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Feasibility of Transferring Fluorescent In Situ Hybridization Probes to an 18S rRNA Gene Phylochip and Mapping of Signal Intensities▽

Katja Metfies and Linda K. Medlin*

Alfred Wegener Institute for Polar and Marine Research, Am Handelshafen 12, D-27570 Bremerhaven, Germany

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DNA microarray technology offers the possibility to analyze microbial communities without cultivation, thus benefiting biodiversity studies. We developed a DNA phylochip to assess phytoplankton diversity and transferred 18S rRNA probes from dot blot or fluorescent in situ hybridization (FISH) analyses to a microarray format. Similar studies with 16S rRNA probes have been done determined that in order to achieve a signal on the microarray, the 16S rRNA molecule had to be fragmented, or PCR amplicons had to be <150 bp in length to minimize the formation of a secondary structure in the molecule so that the probe could bind to the target site. We found different results with the 18S rRNA molecule. Four out of 12 FISH probes exhibited false-negative signals on the microarray; eight exhibited strong but variable signals using full-length 18S RNA molecules. A systematic investigation of the probe's accessibility to the 18S rRNA gene was made using *Prymenisum parvum* as the target. Fourteen additional probes identical to this target covered the regions not tested with existing FISH probes. Probes with a binding site in the first 900 bp of the gene generated positive signals. Six out of nine probes binding in the last 900 bp of the gene produced no signal. Our results suggest that although secondary structure affected probe binding, the effect is not the same for the 18S rRNA gene and the 16S rRNA gene. For the 16S rRNA gene, the secondary structure is stronger in the first half of the molecule, whereas in the 18S rRNA gene, the last half of the molecule is critical. Probe-binding sites within 18S rRNA gene molecules are important for the probe design for DNA phylochips because signal intensity appears to be correlated with the secondary structure at the binding site in this molecule. If probes are designed from the first half of the 18S rRNA molecule, then full-length 18S rRNA molecules can be used in the hybridization on the chip, avoiding the fragmentation and the necessity for the short PCR amplicons that are associated with using the 16S rRNA molecule. Thus, the 18S rRNA molecule is a more attractive molecule for use in environmental studies where some level of quantification is desired. Target size was a minor problem, whereas for 16S rRNA molecules target size rather than probe site was important.

With the introduction of molecular techniques into marine microbial biology, microbial biodiversity is easier to assess. rRNA-targeted probes can identify unculturable cells (2). With the routine application of these probes, it quickly became apparent that there was no uniform intensity to the target signals for all the probes utilized in a fluorescent in situ hybridization (FISH) format. Binding sites in the rRNA genes were sequentially analyzed for their accessibility by probes. First, the bacterial 16S rRNA gene was mapped (8), followed by the bacterial 23S rRNA gene (9). Finally, the accessibility of archeal 16S rRNA and eukaryotic 18S rRNA genes was mapped (3). All probes must be empirically tested because not all sites behave the same in all species, although some generalizations can be drawn (compare the intensity maps from the sources named above and see also reference 10). Although FISH rRNA probes are used fairly routinely, only a limited number of species can be examined simultaneously because of the small number of fluorochromes available.

Over the past decade, microarray technology has become an important tool to assess all biological problems. DNA microarrays are widely used for gene expression (6, 19, 28, 32); however, DNA microarrays, or the so-called phylochips, used to study species biodiversity (4, 24) or genetic variation (16, 33) are increasing. DNA phylochips consist of ordered sets of molecular probes fixed to solid surfaces. The continually growing number of available algal 18S or 28S rRNA gene sequences makes it possible to design probes that target species or strains.

The design of molecular probes dedicated to microbial identification is challenging; probe specificity in theory and practice must be carefully and critically evaluated because in the environment, probes have to be specific against the presence of a large, mostly unknown, diversity. With microarrays, rRNA probes are spotted onto glass slides, and target DNA is labeled with the fluorochrome. Only one fluorochrome is needed, but thousands of species and higher taxa can be spotted onto the glass slides for target hybridization. Only target DNA hybridized to probe complexes will fluoresce. Many 18S rRNA gene probes for the identification of phytoplankton are available using FISH or dot blot analysis (10, 12, 14, 15, 29, 30).

If probes from other formats could be transferred to DNA microarrays, it would greatly accelerate the development of a comprehensive probe set that would facilitate the analysis of complex microbial samples. The first transferability of a standard FISH protocol onto phylochips was shown for 16S rRNA gene targeting probes (27). As rRNA probes were adapted to a microarray format, it became apparent that sites were not accessible to the probes (13, 18). Secondary structure and length of the target were implicated as the causes for the

* Corresponding author. Mailing address: Alfred Wegener Institute for Polar and Marine Research, Am Handelshafen 12, D-27570 Bremerhaven, Germany. Phone: 49 471 4831 1655. Fax: 49 471 4831 1425. E-mail: lkmedlin@awi-bremerhaven.de.

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TABLE 1. List of PCR primers used in this study

Primer	Sequence	Reference	Primer combination for PCR fragment amplification (length [bp])[a]
1F-Biotin	AACCTGGTTGATCCTGCCAGT	22	1F-Biotin + 1528R (1,800)
690 F-Biotin	TCAGAGGTGAAATTCTTGGAT	7	**690F-Biotin** + **1528R** (900)
690 R	ATCCAAGAATTTCACCTCTGA	7	**1F-Biotin** + **690R** (900)
528F-Biotin	GCGGTAATTCCAGCTCCAA	7	**528F** + **1528R** (1,200)
1528R	TGATCCTTCTGCAGGTTCACCTAC	22	

[a] Primers in boldface were used to amplify the truncated version of the 18S rRNA gene.

inaccessibility to the 16S rRNA gene in the microarray format (13, 18), whereas proteins were assumed to be blocking the sites for FISH hybridization (3, 8, 9). In the present study, we determined whether a similar problem occurred in the 18S rRNA gene by using probes targeting eukaryotic phytoplankton. Our data suggest that probe specificity has to be reevaluated if probes are exchanged between different hybridization formats; moreover, the binding site of the probe in the 18S rRNA gene molecule is an important criterion in probe design when phylochips are used. The implementation of a taxonomic hierarchical approach with the DNA phylochips increases the specificity of microarray analysis in the presence of a largely unknown genetic background. Thus, for a species to be present, it must also have a signal with probes from its genus, family, clade, or class level. Such an approach will reduce the number of false positives. In the present study, we determined whether this methodology transfer could also be applied to 18S rRNA gene targeting probes for phytoplankton identification.

MATERIALS AND METHODS

Algal strains and templates. Genomic DNA isolated from phytoplankton laboratory strains or plasmid DNA was used as a template for the PCR amplification of the 18S rRNA gene for use as a target in hybridization experiments. The following algal strains were used: *Prymnesium parvum* f. *patelliferum* RL10, *Bolidomonas mediterranea* CCMP 1867, and *Rhinomonas reticulata* PLY 358. Plasmid DNA consisted of an ~1,800-bp PCR fragment of the environmental picoplankton 18S rRNA gene cloned into the vector pCR-XL-TOPO (Invitrogen, Groningen, The Netherlands) originally analyzed in Medlin et al. (23). Plasmid DNA was isolated with Qiagen plasmid mini kits (Qiagen, Hilden, Germany).

DNA extraction. DNA was extracted from pure cultures with DNeasy plant mini kits (Qiagen).

PCR amplification and labeling. Biotinylated PCR fragments were amplified from genomic DNA or cloned 18S rRNA genes of algal targets (22) (Table 1). Incorporation of biotin on one of the primer pairs provided the label for later staining of the PCR product after hybridization with the phylochip probes. PCR was carried out for all primer combinations in an Eppendorf cycler (Hamburg, Germany) with the following protocol, optimized for amplifying from genomic DNA: 5 min at 94°C; 35 cycles of 2 min at 94°C, 1 min at 54°C, 3 min at 72°C; and a final step for 10 min at 72°C. A 250-bp fragment from the *Saccharomyces cerevisiae* TATA box binding protein (TBP) gene was amplified with the primer pair TBP-F (5′-ATGGCCGATGAGGAACGTTTAA-3′) and TBP-R-biotin (5′-TTTTCAGATCTAACCTGCACCC-3′). The amplification of this PCR fragment was done as follows: 5 min at 94°C; 35 cycles of 1 min at 94°C, 1 min at 52°C, and 1 min at 72°C; and a final extension step for 10 min at 72°C. PCR fragments were purified with a QIAquick PCR purification kit (Qiagen). DNA was quantified spectrophotometrically (Eppendorf).

Probe set. For the first phylochip experiments, 12 probes (18- to 20-mer) were chosen that specifically target the 18S rRNA gene of a subset of phytoplankton groups at division, class, or genus level. This probe set implemented a hierarchical approach for Prasinophyceae, which are targeted by two probes, one each at division and class level. For other taxa, this probe set contained two specific probes at the same taxonomic level in order to discover whether taxonomically identical probes could result in comparable hybridization intensities on the phylochip (Table 2). All of these probes work specifically with dot blot or FISH detection systems with strong signals (Table 2). All had similar lengths, thermal denaturation midpoint temperatures, and GC-content, and minimal self-annealing properties. A second probe set was constructed from the sequence of *P. parvum* to cover hybridization of the 18S rRNA gene molecule in those regions not hybridized by the FISH probes from the first set (Table 2).

One positive control with a perfect match in the gene of the TBP of *S. cerevisiae* was present to estimate hybridization efficiency, and a second negative control probe, for which a BLAST search of the GenBank database did not find any match (1), was used to evaluate nonspecific binding. Equal amounts of the positive control were added to each hybridization reaction mixture.

Microchip fabrication. Oligonucleotide probes (18- to 20-mer) for phylochip printing were obtained from Thermo Hybaid, Interactive Division (Ulm, Germany) with a C_6/MMT Aminolink at its 5′ end. Slide manufacturing and oligonucleotide printing onto the chip were carried out by PicoRapid GmbH (Bremen, Germany). After the printing step, microchips were stored at −20°C. The probe array was replicated four times on each phylochip.

Hybridization of 18S PCR fragments to DNA microchips. The hybridization mixture contained biotinylated 18S rRNA gene PCR fragments at different concentrations. One positive target DNA was included for each probe immobilized on the phylochip. Hybridizations were repeated twice. Depending on the experiment, the target DNA had a length of either ~1,800 bp or ~900 bp. Additionally, a 250-bp PCR fragment of *S. cerevisiae* TBP at a final concentration of 4 ng/μl in hybridization buffer (1 M NaCl, 10 mM Tris, pH 8, 0.005% Triton X-100, 1 mg/ml bovine serum albumin, 0.1 μg/μl herring sperm DNA) was added to the hybridization mixture, for a final volume of 100 μl. Prehybridization (equilibration) of the phylochips was carried out in hybridization buffer for 60 min at the hybridization temperature (see below). The hybridization mixture was incubated for 5 min at 94°C. Following denaturation, the hybridization mixture was applied directly onto the equilibrated phylochips. The hybridization was done under a coverslip in a wet chamber at 58°C for 60 min. Phylochips were washed immediately for 15 min in 50 ml of buffer 1 (2× SSC–10 mM EDTA–0.05% sodium dodecyl sulfate [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate]) and for an additional 15 min in 50 ml of buffer 2 (1× SSC–10 mM EDTA).

Staining of the hybridized DNA microchips. Hybridized biotinylated target DNA was visualized by staining the phylochips for 30 min with streptavidin-Cy5 (Amersham Biosciences, Germany) in hybridization buffer at a final concentration of 50 ng/ml. Subsequently, the phylochips were washed two times for 5 min (each) in 50 ml of buffer 1 and for an additional 5 min in 50 ml of buffer 2.

Scanning and quantification of phylochips. Fluorescence images of the hybridized phylochips were taken with a Genepix 4000B scanner (Axon Instruments Inc.). Signal intensities were quantified using GenePix, version 4.0, software (Axon Instruments Inc.). To quantify each spot, a grid of individual circles defining the location of a spot was superimposed onto the image. Mean signal intensity and the intensity of the local background area were determined for each spot. Raw data were analyzed according to Loy et al. (20) and normalized to the signal of the positive control and for each experiment to a target concentration of 30 ng/μl.

RESULTS AND DISCUSSION

Transferability of FISH probes to the microarray format. The transferability of the FISH probes on the phylochips was evaluated by separate hybridizations of at least one perfectly matching target 18S rRNA gene PCR fragment. A set of probes was chosen that represented the most relevant groups

TABLE 2. Probes spotted on the DNA chip

Probe[a]	Target	Sequence	Reference or source	Position in 18S DNA (nt)[b]	Hybridization signal[c]
Prymparv1	***P. parvum***	**GGCAGGATCAACCAGGTT**	**This work**	**1**	**+**
Prymparv3	***P. parvum***	**TAGAATTACTACGGTTATCC**	**This work**	**141**	**+**
Prymparv5	***P. parvum***	**CGAGGCCATGCGATTCGA**	**This work**	**261**	**+**
DinoE-12	Division Dinophyta	CGGAAGCTGATAGGTCAGAA	This work	305	+++
Prymparv7	***P. parvum***	**GGTAGCCATTTCTCAGGC**	**This work**	**391**	**+**
Prymparv9	***P. parvum***	**TCCTCGTGAAGAGATGTAAAT**	**This work**	**520**	**+**
Prymparv11	***P. parvum***	**CCGGAAGGAAGGACGCGC**	**This work**	**681**	**+**
Pras04	Class Prasinophyceae	CGTAAGCCCGCTTTGAAC	26	747	++
Prymparv13	***P. parvum***	**AGTCCTATTTCATTATCCCAT**	**This work**	**801**	**+**
CryptoB	Division Cryptophyta	ACGGCCCCAACTGTCCCT	This work	820	+++
Boli02	Class Bolidophyceae	TACCTAGGTACGCAAACC	11	841	+++
NS04	New Stramenopiles, clade 4	TACTTCGGTCTGCAAACC	20	847	++
Prym02	Division Prymnesiophyta	GGAATACGAGTGCCCCTGAC	29	871	++
Prymparv15	***P. parvum***	**CCTGGCAAATGCTTTCGC**	**This work**	**937**	**+**
Prym01	Division Prymnesiophyta	ACATCCCTGGCAAATGCT	14	940	++
Chlo02	Division Chlorophyta	CTTCGAGCCCCCAACTTT	29	967	++
Prymparv17	***P. parvum***	**CCAAAGACTATAGTTTCCCT**	**This work**	**1081**	**−**
Chlo01	Division Chlorophyta	GCTCCACGCCTGGTGGTG	28	1100	−
Prymparv18	***P. parvum***	**CACTCCTGGTGGTGCCCT**	**This work**	**1151**	**−**
Prymparv19	***P. parvum***	**CAATCTGTCAATCCTCACAA**	**This work**	**1221**	**−**
Prymparv21	***P. parvum***	**GAAGTGCTCGCCAACGAG**	**This work**	**1361**	**+**
DinoB	Division Dinophyta	CCTCAAACTTCCTTGCITTA	12	1392	−
Euk1209	Domain Eukaryota	GGGCATCACAGACCTG	15	1430	−
Prymparv23	***P. parvum***	**GGTTTCCCGGACCTTTCG**	**This work**	**1501**	**+**
Boli01	Class Bolidophyceae	CAGTCTGATTGAACTGCGT	11	1598	−
Prymparv25	***P. parvum***	**CAATCGGTAGGAGCGACG**	**This work**	**1641**	**+**
Positive control	*S. cerevisiae*	ATGGCCGATGAGGAACGT	This work		
Negative control		TCCCCCGGGTATGGCCGC	This work		

[a] Probes highlighted in boldface represent the second probe set used to map the hybridization efficiency along the length of the 18S rRNA gene in the areas not covered by the first set of probes originally designed for FISH hybridizations. Intervening probes, i.e., Prymparv2, were designed but not tested.
[b] nt, nucleotide.
[c] Signal-to-noise ratios are indicated as follows: +++, above 10; ++, 4 to 10; +, 2 to 4; −, below threshold.

in the phytoplankton. Mixed targets were not tested here, but the principle has previously been proven to work (23). PCR fragments were amplified either from genomic DNA isolated from laboratory algal strains or from environmental clone libraries. This approach was chosen to keep the system as simple as possible for data interpretation. Initially, the complete 18S rRNA gene of approximately 1,800 bp was hybridized to the DNA chip because the binding sites of the probes were distributed randomly over the entire sequence (Table 2). A 5′ end biotinylated labeled primer was used to label the target DNA. This method was chosen because it is relatively cheap in comparison to the incorporation of biotinylated nucleotides. Subsequent to determining the signal-to-noise ratio (20), the signal of the positive control was used to normalize hybridizations. The signal-to-noise ratio gives information about the ratio between the perfect match hybridization signal and the background signal. A high signal-to-noise ratio indicates a strong specific hybridization signal. A signal was determined specific if the signal-to-noise ratio exceeded a threshold value of 2.0 (20).

Thus, 8 out of 12 probes from the first probe set tested resulted in specific signals with a signal-to-noise ratio above this threshold (Table 3) and no nonspecific binding. Probe DinoE-12 had a very strong hybridization signal for the perfectly matching target sequence, with a signal-to-noise ratio of 25.63. However, for one nontarget species, a diatom, the signal-to-noise ratio had a value of 2.19, which was slightly above our cutoff threshold of 2.0. This was interpreted as a weak cross-reaction to a nontarget organism, a result which could lead to an overestimation of diversity if environmental samples with an unknown genetic background are analyzed. This problem can be overcome by using multiple probes for one target or taxonomic level on the phylochips, the so-called hierarchical approach to probe design (17). Thus, if one probe produces a weak signal just above the threshold and another probe at the same taxonomic level or at a higher or lower taxonomic level gives no signal, then it can be assumed that the weak signal just above the threshold is nonspecific. Such an approach was partially demonstrated on our phylochips. For the Prasinophyceae, the phylochips contained probes at two different taxonomic levels (division and class). Additionally, other targets, such as the Chlorophyta and Dinophyceae, had two probes at the same taxonomic level (Table 3). The presence of the duplicates in a hierarchical system helps clarify whether weak signals are positive or negative.

As observed elsewhere, differences in signal intensities were found for probes with the same target or taxonomic hierarchical level (20, 25, 31). Strong hybridization signals were observed for Boli02, Chlo02, and DinoE-12, and no significant signals were found for duplicate probes at the same taxonomic level (Boli01, Chlo01, and DinoB, respectively). Notably, all probes worked very well in FISH (12, 29). The differences in the results of the FISH analysis could be explained by the nature of the target in the different hybridization formats. If the probes are used for FISH, the probes are in solution and bind to ribosomes in intact cells. In contrast, if they are used in the microarray format, the probes are immobilized on the

TABLE 3. Probe specificity[a]

Probe	Target species	Highest signal/noise value for:	
		Target	Nontarget
Euk1209	All eukaryotes	1.58	
Chlo01	HE001005-53 (Chlorophyceae, *Micromonas*)	0.63	1.21
Chlo02	He001005-53 (Chlorophyceae, *Micromonas*)	4.51	1.37
Boli01	*B. mediterranea* CCMP 1867 L. Guillou and M.-J. Chrétiennot-Dinet	0.18	1.18
Boli02	*B. mediterranea*	16.40	1.65
Prym01	*P. parvum* f. *patelliferum* (J. C. Green, D. J. Hibberd, and R. N. Pienaar) A. Larsen	3.58	1.2
Prym02	*P. parvum* f. *patelliferum* (J. C. Green, D. J. Hibberd, and R. N. Pienaar) A. Larsen	4.66	1.62
DinoB	HE001005-127 (Dinophyceae)	0.68	1.33
DinoE-12	HE001005-127 (Dinophyceae)	25.63	2.19
NS04	HE001005-47 (New Stramenopiles)	9.11	1.12
Pras04	HE001005-53 (Chlorophyceae, *Micromonas*)	4.91	1.24
CryptoB	*R. reticulata* PLY 358	11.06	1.53

[a] The specificity of the molecular probes was tested by a hybridization of all probes listed in this table against all targets listed in this table. The results of these hybridizations are displayed as signal-to-noise ratios. The results of the perfect match signal and the highest signal-to-noise ratio to a nontarget sequence are given. All other nontarget sequences had a lower signal-to-noise ratio than the one listed here.

surface of a glass slide. The probes in solution are probably more accessible for the target nucleic acid than the immobilized probes. Additionally, the in situ format contains proteins that bind to the rRNAs and thereby influence their secondary structure by blocking some of the binding sites of the molecule (3, 8, 9). It is also conceivable that binding sites that are accessible in situ are inaccessible if pure nucleic acids without accompanying proteins are used for hybridizations on phylochips and vice versa. In phylochips, the natural secondary structure of the rRNA is allowed to form without any protein interaction to close or open any site.

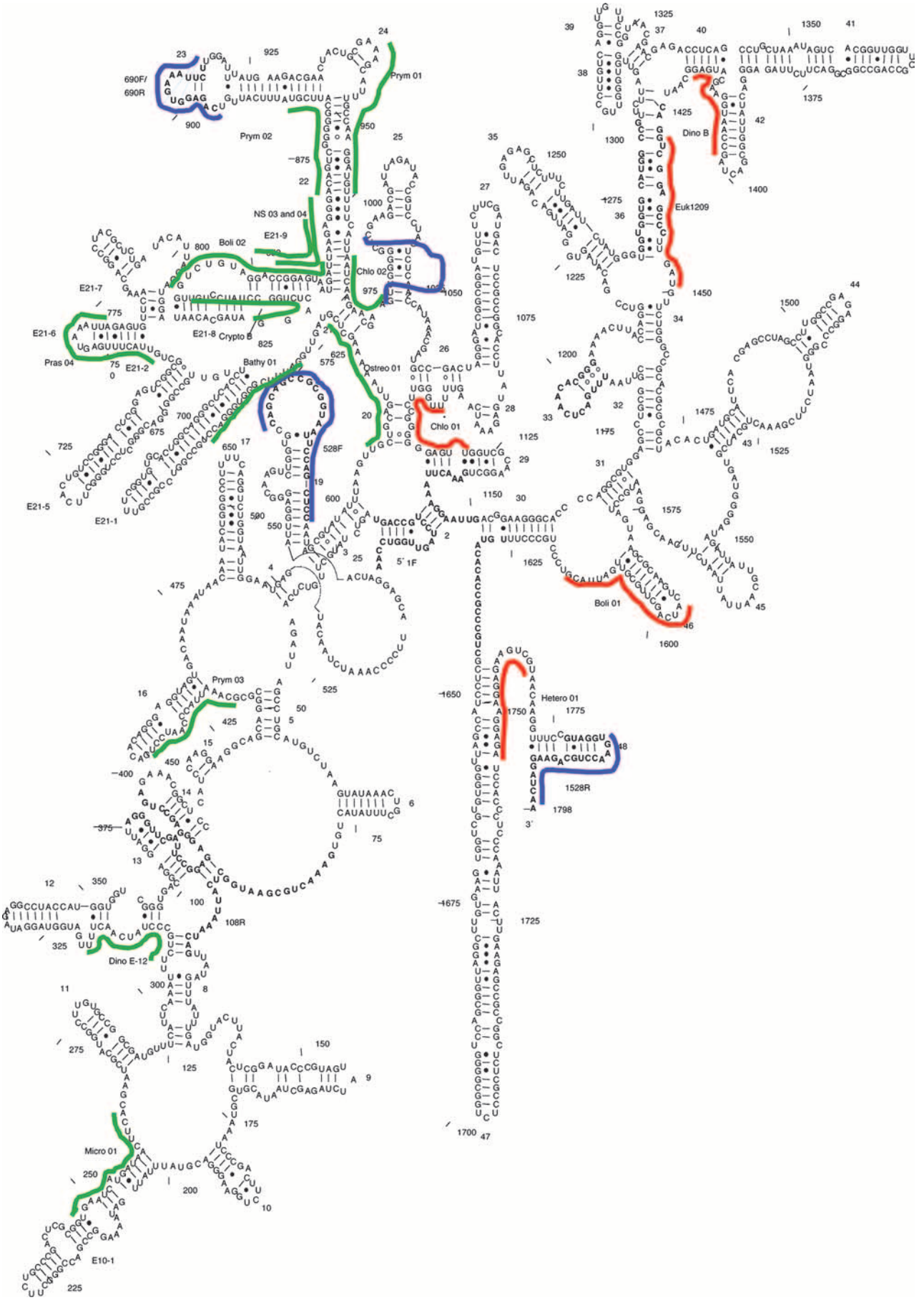
Correlation of binding sites with signal intensities. The signal intensities of the hybridization experiments have been matched with the binding sites of the probes (Table 2). There is a correlation between strength of signal and the probe binding site. Probes with poor signal intensities were located at a distance of >1,000 bp from the 5′ end of the 18S rRNA gene (Table 2; see also Fig. 3). The first probe set contained probes with binding sites randomly distributed over the entire sequence but with most probes located in the first part of the molecule. This left some regions of the molecule untested for probe accessibility.

In order to map the accessibility of probe hybridization in the entire 18S rRNA gene, a second set of probes was designed to bind at regular intervals to the 18S rRNA gene of *P. parvum* (Table 2) between the regions covered by the first set. This systematic analysis revealed that all probes that bind in the first 1,000 bp of the 18S rRNA gene produced positive hybridization signals. In contrast, 66% of the probes with a binding site further downstream in the 18S rRNA gene showed a false-negative hybridization signal even though the probes were identical to their target.

One reason for the differences in this hybridization efficiency could be the molecule's secondary structure. A consensus secondary structure was assembled from the 18S genes of different algal classes (Fig. 1). The sequence between bp 1000 and bp 1300 is located in the center of the 18S rRNA molecule (Fig. 1). None of the probes that were located in this area resulted in any hybridization signal. In contrast, some probes (Prymparv21, Prymparv23, and Prymparv25) with binding sites further downstream, and thus located at more exposed positions of the 18S rRNA secondary structure (Fig. 1), resulted in hybridization signals. This indicates an influence of the secondary structure on the hybridization efficiency of phylochip molecular probes.

Influence of target length on the hybridization efficiency. Size or length of the target might also account for different probe hybridization intensities at the different binding sites (13, 20). However, the observation that some probes downstream of bp 1000 resulted in a hybridization signal indicated that target size is only a minor factor for the probe hybridization efficiency for the 18S rRNA molecule. Hybridizations were done with two smaller PCR fragments (900 bp). Together, these two fragments add up to the complete 18S rRNA gene (Table 1). It was possible to observe a signal for Chlo01 and Euk1209 if either fragment was hybridized (Fig. 2). However, signals of Boli01 and DinoB probes, which are further downstream in the molecule, did not improve with the shorter fragments and never gave a signal even though they gave clear bright signals in FISH formats (data not shown). It is likely that decreasing the amplicon size even further could result in a signal on the microarray. However, resorting to fragmentation or short amplicons for the 18S rRNA molecule is not necessary because we have demonstrated that a signal can be achieved with a full-length molecule if the probe design is limited to the first 1,000 bp. Decreasing the hybridization temperature to 52°C or increasing to 60°C influenced the signal intensity of the specific signals but not the nonspecific signals (data not shown). Thus, target size was in this case only a minor factor for hybridization efficiency in the 18S rRNA molecule. The secondary structure of the molecule is suggested to cause the false-negative hybridization signals when full-length molecules are used.

Influence of the secondary structure on probe binding. Smaller fragments led to a hybridization signal for Chlo01. Therefore, the binding site of Chlo01 was examined more closely. Two truncated versions of the 18S rRNA gene were hybridized. One version was truncated for the first 566 bp, and the second version was truncated for the first 899 bp. The binding site of Chlo01 is located at ca. base 1100 in the theo-


690F/
690R
Prym 01
Prym 02
NS 03 and 04
E21-9
Boli 02
Chlo 02
E21-7
E21-6
E21-8
Crypto 8
Pras 04
E21-2
Bathy 01
Ostreo 01
Chlo 01
528F
E21-5
E21-1
Prym 03
Dino E-12
108R
Micro 01
E10-1
Dino B
Euk1209
Boli 01
Hetero 01
1528R

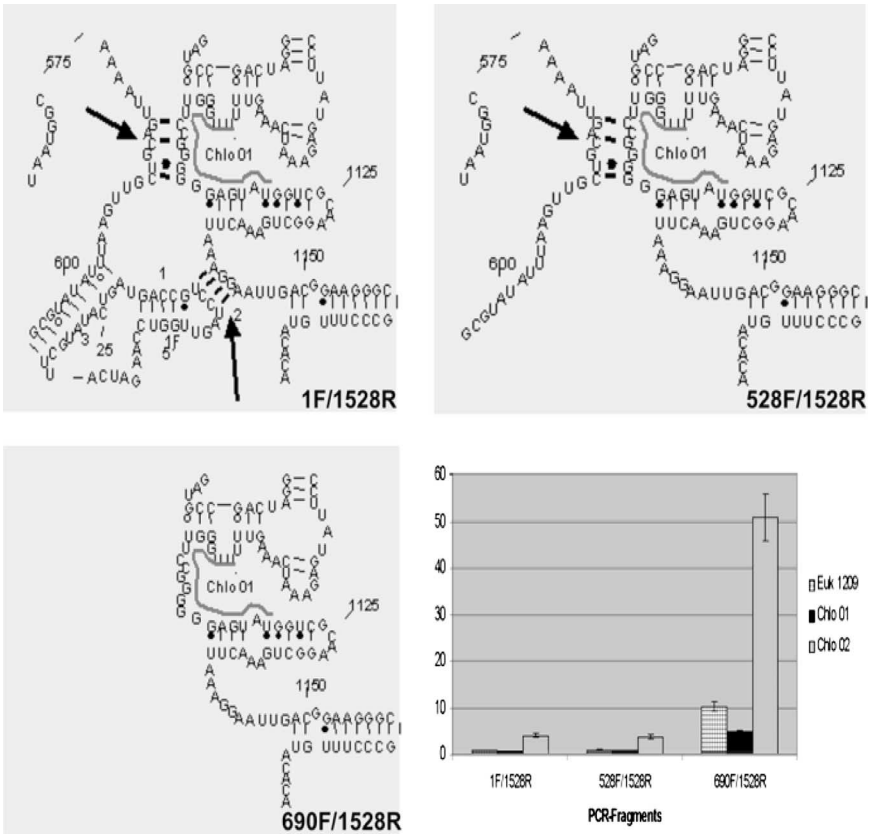


FIG. 2. The secondary structure formed from three different fragments showing the accessibility of the Chlo01 probe site and the hybridization of three different fragments of the 18S DNA amplified from clone HE001005-53, a chlorophyte alga, isolated in PICODIV (23).

retically determined secondary structure sensu Behrens et al. (3) (Fig. 1 and 2b). Using the program Mfold (34), the secondary structures of the truncated fragments were determined to be nearly identical (data not shown). If the first fragment was hybridized to the phylochips, no signal could be observed for Chlo01. In contrast, if the gene was truncated for the first 899 bp, a signal was observed. This difference could be explained by the secondary structure at the binding site of the probe. If the complete 18S rRNA gene or a PCR fragment truncated for the first 566 bases is used for hybridization, then bases 612 to 617 are paired in the secondary structure to bases 1106 to 1111. Consequently, the binding site of Chlo01 is not fully accessible (Fig. 2b) because it is involved in helix formation. However, if the possibility to form a higher-order structure at the binding site of Chlo01 is removed by truncating bases 1 to 899, then the binding site for Chlo01 is accessible, resulting in a hybridization signal on the phylochips. This result strongly indicates that the secondary structure of the 18S rRNA gene is the major impact factor on the hybridization efficiency of phylochip probe binding. Similarly to Chlo01, no signal was observed for Euk1209 if the 18S rRNA gene used in the hybridization was complete or if bases 1 to 566 were truncated. However, it was possible to detect a signal if bases 1 to 899 were truncated from the target molecule, even though these bases are not directly involved in forming the hairpin structure at the binding site (Fig. 1); this indicates that secondary structure at the binding site of Euk1209 is more accessible with the truncation of the first 899 bases. In contrast to the results for Chlo01 and Euk1209, it was impossible to observe a signal for DinoB or Boli01, regardless of the truncated fragment used as a target (data not shown). The accessibility of the binding sites of these probes does not seem to be influenced by a truncation. Thus, fragmentation of the 18S rRNA gene results in improvement of a signal if that fragmentation results in small enough pieces to open the probe binding site. But because fragmentation cannot be controlled reliably, it reduces the possibility of using this method to attain signals in regions of the molecule where secondary structure prohibits probe binding. However, fragmentation of the 18S rRNA molecule is not necessary. It is more desirable to restrict the regions of probe design to the first 1,000 bp, thus avoiding the need for fragmentation or short PCR amplicons, which would

FIG. 1. Consensus secondary structure of the 18S rRNA gene according to previous studies (3, 8, 9). The binding sites of the probes are displayed as red lines for the probes that resulted in no signals and in green lines for the probes that resulted in signal-to-noise ratios above 2.0. Blue lines indicate the primers that were used to amplify the target DNA.

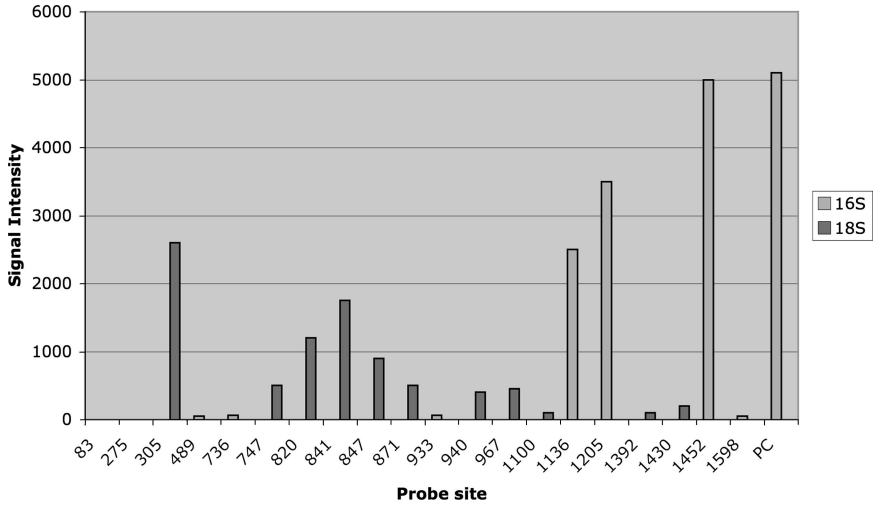


FIG. 3. Comparison of the signal intensity of the full-length amplicon hybridized with published probes from 18S and 16S rDNA. 16S rDNA data are taken from the study of Lane et al. (13). 18S rDNA data are from Table 3 and represent normalized signals.

eliminate the possibility of using the microarray quantitatively. The most attractive aspect of our study has been that we can show that the 18S rRNA molecule has the potential to be used quantitatively on the microarray because its secondary structure in the first half of the molecule does not seem to be as strong as that in the 16S rRNA molecule.

The results from this study indicate that there are certain areas in the 18S rRNA gene that should not be considered for the design of DNA phylochip probes. These areas form stable secondary structures in the absence of proteins. They include the sequence of the 18S rRNA between bp 1000 and bp 1200 located at the center of the molecule (Fig. 1) and less exposed areas downstream. The in situ accessibility of the 18S rRNA gene of the eukaryote *S. cerevisiae* has previously been depicted (3), and it has been shown that the area downstream of base 1000 in the molecule holds more regions that are not accessible or are only poorly accessible to in situ oligonucleotide probes. Thus, it is potentially more difficult for probes to access the binding site in this area than in other areas, likely because proteins block the sites. We compared our data with data shown in this previous study. Even though two different hybridization formats were used, we found that the in situ mapping of the 18S rRNA is basically similar to the mapping that has been done in the prior study. Some regions of the ribosomal sequence that displayed poor signal intensities with FISH also had poor microarray signal intensities. Sites suggested to be closed for FISH hybridization are actually open in some species, e.g., DinoB. Thus, our experiments show that because of the nature of different hybridization formats, the map of the 18S rRNA generated by FISH cannot be copied base by base to phylochips. Whether or not restriction of probe development to the first half of the molecule inhibits a hierarchical approach to probe development has not been fully explored. Helix 23 does contain four class-level probes, but whether other helices can be shown to produce probes at specific taxonomic hierarchies remains to be investigated. The first FISH probes transferred to a phylochip were probes successful in an in situ format, and there was no a priori reason to believe that they would not work in a phylochip format. Lane et al. and Liu et al. (13, 18) decreased amplicon length, which improved probe accessibility. In their study the probes at the beginning of the 16S rRNA produced no signal with the longest amplicon, whereas downstream regions did produce a signal. In the present study, amplicon length had a negligible effect; the actual probe site was more important. Thus, the secondary structure of the 18S rRNA gene is stronger in the second half of the molecule, whereas the secondary structure of the 16S rRNA gene is stronger in the first half of the molecule (Fig. 3). Access to the 16S rRNA gene was achieved by nick translation of the entire molecule or reducing the size of the amplicon target DNA (13, 18). In the 18S rRNA gene, avoiding the second half of the molecule for probe design seems to circumvent site inaccessibility. Only the work by Behrens et al. (3), in which the binding efficiency in situ was systematically investigated, is available to guide probe design, and it has been shown that all sites must be tested empirically for in situ work (10). For phylochips we can predict thus far that the second part of the 18S rRNA gene should not be used for probe development, regardless of the species. Most importantly, the 18S rRNA molecule has the potential to be used quantitatively if total RNA is extracted from environmental samples and applied to a phylochip. This feature makes this molecule more attractive as a means of monitoring biodiversity (sensu Caron et al. [5]) on a nanoscale and in an automated fashion.

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