or no depletion of the pre-rRNA pool. Therefore, inhibition of pre-rRNA synthesis in stationary-phase *Pseudo-nitzschia* cells did not exceed inhibition of pre-rRNA processing, resulting in a stable pre-rRNA pool under these conditions. This pattern of coordinated down-regulation can be described as coupled, in contrast to the uncoupled pattern observed in bacteria. Coupled down-regulation has been reported in other eukaryotic cell types analyzed by individual measurements of pre-rRNA synthesis and processing activities (8, 9, 22). These data suggest that there exists a general difference between these eukaryotes and some bacteria; however, this difference may simply be one of degree. Moreover, conditions other than those used in this

study could lead to uncoupled down-regulation and more significant pre-rRNA depletion in *Pseudo-nitzschia* cells.

In summary, pre-rRNA in *Pseudo-nitzschia* cells cultured in f/2-enriched seawater is of sufficient abundance and stability to render it detectable by simple, direct DNA probe hybridization analysis. *P. multiseries* cells, both growing and nongrowing, cultured under these conditions maintain a stable pool of pre-rRNA, presumably by coupling the regulation of pre-rRNA synthesis and processing. Characterization of pre-rRNA pools under additional conditions, and in additional species, will further define the distribution of coupled and uncoupled regulation patterns and the types of information which can be obtained by direct detection of pre-rRNA in microorganisms.

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