Methodological Aspects of Fluorescence In Situ Hybridization for the Identification of Microorganisms

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Summary

Fluorescence in situ hybridization (FISH) with rRNA-targeted oligonucleotide probes facilitates the rapid and specific identification of individual microbial cells in their natural environments. Detailed knowledge of methodological aspects of the FISH protocol is of great importance for understanding the potential and limitations of the technique.

In this thesis the regional differences in hybridization efficiency of Cy3-labeled oligonucleotides to small subunit ribosomal RNA have been systematically studied. The in situ accessibility of small-subunit rRNA was investigated for the bacteria *Escherichia coli* and *Pirellula* sp. strain 1, the archaeon *Metallosphaera sedula*, and the yeast *Saccharomyces cerevisiae*. Additionally, the efficiency of probe binding to the D1/D2 domains, located at the 5' end of the 26S rRNA of *Saccharomyces cerevisiae*, has been evaluated. The in situ accessibility data are more similar for phylogenetically more closely related organisms. Differences in probe-conferred fluorescence correlated most strongly with intra-helix secondary rRNA-rRNA interactions. Future updates of the probe design software package ARB will include information on the in situ accessibility of ribosomal RNA to oligonucleotide probes.

Data from the probe binding study of *Escherichia coli* 16S rRNA has been systematically evaluated with respect to the currently available models of the three-dimensional structure of the small ribosomal subunit. Although the small-subunit rRNA is a highly conserved molecule, the data presented in this study show that the 3D-structure of the native small ribosomal subunit is not relevant to probe hybridization, likely because FISH is performed in a strongly denaturing environment. Probe hybridization without sodium dodecylsulfate in the hybridization buffer was very low.

In the third part of this thesis the relevance of nucleotide specific quenching for FISH was addressed for the first time. The 5' end sequence of carboxyfluorescein-labeled oligonucleotide probes was investigated for their influence on probe fluorescence intensity before and after duplex formation. Probes quenched upon hybridization to their complementary sequence in solution were not quenched upon FISH. A statistical analysis of 113 carboxyfluorescein-labeled oligonucleotide probes hybridized to whole fixed *Escherichia coli* cells suggested that the nucleotide sequence in close proximity to the probe's 5' end does not affect probe-conferred fluorescence upon FISH.

Zusammenfassung

Die Fluoreszenz-*In Situ*-Hybridisierung (FISH) ermöglicht die schnelle und spezifische Identifizierung von einzelnen Mikroorganismen in ihrem natürlichen Habitat. Das genaue Studium methodischer Aspekte des FISH Protokolls ist von immenser Bedeutung für das Verständnis der Möglichkeiten und Grenzen dieser Technik.

Der erste Teil dieser Arbeit beschäftigt sich mit der Untersuchung von lokalen Unterschieden in der Hybridisierungseffizienz von Cy3-markierten Oligonukleotidsonden nach Bindung an die RNA der kleinen ribosomalen Untereinheit. Dabei stand die systematische Untersuchung der Zugänglichkeit der 16S ribosomalen RNA (rRNA) der Bakterien *Escherichia coli* und *Pirellula* sp. strain 1, sowie des Archaebakteriums *Metallosphaera sedula* und der 18S rRNA der Hefe *Saccharomyces cerevisiae*, als Vertreter der Eukaryoten, im Mittelpunkt. Desweitern wurde die Sondenbindungseffizienz an die D1/D2 Domäne am 5' Ende der 26S rRNA von *Saccharomyces cerevisiae* betrachtet. Die Unterschiede in der Sonden-vermittelten Fluoreszenz korrelierten am besten mit sekundären intrahelicalen rRNA-rRNA Wechselwirkungen. Die Ähnlichkeit der ermittelten Sondenzugänglichkeit für die verschiedenen rRNAs ist für phylogenetisch näher verwandte Organismen größer. Zukünftige Versionen des weltweit zur Sondenentwicklung genutzten Computerprogramms ARB werden die in dieser Arbeit ermittelten Informationen über die unterschiedlichen Sondenbindungseigenschaften an die rRNA enthalten.

Im zweiten Teil dieser Arbeit wurden die Daten über die unterschiedlichen Sondenbindungseffizienzen an die rRNA von *Escherichia coli* mit dem gegenwärtig aktuellen 3D-Strukturmodell der kleinen ribsomalen Untereinheit verglichen. Obwohl die räumliche Struktur des Ribosoms hoch konserviert ist, konnte gezeigt werden, dass die native 3D-Struktur der kleinen ribsomalen Untereinheit nicht geeignet ist, die lokalen Hybridisierungsunterschiede von Oligonukleotidsonden mit rRNA zu erklären. FISH wird unter stark denaturierenden Bedingungen durchgeführt. Ohne Natriumdodecylsulfat in der Hybridisierungslösung war die Sondenhybridisierung stark eingeschränkt.

Im dritten Teil dieser Arbeit wurde die Bedeutung der Nukleobasen-spezifischen Fluoreszenz-Auslöschung (Quenching) für FISH untersucht. Dazu wurde der Einfluss der Sequenz am 5' Ende von Carboxyfluorescein-markierten Oligonukleotidsonden auf die Sonden-vermittelte Fluoreszenz vor and nach

Doppelstrangbildung untersucht. Selbst für Sonden, die nach Hybridisierung in Lösung eine Reduktion der Fluoreszenz zeigten, konnte kein Quenching der Fluoreszenz bei der FISH nachgewiesen werden. Eine erste Durchsicht von 113 Carboxyfluorescein-markierten Sonden nach Hybridisierung mit fixierten *Escherichia coli* Zellen ergab keinen signifikanten Einfluss der Nukleotidsequenz in der Nähe des Fluorenszenzfarbstoffs auf die Sonden-vermittelte Fluoreszenz. Weitere statistische Untersuchungen sind im Gange.

Part I:

Combined Presentation of Results

A Introduction

1 Ribosomal RNA-Targeted Oligonucleotide Probes for Investigation of Microorganisms

One major objective in applied and environmental microbiology is the accurate identification and quantitative description of microorganisms in their natural environment. The limited morphological detail of most microorganisms prevents the visual identification possible with higher plants and animals. Traditional cultivation-based methods are slow and selective, and normally result in an incomplete picture of the real community composition (Wagner *et al.*, 1993). In recent years, there have been attempts to use PCR-based assays for the rapid and sensitive detection of microorganisms that have so far proven impossible to culture (Liu *et al.*, 1997; Muyzer *et al.*, 1993). Although quantitative PCR allows quite precise measurements of gene copy numbers, PCR-based techniques provide only semi-quantitative estimates of cell numbers and the spatial distribution of microorganisms (Everett *et al.*, 1999; Ludwig & Schleifer, 2000; Pahl *et al.*, 1999; Pusterla *et al.*, 1999). In the past 25 years the comparative analysis of homologous ribosomal RNA (rRNA) sequences and the genes encoding them has fundamentally revolutionized the field of microbial taxonomy. rRNA sequences can be retrieved directly from the environment without prior cultivation of the organism of interest (Amann *et al.*, 1995; Giovannoni *et al.*, 1990; Hugenholtz *et al.*, 1998; Olsen *et al.*, 1986). Fluorescence in situ hybridization (FISH) with rRNA-targeted oligonucleotide probes, combines the precision of nucleic acid hybridization with the visual information of microscopy, permitting visualization and quantification of the microorganism behind an rRNA sequence (Figure 1). Since the initial application of fluorescently labeled rRNA-targeted oligonucleotide probes as 'phylogenetic stains' for the in situ detection of whole fixed cells (DeLong *et al.*, 1989), FISH has become a common and reliable method for the direct, cultivation-independent identification of individual microbial cells in natural samples (Amann *et al.*, 1995; Moter & Gobel, 2000).

rRNA-targeted oligonucleotide probes are ideally suited to investigation of the composition of complex microbial communities. There are many good reasons for targeting probes to 16/18S rRNA of the small subunit of the ribosome (SSU rRNA) or to the 23S/28S rRNA of the large subunit of the ribosome (LSU rRNA) (Woese, 1987): rRNA molecules are found in all living organisms. They occur in high copy

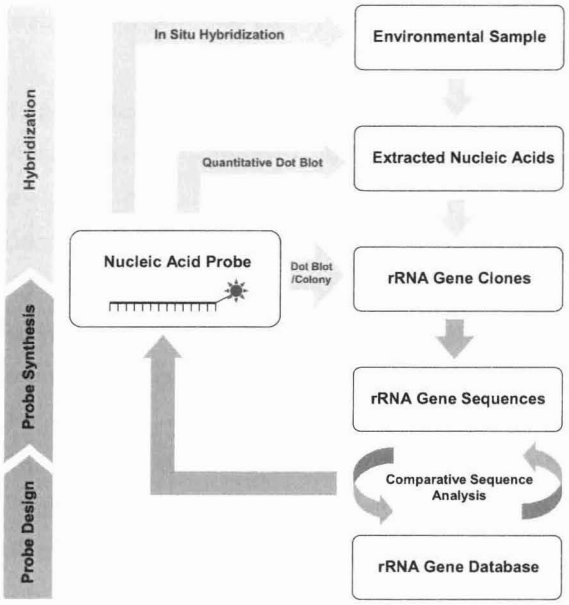

FIG. 1. Flow chart showing the application of rRNA-targeted nucleic acid probes for the cultivation-independent identification of microorganisms in an environmental sample. Modified after Amann *et al.*, 1995.

numbers, are relatively stable, and have a length of about 1500 (16S) and 3000 (23S) nucleotides comprising variable and highly conserved sequence domains (Amann *et al.*, 1990; Amann *et al.*, 1995). However, the major advantage is the availability of comprehensive rRNA sequence databases (Cole *et al.*, 2003; Ludwig *et al.*, 2003; Wuyts *et al.*, 2002). Currently the ARB database contains 30,000 aligned SSU rRNA sequences and 1,500 LSU rRNA sequences. Comparative sequence analysis allows the identification of short sequence stretches (15 to 20 nucleotides) unique to a group of microorganisms ranging from whole phyla to individual species. Computer-based algorithms (Ashelford *et al.*, 2002; Ludwig *et al.*, 2003; Pozhitkov & Tautz, 2002) have been used to design several hundred rRNA-targeted oligonucleotides applicable for FISH. Recently, an online database has been established that contains more than 700 published probes and their characteristics (Loy *et al.*, 2003).

2 Limitations of rRNA-Targeted Probes and Methodological Improvements to Overcome Them

While the importance of FISH for microbiology and biotechnology is undisputed, the standard protocol has certain limitations. The procedure must be optimized for each target group to achieve good probe penetration of cells while maintaining morphological integrity. For example, Gram-positive bacteria need different fixation protocols than Gram-negative bacteria (Beimfohr *et al.*, 1997; Erhart *et al.*, 1997; Roller *et al.*, 1994; Sekar *et al.*, 2003) to sufficiently permeabilize their more rigid cell wall of multiple peptidoglycan layers, lipoteichoic acids, and teichuronic acids.

Other limitations of the FISH technique include the limitations on oligonucleotide probe access imposed by rRNA secondary structure, the limited detectability of mono-labeled oligonucleotide probes and the lack of automation. In the following sections the current limitations of the standard FISH protocol are described and methodological improvements to overcome many of them are discussed.

2.1 The Target Molecule

rRNA has a relatively slow mutation rate. Therefore it is generally impossible to find target sites on the 16S rRNA that allow strains of a prokaryotic species to be distinguished, and sometimes even different species will have identical 16S rRNA (Fox *et al.*, 1992). The 23S rRNA molecule, which is approximately twice as long, may be a useful target in such cases. Another problem can be the heterogeneity among rRNA operons of a single organism. For the archaeon *Haloarcula marismortui*, 16S rRNA sequence dissimilarities of 5% have been found for the two *rrn* operons (Mylvaganam & Dennis, 1992). Interoperon differences have also been described in bacterial species (Nübel *et al.*, 1996; Wang *et al.*, 1997).

There are also problems with probes designed to target the rRNA sequences of yet uncultured organisms. Optimization of hybridization and washing conditions for these probes cannot precisely be done. Recently, Schramm and coworkers (2002) (Schramm *et al.*, 2002) reported a FISH protocol for detection of heterologous rRNA transcribed in *Escherichia coli* (Clone-FISH). 16S rRNA genes cloned from the environment are transcribed *in vivo* using plasmids with a T7 RNA polymerase promotor and *Escherichia coli* host cells with an isopropyl-beta-D-thiogalacto-pyranoside (IPTG)-inducible T7 RNA polymerase. Heterologous rRNA transcripts can

be detected by FISH within the *E. coli* cells. Clone-FISH is generally applicable for the validation of probes that target organisms for which no pure cultures are available and for the rapid screening of environmental rRNA gene libraries for those clones carrying the genes of interest.

Further limitations come from the fact that rRNA diversity has only partially been described (Amann *et al.*, 1995; Olsen *et al.*, 1986). If a probe is designed to be specific for a defined group of organisms based on the currently available data set, it may also hybridize with as yet unknown organisms. Therefore, it is recommended to target one population with more than one probe (Amann & Ludwig, 1994; Amann, 1995). The multiple probe approach (Ludwig *et al.*, 1998) involves the use of nested probes specific for the genus, species, and sequences of interest, or of two or three probes that target the same population. The simultaneous application of two or three differently labeled probes allows the identification of 'cross-hybridizing' populations in a sample (Amann *et al.*, 1996). Finally, the ability to quantify populations in complex microbial communities is dependent on a high-quality rRNA sequence database, which must be continually enlarged and updated.

2.2 Cellular Ribosome Content

The ribosome content of bacterial cells varies among species or, within one species, for cells in different physiological states. The influence of cellular growth rate and nutritional status on cell detection by FISH has extensively been studied (Amann *et al.*, 1995; Oda *et al.*, 2000). Since the FISH signal directly depends on the number of target molecules present within a cell, cells with low ribosome content are difficult to detect with mono-labeled rRNA-targeted oligonucleotide probes. To improve FISH detection of cells with low ribosome content, attempts have been made to boost cellular ribosome content before cell fixation, by preincubation in a mixture of substrate and antibiotics to activate rRNA synthesis without cell division. Increased fluorescence signals have been reported for bacterial biofilms in oligotrophic drinking and cooling water systems after pretreatment with different substrates and chloramphenicol or pipemidic acid (Kalmbach *et al.*, 1997; McDonald & Brözel, 2000). The problem with this approach is that community shifts may be induced by the selectivity of substrates and antibiotics.

2.3 Polyribonucleotide Probes

FISH studies with mono-labeled oligonucleotide probes in oligotrophic environments are often hampered by the low rRNA content of the target cells. Therefore, slow-growing or starving cells frequently remain undetected. Polyribonucleotide probes are 16S and/or 23S rRNA gene transcripts, usually several hundred nucleotides long, labeled with several fluorophores. Polyribonucleotide probes are produced by incorporation of labeled nucleotides during *in vitro* transcription (DeLong *et al.*, 1999; Karner *et al.*, 2001; Trebesius *et al.*, 1994) or PCR (Zimmermann *et al.*, 2001). They have proven to detect a significantly higher percentage of prokaryotes than FISH with mono-labeled oligonucleotide probes (Pernthaler *et al.*, 2002b). Domain-specific polyribonucleotide probes have successfully been applied to differentiate between bacteria and archaea (DeLong *et al.*, 1999; Karner *et al.*, 2001; Pernthaler *et al.*, 2002b). However, polyribonucleotide probes also target many conserved sites, and therefore do not allow discrimination between closely related groups of microorganisms. Shorter polyribonucleotide probes that target a variable region of 250 nucleotides of the 23S rRNA enable differentiation among genera (Trebesius *et al.*, 1994). Another disadvantage of polyribonucleotide probes is that they cannot be commercially purchased. Their production in the laboratory is expensive and time-consuming, may result in uneven quality, and the probes are susceptible to enzymatic or chemical degradation.

2.4 Peptide Nucleic Acids

Peptide nucleic acid (PNA) molecules are DNA analogs with an uncharged polyamide backbone. They bind to nucleic acids much more strongly than oligonucleotide probes because there is no electrostatic repulsion between the PNA probe and the negatively charged sugar-phosphate backbone of the target molecule (Egholm *et al.*, 1993; Perry-O'Keefe *et al.*, 2001; Ray & Nordén, 2000). Hybridizations can be performed at low salt concentrations and high temperatures, which decreases the stability of the rRNA secondary structure and makes probe target sites more accessible. The application of PNA probes for FISH has improved the staining of Gram-positive bacteria (Oliveira *et al.*, 2002; Stender *et al.*, 1999), marine cyanobacteria (Worden *et al.*, 2000), and tap water samples (Prescott & Fricker, 1999). Currently, relatively high prices and the fact that oligonucleotide probes can not simply be converted into PNA probes limit the application of peptide

nucleic acids to FISH. PNA probes need to be shorter than DNA probes (≤15 nucleotides, compared to 17-30 nucleotides) because the high dissociation temperatures required for longer probes can destroy fixed microbial cells.

2.5 Enzymatic Signal Amplification

Schönhuber and coworkers (1997) significantly enhanced the signal intensities of hybridized cells by the application of horseradish peroxidase (HRP)-labeled oligonucleotide probes and tyramide signal amplification (TSA) (Schönhuber *et al.*, 1997). This technique is also known as catalyzed reporter deposition (CARD)-FISH. The critical step in this approach is the diffusion of the large HRP-labeled oligonucleotide into whole fixed cells. The protocol requires a very carefully controlled permeabilization step prior to the enzymatic signal amplification, balancing permeability with cellular integrity (Schönhuber *et al.*, 1999). Therefore, the universal applicability of this technique was limited, because cell wall composition varies greatly among prokaryotes (Schönhuber *et al.*, 1997). Recently, Pernthaler and colleagues modified the permeabilization procedure of the protocol for FISH with HRP-labeled oligonucleotide probes for the quantification of planktonic and benthic marine bacteria (Pernthaler *et al.*, 2002a). In this protocol the cells are embedded in low-gelling point agarose to prevent cell loss during permeabilization by controlled lysozyme digestion. Application of the CARD-FISH protocol for the analysis of North Sea bacterioplankton samples showed that 94% of the total cell counts were detectable with a universal bacterial probe. The fluorescently labeled derivative of the same probe revealed detection rates of only 48%. For the permeabilization of Gram-positive cell walls an additional digestion step with the enzyme achromopeptidase has been recommended (Sekar *et al.*, 2003).

2.6 Self-Ligating Probes

The application of standard oligonucleotide probes requires stringent washing conditions after hybridization to decrease background fluorescence caused by unspecific binding of labeled probes to sample material. It has been reported that synthetic quenched DNA probes (QUAL probes) have the potential to reduce this problem (Sando & Kool, 2002a). QUAL probes are oligonucleotide pairs that target adjacent regions on the rRNA. One probe carries a fluorophore that is quenched by a 5'-dabsylthymidine group. The other probe has a phosphorothiolate group at its 3'

end. Hybridization of both probes brings the two reactive groups into close proximity. A nonenzymatic self-ligation reaction covalently connects both probes leading to the release of the quenching group (Sando & Kool, 2002b) (Figure 2). The fluorescence unquenching results in a strong signal change when the two oligonucleotide probes are joined. The technique allows detection of bacterial rRNA in whole cells without washing steps because fluorescence is only achieved upon specific hybridization of both probes. However, probe design may be more complicated, since two probes that target adjacent regions are needed, and the synthesis of the self-ligating probes is costly.

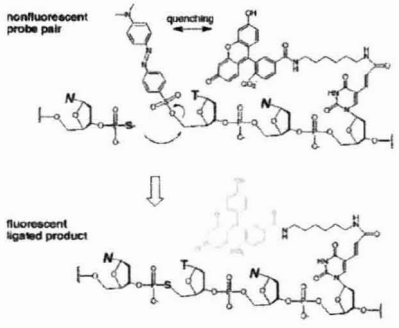

FIG. 2. The use of quenched autoligation (QUAL) probe pairs in bacterial RNA sensing. Loss of dabsyl results in "lighting up" of the fluorescent signal, reporting on the bond-formation reaction in real time. Figure taken from Sando and Kool (2002), J. Am. Chem. Soc. **124**, 9686-9687.

2.7 Nucleotide-Specific Quenching of Fluorescent Dyes

In multiple biophysical and biochemical applications of fluorescently labeled oligonucleotides it has been observed that the fluorescence of several conjugated dyes is sensitive to the sequence environment around the point of attachment (Cooper & Hagerman, 1990; Lee *et al.*, 1994; Sauer *et al.*, 1998; Walter & Burke, 1997). The fluorescence quenching of fluorescent dyes conjugated to oligonucleotides can be attributed to interactions between the dye and nucleobases (Cooper & Hagerman, 1990; Draganescu *et al.*, 2000; Edman *et al.*, 1996; Eggeling *et al.*, 1998; Fukui *et al.*, 1999; Horn *et al.*, 1997; Jia *et al.*, 1997; Lee *et al.*, 1994; Walter & Burke, 1997). Most dyes are quenched by the nucleobase guanine (Atherton & Harriman, 1993; Crockett & Wittwer, 2001; Marras *et al.*, 2002; Nazarenko *et al.*, 2002b; Torimura *et al.*, 2001; Widengren *et al.*, 1997; Zahavy & Fox, 1999). Guanine, as the most oxidizable nucleobase, has excellent electron

donating properties (Seidel *et al.*, 1996; Steenken & Jovanovic, 1997). Generally, fluorophores emitting light in the green and yellow wavelengths are more affected by nucleotide quenching than dyes that fluoresce in the blue and red spectral range. Fluorescence quenching based on interactions between nucleobases and a fluorochrome involve a photoinduced electron transfer mechanism between a nucleotide residue and the dye (Edman *et al.*, 1996; Eggeling *et al.*, 1998; Fukui *et al.*, 1999; Lewis *et al.*, 2001; Lewis *et al.*, 2000; Seidel *et al.*, 1996; Torimura *et al.*, 2001; Walter & Burke, 1997). Therefore, the fluorescence quenching mechanism is considered to be distance-dependent.

Fluorescence quenching can also occur upon hybridization of fluorescently labeled oligonucleotides to their complements. It has been reported that the fluorescence intensity of oligonucleotides labeled with carboxyfluorescein (FAM) is decreased after binding to their complementary sequence (Crockett & Wittwer, 2001; Kurata *et al.*, 2001; Lee *et al.*, 1994; Nazarenko *et al.*, 2002a; Nazarenko *et al.*, 2002b; Torimura *et al.*, 2001). The reduction in fluorescence was attributed to the presence of guanine bases in close proximity to the dye on the target strand. Torimura and co-workers (2001) demonstrated that the decrease in signal intensity using artificial probe-target duplexes could be up to 86% for FAM labeled oligonucleotides. Whether nucleotide-specific quenching is of relevance for FISH needs to be proven (Wagner et al., 2003). Experimental data on the effect of nucleobase-mediated quenching for FISH with rRNA-targeted oligonucleotide probes were not available before this thesis.

2.8 Accessibility of Probe Target Sites

The probe-conferred fluorescence depends, in addition to cell wall permeability and cellular ribosome content, on the in situ accessibility of the probe target site. Not all target sites on the 16S rRNA are equally accessible. The access of fluorescently labeled oligonucleotide probes to their targets may be hindered by the three-dimensional structure of the ribosome which includes rRNA-rRNA interactions as well as interactions of the rRNA with ribosomal proteins.

The variable accessibility was addressed in preliminary studies, by either oligonucleotide binding assays on filters (Hill *et al.*, 1990; Lasater *et al.*, 1988) or FISH (Frischer *et al.*, 1996). In the following years, two systematic studies on the accessibility of rRNA target sites in whole fixed cells for fluorescent oligonucleotides

have been performed. In 1998, Fuchs and co-workers quantified the fluorescence signals conferred by 171 carboxyfluorescein labeled oligonucleotides targeted to the 16S rRNA of *Escherichia coli* (Fuchs *et al.*, 1998). Since then the use of carbocyanine dyes like Cy3 and Cy5 (Southwick *et al.*, 1990) has greatly increased the sensitivity of FISH (Glöckner *et al.*, 1996). A second study on the in situ accessibility of the 23S rRNA of *E. coli* has therefore been performed with Cy3-labeled oligonucleotide probes (Fuchs *et al.*, 2001). However, rRNA target sites yielding bright fluorescent signals in *E. coli* might not work well for other organisms. The transferability of the *E. coli* accessibility data to other organisms remained an open question when this thesis started.

The problem of low signal intensity due to the inaccessibility of probe target sites can be overcome by the use of helper oligonucleotides. The application of unlabeled helper oligonucleotides that bind adjacent to the probe target site has been shown to significantly increase weak probe hybridization signals (Fuchs *et al.*, 2000). Niemeyer and colleagues suggested a mechanism for the 'helper-effect' in which the unlabeled oligonucleotide binds to the denatured RNA during hybridization and prevents the reestablishment of the native secondary structure, thereby keeping the probe target site open (Niemeyer *et al.*, 1998).

2.9 Automation

One of the most important practical limitations for the wide application of rRNA-targeted oligonucleotide probes in applied and environmental microbiology is the lack of automation. Quantitative data on the abundance of in situ stained microorganism are mostly obtained by time consuming manual microscopic counting. In contrast, digital image analysis allows the quantification of several hundred stained objects per sample within a few hours (Daims *et al.*, 2001b; Schmid *et al.*, 2000). Hybridized cells are pictured using a confocal laser scanning microscope or an epifluorescence microscope. The abundance of probe target cells is measured as percentage of the number of total cells in the sample. Image analysis can be fully automated if motorized computer-controlled microscopic stage drives are available (Kuehn *et al.*, 1998; Pernthaler *et al.*, 2003).

Another drawback of the FISH technique is the limited number of probes that can be applied in one hybridization experiment, especially when the protocol is used for community analysis on a high level of phylogenetic resolution. In addition, the

multiple probe concept (Ludwig *et al.*, 1998) recommends the use of more than one probe per population to check for false-positive and false-negative results (see chapter 2.1). DNA chips or DNA microarrays allow the simultaneous application of a nearly unlimited number of probes in a single hybridization experiment (Gupta *et al.*, 1999; Hoheisel, 1997). They are based on a reverse hybridization format that makes use of matrix-immobilized oligo- or polynucleotide probes for the specific capture of labeled target molecules. The DNA microarray technology has the potential to facilitate the parallel application of multiple probe sets for environmental studies (Gupta *et al.*, 1999; Peplies *et al.*, 2003; Rudi *et al.*, 2000; Small *et al.*, 2001), thereby complementing FISH.

3 Linking FISH to Function and Activity

A major challenge for contemporary microbial ecology is the development of methods that allow the activity and function of microbial cells in natural samples to be determined with single cell resolution. FISH with rRNA-targeted oligonucleotide probes has found numerous applications for the identification and localization of individual microbial cells in their natural environment (Amann *et al.*, 2001; Moter & Gobel, 2000). However, to gain information on the physiology of the detected cells, the microbial community composition analysis by rRNA probing has to be combined with in situ measurements of function.

FISH and microsensor measurements can be combined to study the distribution and activities of specific microbial populations with similar spatial resolution (Amann & Kühl, 1998). The first such study focused on the distribution and activity of sulfate-reducing bacteria in biofilms (Ramsing *et al.*, 1993). FISH in combination with microelectrode measurements has also extensively been used to study the structure and function of nitrifying biofilms (Gieseke *et al.*, 2002; Gieseke *et al.*, 2001; Schramm *et al.*, 2000; Schramm *et al.*, 1999; Schramm *et al.*, 1998; Schramm *et al.*, 1996).

Another trend is the combination of FISH and microautoradiography (Lee *et al.*, 1999; Ouverney & Fuhrman, 1999). The simultaneous application of these techniques allows general physiological activity to be determined, as does the alternative approach of combining FISH with CTC (5-cyano-2,3-tolyl-tetrazolium chloride) (Nielsen *et al.*, 2003a). Furthermore, microautoradiography in combination with FISH is increasingly being used to monitor substrate uptake patterns of probe-stained bacteria on a single-cell level (Daims *et al.*, 2001a; Ito *et al.*, 2002; Wagner & Loy, 2002). In addition, the combination of FISH and microautoradiography has the potential to identify defined physiological groups of microorganisms in a complex microbial community. Recently, Nielsen and co-workers quantified the abundance and phylogenetic affiliation of iron-reducing bacteria in activated sludge by FISH-microautoradiography (Nielsen *et al.*, 2003b). The use of specific inhibitors excluded consumption of radio-labeled acetate by other physiological groups such as sulfate reducers and methanogenic prokaryotes.

Improvements in the FISH technique have led to the in situ visualization of gene expression by probing of mRNA (Hahn *et al.*, 1993; Hönerlage *et al.*, 1995). Due to the low copy number of mRNA as compared to rRNA, digoxygenin (DIG)-

labeled polyribonucleotide probe have been used. Detection has been realized by the use of HRP-labeled anti-DIG antibodies and the fluorogenic TSA system (Wagner *et al.*, 1998).

Recently, Pernthaler and colleagues reported a method for the microscopic identification of actively DNA-synthesizing cells in bacterioplankton samples. After incubation with the halogenated thymidine analogue bromodeoxyuridine (BrdU), the identification of environmental bacteria by FISH with HRP-labeled oligonucleotide probes was possible. However, data has been published describing the insufficient up take of BrdU by wild-type bacteria and its toxicity for different bacterial groups (Coote & Binnie, 1986; Urbach *et al.*, 1999). Therefore, the general applicability of the method for different environmental samples needs to be demonstrated.

4 Outline of the Present Study

The in situ accessibility of the 16S and 23S rRNA of *E. coli* for fluorescently labeled oligonucleotide probes has extensively been studied. However, in contemporary FISH applications the carbocyanine dye Cy3 has almost completely replaced fluorescein dye derivates as a fluorescent label for oligonucleotide probes. Hence, a systematic study on the accessibility of the 16S rRNA of *E. coli* for Cy3-labeled oligonucleotides that updates the old data set was needed. Furthermore, the question of the transferability of the *E. coli* accessibility data to other organisms has so far not been sufficiently addressed. Therefore, the present study undertook the flow cytometric quantification of fluorescent signals conferred by oligonucleotides targeting the 16S rRNA of the bacterium *Pirellula* sp. strain 1 and the archaeon *Metallosphaera sedula* as well as the 18S rRNA of the yeast *Saccharomyces cerevisiae*. The organisms were chosen to cover all three domains of life. The *Planctomycete Pirellula* sp. was included in the study as another bacterium because of its distant relationship to *E. coli*. By integration of the in situ accessibility data into the commonly used software package ARB, these data became publicly available for probe design.

Although FISH has extensively been used in ecological studies of bacteria (Amann *et al.*, 2001), only a limited number of publications describe its successful application for the in situ identification of fungi (Kempf *et al.*, 2000; Kosse *et al.*, 1997; Li *et al.*, 1997; Lischewski *et al.*, 1996; Lischewski *et al.*, 1997; Oliveira *et al.*, 2001; Rigby *et al.*, 2002; Spear *et al.*, 1999; Stender *et al.*, 2001). To promote a more frequent use of FISH for the identification of yeast, the accessibility of the D1/D2 domains, located at the 5' end of the 26S rRNA of *Saccharomyces cerevisiae*, was evaluated for Cy3-labeled oligonucleotide probes. These regions show a high degree of inter-species sequence variations among yeasts, which make them excellent target sites for the design of species-specific probes (Fell *et al.*, 2000; Kurtzman & Robnett, 1998).

During the past three years, numerous studies of the higher order structure of the ribosome have advanced our understanding of its spatial conformation (Ban *et al.*, 2000; Harms *et al.*, 2001; Schluenzen *et al.*, 2000; Tung *et al.*, 2002; Wimberly *et al.*, 2000). These studies comprise the identification of rRNA-rRNA interactions based on covariation analyses (Gutell *et al.*, 2002) and physical imaging of the ribosome to precisely determine protein-rRNA interactions (Ban *et al.*, 2000;

Wimberly *et al.*, 2000). Data from the in situ accessibility studies have not hitherto been systematically evaluated with respect to the currently available models of the 3D-structure of the ribosome. Therefore it was another goal of this thesis to compare the *E. coli* 16S rRNA in situ accessibility for Cy3-labeled oligonucleotides to a three-dimensional structure model of the 30S ribosomal subunit. This comparison is complicated by the fact that in situ accessibility studies were performed on paraformaldehyde fixed cells, whereas structure analysis is based on native crystallized ribosomal subunits. For this reason further studies were performed to clarify the effect of different fixation methods and hybridization protocols on the 16S rRNA in situ accessibility of *E. coli* for a selected probe set.

Beside probe target site accessibility, photo-induced electron transfer between a fluorescent dye used for probe labeling and nucleobases of the probe or the target sequence might affect signal intensity of FISH (Marras *et al.*, 2002; Nazarenko *et al.*, 2002b; Torimura *et al.*, 2001). It has been shown for in vitro hybridization experiments of artificial probe-target duplexes that guanine nucleotides in close proximity to the fluorophore act as quenchers of probe-conferred fluorescence. At the start of this thesis, the relevance of nucleotide specific quenching for FISH had not been experimentally evaluated. We statistically analyzed the fluorescent signals of 113 carboxyfluorescein-labeled probes upon in situ hybridization to explore the influence of nucleobase quenching on FISH. Furthermore, fifteen fluorescein labeled probes were intentionally designed to investigate quenching upon duplex formation with purified RNA, isolated 30S ribosomal subunits or whole fixed *E. coli* cells.

B Results and Discussion

1 In Situ Accessibility of Small-Subunit rRNA to Cy3-Labeled Oligonucleotide Probes

The major objective of this study was to compare the in situ accessibility of small-subunit rRNA from three different prokaryotes and a eucaryote for Cy3-labeled oligonucleotide probes. The probes were grouped according to their relative fluorescence hybridization signals into six arbitrary brightness classes (relative intensity units are given in parentheses): class I (>0.81), class II (0.8 to 0.61), class III (0.6 to 0.41, class IV (0.4 to 0.21), class V (0.2 to 0.06), and class VI (0.05 to 0). Table 1 lists the distribution of probes over the different brightness classes for the four investigated strains.

TABLE 1. Distribution of probes over the different brightness classes for the four investigated strains.

Strain	No. of Probes in Brightness Classes:						Total No. of Probes
	I	II	III	IV	V	VI	
Escherichia coli	17	48	45	35	20	11	176
Pirellula sp. strain 1	0	37	26	12	10	3	88
Metallosphaera sedula	34	18	14	21	52	22	161
Saccharomyces cerevisiae	25	20	21	14	22	10	112

For *E. coli* 37% of all probes belong to brightness class I and II. Almost 18% showed weak or no signals (classes V and VI). The signal-to-noise ratios even for the less-bright probes of class IV were still >20 for exponential-phase *E. coli* cells. The normalized probe-conferred fluorescence data obtained for *Pirellula* sp. showed that none of the probes could be assigned to brightness class I. Nevertheless, the in situ accessibility of the 16S rRNA of *Pirellula* sp. strain 1 for oligonucleotide probes seems to be high, since only 15% of all probes were grouped into class V and VI. For *M. sedula* about one-half (46%) of all probes showed low signal intensities at or just barely above background fluorescence (class V and VI). However, brightness classes I and II comprised 32% of all probes and the signal-to-noise ratio of the less-bright probes of class IV was about 21 for exponential-phase *M. sedula* cells. In comparison to the three prokaryotes, 40% of all probes targeting the 18S rRNA of *S. cerevisiae* could be assigned to brightness class I and II. Dim or no fluorescence was measured for almost 30% of all 18S rRNA probes. The signal-to-noise ratios even for

the less bright probes of class IV were still 22 for exponential-phase *S. cerevisiae* cells.

The highest correlation between two data sets was achieved for the 77 probes targeting homologous positions in *E. coli* and *Pirellula* sp.. The statistical *P* test ($P < 0.01$) revealed a highly significant r^2 value of 0.47 (Figure 3a). When members of the domain *Bacteria* were compared to the archaeon *M. sedula* the correlation coefficient was lower indicating that in situ accessibility of the small-subunit rRNA may be more similar for phylogenetically more closely related organisms. Between *E. coli* and *M. sedula* the correlation coefficient was 0.22 (Figure 3 B). The significance of this value was supported by the *P* test ($P < 0.01$) because these organisms shared an extensive set of 131 homologous probes. No significant correlation was found between *Pirellula* sp. and *M. sedula*. This may be because there are only 60 probes targeting homologous sites on the 16S rRNA of these organisms (Figure 3 C).

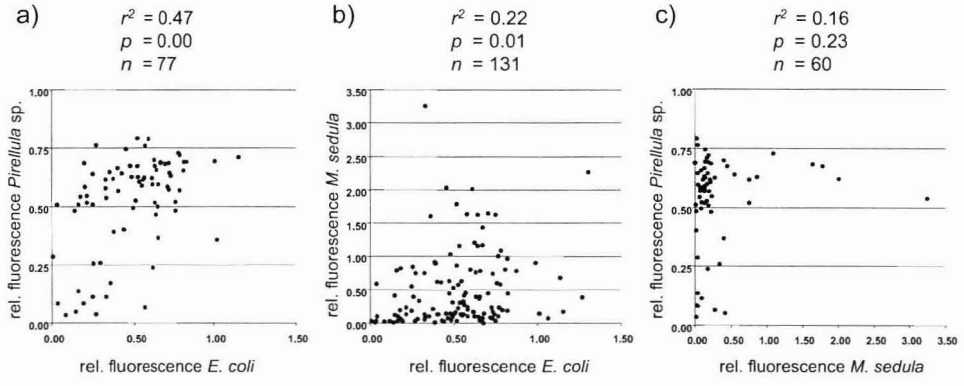

FIG. 3. Correlation of relative fluorescence intensities on fully homologous target sites (*n*). (a) *E. coli* versus *Pirellula* sp. strain 1. (b) *E. coli* versus *M. sedula*. (c) *Pirellula* sp. strain 1 versus *M. sedula*. Linear correlation coefficients (r^2) and *P* test values were calculated for each pairwise comparison.

The data on the 60 fully homologous target sites in all three prokaryotes were used to calculate a consensus in situ accessibility map for prokaryotes. For each organism, the probes were assigned rank sum values from 60 for the brightest probe down to 1 for the least bright. Rank sum values were calculated for each of the 60 target sites. According to their rank sum values the target sites were arbitrarily grouped into six brightness classes: class I, rank sum values of >150; class II, rank

sum values of 120 to 149; class III, rank sum values of 90 to 119; class IV, rank sum values of 60 to 89; class V, rank sum values of 30 to 59; and class VI, rank sum values of <30. Figure 4 shows the distribution of the different brightness classes over the 16S rRNA secondary structure model (Cannone *et al.*, 2002). Regions with high accessibility in all three prokaryotes are (i) position 285 to 338 (helices 13 and 14), (ii) position 871 to 925, except helix 30 target positions, and (iii) positions 1248 to 1283 (uppermost part of helix 46).

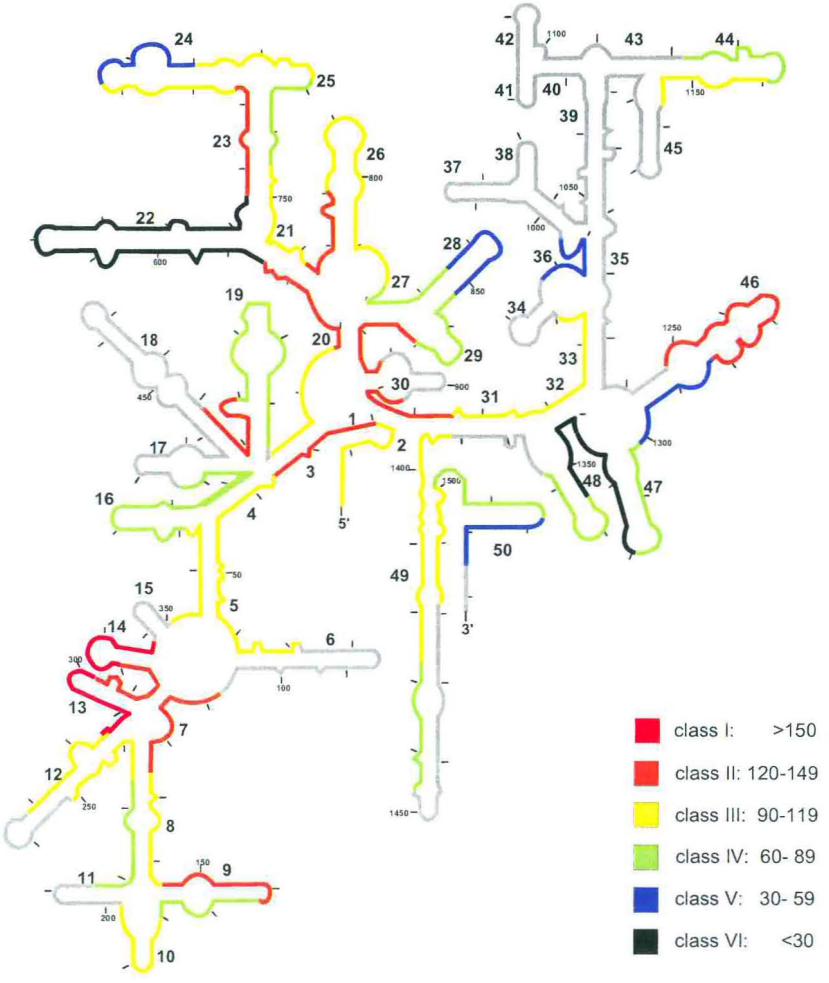

FIG. 4. Consensus accessibility map for prokaryotes. The color coding on a 16S rRNA secondary structure model of *E. coli* is based on rank sums for homologous target sites. Grey areas could not be covered with fully homologous probes. The different colors indicate different levels of brightness (classes I though VI). Numbers in small type indicate nucleotide positions. Numbers in larger type reflect helix numbering according to Brosius et al. (Brosius *et al.*, 1981).

For the 18S rRNA of *S. cerevisiae*, an independent probe set was created, because the long inserts in the eukaryotic sequence made it very difficult to design a large set of probes homologous to the prokaryotic data set. Therefore, the relative fluorescence values of the *S. cerevisiae* accessibility data set were compared to the prokaryotic consensus rank sum values over the whole 16S rRNA sequence (Figure 5). In accordance with the prokaryotic consensus data, in situ accessibility of *S. cerevisiae* 18S rRNA was low for position 587 to 651.

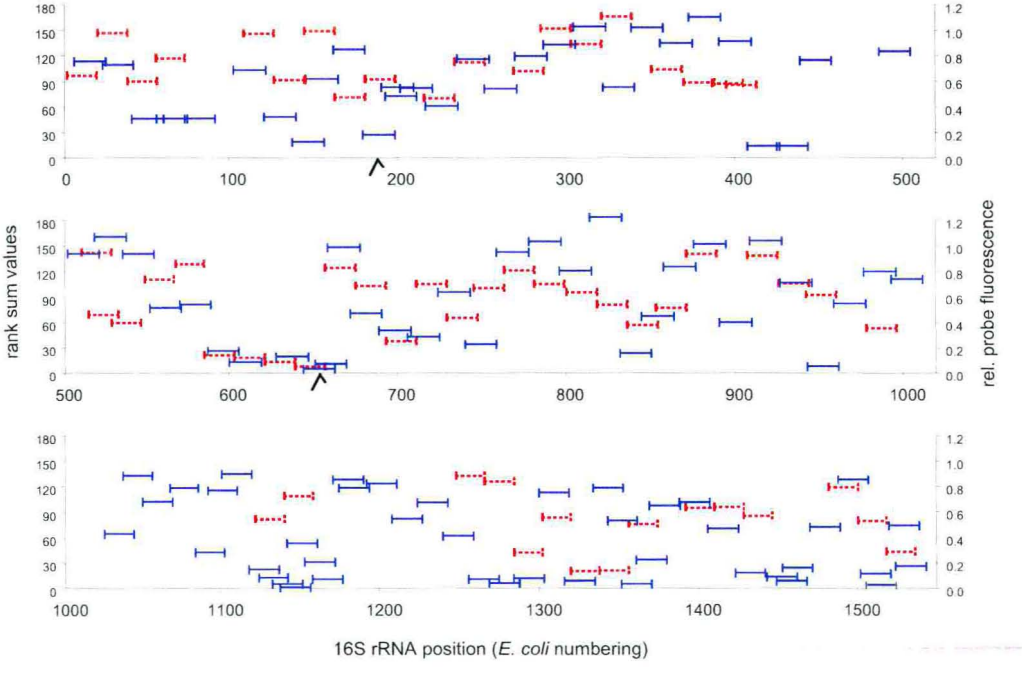

FIG. 5. Comparison of the relative fluorescence of *S. cerevisiae* 18S rRNA (solid blue lines) with the rank sum values of the prokaryotic 16S rRNA consensus model (dotted red lines). Inverted check marks on the sequence axis indicate two large inserts of 72 and 166 nucleotides in the 18S rRNA sequence of *S. cerevisiae* for which not all probes are shown. The length and exact positions of probes with respect to *E. coli* numerbing (Brosius *et al.*, 1981) are indicated on the *x* axes.

In addition to the 18S rRNA, we studied the in situ accessibility for Cy3-labeled oligonucleotide probes covering the full length of the D1/D2 domains of the 26S rRNA of *S. cerevisiae*. Figure 6 summarizes the distribution of probe-conferred fluorescence values over the D1/D2 secondary structure model of *S. cerevsiae*. Fluorescence intensities were expressed as percentage of the fluorescence signal of the brightest probe detected (D-223). Probes were grouped into brightness classes according to their relative fluorescence intensity (see above).

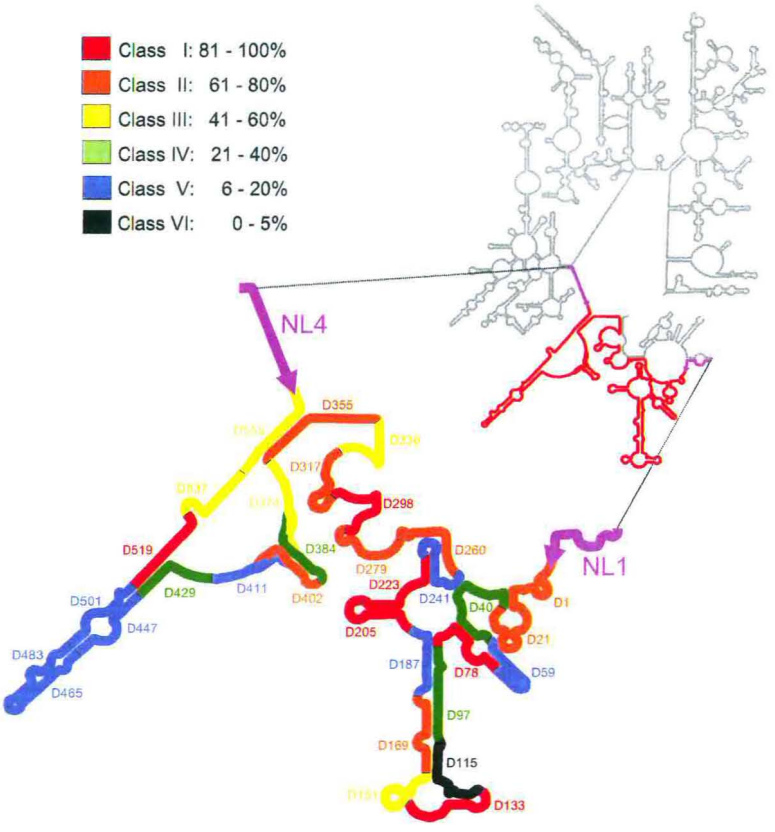

FIG. 6. Distribution of relative fluorescence hybridization intensities of oligonucleotide probes targeting the D1/D2 domains on the 26S rRNA of *Saccharomyces cerevisiae*. The D1/D2 domains (shown in detail) are enclosed by the NL1 and NL4 primer binding sites. The different colors indicate different levels of brightness (classes I through VI).

About 44% of all probes belong to brightness class I and II. For almost 28% only weak or no fluorescence could be measured. However, despite their short length of only 600 nucleotides the D1/D2 domains include potentially good target sites for yeast probe design. A comparative analysis with homologous target sites on the 23S rRNA of *E. coli* revealed some striking similarities (Figure 7). The in situ accessibility followed the same general trends in both microorganisms.

In general the in situ accessibility of ribosomal RNA for Cy3-labeled oligonucleotide probes is higher for more conserved regions, whereas the most variable areas often show only medium to low accessibility. A similar trend has been observed in previous accessibility studies (Fuchs *et al.*, 2001; Fuchs *et al.*, 1998). However, regions of high sequence variability are the most interesting for the

selection of specific probes. Probe design to these regions should therefore be done with great care. In some cases shifting a selected target site by only a few

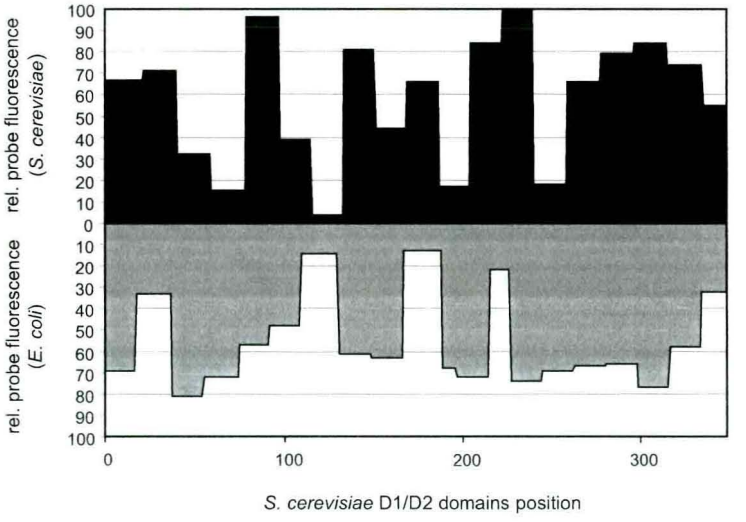

FIG. 7. Comparison of the accessibilities of homologous regions in *Saccharomyces cerevisiae* 26S rRNA and *Escherichia coli* 23S rRNA to Cy3-labeled oligonucleotide probes.

nucleotides can result in a distinct increase in probe-conferred fluorescence signal. The use of helper oligonucleotides should also be considered if targeting these regions is unavoidable. The accessibility data presented should help make probe design more reliable. However, the low correlation between the data sets of the four studied microorganisms makes clear that it is still necessary to test every newly developed probe on reference organisms before it is used with natural samples for the quantification and in situ identification of individual microbial cells.

In 1998 the *E. coli* 16S rRNA in situ accessibility was examined with carboxy-fluorescein-labeled probes (Fuchs *et al.*, 1998). The present study was performed with Cy3-labeled probes. Due to their superior fluorescence, carbo-cyanine dyes, (e.g. Cy3 and Cy5) have almost completely replaced fluorescein and rhodamine dyes in FISH applications. Therefore, and for comparative purposes, we also reexamined *E. coli*. When the Cy3- and fluorescein-labeled probe data sets are normalized in the same way, the data are generally consistent (Figure 8). 37% of the probes are in the same brightness class, while one-third of the Cy3-labeled probes are one or two brightness classes higher. The linear structure of the carbo-cyanine dye derivative Cy3 is, relative to the structure of the triphenylmethane derivative carboxy-

fluorescein, believed to reduce steric hindrance and thereby facilitate probe binding to the target (Fuchs *et al.*, 2000). Other reasons for the superior performance of Cy3

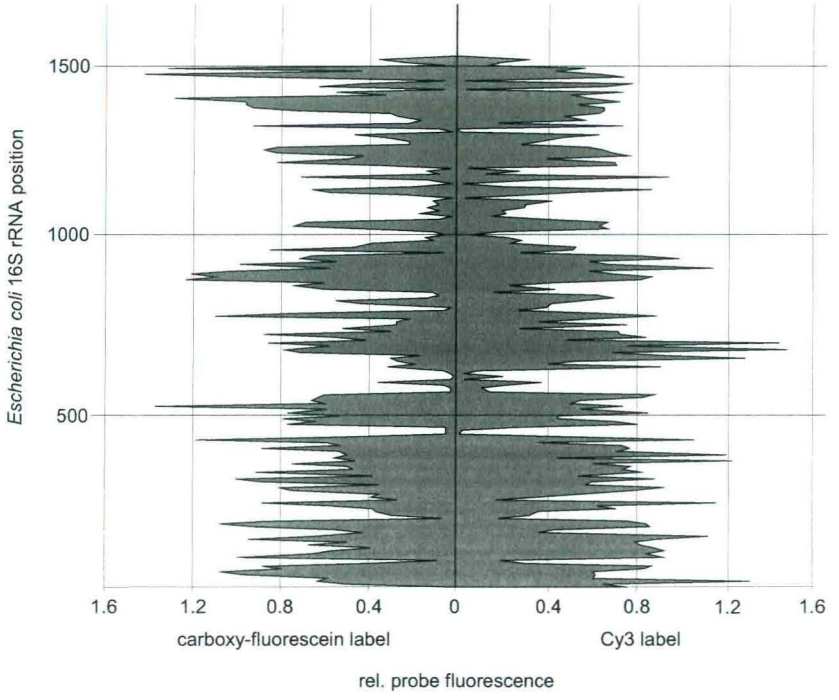

FIG. 8. Comparison of relative fluorescence intensities of carboxy-fluorescein- (left side) and Cy3-labeled (right side) oligonucleotide probes targeting the same regions on the 16S rRNA of *Escherichia coli*.

are its pH independence and its relative immunity to nucleobase-specific fluorescence quenching (Marras *et al.*, 2002; Torimura *et al.*, 2001).

The recent methodological improvements of the CARD-FISH protocol will in the future significantly increase the application of horseradish peroxidase (HRP)-labeled oligonucleotide probes and tyramide signal amplification (TSA) for the in situ detection and identification of environmental bacteria (Pernthaler *et al.*, 2002). So far the in situ accessibility of small ribosomal subunit rRNA has only been described for fluorescently labeled oligonucleotide probes. The commonly used fluorescent dyes, e. g. fluorescein (MW 389 Dalton) or Cy3 (MW 766 Dalton), are relatively small molecules compared to HRP (MW 40 kDalton). Our hypothesis was that the steric hindrance of oligonucleotides labeled with HRP should be drastically increased, resulting in a reduced probe binding efficiency. However, the enzymatic activity of the HRP amplifies the tyramide-mediated fluorescence signal. Therefore only a few

hybridized oligonucleotides should be needed for a strong fluorescence signal. The question was therefore whether differences in probe-conferred fluorescence would also occur for HRP-labeled probes.

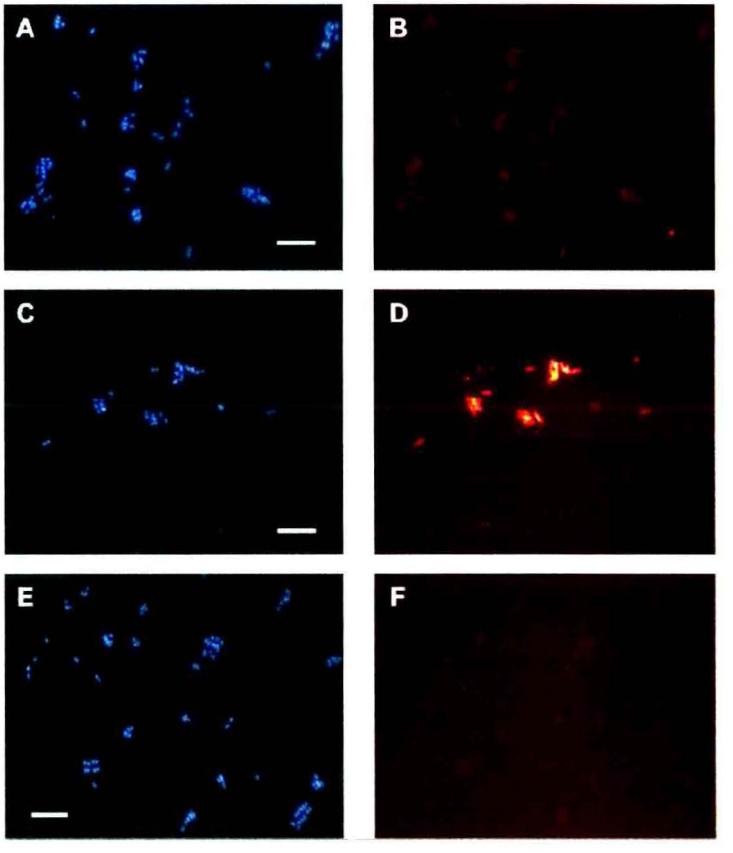

FIG. 9. Photomicrographs of FISH-stained *E. coli* cells using HRP-labeled oligonucleotide probes that target differently accessible regions on the 16S rRNA. Left, DAPI staining in blue (A, C, E,) and right, probe staining in red (B, D, F). Exposure times were kept constant for all images. (B) probe Eco632, brightness class VI. (D) probe Eco681, brightness class 1. (F) probe Eco1320, brightness class VI. Scale bar 10 µm.

Three oligonucleotide probes from the *E. coli* probe data set were ordered with an HRP label. Probe Eco681, as the brightest probe of the Cy3-data set, belongs to brightness class I. The two probes Eco632 and Eco1320 were among the least bright probes of the *E. coli* probe set. They belong to brightness class VI. All probes were hybridized under standardized conditions to paraformaldehyde-fixed *E. coli* cells following the protocol of Pernthaler and co-workers (Pernthaler *et al.*, 2002). The samples were qualitatively analyzed using an epifluorescence microscope and

computer-supported image acquisition software. In Figure 9 the epifluorescence micrographs of the hybridized *E. coli* cells are shown. The fluorescence signals obtained for the class VI probes Eco632 and Eco1320 (Figure 9 B and F) are significantly lower than the signals recorded for class I probe Eco681 (Figure 9 D). Obviously, regional differences in probe binding efficiency also affect signal intensities upon hybridization with HRP-labeled probes and tyramide signals amplification. The easiest way to overcome these limitations would probably be to increase tyramide concentration and incubation times for the HRP catalyzed signal amplification. The good match between HRP- and Cy3-probe hybridization data already suggests that FISH does not occur on native, highly condensed ribosomes but rather on looser, more denatured structures.

2 Effects of the 3D-Structure of the Small Ribosomal Subunit on Fluorescence In Situ Hybridization

In the second major part of this thesis, the in situ accessibility of *E. coli* 16S rRNA for Cy3-labeled oligonucleotides was compared to the three-dimensional structure model of the 30S ribosomal subunit. Therefore, 176 probe target sequences that have previously been studied for their in situ accessibility to oligonucleotide probes were visualized within a three-dimensional model of the small ribosomal subunit. Figure 10 shows the probe target sequences on a spatial model of the 30S ribosomal subunit. The six brightness classes seem to be quite evenly distributed over the three-dimensional structure model of the small ribosomal subunit. No evidence was found for a clustering of highly accessible sites on the surface or the predominance of less accessible sites within the small subunit or at the small/large subunit interface. Although the highest level of conservation is realized in the tertiary structure of ribosomal RNA (Tung *et al.*, 2002), the low correlation between the in situ accessibility data sets already suggested that in situ accessibility does not exclusively depend on probe target site location within or on the surface of the ribosome.

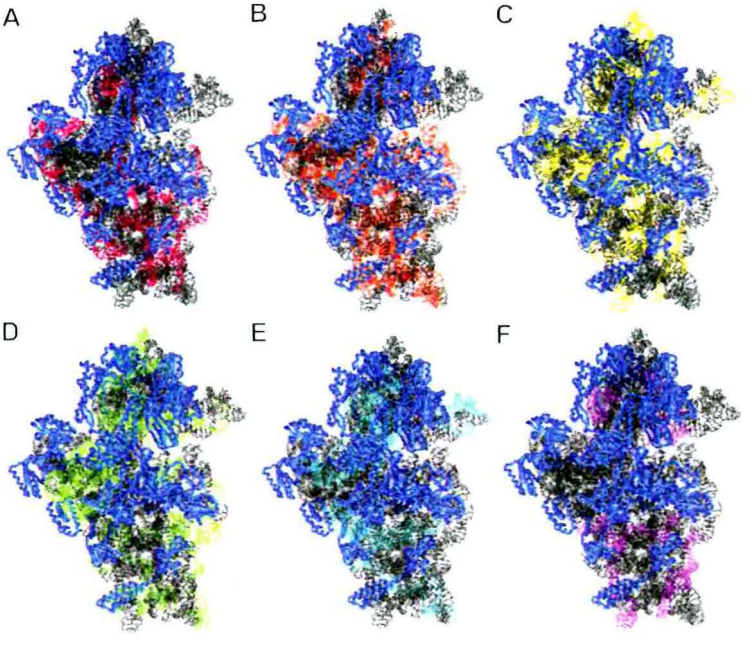

FIG.10. Target sequences of fluorescently labeled oligonucleotide probes are shown within a 3D structure model of the 30S ribosomal subunit of *E. coli*. Ribosomal proteins are shown in blue. (A) Red, (B) orange, (C) yellow, (D) green, (E) light blue, and (F) magenta indicate target sequences belonging to probe brightness class I (highest fluorescence signal) to VI (lowest fluorescence signal).

In general the 3D model of the small ribosomal subunit is not suited to explain the differences in probe-conferred hybridization signals measured in the in situ accessibility studies. Neither rRNA regions that show extensive interactions with ribosomal proteins (Figure 11), nor tertiary RNA-RNA interactions could be linked to target sites of low probe accessibility. These results may be explained by the fact that the flow cytometric quantification of probe-conferred fluorescence signals in the accessibility studies was done on paraformaldehyde (PFA) fixed cells. Upon PFA treatment, ribosomes undergo massive conformational changes, including protein denaturation. In addition, formaldehyde is able to form Schiff bases with primary amino groups of nucleobases (adenine, guanine, and cytosine), thereby influencing RNA-RNA interactions.

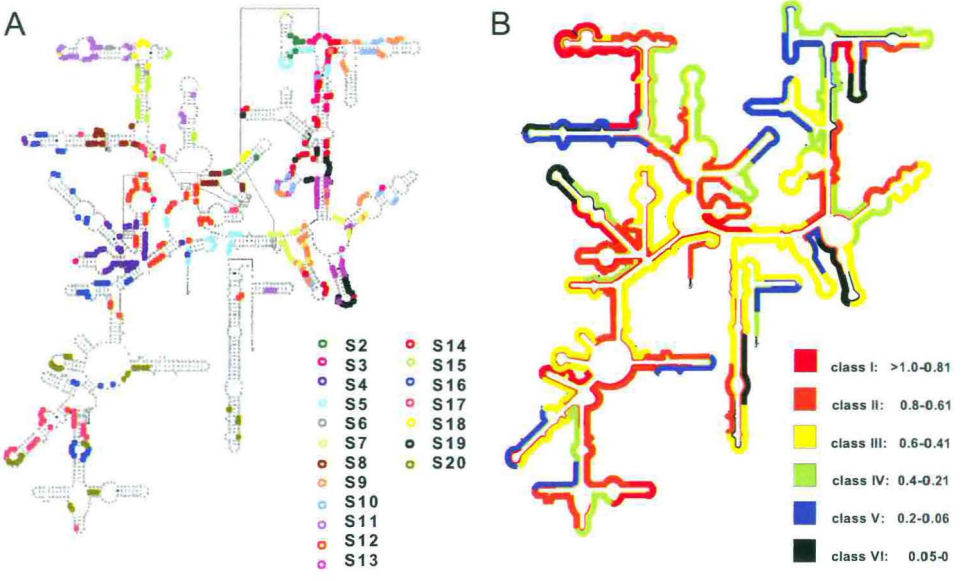

FIG.11. Predicted secondary structures of *E. coli* 16S rRNA (Cannone *et al.*, 2002). A. Regions of the 16S rRNA of *E. coli* contacted by ribosomal proteins S2 to S20 are highlighted. Proteins S1 and S21 are not included. Actual contacts as observed in the crystal structure of the 30S ribosomal subunit of *Thermus thermophilus* are shown with colored circles around the RNA residues in question. No distinction was made between backbone-only, base-only, and contacts to both backbone and base. B. Distribution of relative fluorescence hybridization intensities of 176 oligonucleotide probes targeting the 16S rRNA of *E. coli*. The different colors indicate different brightness classes (class I: red, though VI: black).

To determine the influence of different fixation protocols, storage of fixed cells, and SDS in the hybridization buffer on target site accessibility, we quantified the probe-conferred fluorescence intensity for a limited set of Cy3-labeled probes after

hybridization to differently treated *E. coli* cells (Figure 12). No clear difference in probe-mediated fluorescence was found between ethanol- and PFA-fixation of *E. coli* cells. Although ethanol fixation mainly works by dehydration, whereas PFA is able to interact covalently with primary amino groups, both methods seemed to lead to similar denatured states of the ribosome. The effect of SDS in the hybridization buffer was pronounced. Without SDS hybridization was very low. SDS can affect cell wall permeability, ribosomal protein conformation, and/or ribosome folding. Specific RNA-protein interactions based on shape and charge complementarity will most likely be interrupted in the presence of SDS. This is consistent with our observation that in situ accessibility of the 16S rRNA does not match the rRNA-protein interaction data (Figure 11).

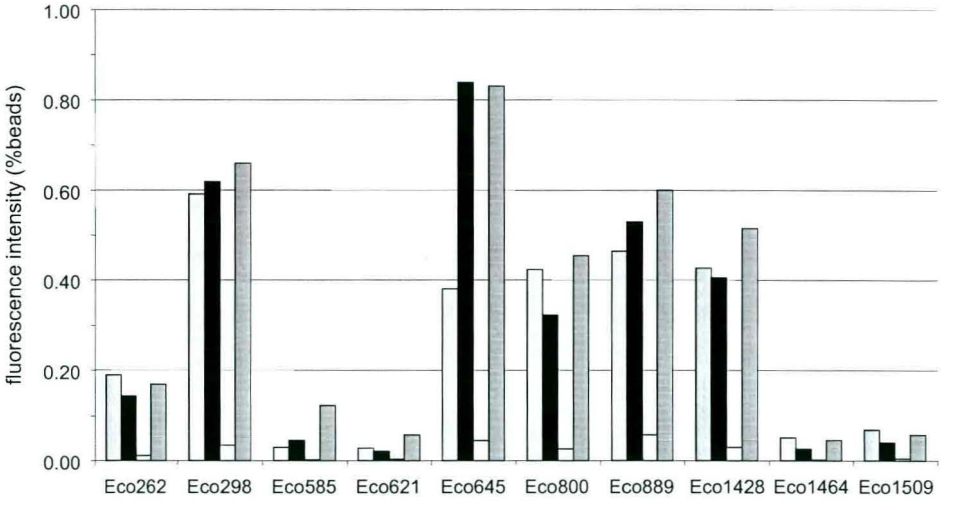

FIG.12. Comparison of fluorescence intensities of Cy3-labeled oligonucleotides probes hybridized with and without SDS in the hybridization buffer to differently fixed *E. coli* cells. Fluorescence intensity is expressed as percentage of standard beads. Gray bars: Ethanol fixation, storage in ethanol/1xPBS mixture, standard hybridization with 0.01% SDS; black bars: PFA fixation, storage in ethanol/1xPBS mixture, standard hybridization with 0.01% SDS; white bars: PFA fixation, storage in ethanol/1xPBS mixture, hybridization without SDS; horizontally striped bars: PFA fixation, storage in 1x PBS, standard hybridization with 0.01% SDS.

Apart from the changes induced by our FISH protocol, oligonucleotide hybridization itself likely causes massive conformational changes within the ribosome. A 18mer oligonucleotide has a length of 55 Å (Figure 13). The duplex of an oligonucleotide of that size with its rRNA target comprises more than 1.5 helix turns. The 30S ribosomal subunit has a width of roughly 70 Å. Hybridization of a 18mer

oligonucleotide must result in enormous distortions of the ribosome structure. The strong conformational effect of oligonucleotide hybridization has also been described

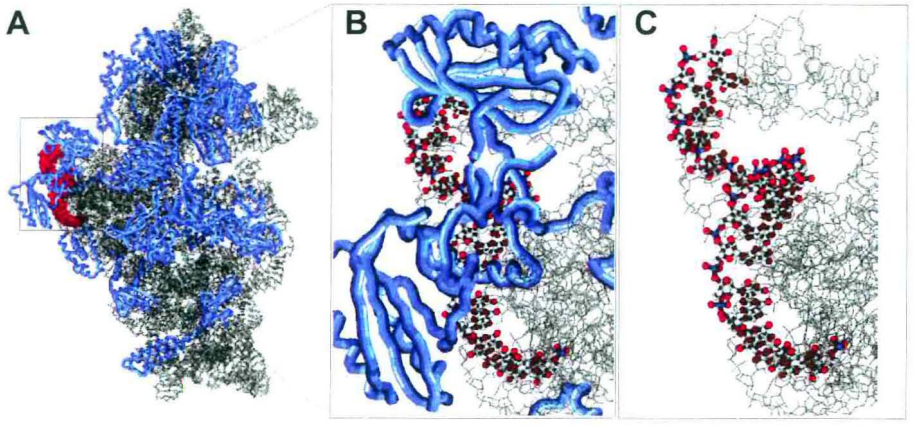

FIG.13. Detailed view of the transition zone between the 5' end of helix 23 and 24 (helix numbering according to Brosius et al. (Brosius *et al.*, 1981)) within the 30S ribosomal subunit of *E. coli*. A. Overview of the whole 30S subunit with the region shown on large scale highlighted in red. Proteins are shown as blue tubes. B. Marked as ball and stick model is the target region of probe Eco 668 (position 668 to 685). Proteins are shown as blue tubes. C. The same as B but without ribosomal proteins.

by Fuchs and coworkers (Fuchs *et al.*, 2000). They were able to open up inaccessible target sites by the use of unlabeled helper oligonucleotides. This indicates that a significant proportion of site-specific hindrance may originate RNA-RNA secondary structure interactions.

3 Nucleobase-Specific Quenching of Fluorescence In Situ Hybridization

The next addressed question was whether fluorescence in situ hybridization with rRNA-targeted oligonucleotide probes is affected by nucleobase-specific fluorescence quenching. Because fluorophores that emit in the green and yellow wavelength are more affected by nucleotide quenching than dyes that fluoresce in the blue and red spectral range, we focused on oligonucleotide probes labeled with carboxyfluorescein. Before carbo-cyanine dyes like Cy3 became available, fluorescein and rhodamine dyes were the most commonly used labels for FISH. They are still widely used in multicolor applications. For carbo-cyaninde dye derivatives no quenching by nucleobases has been reported so far (Figure 14).

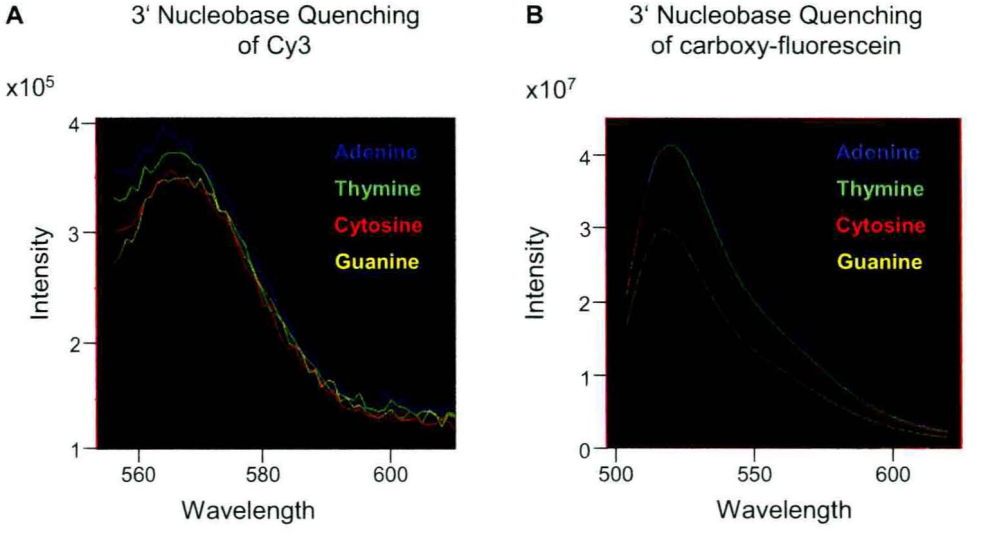

FIG.14. Fluorescence quenching of Cy3 (A) and carboxyfluorescein (B) conjugated to the 3' end of an oligonucleotide by the 3' proximal nucleobase. The oligonucleotide sequence was 5'-GGAAACAGCTATGACCATX-3'. The X indicates each of the four nucleobases. The concentration of each oligonucleotide was normalized by OD_{260} in buffer (10 mM Tris HCl pH 8.3, 50 mM KCl, 5 mM $MgCl_2$). Fluorescence measurements for a 200 nM solution of each oligonucleotide were made using a PTI (Photon Technologies International) scanning fluorometer. Fluorescence intensity at the emission maximum for each dye was recorded and normalized relative to the value obtained when adenine was the proximal base. (A) No significant quenching of Cy3 emission by any of the adjacent nucleotide residues. (B) An adjacent guanine residue significantly quenched fluorescein emission. Figures taken from http://www.idtdna.com. (Integrated DNA Technologies, Coralville, USA).

Recent studies on the electronic interaction of fluorescent dyes with guanine nucleotides have shown that the quantum yield and the fluorescence lifetime of certain dyes is changed due to electron transfer from the nucleobase to the dye (Knemeyer *et al.*, 2000; Nazarenko *et al.*, 2002; Seidel *et al.*, 1996; Torimura *et al.*, 2001). This process is referred to as photoinduced electron transfer. In the presence

of guanine the excitation of the dye molecule takes place as usual, but the fast dark reaction of the electron transfer from guanine to the dye competes with the fluorescence reaction. Hence the number of photons emitted (quantum yield) and the fluorescence lifetime are dramatically decreased in the presence of guanine in comparison to the free dye fluorescence properties. When these measurements are performed in solution, the resulting fluorescence intensity and the averaged fluorescence lifetime depend on the concentrations of guanine and fluorophor (Figure 14).

In this study, we therefore investigated two forms of nucleotide quenching: (i) sequence-specific variations in probe fluorescence in solution monitored through the ratio of oligonucleotide and dye absorption maxima at 260 and 496 nm; (ii) the 'hybridization effect', quenching of probe-conferred fluorescence upon hybridization to its complementary sequence. A statistical analysis of 113 5' carboxyfluorescein-labeled oligonucleotides for sequence-specific variations in probe fluorescence, monitored through probe and dye absorption maxima, is in preparation.

Fluorophores that are conjugated to the end of single-stranded oligonucleotides can also be quenched upon hybridization to their complementary sequence. We observed that the fluorescence of probe Eco654 (5'-CCCCCCTCTACGAGACTC-3'), labeled with carboxyfluorescein at the 5' end, was quenched up to 30% upon hybridization to purified RNA or small ribosomal subunits (Figure 15 B and C).

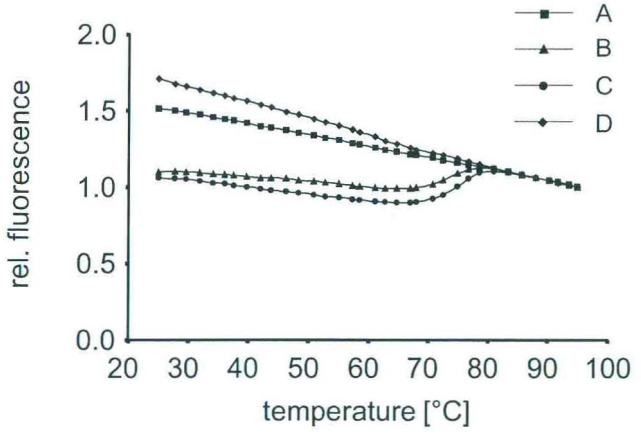

FIG.15. Melting curves of the fluorescein-labeled oligonucleotide Eco654 hybridized to different targets. (A) 100 nM probe without target. (B) Probe hybridized to isolated RNA. (C) Probe hybridized to prepared 30S ribosomal subunits. (D) Probe hybridized to whole fixed *E. coli* cells (in situ hybridization).

We also recorded melting curves of probe Eco654 upon hybridization to whole fixed *E. coli* cells. Surprisingly, the probe revealed no decrease in fluorescence during FISH (Figure 15 D).

A statistical analysis of 113 carboxyfluorescein-labeled probes upon in situ hybridization to whole fixed *E. coli* cells suggested that the probe-conferred fluorescence is directly dependent on the nucleotide sequence close the probe's 5' end (Figure 16). The current state of the statistical tests shows that probe fluorescence is enhanced rather than quenched upon FISH, but influences on the probe fluorescence resulting from regional differences in probe binding efficiency cannot be excluded. Furthermore, the applied statistical analysis does not consider the possibility of combined effects of adjacent nucleotides. At the moment we believe that nucleotide specific quenching does not significantly affect probe-conferred fluorescence after in situ hybridization, but further experiments are needed to prove this hypothesis.

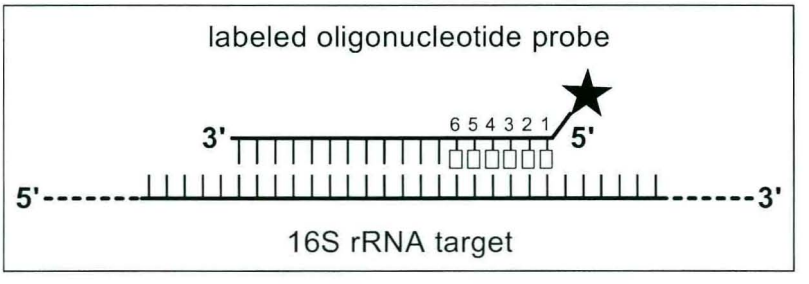

FIG.16. Schematic representation of a labeled oligonucleotide probe bound to its complementary sequences on the 16S rRNA. The black star indicates a fluorescent dye conjugated to the 5' end of the probe by short carbon linker. The six bases adjacent to the 5' end of the probe are highlighted by white boxes and numbered starting with position one for the 5' proximal nucleotide.

The absence of nucleotide specific quenching upon FISH may be due to the paraformadehyde (PFA) fixation of the *E. coli* cells prior to hybridization. PFA is able to form Schiff bases with primary amino groups of nucleotides. As a result, the electron donating properties of PFA-modified nucleobases may be decreased, leading to unhindered fluorescence development. We have shown that treatment of isolated RNA with formaldehyde resulted in a decreased quenching efficiency upon duplex formation with probe Eco654 (Figure 17). High concentrations of proteins also seemed to partially reduce nucleotide quenching. Hybridization of probe Eco654 to

isolated RNA in the presence of 50 µg bovine serum albumine (BSA) decreased quenching up to 37% (Figure 17).

Since photoinduced electron transfer is distance dependent, we extended the 5' end of probe Eco654 by four non-hybridizing adenine nucleotides to reduce quenching of the probe upon hybridization. As shown in figure 17, duplex formation of probe Eco654_4A (5'-AAAACCCCCCTCTACGAGACTC-3') with purified RNA in vitro reduced quenching by 44%.

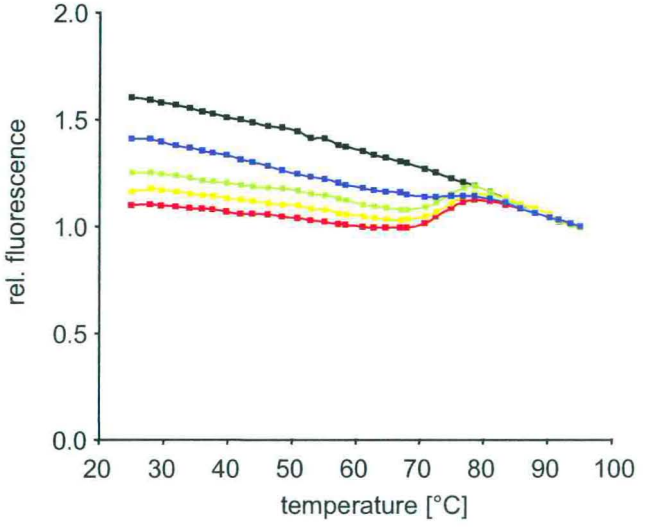

FIG.17. Melting curves of the fluorescein-labeled oligonucleotides. (**Black**) 100 nM probe Eco 654 without target. (**Red**) Probe Eco654 hybridized to purified RNA. (Yellow) Probe Eco654 upon duplex formation with formaldehyde fixed RNA. (Green) Hybridization of probe Eco654 to isolated RNA in the presence of 50 µg bovine serum albumine (BSA). (**Blue**) Probe Eco654_4A, supplemented with 4 additional adenine nucleotides at the 5' end, upon duplex formation with purified RNA.

The phenomenon of fluorescence quenching upon hybridization can also be applied to melting point determinations. The formation of nucleic acid hybrids is a reversible process and an understanding of the parameter that affect their stability enables one to derive the optimal conditions for discriminating between perfect and imperfect hybrids. The melting temperature (T_m) is defined as the temperature when half the duplex molecules have dissociated into their constituent single strands. It is affected by the monocovalent cation concentration (*M*, in moles per liter), the base composition expressed as mole fraction of guanine and cytosine residues, the length in nucleotides of the shortest chain in the duplex (*L*), and the concentration of helix-destabilizing agents such as formamide (Lathe, 1985). Wahl and coworkers (1987)

published an equation that has been derived from analyzing the influences of these factors on the stability of DNA oligonucleotide probes (14-20 nucleotides) hybridized to RNA targets (Wahl *et al.*, 1987):

$$T_m = 79.8°C + 18.5 \log M + 58.4 \text{ (mole fraction GC)} - 820/L - 0.5 \text{ (\% formamide)}$$

From this expression it follows that the T_m of DNA-RNA hybrids is decreased by 0.5 °C with each percent increase in formamide concentration. Carboxy-fluorescein-labeled oligonucleotides that are quenched upon hybridization to their complementary sequence can be used to study the interdependence of T_m and formamide concentration. We recorded melting curves of probe Eco653 (5'-CCCCCTCTACGAGACTCA-3') upon duplex formation with its complement in purified RNA at different formamide concentrations. With increasing formamide concentration the melting temperature of the probe-RNA duplex decreased indicated by the shift of the fluorescence minima towards lower temperatures (Figure 18).

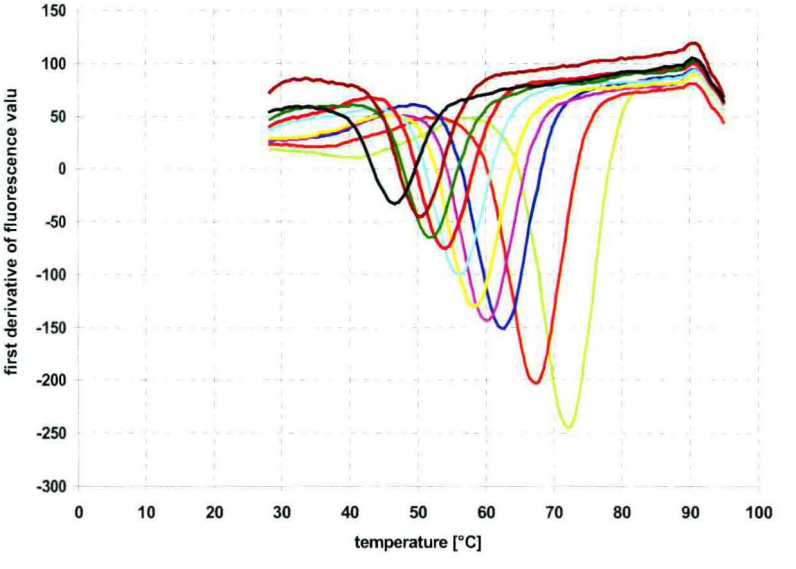

FIG.18. Melting curves of probe Eco653 (5'-CCCCCTCTACGAGACTCA-3') at different formamide concentrations upon hybridization to its complementary sequence within purified RNA. The probe-conferred fluorescence is quenched upon duplex formation. On the y-axis the first derivative of the probe fluorescence is shown. Fluorescence minima indicate the melting temperature [T_m] of the DNA-RNA duplex. Light green: hybridization with 0% formamide. **Orange**: 10%. **Dark blue**: 20%. Magenta: 25%. Yellow: 30%. Light blue: 35%. **Red**: 40%. **Dark green**: 45%. **Brown**: 50%. **Black**: 60%.

For probe Eco653 we calculated that a one percent increase in formamide concentration results in a decrease of the melting temperature of the probe target duplex of 0.42 °C (Figure 19). This value should be tested on other probes before it is considered to substitute for -0.5°C.

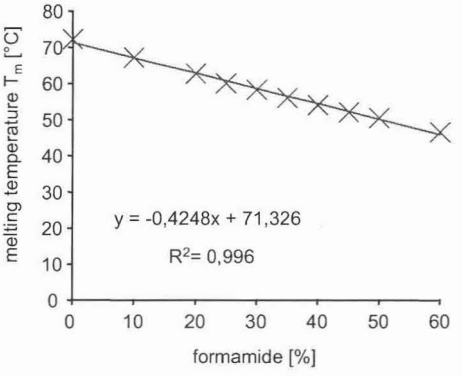

FIG.19. Relation of melting temperature (T_m) and formamide concentration for the hybrid formation of the DNA oligonucleotide probe Eco653 (5'-CCCCCTCTACGAGACTCA-3') with its complementary sequence in total purified RNA. The linear regression revealed a negative slope of –0.4248 indicating a decrease of the melting temperature by approx. 0.42 °C with each percent increase in formamide concentration. R^2 = Linear correlation coefficient.

4 Toward More Rational Probe Design

The ability to precisely quantify microbial communities in complex environmental samples is directly correlated to the effort, which is spent on continuous development of methods for more rational probe design. The work presented in this study can be summarized in some informal statements regarding probe development:

(1) Although the small-subunit rRNA is a highly conserved molecule, the data presented in this study show that the 3D-structure of the native small ribosomal subunit is not relevant to probe hybridization. This is not surprising, since FISH is performed in a strongly denaturing environment. Influences of protein-RNA interactions on the target site accessibility can therefore generally be neglected. The differences in probe-conferred fluorescence correlate most strongly with intra-helix rRNA-rRNA interactions. Because the in situ accessibility maps presented in this study are more similar for phylogenetically more closely related organisms, extrapolation of the accessibility data to other organisms should be based on the data available for the closest relative.

Future updates of the probe design software package ARB (http://www.arb-home.de) (Ludwig *et al.*, 2003) will include information on the accessibility of ribosomal RNA to oligonucleotide probes (Figure 20). The integration of the in situ accessibility data into this commonly used probe design software tool will help to make *in silico* probe selection more reliable with regards to a successful use of the designed probes for FISH studies.

(2) Probes should not target long, smooth helical regions. These regions are consistently among the least accessible sites in all organisms investigated in this study. Perfect helices that are not interrupted by internal loops or bulges are stabilized by strong secondary rRNA-rRNA interactions. Therefore probe binding to these sites is limited. However, stem regions of long helices show the highest sequence variability and are therefore of particular interest for the design of specific probes. If targeting these regions is unavoidable, the use of helper probes should be considered (Fuchs *et al.*, 2000).

(3) Nucleotide-specific quenching of fluorescent dyes has been described to account for reduced probe-conferred fluorescence intensities of artificial probe-target duplexes upon hybridization in solution. At the current state of the study, nucleotide quenching seems not to affect probe-conferred fluorescence upon FISH. Further

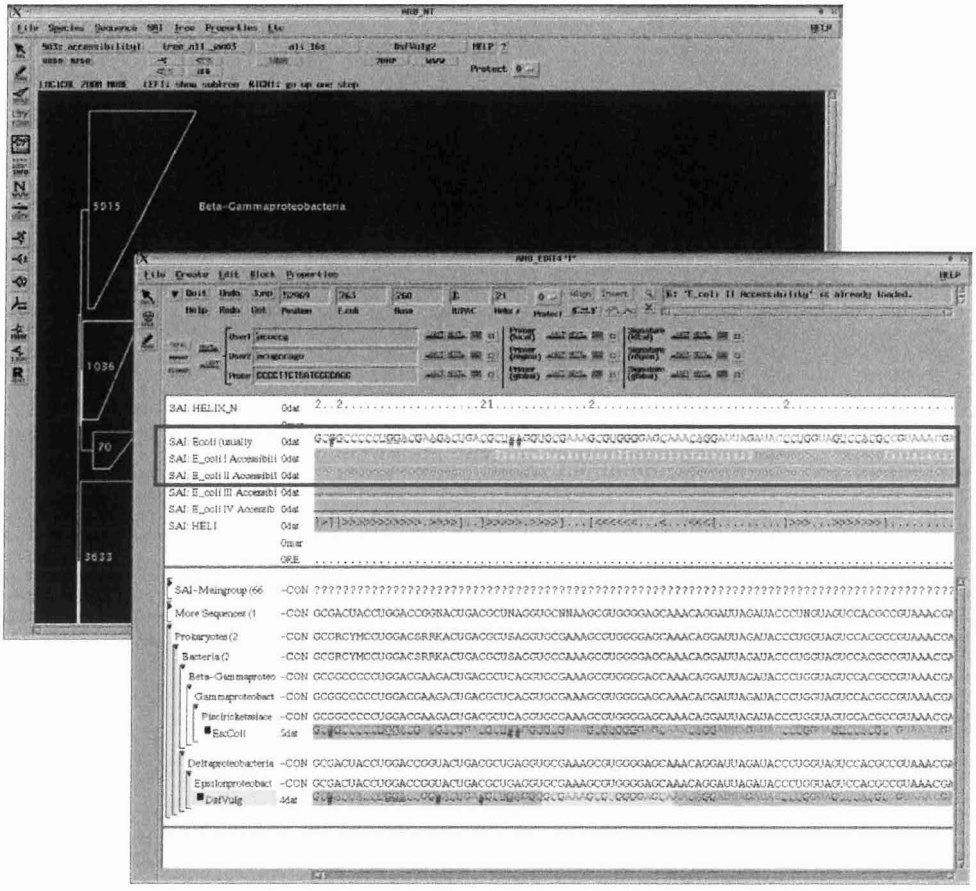

FIG.20. Screenshots taken from the probe design software package ARB. The red box in the alignment editor window shows the integration of the accessibility data as sequence associated information (SAI). In the SAI's probes are shown as colored letters. The colors were chosen according to the fluorescence brightness classes defined in the accessibility studies. (**E and e**) brightness class I, highest fluorescence signals. (**F and f**) class II; ( ) class III; (O and o) class IV; (**P and p**) class V; and (**X and x**) class VI, lowest fluorescence signals. Capital letters indicate the 5' and 3' rRNA-target positions of the probes. Selected probe target sites, highlighted in light blue, can easily be check for their in situ accessibility by comparing it to the available 'accessibility-SAI' of the closest relative.

statistical analysis and wet-lab experiments are in preparation to clarify the interdependence of probe sequence and fluorescence of the conjugated dye molecule in more detail.

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Part II:

Publications

A List of Publications

Contributions to the manuscripts presented in this thesis

(1) **Sebastian Behrens, Caroline Rühland, João Inácio, Harald Huber, Álvaro Fonseca, Isabel Spencer-Martins, Bernhard M. Fuchs, and Rudolf Amann.** 2003. In Situ Accessibility of Small-Subunit rRNA of Members of the Domains *Bacteria*, *Archaea*, and *Eucarya* to Cy3-Labeled Oligonucleotide Probes. Applied and Environmental Microbiology. **69**: 1748-1758.

(2) **João Inácio, Sebastian Behrens, Bernhard M. Fuchs, Álvaro Fonseca, Isabel Spencer-Martins, and Rudolf Amann.** 2003. In Situ Accessibility of *Saccharomyces cerevisiae* 26S rRNA to Cy3-Labeled Oligonucleotide Probes Comprising the D1/D2 Domains. Applied and Environmental Microbiology. **69**: 2899-2905.

(3) **Sebastian Behrens, Bernhard, M. Fuchs, Florian Mueller, and Rudolf Amann.** 2003. Is the in Situ Accessibility of the 16S rRNA of *Escherichia coli* for Cy3-Labeled Oligonucleotide Probes Predicted by a 3D-Structure Model of the 30S Ribosomal Subunit? Applied and Environmental Microbiology. **69**: 4935-4941.

(4) **Sebastian Behrens, Bernhard M. Fuchs, Werner Wosniok and Rudolf Amann.** 2003. Nucleobase-Specific Quenching Effects on Fluorescence in Situ Hybridization with rRNA-Targeted Oligonucleotide Probes. *Manuscript in preparation.*

(5) **Rudolf Amann, Bernhard M. Fuchs, and Sebastian Behrens.** 2001. The Identification of Microorganisms by Fluorescence in Situ Hybridization. Current Opinion in Biotechnology. **12**: 231-236.

B Publications

1

In Situ Accessibility of Small-Subunit rRNA of Members of the Domains *Bacteria*, *Archaea*, and *Eucarya* to Cy3-Labeled Oligonucleotide Probes

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In Situ Accessibility of Small-Subunit rRNA of Members of the Domains *Bacteria*, *Archaea*, and *Eucarya* to Cy3-Labeled Oligonucleotide Probes

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Low accessibility of the rRNA is together with cell wall impermeability and low cellular ribosome content a frequent reason for failure of whole-cell fluorescence hybridization with fluorescently labeled oligonucleotide probes. In this study we compare accessibility data for the 16S rRNA of *Escherichia coli* (gamma *Proteobacteria*, *Bacteria*) with the phylogenetically distantly related organisms *Pirellula* sp. strain 1 (*Planctomycetes*, *Bacteria*) and *Metallosphaera sedula* (*Crenarchaeota*, *Archaea*) and the 18S rRNA accessibility of *Saccharomyces cerevisiae* (*Eucarya*). For a total of 537 Cy3-labeled probes, the signal intensities of hybridized cells were quantified under standardized conditions by flow cytometry. The relative probe-conferred fluorescence intensities are shown on color-coded small-subunit rRNA secondary-structure models. For *Pirellula* sp., most of the probes belong to class II and III (72% of the whole data set), whereas most of the probes targeting sites on *M. sedula* were grouped into class V and VI (46% of the whole data set). For *E. coli*, 45% of all probes of the data set belong to class III and IV. A consensus model for the accessibility of the small-subunit rRNA to oligonucleotide probes is proposed which uses 60 homolog target sites of the three prokaryotic 16S rRNA molecules. In general, open regions were localized around helices 13 and 14 including target positions 285 to 338, whereas helix 22 (positions 585 to 656) and the 3′ half of helix 47 (positions 1320 to 1345) were generally inaccessible. Finally, the 16S rRNA consensus model was compared to data on the in situ accessibility of the 18S rRNA of *S. cerevisiae*.

Fluorescence in situ hybridization (FISH) is an integral part of the rRNA approach to microbial ecology and evolution (14). Since the first application as phylogenetic stains in 1989 (8), fluorescence-labeled, rRNA-targeted oligonucleotide probes have evolved to become a widely used tool for the direct, cultivation-independent identification of individual microbial cells in complex environmental samples.

FISH is often hampered by low signal intensities. The probe-conferred fluorescence is, in addition to cell wall permeability and the cellular ribosome content, dependent on the in situ accessibility of the probe target site. The access of oligonucleotide probes to their target site may be hindered by the three-dimensional structure of the ribosome which includes rRNA-rRNA interactions as well as interactions of the rRNAs with ribosomal proteins (2, 6).

Until now, there have been only two systematic studies on the accessibility of rRNA target sites. In 1998, Fuchs et al. quantified the fluorescence signals conferred by 171 carboxyfluorescein-labeled oligonucleotides targeted to the 16S rRNA of *Escherichia coli* (11). Three years later, a study was published on the in situ accessibility of the 23S rRNA of *Escherichia coli* for Cy3-labeled oligonucleotide probes (10). The question of the transferability of the *E. coli* accessibility data to other organisms remained open. Here, we address this question by flow cytometric quantification of fluorescent signals conferred by oligonucleotides targeting the 16S rRNAs of the bacterium *Pirellula* sp. strain 1 and the archaeon *Metallosphaera sedula* as well as the 18S rRNA of the yeast *Saccharomyces cerevisiae*.

The organisms were chosen to cover all three domains of life. *Pirellula* sp. belongs to the bacterial phylum *Planctomycetes* and was included in the study because of the distant relationship to *E. coli*. Furthermore, the 16S rRNA accessibility of *E. coli*, which was initially studied with carboxy-fluorescein-labeled oligonucleotides, was reexamined with Cy3-labeled oligonucleotides to exclude any dye effects.

MATERIALS AND METHODS

Microorganisms and fixation. The following type strains were grown: *E. coli* strain K-12 DSM 30083[T] (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany), *M. sedula* DSM 5348[T], *Pirellula* sp. strain 1, and *S. cerevisiae* PYCC 4455[T] (Portuguese Yeast Culture Collection, Caparica, Portugal). In a slight modification of the protocol described by Huber et al. (12), *M. sedula* was grown without any sulfur particles in the media. *Pirellula* sp. strain 1 was grown as described previously (16). *S. cerevisiae* was grown aerobically under continuous shaking in YM broth (0.3% [wt/vol] malt extract, 0.2% yeast extract, 0.5% peptone, and 1% glucose) at 25°C. Cells were harvested in the exponential growth phase (prokaryotes, optical density at 600 nm of ~0.5; yeast, optical density at 600 nm of ~2.5), washed once with 1× phosphate-buffered saline (130 mM sodium chloride, 10 mM sodium phosphate buffer [pH 7.2]), and fixed with 4% paraformaldehyde as described before (1).

Sequencing. Almost full-length 16S rRNA gene sequences of *M. sedula* and *Pirellula* sp. were amplified directly from freshly harvested cells by PCR as described previously (22). After the subsequent purification with a QIAquick PCR purification kit (Qiagen, Düsseldorf, Germany) both strands of the PCR product were sequenced with an Applied Biosystems 3100 DNA sequencer which

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used the Applied Biosystems DNA BigDye Terminator, version 3.0, Cycle Sequencing ready reaction kit (Applied Biosystems, Warrington, United Kingdom) supplied with AmpliTaq DNA polymerase in order to corroborate that all probes were indeed targeted to fully complementary target sites. To obtain the 18S rRNA gene sequence of *S. cerevisiae*, DNA was extracted from yeast cells following the method described by Sampaio et al. (15). PCR amplification and the primers used were previously described by Cai et al. (4). After purifying the PCR products with the GFX PCR DNA and gel band purification kit (Amersham Pharmacia Biotech, Piscataway, N.J.), both strands of the 18S rRNA gene were sequenced with an ALFexpressII automated sequencer (Amersham Pharmacia) (4). The sequences we obtained were identical to those already known and registered with the following accession numbers: *E. coli*, X80725; *Pirellula* sp. strain 1, X81938; *M. sedula*, X90481; and *S. cerevisiae*, J01353.

The operon diversity of *E. coli* has been investigated by Fuchs et al. (11). They could not find any effect on the fluorescence intensities in the comparison between operon-specific probes and probes from the standard set. For *Pirellula* sp. strain 1 and *M. sedula*, only one rRNA gene operon has been described. *S. cerevisiae* reveals more than 140 rRNA transcription units. We have not checked for sequence heterogeneity for the numerous copies in *S. cerevisiae* because the *E. coli* data suggested no relevant influence on the fluorescence signal intensities.

Probe design. All oligonucleotide probes were designed to be fully complementary to the respective small-subunit rRNA sequences. The oligonucleotide probes for the 16S rRNA of *E. coli* were adapted from a study by Fuchs et al. (11). For *E. coli*, *M. sedula*, and *Pirellula* sp., care was taken that probes were designed to homolog target sites. Because of inserts, deletions, and differences in the GC contents of the 16S rRNA sequences (*E. coli*, 54.6%; *Pirellula* sp., 54.6%; and *M. sedula*, 62.6%), it was not possible to find more than 60 oligonucleotide probes that target fully homologous target sites in the three investigated prokaryotes.

For *M. sedula*, a set of 161 partly overlapping oligonucleotide probes was designed, 131 of which were homologous to those of *E. coli*. For *Pirellula* sp. strain 1, a single set of 88 probes was designed, 77 of which were homologous to those of *E. coli*. The probe sets of *M. sedula* and *Pirellula* sp. share 60 target positions. The 18S rRNA of *S. cerevisiae* was covered with a set of 112 mostly adjacent probes. This set was not homologous to target sites in any of the prokaryotic strains. For better comparability, *S. cerevisiae* probe designations are based on *E. coli* numbering (3). All helix numbers were directly taken from the *E. coli* secondary-structure model according to the ARB software package (http://www.arb-home.de).

Each single probe set covers the full length of the 16S or 18S rRNA of the respective organism. The standard probe length was 18 nucleotides. If the theoretical melting point according to the 4 + 2 formula of Suggs et al. (18), $T_d = [4 \cdot (G+C) + 2 \cdot (A+T)]$, exceeded 60°C or was below 48°C, the probe length was varied accordingly. Probe lists are available at http://www.mpi-bremen.de/~sbehrens.

Probe labeling and quality control. Probes were synthesized, monolabeled at the 5′ end with Cy3 [5,5′-disulfo-1,1′-(-γ-carbopentynyl)-3,3,3′,3′-tetramethylindolocarbocyanin-*N*-hydroxysuccinimidester] in the last step of solid-phase synthesis, and high-performance liquid chromatography purified by ThermoHybaid Interactiva Division GmbH (Ulm, Germany). Since differences in the quality of labeling directly influenced the amount of probe-conferred fluorescence (data not shown), aliquots of each probe were analyzed in a spectrophotometer (DU530; Beckmann, München, Germany) as described by Fuchs et al. (11).

Absorption peaks at 550 nm (Cy3) and 260 nm (oligonucleotide) were recorded. According to the Lambert-Beer law, the ratio of absorption at 550 nm (A_{550}) versus 260 nm (A_{260}) of a monolabeled oligonucleotide should match the ratio of the extinction coefficients (ε) of Cy3 and oligonucleotide. Values of <1 indicate an incomplete labeling of a probe, whereas values of >1 point to the presence of additional, potentially unbound Cy3 dye. Considering inaccuracies in the estimation of the extinction coefficients of oligonucleotides, we accepted values ranking from 0.7 and 1.3, assuming that these oligonucleotides were monolabeled.

FISH. Approximately 10^8 fixed cells were hybridized in 100 μl of buffer containing 0.9 M sodium chloride, 0.1% sodium dodecyl sulfate, 20 mM Tris-HCl (pH 7.2), and 1.5 ng of fluorescent probe μl^{-1} at 46°C for 3 h (21). Subsequently, cells were pelleted by centrifugation for 2 min at 4,000 × *g* and resuspended in 100 μl of hybridization buffer containing no probe. After washing for 30 min at 46°C, samples were mixed with 500 μl of 1× phosphate-buffered saline (pH 8.4), immediately placed on ice, and analyzed within 3 h.

Flow cytometry. The fluorescence intensities of hybridized cells were quantified by a FACStar Plus flow cytometer (BD Lifesciences, Mountain View, Calif.). The 514-nm emission line of an argon ion laser was used as a light source and tuned to an output power of 750 mW. Forward-angle light scatter (FSC) was detected with a 530 ± 30 nm (BD Lifesciences) band-pass filter. Fluorescence was detected with a 620 ± 60 nm band-pass filter (FL1; Hugo Anders, Gesellschaft für dünne Schichten mbH, Nabburg, Germany). All measurements were calibrated to polychromatic, 0.5-μm-diameter polystyrene beads (Polysciences, Warrington, Pa.) both to check the stability of the optical alignment of the flow cytometer and to standardize the fluorescence intensities of the probes.

Data acquisition and processing. The parameters FSC, side scatter, and FL1 were recorded as pulse height signals (four decades in logarithmic scale each), and for each measurement, 10,000 events were stored in list mode files. Subsequent analysis was done with CellQuest software (BD Lifesciences). Probe-conferred fluorescence was determined to be the median of the FL1 values of single cells lying in a gate that was defined in an FSC-versus-FL1 dot plot. Probe-conferred fluorescence intensities were recorded from triplicate samples. Each replicate represents independent cell hybridization. Only triplicates with a coefficient of variation of less than 10% were accepted, otherwise the quantification was repeated. No standard deviations are given, since the coefficient of variation in all cases were <10%.

The fluorescence of cells was corrected by subtraction of background fluorescence of negative controls and standardized to the fluorescence of reference beads. The probe-conferred fluorescence was finally expressed as the percentage of the mean of the whole data set of each organism (mean = 50%). Thereby, effects caused by differences in autofluorescence and ribosome content of the four microorganisms examined in this study can be excluded.

RESULTS

Accessibility of *E. coli* 16S rRNA for Cy3-labeled oligonucleotide probes. All probes were arbitrarily grouped according to their relative fluorescence hybridization signals into six classes of brightness (relative intensity units are given in parentheses): class I (>0.81), class II (0.8 to 0.61), class III (0.6 to 0.41), class IV (0.4 to 0.21), class V (0.2 to 0.06), and class VI (0.05 to 0). Figure 1 shows the distribution of the different brightness classes over the 16S rRNA secondary-structure model (5).

Of a total of 176 probes, only 17, i.e., Eco20 (1.15), Eco91 (0.82), Eco109 (0.82), Eco155 (0.99), Eco252 (1.02), Eco298 (0.82), Eco378 (1.09), Eco395 (1.06), Eco440 (0.94), Eco645 (0.81), Eco665 (1.14), Eco668 (0.82), Eco681 (1.30), Eco690 (1.27), Eco907 (1.01), Eco934 (0.88), and Eco1176 (0.84) (brightness values are in parentheses), are in the brightest class, class I, and 48 belong to class II (Table 1). Most probes of class I are directed against five regions where accessibility for oligonucleotide probes in *E. coli* seems to be very high: (i) positions 91 to 172 (the last few nucleotides of the 3′ half of helix 6 and the 5′ half of helices 7, 8, and 9); (ii) positions 285 to 315 (helix 13); (iii) positions 395 to 439 (the 3′ half of helix 4 and the complete helix 17); (iv) positions 645 to 728 (the 5′ half of helix 23, the complete helix 24, and the 5′ half of helix 25), except for the probe Eco650, Eco657, and Eco693 target positions; (v) positions 907 to 959 (the 5′ halves of helices 31, 32, and 33), except for the probe Eco917 and Eco926 target positions. Five smaller regions with very good accessibility are spread over the whole 16S rRNA. About half of all probes are in classes III (45 probes) and IV (35 probes). The signal-to-noise ratios even for the less-bright probes of class IV were still >20 for exponential-phase *E. coli* cells.

About 17% of all probes showed weak or no signals (classes V and VI; 0 to 0.2). Apparently, totally blocked sites (class VI) include the loop regions and the 3′ half of helix 47, the 3′ half of helix 22 and the loop regions of helices 18 and 45, and the target sites of probes Eco1113, Eco1202, Eco1437, and Eco1464. Target regions which are apparently only partially accessible to oligonucleotides (class V) include the 5′ halves

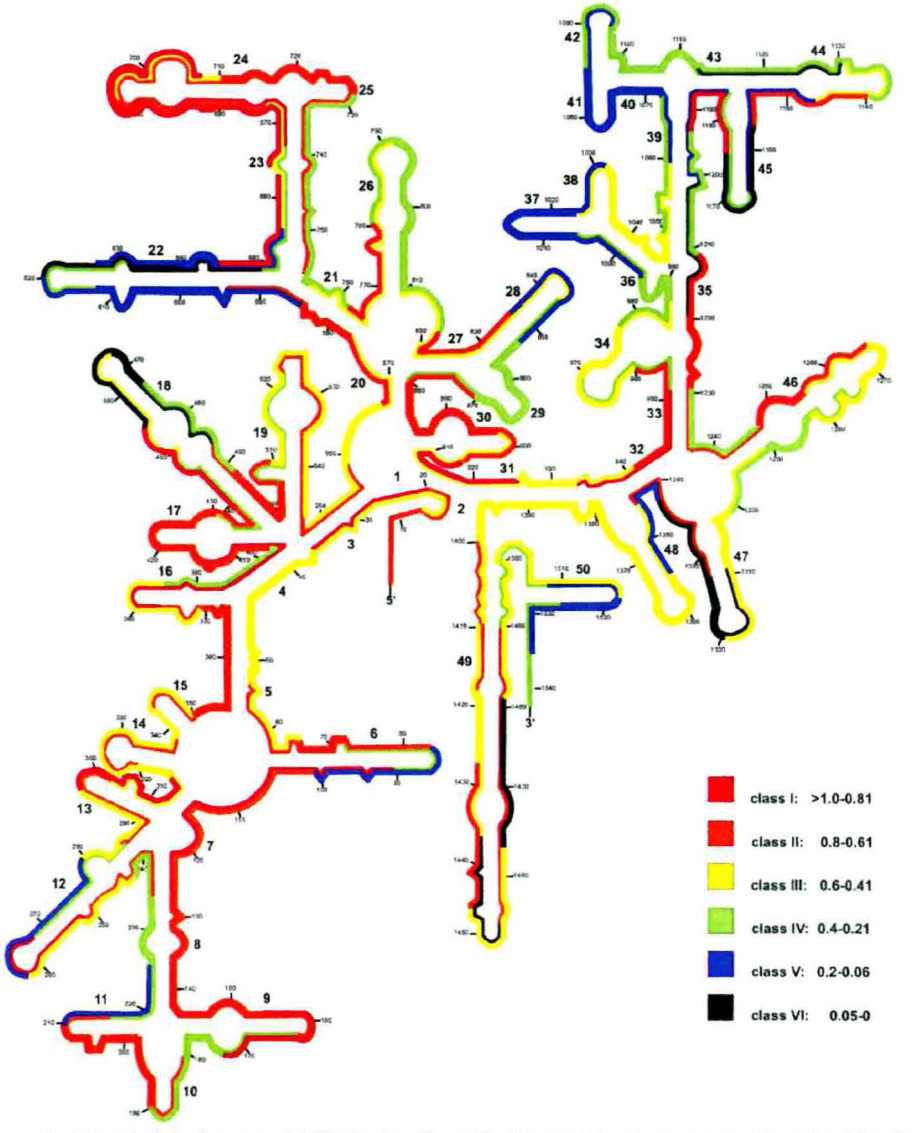

FIG. 1. Distribution of relative fluorescence hybridization intensities of 171 oligonucleotide probes targeting the 16S rRNA of *E. coli*. The different colors indicate different brightnesses (classes I through VI). Numbers in small type indicate nucleotide positions. Numbers in larger type reflect helix numbering according to Brosius et al. (3).

of helices 36, 38, and 40; almost the complete helices 22, 37, and 41; the 3' half of helix 50; and the target sites of probes Eco84, Eco210, Eco262, Eco836, Eco1147, Eco1184, and Eco1338.

Accessibility of *Pirellula* sp. strain 1 16S rRNA for Cy3-labeled oligonucleotide probes. Normalized probe-conferred fluorescence data obtained for *Pirellula* sp. are summarized in Fig. 2, once again color coded into a 16S rRNA secondary-

TABLE 1. Distribution of probes over the different brightness classes for the four investigated strains

Strain	No. of probes in brightness class:					
	I	II	III	IV	V	VI
E. coli	17	48	45	35	20	11
Pirellula sp. strain 1	0	37	26	12	10	3
M. sedula	34	18	14	21	52	22
S. cerevisiae	25	20	21	14	22	10

structure model (5). Of a total of 88 probes, 42% are in class II (37 probes). None of the probes could be assigned to class I. Nevertheless, the in situ accessibility of the 16S rRNA of *Pirellula* sp. strain 1 for oligonucleotide probes seems to be high. The brightest probes cover target sites including the complete helices 1, 2, 3, 4, 7, 13, 14, 15, 30, and 41 as well as large parts of helices 26, 46, and 49; the 3′ halves of helices 6, 12, 16, 35, and 38; and the 5′ halves of helices 9, 23, 25, 31, 39, 40, and 44.

Overall, 38 of 88 probes are in classes III (26 probes) and IV (12 probes). The signal-to-noise ratio of the less-bright probes of class IV, for exponential-phase *Pirellula* sp. strain 1 cells, was about 6. About 15% of all probes showed weak or no signals (classes V and VI). Totally blocked sites include the 3′ half of helix 22, the loop region of helix 28, and helix 42. Apparently, only partially accessible sites for oligonucleotide probes are located at the loop region of helix 6, the upper 5′ half and nearly the complete 3′ half of helix 18, the loop region of helix 22, helix 34, the 5′ and 3′ basal part of helix 46, the 5′ half of helix 47, and probe Pir1338 target sites.

Accessibility of *M. sedula* 16S rRNA for Cy3-labeled oligonucleotide probes. Fig. 3 summarizes the distribution of probe-conferred fluorescence values over the 16S rRNA secondary-structure model of *M. sedula* (5). Of a total of 161 probes, 34 are in the brightest class, class I, and 18 belong to class II. Most probes of these two classes are directed against five target regions: (i) positions 234 to 338 (helices 12, 13, and 14), except for the Met244 and Met316 target sites; (ii) positions 645 to 682 (the distal part of the 3′ half of helix 22 and the complete 5′ half of helix 23), except for the Met657 target sites; (iii) positions 704 to 772 (the 3′ half of helix 24, helix 25, and the 3′ half of helices 23 and 21), except for the Met711 and Met729 target sites; (iv) positions 850 to 906 (helices 29 and 30 and the 3′ half of helix 20), except for the Met853, Met871, and Met885 target sites; (v) positions 1248 to 1282 (the loop region of helix 46), except for the probe Met1274 target sites. Twenty-three smaller accessible spots are spread over the whole 16S rRNA. About one-fifth of all probes is in class III (14 probes) and IV (21 probes). Probes of class IV had a signal-to-noise ratio of about 21.

About one-half (46%) of all probes showed low signal intensities down to only background fluorescence (class V and VI). Apparently, totally blocked sites (class VI) include the 5′ halves of helices 43 and 45 and the complete helix 44, except for the Met1140 target sites. Eighteen smaller spots of totally blocked sites are distributed over the whole 16S rRNA secondary-structure model. Target regions which are apparently only partially accessible to oligonucleotides (class V) include the 5′ halves of helices 5, 22, 39, and 40; the complete helices 6, 16, 17, 18, 41, and 50; and the 3′ halves of helices 33, 35, 37, 38, and 45. Thirty-four other oligonucleotides belonging to class V are located all over the 16S rRNA.

Accessibility of *S. cerevisiae* 18S rRNA for Cy3-labeled oligonucleotide probes. In analogy to the three prokaryotes, the distribution of the six brightness classes over the *S. cerevisiae* 18S rRNA secondary-structure model is shown in Fig. 4 (5). Of a total of 112 probes, 25 are in class I and 20 belong to class II. Most of these probes cover six major hot spots of good in situ accessibility: (i) positions 270 to 408 (the 5′ half of helix 12 and helices 13, 14, 15, and 16), except for probe Sac322 target positions; (ii) positions 439 to 552 (helices 18 and 19); (iii) positions 759 to 832 (helix 26 and the 3′ half of helix 27); (iv) positions 858 to 927 (helices 29 and 30, the 5′ half of helix 2, and the first few nucleotides of the 3′ half of helix 31), except for probe Sac891 target sites; (v) positions 1037 to 1118 (the 5′ half of helices 37 and 38; helices 39, 40, 41, and 42, and the 3′ half of helix 43), except for probe *Sac*1084 target sites; and (vi) positions 1171 to 1208 (the 5′ half of helices 45 and 39).

Nearly one-third of all probes are in class III (21 probes) and IV (14 probes). The signal-to-noise ratios even for the less-bright probes of class IV were still 22 for exponential-phase *S. cerevisiae* cells. Nearly 30% of all probes showed only dim or no fluorescence. Obviously, only partially accessible target regions (class V) include helices 17 and 22, the 5′ half of helices 44 and 45, the distal part of helix 49, except for the probe *Sac*1449 target positions, and the target sites of probes *Sac*138, *Sac*180, Sac651c, Sac651h, Sac832, *Sac*1256, *Sac*1284, *Sac*1502, and *Sac*1524. Completely blocked regions (class VI) enclose most of the 3′ half of helix 44 and the target sites of probes Sac644, Sac651b, Sac943, *Sac*1269, *Sac*1316, *Sac*1352, *Sac*1449, and *Sac*1506.

DISCUSSION

The aim of this study was to compare the in situ accessibility of the small-subunit rRNA of three different prokaryotes and a eucaryote for Cy3-labeled oligonucleotide probes. Care was taken that the probe-mediated fluorescence was not affected by parameters like quality of probe synthesis or dissociation temperature. Effects caused by differences in ribosome content or autofluorescence were minimized by the standardization procedures applied. The best correlation between two data sets was achieved for the 77 probes targeting homolog positions in *E. coli* and *Pirellula* sp. The applied *F* test ($P \ll 0.01$) revealed a highly significant r^2 value of 0.47 (Fig. 5a). The correlation coefficient decreased when the members of the domain *Bacteria* were compared to the archaeon *M. sedula*. Between *E. coli* and *M. sedula* the correlation coefficient was 0.22. The *F* test supports the significance of this value ($P < 0.01$) because of the extensive set of 131 homolog probes shared by these two organisms (Fig. 5b). The correlation between *Pirellula* sp. and *M. sedula* was not significant ($P > 0.01$). This might be due to the limited number of only 60 probes targeting homolog sites within these organisms (Fig. 5c). The correlation analysis clearly shows that the in situ accessibility maps are more similar for phylogenetically more-related organisms. Extrapolations of our data to other organisms should therefore be based on the data available for the closest relative.

The consensus in situ accessibility map (Fig. 6) of the three

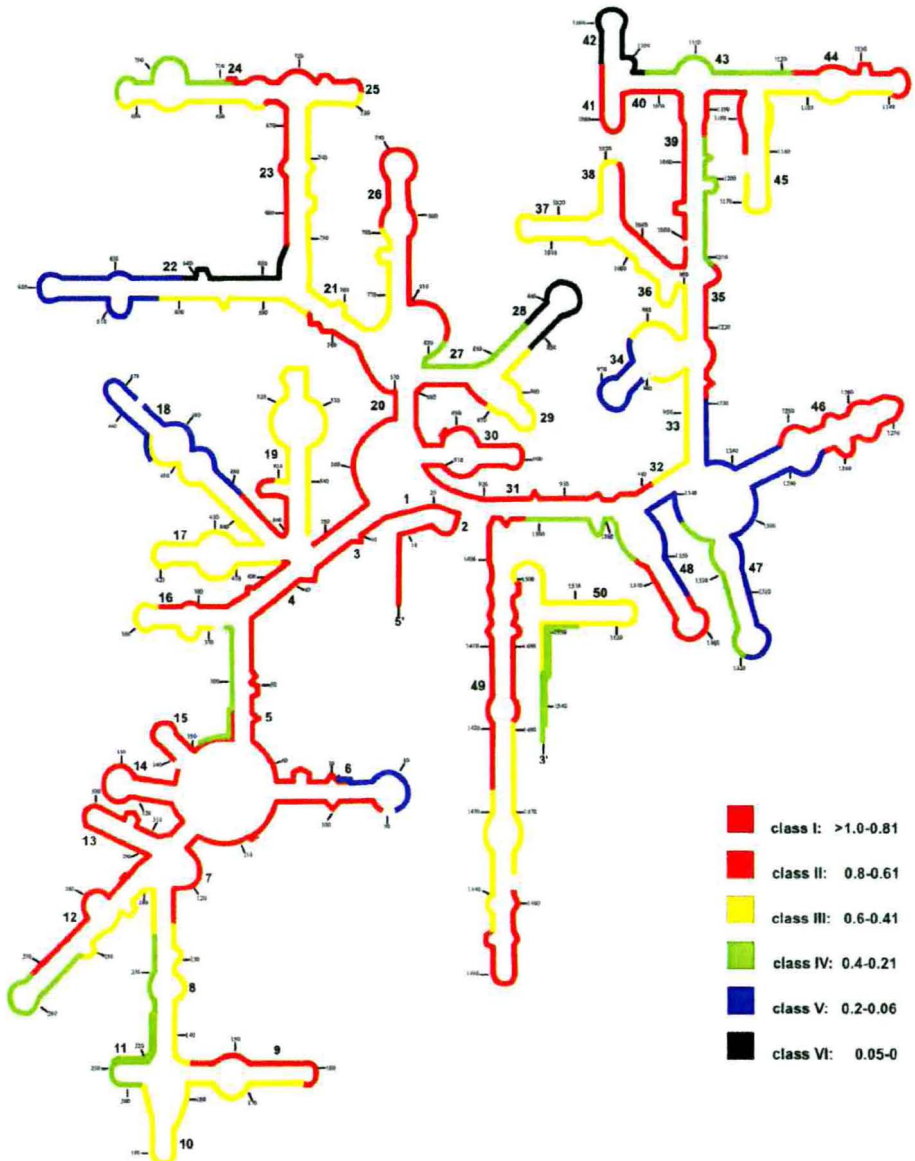

FIG. 2. Distribution of relative fluorescence hybridization intensities of 88 oligonucleotide probes targeting the 16S rRNA of *Pirellula* sp. strain 1. The different colors indicate different brightnesses (classes I through VI). Numbers in small type indicate nucleotide positions. Numbers in larger type reflect helix numbering according to Brosius et al. (3).

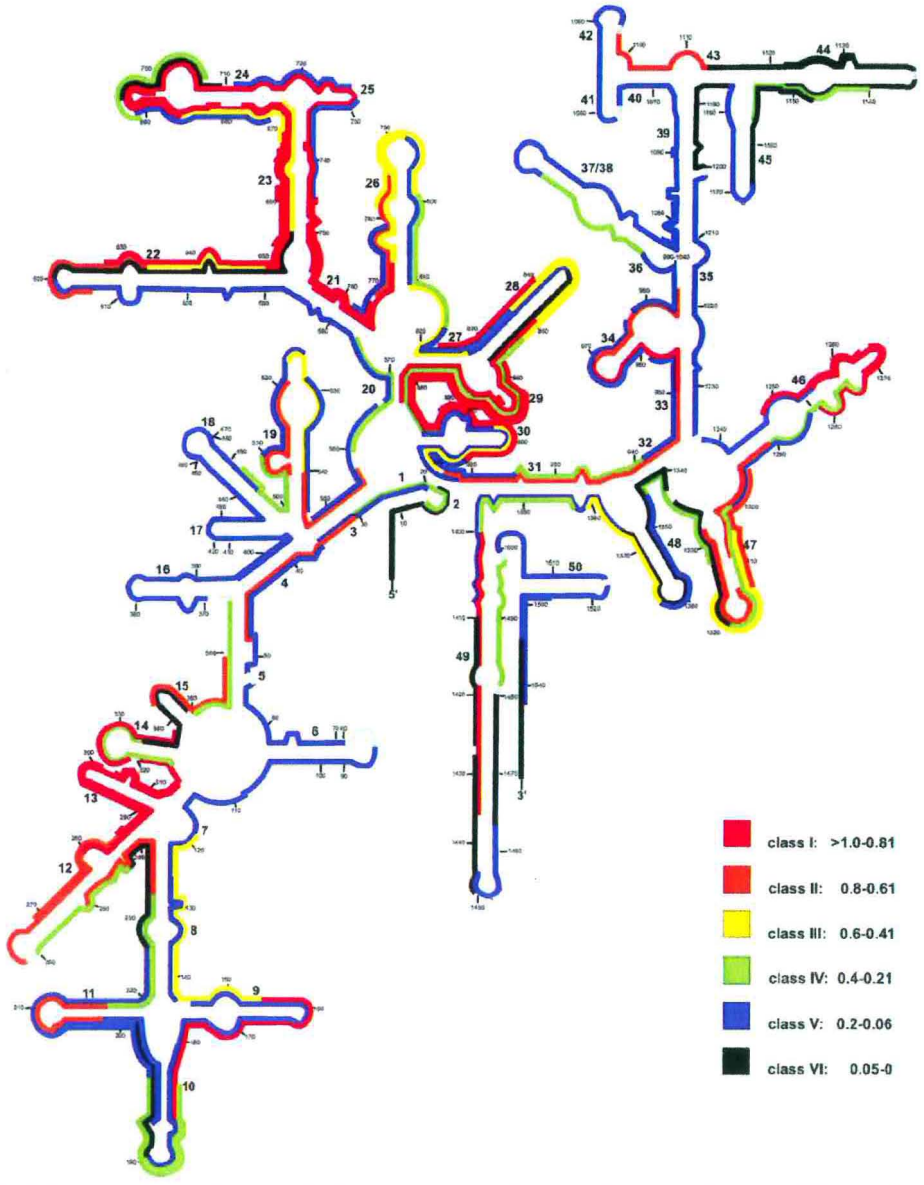

FIG. 3. Distribution of relative fluorescence hybridization intensities of 161 oligonucleotide probes targeting the 16S rRNA of *M. sedula*. The different colors indicate different brightnesses (classes I through VI). Numbers in small type indicate nucleotide positions. Numbers in larger type reflect helix numbering according to Brosius et al. (3).

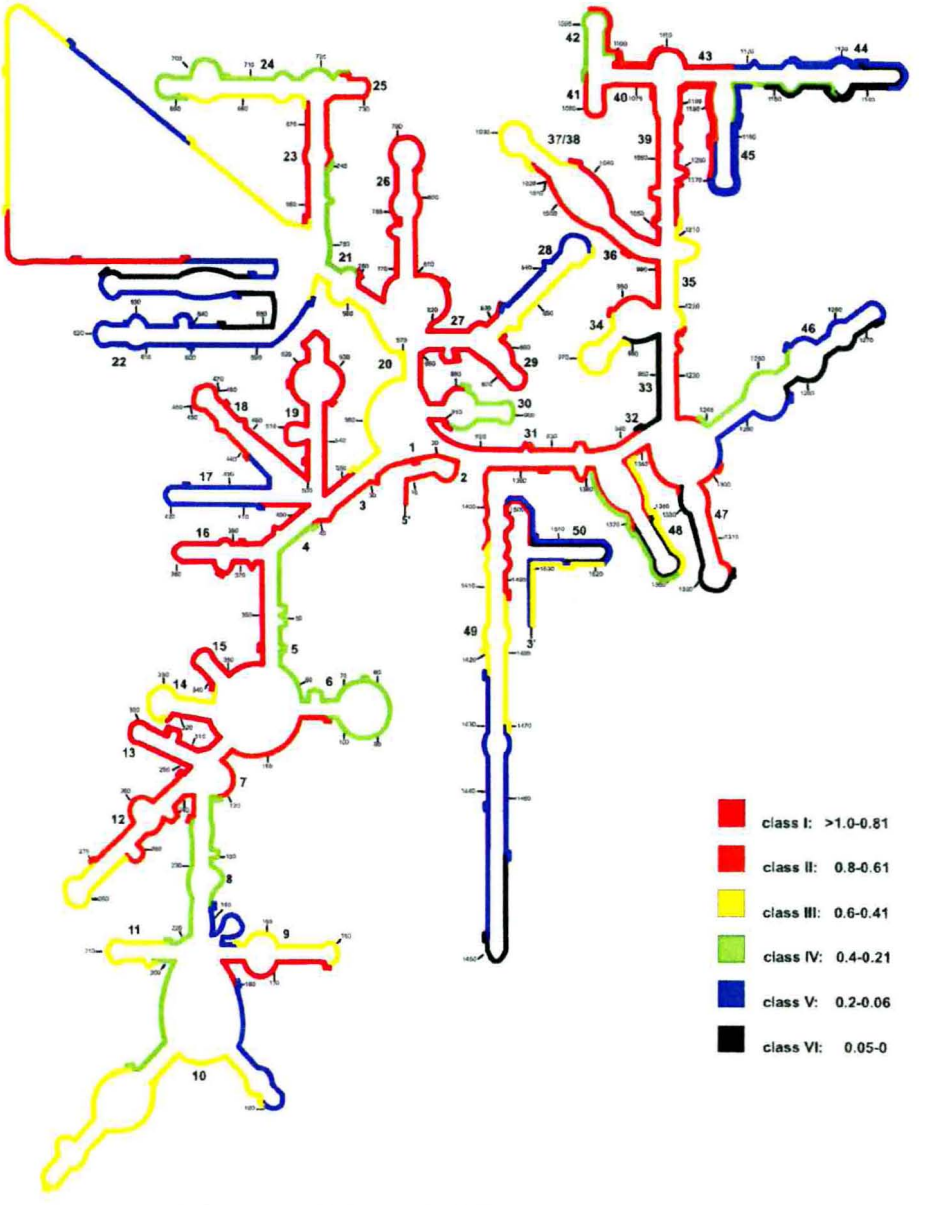

FIG. 4. Distribution of relative fluorescence hybridization intensities of 112 oligonucleotide probes targeting the 16S rRNA of *S. cerevisiae*. The different colors indicate different brightnesses (classes I through VI). Numbers in small type indicate nucleotide positions. Numbers in larger type reflect helix numbering according to Brosius et al. (3).

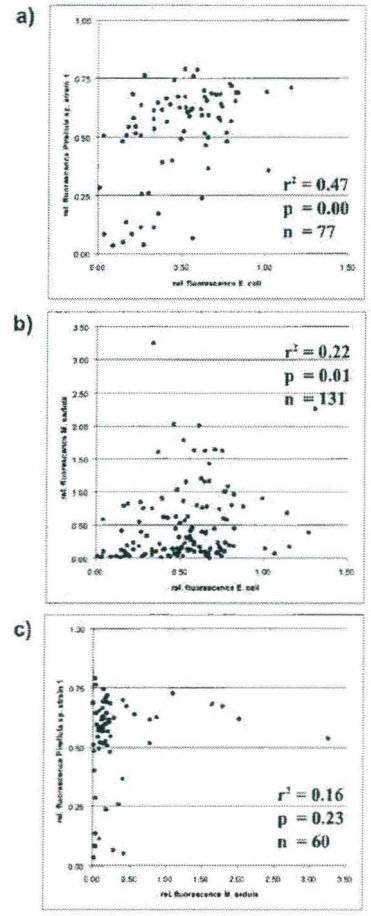

FIG. 5. Correlation of relative (rel.) fluorescence intensities on fully homologous target sites (n). (a) *E. coli* versus *Pirellula* sp. strain 1. (b) *E. coli* versus *M. sedula*. (c) *Pirellula* sp. strain 1 versus *M. sedula*. Linear correlation coefficients (r^2) and P test values (P) were calculated for each pairwise comparison.

prokaryotes considers only the data on the 60 fully homologous target sites. To each organism, rank values were given beginning with 60 for the brightest probe down to 1 for the less bright probe. Rank sum values for each of the 60 target sites were calculated and arbitrarily grouped into six classes of brightness as follows: class I, rank sum values of >150; class II, rank sum values of 120 to 149; class III, rank sum values of 90 to 119; class IV, rank sum values of 60 to 89; class V, rank sum values of 30 to 59; and class VI, rank sum values of <30. Only two of the target sites (positions 285 to 302 and 321 to 338) are in class I. Regions with high accessibility in all three prokaryotes are (i) positions 285 to 338 (helices 13 and 14), (ii) positions 871 to 925, except helix 30 target positions, and (iii) positions 1248 to 1283 (most of the upper part of helix 46). Seven smaller regions of good accessibility are located on helices 2, 3, 7, 9, 20, 23, 26, 27, and 31.

Recently a homology model of the 30S ribosomal subunit of *E. coli* became available (20). It is based on the high-resolution three-dimensional structure model of the small ribosomal subunit of *Thermus thermophilus* (17, 23). We plan to compare our in situ accessibility data to three-dimensional structure models of the 30S ribosomal subunit. It must, however, be considered that this comparison might not be straightforward because the ribosomes of whole paraformaldehyde-fixed cells may be in a denatured conformational stage that does not mimic the native ribosome structure.

Due to long inserts, it was very difficult to design a larger set of probes for the 18S rRNA of *S. cerevisiae* that is a homolog to the prokaryote data sets. An independent probe set was created. Therefore, we compared the prokaryotic consensus rank sum values with the relative fluorescence values of each probe of the *S. cerevisiae* data set over the whole 16S rRNA sequence (Fig. 7). At least for selected regions, in situ accessibilities were similar, e.g., positions 587 to 651 were low both in the prokaryotic consensus and in *S. cerevisiae*.

Differences in the *E. coli* in situ accessibility for carboxyfluorescein- and Cy3-labeled oligonucleotides. *E. coli* 16S rRNA accessibility had been examined in 1998 with carboxyfluorescein-labeled probes (11). This study was performed with Cy3-labeled oligonucleotides, which have, due to their superior fluorescence, almost fully replaced fluorescein- and rhodamine-labeled probes. For comparative purposes, we also reexamined *E. coli*. The data are generally very consistent, although the probe-conferred fluorescence signals of the fluorescein-labeled probe data set has been normalized differently (11). This is most evident for blocked sites (classes V and VI). When the fluorescein-labeled probe data of Fuchs et al. are normalized the same way we analyzed our data in this study, 37% of the probes are in the same brightness class. One-third of the Cy3-labeled probes is listed in one or two brightness classes higher. The carbo-cyanine dye derivative Cy3 has, in comparison to the triphenylmethane derivative carboxy-fluorescein, a more-linear structure that could reduce steric hindrance and thereby facilitate probe binding to the target (9). Another reason for the superior performance of Cy3 is its pH independence. Interestingly, five of the Cy3-labeled probes were grouped into brightness classes three or four categories higher than the same oligonucleotides carrying a fluorescein label. Three of these probes target the 5′ half of helix 23 (Eco645, Eco665, and Eco668). The decrease in the fluorescein fluorescence for these target regions might be caused by base-specific quenching (7, 13, 24). Torimura et al. described the sequence-specific quenching of fluorescein with special attention to guanine bases (19). For the three mentioned probes targeting the 5′ half of helix 23, 5′-GG (positions 645 and 646) and 5′-GAG_6 (positions 664 to 670) can be found on the probe target sequence in spatial proximity to the dye-labeled 5′ end of the hybridized oligonucleotide.

Although fluorescence-quenching has not yet been described for the cyanine dyes Cy3 and Cy5, we argue that positional effects on probe-conferred fluorescence may also be accountable for single cases where effects occur that cannot be

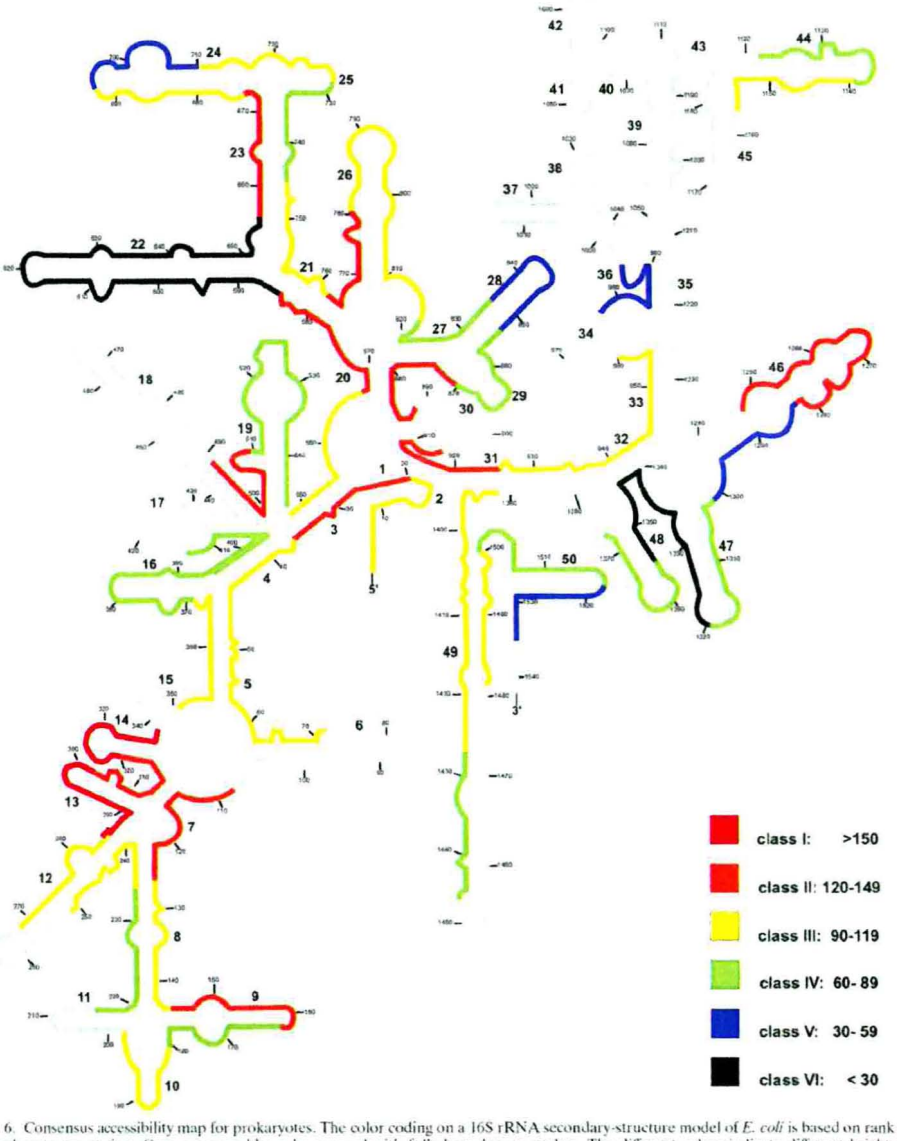

FIG. 6. Consensus accessibility map for prokaryotes. The color coding on a 16S rRNA secondary-structure model of *E. coli* is based on rank sums for homologous target sites. Grey areas could not be covered with fully homologous probes. The different colors indicate different brightnesses (classes I through VI). Numbers in small type indicate nucleotide positions. Numbers in larger type reflect helix numbering according to Brosius et al. (3).

explained by hindered or unimpeded probe access to its target position, for example, the 3′ half of helix 22 or the 5′ half of helix 49 in *M. sedula*, where overlapping probes vary in their fluorescence intensities from class I to class VI. In these regions, the probe-conferred fluorescence may also be influenced by position effects such as sequence-specific quenching of the fluorescence signal by electron energy transfer, as previously described (7, 19, 24).

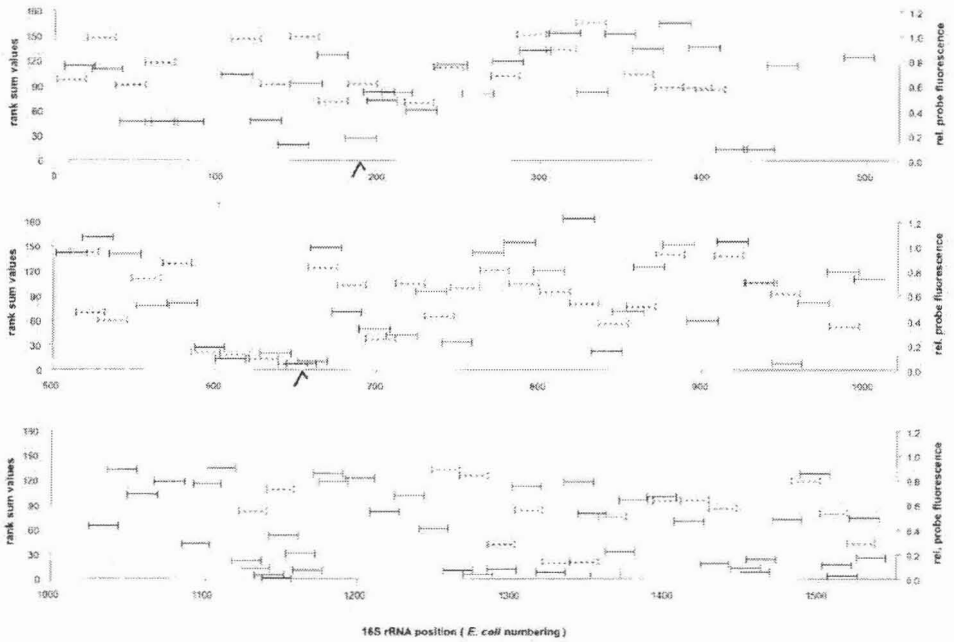

FIG. 7. Comparison of the relative (rel.) fluorescence of *S. cerevisiae* 18S rRNA probes (solid lines) with the rank sum values of the prokaryotic 16S rRNA consensus model (dotted lines). Hooks on the sequence axis indicate two large inserts of 72 and 166 nucleotides on the 18S rRNA sequence of *S. cerevisiae* for which not all probes are shown. The lengths and exact positions of probes with respect to *E. coli* numbering (3) are indicated on the *x* axes.

Although the small-subunit rRNA is a highly conserved molecule, our data show that there are differences in higher-order structures that influence target site accessibility to oligonucleotide probes. By referring to our accessibility maps, probe design should become more reliable. We intend to incorporate the in situ accessibility data into future updates of the commonly used probe design software package ARB (http://www.arb-home.de). Nevertheless, it is still necessary to test every newly designed probe on reference organisms before it is used with environmental samples for the quantification and in situ identification of individual microbial cells. In addition to in situ accessibility effects on FISH results, we found clear indications for positional effects on dye fluorescence that should be further investigated in the future.

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2

In Situ Accessibility of *Saccharomyces cerevisiae* 26S rRNA to Cy3-Labeled Oligonucleotide Probes Comprising the D1/D2 Domains

João Inácio, Sebastian Behrens, Bernhard M. Fuchs, Álvaro Fonseca, Isabel Spencer-Martins, and Rudolf Amann

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In Situ Accessibility of *Saccharomyces cerevisiae* 26S rRNA to Cy3-Labeled Oligonucleotide Probes Comprising the D1/D2 Domains

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ABSTRACT

Fluorescence *in situ* hybridization (FISH) has proven most useful for the identification of microorganisms. However, species-specific oligonucleotide probes often fail to give satisfactory results. Among the causes leading to low hybridization signals is the reduced accessibility of the targeted rRNA site to the oligonucleotide, mainly for structural reasons. In this study we used flow cytometry to determine whole cell fluorescence intensities with a set of 32 Cy3-labeled oligonucleotide probes covering the full length of the D1/D2 domains in the 26S rRNA of *Saccharomyces cerevisiae* PYCC 4455^{T}. The brightest signal was obtained with a probe complementary to positions 223 to 240. Almost half of the probes conferred a fluorescent intensity above 60% of the maximum, whereas only one probe could hardly detect the cells. The accessibility map based on the results obtained can be extrapolated to other yeasts, as shown experimentally with 27 additional species (14 ascomycetes and 13 basidiomycetes). This work contributes to a more rational design of species-specific probes for yeast identification and monitoring.

INTRODUCTION

In the last decade, fluorescence *in situ* hybridisation (FISH) became the method of choice for the direct detection and identification of microorganisms in their natural environments (1, 3, 15). Even though FISH has been extensively used in ecological studies of bacteria (3) and other organisms (17), the work with fungi has been restricted to the detection of *Aureobasidium pullulans* on the phylloplane (12, 19) and either clinically relevant or food spoilage yeasts (9, 10, 13, 14). Recently, a method using fluorescently labelled peptide nucleic acid probes was applied with success to the detection of *Dekkera bruxellensis* in wine (20), to the differentiation

between *Candida albicans* and *C. dublinensis* (16) and direct detection of *C. albicans* in blood culture bottles (18).

Preliminary studies with yeasts have shown that FISH assays are rapid and simple to carry out, do not require special cell permeation treatments and result in a high signal to noise ratio even when the cellular ribosome content is low, e.g. in late stationary phase cells (Inácio *et al.*, unpublished data). However, a significant fraction of the probes designed yield low or no hybridization signals under optimal experimental conditions as assessed with a universal probe (10). One possible limitation of the method is associated with the target molecule, the rRNA. The targeted region of the ribosomes, which remain in the intact cell, might be structurally hindered or involved in molecular interactions, rendering it inaccessible to probe hybridization (3). Despite the development of procedures to improve the accessibility of those regions using unlabelled helper oligonucleotides (6), a very useful clue when trying to design a good probe is to look for target sites located in rRNA regions already known to be accessible (7, 8).

The D1/D2 domains at the 5' end of 26S rRNA show a high degree of inter-species sequence variation for yeasts and are, therefore, frequently used for identification as well as in phylogenetic studies (5, 11). Due to the nucleotide sequence variability and to the large number of sequences available in public databases, this region provides an excellent basis to design species-specific FISH probes targeting the rRNA of yeasts (16, 20).

The aim of the present study was to evaluate the accessibility of the D1/D2 domains in the 26S rRNA to fluorescently labeled probes using *Saccharomyces cerevisiae* as a model.

MATERIAL AND METHODS

Cultivation. *Saccharomyces cerevisiae* PYCC 4455^T (PYCC - Portuguese Yeast Culture Collection, Caparica, Portugal) was grown aerobically under continuous shaking in YM broth (Malt Extract 0.3% w/v, Yeast Extract 0.2%, Peptone 0.5% and Glucose 1%) at 25ºC. Cells were harvested in the exponential growth phase (optical density of 2.5 at 600 nm) by centrifugation for 5 min at 4,500 × g. Cells were washed once with 1x phosphate-buffered saline (PBS - 130 mM sodium chloride, 10 mM sodium phosphate buffer, pH 7.2) and fixed for 4 h with 4% (v/v) paraformaldehyde at 4º C (2).

Probe design. Oligonucleotide probes were designed in order to cover the full length of the 26S rRNA D1/D2 domains of *Saccharomyces cerevisiae* (Fig. 1, sequence retrieved from Genbank with accession number U44806). The sequences, and position, of the 32 probes in the D1/D2 domains, are listed in Table 1. The standard probe length of 18 nucleotides was varied if the estimated dissociation temperature (T_d), according to the formula of Suggs *et al.* (21) [$T_d = 4 \times (G + C) + 2 \times (A + T)$], exceeded 60ºC or was below 48ºC.

Probe labeling and quality control. Probes were synthesized monolabelled at the 5' end with Cy3 by Interactiva GmbH (Ulm, Germany). Aliquots of each probe were analyzed in a spectrophotometer (UV-1202, Shimadzu, Duisburg, Germany). The peak ratios of the absorption of DNA at 260 nm and the dye at 545 nm were determined in order to check the labeling quality of the oligonucleotides (7).

Fluorescence *In Situ* Hybridization (FISH). Approximately 10^6 cells were hybridized in 80 μl of hybridization buffer (0.9 M sodium chloride, 0.01% w/v sodium dodecyl sulfate, 20 mM Tris-HCl, pH 7.2) with 1.5 ng μl^{-1} of Cy3 labeled probe at 46ºC for 2h. After incubation, cells were pelleted by centrifugation and the supernatant was discarded. Cells were resuspended in 100 μl of prewarmed

hybridization buffer without probe. After washing for 30 min at 46°C, the suspension was mixed with 200 μl of 1 × PBS, placed on ice, and analyzed within 3 h.

TABLE 1. Sequences, relative fluorescence intensities and brightness class of a set of Cy3-labeled oligonucleotide probes targeting the *Saccharomyces cerevisiae* 26S rRNA D1/D2 domains.

Probe name	*S. cerevisiae* D1/D2 position (5' → 3')	Probe sequence (5' → 3')	Relative probe fluorescence (%)[a]	Brightness class
D-1	1-20	AAGGCAATCCCGGTTGGTTT	67	II
D-21	21-39	CGCTTCACTCGCCGTTACT	71	II
D-40	40-58	TTCAAATTTGAGCTTTTGC	32	IV
D-59	59-77	GGCACCGAAGGTACCAGAT	15	V
D-78	78-96	CTCTCCAAATTACAACTCG	96	I
D-97	97-114	AACGGCCCCAAAGTTGCC	39	IV
D-115	115-132	CAAGGAACATAGACAAGG	4	VI
D-133	133-150	CTCTATGACGTCCTGTTC	81	I
D-151	151-168	CCACACGGGATTCTCACC	44	III
D-169	169-186	AAAGAACCGCACTCCTCG	66	II
D-187	187-204	TCTTCGAAGGCACTTTAC	17	V
D-205	205-222	ATTCCCAAACAACTCGAC	84	I
D-223	223-240	CCACCCACTTAGAGCTGC	100	I
D-241	241-259	TAGCTTTAGATGGAATTTA	18	V
D-260	260-278	TCGGTCTCTCGCCAATATT	66	II
D-279	279-297	TCACTGTACTTGTTCGCTA	79	II
D-298	298-316	AGTTCTTTTCATCTTTCCA	84	I
D-317	317-335	TTTTTCACTCTCTTTTCAA	74	II
D-336	336-354	TTTCAACAATTTCACGTAC	55	III
D-355	355-373	CTGATCAAATGCCCTTCCC	69	II
D-374	374-392	AGGGCACAAAACACCATGT	48	III
D-384	384-401	AAGGAGCAGAGGGCACAA	30	IV
D-402	402-419	CGAGATTCCCCTACCCAC	61	II
D-411	411-428	AGTGAAATGCGAGATTCC	13	V
D-429	429-446	CAAAACTGATGCTGGCCC	24	IV
D-447	447-464	ATGGATTTATCCTGCCAC	7	V
D-465	465-482	GAGGCAAGCTACATTCCT	16	V
D-483	483-500	CAGGCTATAATACTTACC	6	V
D-501	501-518	CAGCTGGCAGTATTCCCA	7	V
D-519	519-536	CGTCGCAGTCCTCAGTCC	83	I
D-537	537-554	GCCAGCATCCTTGACTTA	48	III
D-555	555-572	GCGGCATATAACCATTAT	55	III

[a] Fluorescence intensities expressed as a percentage of the value obtained for the brightest probe detected, D-223.

Flow Cytometry. Fluorescence of hybridized cells was quantified by a FACStar Plus flow cytometer (BD Biosciences, Mountain View, Calif.). The argon ion laser was tuned to an output power of 750 mW at 514 nm. Forward-angle light scatter (FSC) was detected with a 530 ± 30 nm (BD Biosciences) band pass filter. Fluorescence (FL1) was detected with a 620 ± 60 nm band pass filter (Gesellschaft für dünne Schichten mbH; Hugo Anders, Nabburg, Germany). Cy3-probes were

measured with deionised water as sheath fluid, and polychromatic, 0.5 μm polystyrene beads (Ref. 18660, Polysciences, Warrington, Pa.) were used to check the stability of the optical alignment of the flow cytometer and to standardize the fluorescence intensities of hybridized cells (7, 8).

Data acquisition and processing. The parameters FSC and FL1 were recorded, and for each measurement 10,000 events were stored in list mode files. The CellQuest software (BD Biosciences) was used for subsequent analysis. Probe-conferred fluorescence was determined as the mean of the fluorescence values of single cells recorded in a gate that was defined in a FSC versus FL1 dot plot. For every group of 10 measurements, the fluorescence of the reference beads was determined. The standardized cell probe-conferred fluorescence was obtained dividing the probe values by the fluorescence values of the reference beads. All values were finally expressed relatively to the value for the brightest probe detected (Table 1). FISH experiments were performed three times for each probe, in three different days, each experiment with independent triplicates. Only triplicate values with a standard deviation below 10% were accepted. The final value for each probe is the mean of at least two independent experiments, with a standard deviation below 15%. This procedure was adopted to account for the daily variations due to the equipment (e.g. oven temperature and flow cytometer laser power) and user-dependent errors.

Estimation of nucleotide substitution rates for the D1/D2 domains. The nucleotide substitution rate, defined as the number of nucleotide substitutions per site and per unit time in the DNA sequence, provides a relative measure of the conservation/variability of the positions analyzed. An alignment of 145 D1/D2 sequences, reported for yeasts and fungi of different phylogenetic groups (Table 2),

was obtained with Megalign (DNAStar, Madison, USA) and checked visually. Nucleotide

TABLE 2. GenBank accession numbers of the D1/D2 sequences of a variety of yeast species, and related fungi, used to estimate nucleotide substitution rates.

Species	Accession
Ascomycota (65)	
Archiascomycetes	
Schizosaccharomyces pombe	U40085
Taphrina deformans	U94948
Euascomycetes	
Aureobasidium pullulans	AF050239
Hemiascomycetes	
Arxula terrestris	U40103
Blastobotrys nivea	U40110
Candida bombi	U45706
Candida cariosilignicola	U70188
Candida caseinolytica	U70250
Candida castellii	U69876
Candida fennica	U45715
Candida galacta	U45820
Candida humilis	U69878
Candida insectorum	U45791
Candida nemodendra	U70246
Candida norvegica	U62299
Candida quercitrusa	U45831
Candida quercuum	U70184
Candida rugosa	U45727
Candida sake	U45728
Candida santjacobensis	U45811
Candida shehatae	U45761
Candida torresii	U45731
Candida tropicalis	U45749
Candida vini	U70247
Clavispora lusitaniae	U44817
Clavispora opuntiae	U44818
Debaryomyces castellii	U45841
Debaryomyces udenii	U45844
Dekkera anomala	U84244
Dipodascus albidus	U40081
Dipodascus ingens	U40127
Eremothecium coryli	U43390
Galactomyces geotrichum	U40118
Issatchenkia orientalis	U76347
Issatchenkia terricola	U76345
Kluyveromyces lodderae	U68551
Kluyveromyces thermotolerans	U69581
Lipomyces starkeyi	U45824
Metschnikowia reukaufii	U44825
Myxozyma mucilagina	U94945
Myxozyma udenii	U76353
Nadsonia commutata	U73598
Pichia angophorae	U75521
Pichia anomala	U74592
Pichia cactophila	U75731
Pichia euphorbiae	U73580
Pichia farinosa	U45739
Pichia inositovora	U45848
Pichia japonica	U73579
Pichia menbranifaciens	U75725
Pichia onychis	U75421
Pichia opuntiae	U76203
Pichia quercuum	U75416
Pichia toletana	U75720
Saccharomyces cerevisiae	U44806
Saccharomycopsis capsularis	U40082
Saturnispora dispora	U94937
Stephanoascus smithiae	U76531
Torulaspora delbrueckii	U72156
Williopsis mucosa	U75961
Williopsis saturnae	U75966
Yarrowia lipolytica	U40080
Zygoascus hellenicus	U40125
Zygosaccharomyces mellis	U72164
Zygozyma smithiae	U84242
Basidiomycota (80)	
Hymenomycetes	
Agaricus arvensis	U11910
Apiotrichum porosum	AF189833
Auricularia auricula-judae	L20278
Boletus rubinellus	L20279
Bullera crocea	AF075508
Bullera oryzae	AF075511
Bulleromyces albus	AF075500
Calocera cornea	AF291302
Cryptococcus albidus	AF075474
Cryptococcus curvatus	AF189834
Cryptococcus diffluens	AF075502
Cryptococcus gastricus	AF137600
Cryptococcus heveanensis	AF075467
Cryptococcus humicola	AF189836
Cryptococcus laurentii	AF075469
Cryptococcus magnus	AF181851
Cryptococcus skinneri	AF189835
Cryptococcus terreus	AF075479
Cystofilobasidium capitatum	AF075465
Fellomyces borneensis	AF189877
Fellomyces fuzhouensis	AF075506
Filobasidiella neoformans	AF075526
Filobasidium capsuligenum	AF075501
Ganoderma australe	X78780
Mrakia frigida	AF075463
Tremella aurantia	AF189842
Tremella tropica	AF042251
Trichosporum aquatile	AF075520
Trichosporum montevideense	AF105397
Trichosporum mucoides	AF075515
Udeniomyces pyricola	AF075507
Urediniomycetes	
Aurantiosporium subnitens	AF009846
Bensingtonia phyllada	AF189894
Eocronartium muscicola	L20280
Erythrobasidium hasegawianum	AF189899
Helicogloea variabilis	L20282
Kondoa aerea	AF189901
Kurtzmanomyces tardus	AF177410
Leucosporidium fellii	AF189907
Leucosporidium scottii	AF070419
Melampsora lini	L20283
Occultifur externus	AF189909
Pachnocybe ferruginea	L20284
Rhodosporidium kratochvilovae	AF071436
Rhodotorula aurantiaca	AF189921
Rhodotorula bogoriensis	AF189923
Rhodotorula ferulica	AF189927
Rhodotorula fujisanensis	AF189928
Rhodotorula glutinis	AF070430
Rhodotorula hordea	AF189933
Rhodotorula minuta	AF189945
Rhodotorula vanillica	AF189970
Sporidiobolus ruineniae	AF070438
Sporidiobolus salmonicolor	AF070439
Sporobolomyces coprosmae	AF189980
Sporobolomyces coprosmicola	AF189981
Sporobolomyces dracophylli	AF189982
Sporobolomyces falcatus	AF075490
Sporobolomyces gracilis	AF189985
Sporobolomyces roseus	AF070441
Sporobolomyces ruber	AF189992
Sporobolomyces sasicola	AF177412
Sporobolomyces singularis	AF189996
Sporobolomyces tsugae	AF189998
Sterigmatomyces elviae	AF177415
Ustilaginomycetes	
Doassinga callitrichis	AF007525
Entorrhiza aschersonia	AF009851
Entyloma calendulae	AJ235296
Exobasidium rhododendri	AF009856
Malassezia furfur	AJ249955
Melanotaenium endogenum	AJ235294
Pseudozyma fusiformata	AJ235304
Rhodotorula bacarum	AF190002
Rhodotorula phylloplana	AF190004
Thecaphora amaranthi	AF009873
Tilletia caries	AJ235308
Tilletiaria anomala	AJ235284
Tilletiopsis flava	AJ235285
Ustacystis waldsteiniae	AF009880
Ustilago maydis	AJ235275

substitution rate for each position in the alignment was estimated using the software package TREECON (23) and the substitution rate calibration method reported by Van de Peer *et al.* (24).

Comparison of 26S rRNA accessibility in different yeasts. To evaluate whether the accessibility data obtained for the region analyzed in the *S. cerevisiae*

26S rRNA could be extrapolated to other yeast species, a subset of the probes tested in this study were used in FISH experiments with several yeast species that presented a full complementary target site for those probes. Probes and yeast species selected are shown in Fig. 2. EUK 516 (5'- ACCAGACTTGCCCTCC) (2) and NonEUB (5'- ACTCCTACGGGAGGCAGC) (25) probes were used as positive and negative controls, respectively. All the yeast strains were grown, harvested and paraformaldehyde-fixed, as already described. The FISH experiments were carried out as indicated, and 10 μl of the final hybridization mixture was spotted onto microscopic slides, air dried in the dark and mounted with Vectashield solution (Vector, Burlingame, Calif.). The slides were examined with an Olympus BX50 microscope, fitted for epifluorescence microscopy with a U-ULH 100 W mercury high pressure bulb and a U-MA1007 filter set for the fluorochrome Cy3 (Olympus). The fluorescence intensity of the hybridization signal was checked visually. Microphotographs were obtained using a digital camera (Olympus C3030-ZOOM) and edited using standard software (Adobe Photoshop 6.0, Adobe).

RESULTS AND DISCUSSION

The results obtained for the *in situ* accessibility of *S. cerevisiae* 26S rRNA to Cy3-labeled oligonucleotide probes covering the full length of the D1/D2 domains are shown in Fig. 1 and Table 1. Fluorescence intensities for each probe were quantified using flow cytometry, expressed as a percentage of the fluorescence signal of the brightest probe detected (D-223), and grouped into different accessibility classes (7). The fluorescence intensity obtained for probe D-223 was of the same order of magnitude of the signal shown by the universal eukaryote probe EUK 516, which is targeted to the 18S rRNA. About 44% of the probes tested belong to the higher accessibility classes (I and II) and 28% were poorly binding (brightness classes V

and VI). To evaluate whether the probes belonging to the most inaccessible classes (IV, V and VI) would show better fluorescent signals using different hybridization conditions, a subset of these probes was chosen and hybridization reactions were performed at different temperatures. The use of different stringency hybridization conditions did not significantly improve the fluorescence intensities (data not shown), in accordance with previous studies (7).

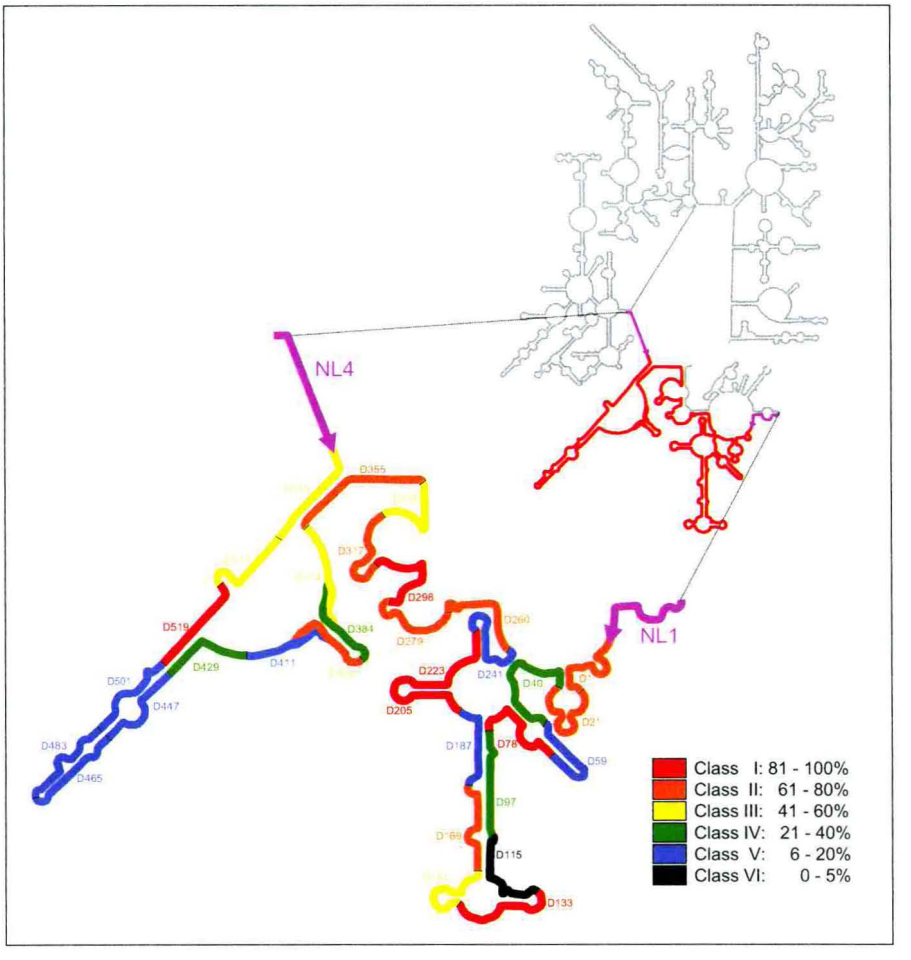

FIG. 1. Fluorescence intensities of all oligonucleotide probes, standardized to that of the brightest probe (D-223), indicated in a model of the *Saccharomyces cerevisiae* 26S rRNA secondary structure in which the D1/D2 domains (delimited by the NL1 and NL4 primer target sites) was zoomed out. The color-coding indicates differences in the level of Cy3 probe-conferred fluorescence. Secondary structure adapted from the European Ribosomal RNA Database (http://rrnuia.ac.be).

The overall results indicate that, despite its short length of approximately 600 nucleotides, the D1/D2 domains include potentially good targets for yeast probe design. However, care should be taken when selecting target sites complementary to the most variable areas of the D1/D2 domains (Fig. 3), where it is easier to find species-specific sequences. The data obtained show that the most conserved stretches of the studied region are more accessible (Fig. 3, e.g. positions 200 to 350), and the most variable areas often show medium to low accessibility (e.g. the region between nucleotides 415 and 510). A similar trend has been observed in a previous accessibility study conducted for *Escherichia coli* 16S rRNA (7).

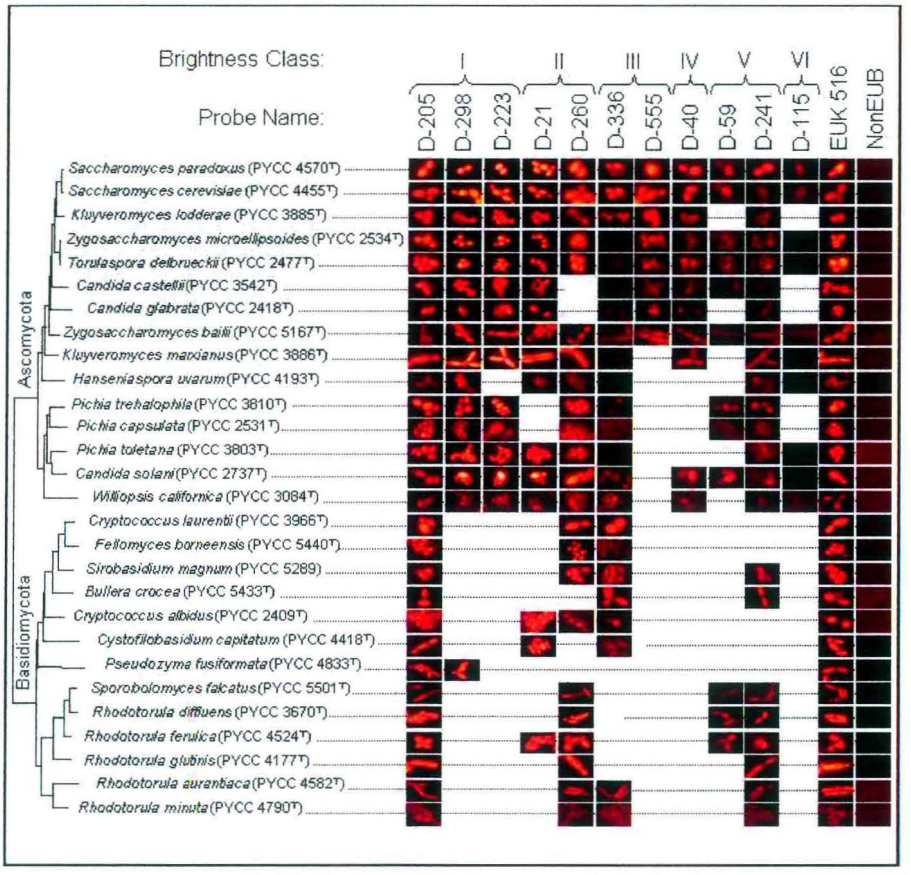

FIG. 2. Comparison of the *in situ* hybridization signals for *Saccharomyces cerevisiae* and other yeast species. The probes selected fall into different accessibility classes in the *S. cerevisiae* 26S rRNA D1/D2 domains and have an identical target site in that region for all the yeasts indicated.

As for other probes belonging to the weaker accessibility classes (IV, V and VI), whose low probe-conferred fluorescence signals may be due to the rRNA secondary structure and/or protein/rRNA interactions, the weaker signal of the probe D-59 can additionally be ascribed to a significant degree of self-complementarity in its sequence, an aspect to be taken into consideration when designing species-specific probes. Another possible explanation for the less intense fluorescence signals observed with some FISH probes is the inherent quenching of the deoxyguanosine nucleotides present mainly in the 3' dangling end of the rRNA targets (4, 22).

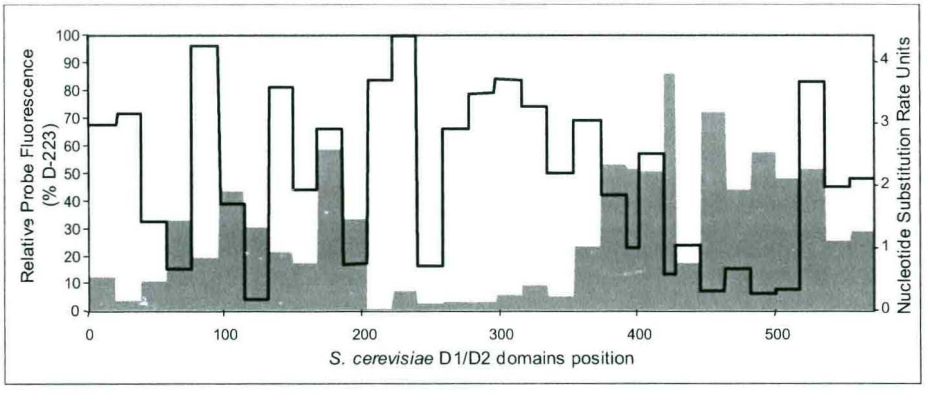

FIG. 3. Comparison of the relative *in situ* accessibility (black line) of the *Saccharomyces cerevisiae* 26S rRNA D1/D2 domains and the average nucleotide substitution rate (gray) in yeasts.

We observed no significant correlation between the probe-conferred fluorescence intensities and the guanine composition of the next five nucleotides in the 3' dangling end of the respective rRNA target (data not shown). This observation agrees with Torimura and colleagues (22), who observed the quenching phenomenon for fluorescein isothiocyanate-labeled oligonucleotides but not for the Cy3-labeled ones. Interestingly, a comparative analysis of the *in situ* accessibility of the first 350 nucleotides in *E. coli* 23S rRNA to Cy3-labeled oligonucleotide probes (8) and the data obtained in this work for *Saccharomyces cerevisiae* shows some

striking similarities (Fig. 4). Although the probes used have different target sequences in both microorganisms, the accessibilities follow the same general trend.

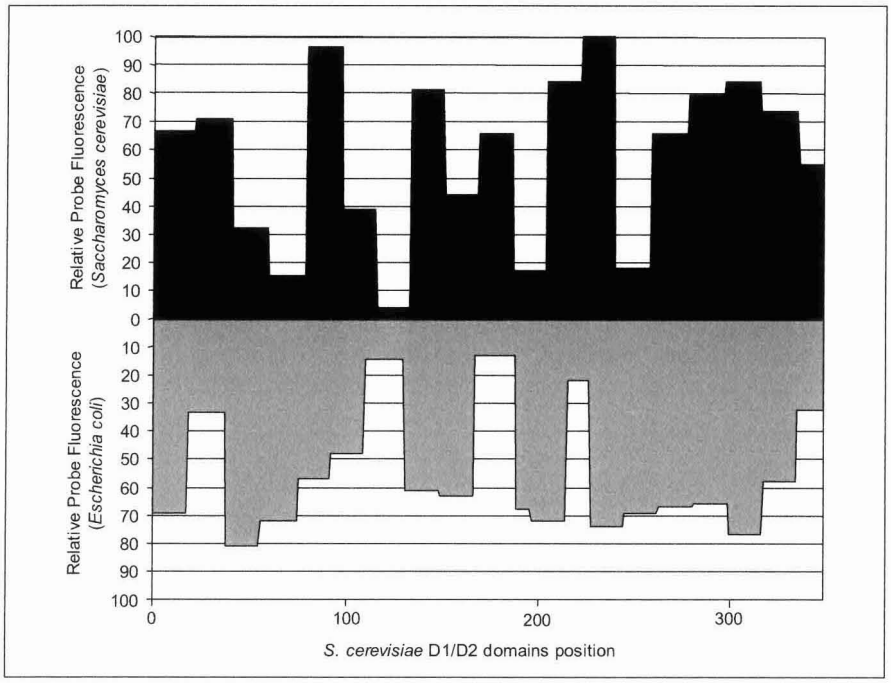

FIG. 4. Comparison of the accessibilities of homologous regions in *Saccharomyces cerevisiae* 26S rRNA and *Escherichia coli* 23S rRNA to Cy3-labeled probes.

On the other hand, the probes belonging to the higher accessibility classes (I and II) in *S. cerevisiae* have also yielded strong hybridization signals with species belonging to different phylogenetic groups including the distantly related basidiomycetous yeasts (Fig. 2). It suggests that the D1/D2 accessibility map presented here for *S. cerevisiae* provides useful guidance for the design of species-specific probes for other yeasts, maybe even for other fungi or eukaryotic microorganisms. However, the design of probes for more distantly related organisms would probably require a different model.

With this study we hope to contribute to a more rational design of fluorescently labeled probes for yeast identification that will stimulate the use of FISH based methods in a wide range of applications, including studies on the ecology of yeasts.

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3

Is the In Situ Accessibility of the 16S rRNA of *Escherichia coli* for Cy3-Labeled Oligonucleotide Probes Predicted by a 3D-Structure Model of the 30S Ribosomal Subunit?

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Is the in Situ Accessibility of the 16S rRNA of *Escherichia coli* for Cy3-labeled Oligonucleotide Probes Predicted by a 3D-Structure Model of the 30S Ribosomal Subunit?

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ABSTRACT

Systematic studies on the hybridization of fluorescently labeled, rRNA-targeted oligonucleotides have shown strong variations in in situ accessibility. Reliable predictions of target site accessibility would contribute to more rational design of probes for the identification of individual microbial cells in their natural environments. During the past three years, numerous studies of the higher order structure of the ribosome have advanced our understanding of its spatial conformation. These studies range from the identification of rRNA-rRNA interactions based on covariation analyses to physical imaging of the ribosome for the identification of protein-rRNA interactions. Here, we re-evaluate our *Escherichia coli* 16S rRNA in situ accessibility data with regard to a tertiary structure model of the small subunit of the ribosome. We localized target sequences of 176 oligonucleotides on a 3.0 Å resolution three-dimensional model of the 30S ribosomal subunit. Little correlation was found between probe hybridization efficiency and proximity of the probe target-region to the surface of the 30S ribosomal subunit model. We attribute this to the fact that fluorescence in situ hybridization is performed on fixed cells containing denatured ribosomes, whereas 3D-models of the ribosome are based on its native conformation. The effects of different fixation and hybridization protocols on the fluorescence signals conferred by a set of ten representative probes were tested. The presence or absence of the strongly denaturing detergent sodium dodecyl sulfate had a much more pronounced effect than a change of fixative from paraformaldehyde to ethanol.

INTRODUCTION

Fluorescence in situ hybridization (FISH) with rRNA targeted oligonucleotide probes has become a commonly used technique for the direct identification of

individual cells in applied and environmental microbiology (2, 10). Low probe-conferred fluorescence is a common problem in FISH. In addition to cellular ribosome content and cell wall permeability the FISH signal depends on the accessibility of the rRNA target site to the fluorescently labeled oligonucleotide. Due to the densely-packed, three-dimensional structure of the ribosome, probe access to target sites may be hindered by rRNA-rRNA interactions as well as by interactions of the rRNAs with ribosomal proteins (3, 25).

One of the first experimental attempts to consider target site-specific effects in the design of rRNA-targeted oligonucleotide probes for FISH applications was published by Frischer et al. (1996) (11). Four additional systematic studies addressing the in situ accessibility of rRNA to fluorescently labeled oligonucleotide probes have since been published. In 1998, Fuchs et al. quantified the fluorescence signals conferred by 171 carboxyfluorescein-labeled oligonucleotides targeting the 16S rRNA of *Escherichia coli* (14). Three years later, a study was published on the *in situ* accessibility of the 23S rRNA of *E. coli* for Cy3-labeled oligonucleotide probes (13). Recently, Inãcio et al. (2003) studied the in situ accessibility of the D1/D2 domains of the 26S rRNA of *Saccharomyces cerevisiae* to Cy3-labeled oligonucleotide probes (17). Also in 2003, Behrens and coworkers reexamined the 16S rRNA accessibility of *E. coli* with Cy3-labeled oligonucleotides and compared it to results obtained for the bacterium *Pirellula* sp. strain 1, the archaeon *Metallosphaera sedula*, and the 18S rRNA of the yeast *Saccharomyces cerevisiae* (4).

During the past three years, major breakthroughs in the determination of atomic-resolution ribosome structures have been made. The structure of the 50S subunit from *Haloarcula marismortui* has been solved to 2.4 Å (Angstrom) resolution (3), and Harms et al. (2001) presented the 3.1 Å resolution structure of the large

ribosomal subunit from *Deinococcus radiodurans* (16). Two high-resolution structures have appeared for the 30S subunit from *Thermus thermophilus*, one from the Yonath group at 3.3 Å resolution (22) and the other from the Ramakrishnan group at 3.0 Å (25). Recently, Tung and coworkers (2002) modeled the all-atom structure of the *E. coli* 30S ribosomal subunit using the *T. thermophilus* structure as a template (23). In this study, we use a computer-generated, atomic homology model of the *E. coli* 30S ribosomal subunit produced by Mueller and Brimacombe (unpublished) based on the 3.0 Å structure of *T. thermophilus* (25).

Data from the in situ accessibility studies have hitherto not been systematically evaluated with respect to the currently available models of the 3D-structure of the ribosome. Here, we compare the *E. coli* 16S rRNA in situ accessibility for Cy3-labeled oligonucleotides with a three-dimensional structure model of the 30S ribosomal subunit. This comparison is complicated by the fact that the in situ accessibility studies were performed on paraformaldehyde fixed cells, whereas structure analysis is done on native ribosomal subunits. Therefore, studies were performed on the influence of different fixation methods and hybridization procedures on the 16S rRNA in situ accessibility of *E. coli* for ten representative Cy3-labeled oligonucleotide probes.

MATERIALS AND METHODS

Microorganisms and fixation. *E. coli*, strain K12, DSM 30083^T (DSM=Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany) was grown as recommended by the strain collection. Cells were harvested in the exponential growth phase (OD_{600nm} ~ 0.5), washed once with 1xPBS (phosphate-buffered saline: 130 mM sodium chloride, 10 mM sodium phosphate buffer, pH 7.2). Different fixation methods were applied. Paraformaldehyde (PFA)

fixation was done as described before (1). In addition one batch of PFA-treated cells was stored at 4°C in 1xPBS, not as in the standard protocol at –20°C in a 1:1 mixture of 1xPBS and absolute ethanol. For ethanol fixation, one volume of cells resuspended in 1xPBS was mixed with 1 volume of cold absolute ethanol. The cells were first incubated at 4°C for 16 h and then stored at –20°C.

Probe design, labeling, and quality control. The oligonucleotide probes were those reported in Behrens et al. (2003) (4). All are fully complementary to 16S rRNA sequences of *E. coli*. The standard length was 18 nucleotides. Each probe was synthesized, monolabeled at the 5' end with Cy3 (5,5´-disulfo-1,1´-(-γ-carbopentynyl)-3,3,3´,3´-tetramethylindolocarbocyanin-N-hydroxysuccinimid-ester) in the last step of solid phase synthesis, and HPLC purified by ThermoHybaid Interactiva Division GmbH (Ulm, Germany). Since differences in the quality of labeling directly influenced the amount of probe-conferred fluorescence (data not shown), aliquots of each probe were analyzed in a spectrophotometer (Beckmann DU530, München, Germany) and estimated as described by Fuchs et al. (1998) (14). A list containing all *E. coli* probes used in this study has been published as a PDF file at www.mpi-bremen.de/~sbehrens. The comparison of different fixation methods was done for the following probes: Eco262, Eco298, Eco585, Eco621, Eco645, Eco800, Eco889, Eco1428, Eco1464, and Eco1509.

Fluorescence *in situ* hybridization. Approximately $1x10^8$ fixed cells were hybridized in 100 µl buffer containing 0.9 M sodium chloride, 0.01% sodium dodecyl sulfate (SDS), 20 mM Tris-HCl (pH 8.4), and 1.5 ng $µl^{-1}$ of fluorescent probe at 46°C for 3 h (24). Alternatively, hybridizations were performed without SDS in the hybridization buffer. After 3 h incubation, cells were pelleted by centrifugation for 3 min at 4,000 x g and resuspended in 100 µl hybridization buffer containing no probe. Washing of the cells was performed for 30 min at 46°C. For flow cytometric analysis,

samples were mixed with 200 µl of 1xPBS (pH 8.4), immediately placed on ice, and analyzed within three hours.

Flow cytometry. The fluorescence intensities of hybridized cells were quantified by a MoFlow flow cytometer (Cytomation Inc., Fort Collins, CO, USA). The 514 nm emission line of an argon ion laser was used as light source and tuned to an output power of 500 mW. Forward angle light scatter (FSC) was detected with a 530 ± 20 nm (Cytomation, Inc.) band pass filter. Fluorescence was detected with a 570 ± 20 nm band pass filter (Cytomation, Inc.). All measurements were calibrated to polychromatic, 0.5 µm polystyrene beads (Polysciences, Warrington, Pa.) to check the stability of the optical alignment of the flow cytometer and to standardize the fluorescence intensities of the probes.

Data acquisition and processing. The parameters FSC, SSC and FL1 were recorded as pulse height signals (four decades in logarithmic scale each), and for each measurement 10,000 events were stored in list mode files. Subsequent analysis was done with the Summit software (Cytomation, Inc.). Probe-conferred fluorescence was determined as median of the FL1-values of single cells lying in a gate that was defined in an FSC versus FL1 dot plot. Probe-conferred fluorescence intensities were recorded of triplicate samples. Each replicate represents independent cell hybridization. Only triplicates with a coefficient of variation (CV) of less than 10% were accepted, otherwise the quantification was repeated. No standard deviations are given, since the coefficient of variation in all cases was <10%.

Fluorescence of cells was corrected by subtraction of background fluorescence of negative controls and standardized to the fluorescence of reference beads.

Ribosomal RNA models. The model for the 3D structure of the *E. coli* 16S rRNA was an atomic homology model (Mueller & Brimacombe, unpublished) based on the 3.0 Å structure of the *T. thermophilus* 16S rRNA (25) and constructed in a similar manner to the *E. coli* model described by Tung et al. (2002) (23). All of the figures in this article showing three-dimensional models of the ribosome were generated using the program ERNA-3D (Editor for RNA in 3-D) (18-20). The 16S rRNA target sites of the probes are shown color-coded according to the six arbitrary brightness classes defined by Fuchs et al. (1998) and Behrens et al. (2003) (4, 14). Because non-target regions are shown in black, light blue instead of blue was chosen for class V probes and magenta replaced black for probes grouped into class VI (Figure 1).

RESULTS AND DISCUSSION

We visualized all 176 target sequences of the probes investigated by Behrens et al. (2003) within a three-dimensional model of the small ribosomal subunit (4). After normalization of the measured fluorescence values, Behrens et al. (2003) grouped the probes according to their relative fluorescence hybridization signals into six arbitrary classes of brightness (relative fluorescence intensity): class I (> 0.81), class II (0.8 to 0.61), class III (0.6 to 0.41), class IV (0.4 to 0.21), class V (0.2 to 0.06), and class VI (0.05 to 0) (4, 14). Figure 1 shows the probe target sequences on a spatial model of the 30S ribosomal subunit. Probes grouped into class I apparently have relatively unhindered access to their target sequence, whereas probes grouped into class VI demonstrate examples for inaccessible binding sites. The six groups all seem to be quite evenly distributed over the three-dimensional structure model of the small ribosomal subunit (4). Neither a clustering of highly accessible sites on the surface, nor predominance of less accessible sites within the small subunit or at the

small/large subunit interface, is evident (Figure 1). Based on the assumption that the highest conservation is found at the tertiary structure level of rRNA (23), the low correlation of the data sets examined by Behrens et al. (2003) already suggested that in situ accessibility does not exclusively depend on probe target site location inside/outside of the ribosome, i.e. on the surface or within more densely-organized structures of the ribosome.

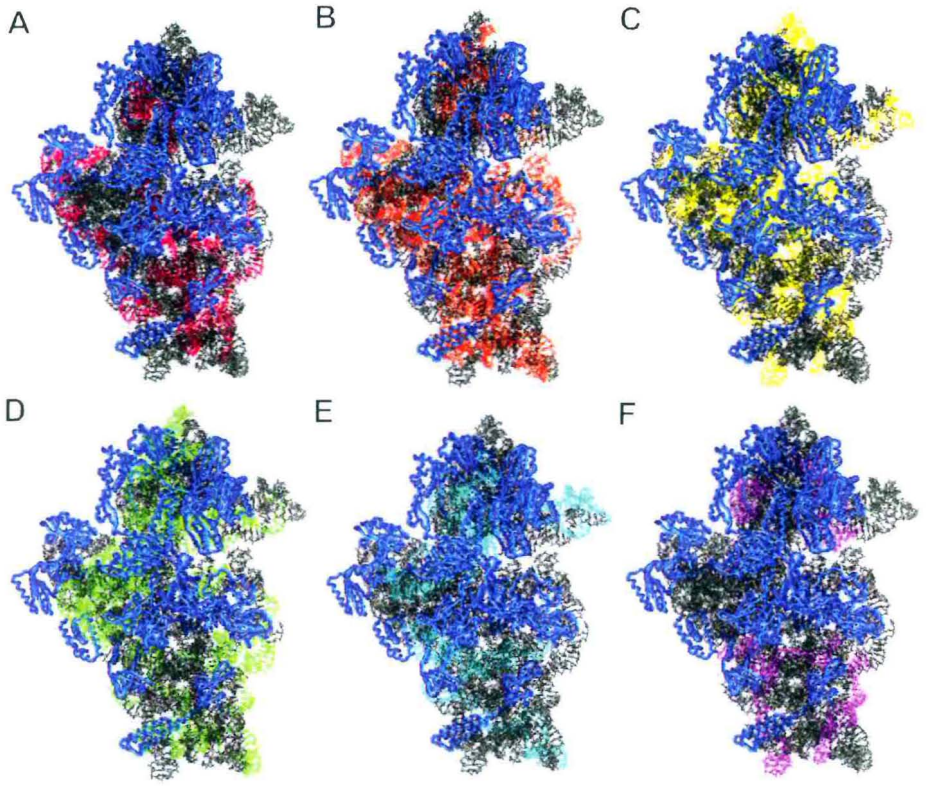

FIG. 1. Target sequences of fluorescently labeled oligonucleotide probes are shown within a 3D structure model of the 30S ribosomal subunit of *E. coli*. Ribosomal proteins are shown in blue. (A) Red, (B) orange, (C) yellow, (D) green, (E) light blue, and (F) magenta indicate target sequences belonging to probe brightness class I (highest fluorescence signal) to VI (lowest fluorescence signal) defined in the study of Behrens et al. (2003) (4).

Further detail is shown for one relatively accessible and one inaccessible region. The target region of the class I probe Eco907 (Figure 2A; position 907 to 925) comprises the 5' end of helix 30, helix 2, and the 3' end of helix 31 (helix numbering according to Brosius et al. (1981) (7)). According to the 3D model, this region is fairly deep within the small subunit and covered by the ribosomal proteins S5 and S12. In contrast, the target site of probe Eco621 (Figure 2B; position 621 to 638) is nearly free from any hindrance by ribosomal proteins and located directly on the outside of the small ribosomal subunit. Nevertheless, hybridization of probe Eco621 (class VI) is strongly hindered. This blocking does not occur only in *E. coli*; rather, helix 22 seems to be fairly inaccessible throughout all three domains of life (4).

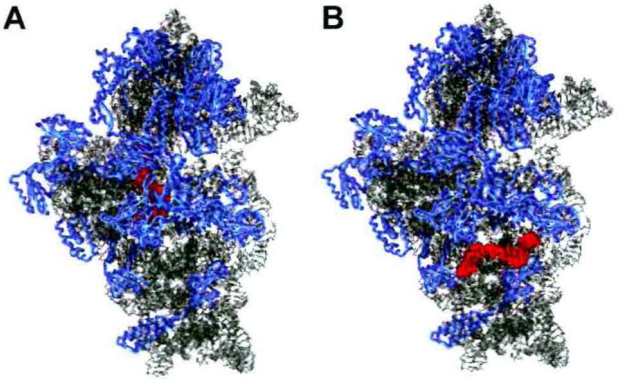

FIG. 2. Three-dimensional structure model of the 30S ribosomal subunit of *E. coli*. A. The target region of class I probe Eco907 (position 907 to 925) is shown in red. The marked region comprises the 5' end of helix 30, helix 2, and the 3' end of helix 31 (helix numbering according to Brosius et al. (1981) (7)). B. Highlighted in red is the target region of class VI probe Eco621 (position 621 to 638) indicating the loop region of helix 22. Proteins are shown in blue.

For better illustration of the in situ accessibility data of Behrens et al. (2003) in the context of the complex interactions observed in the 3D-structure model, we visualized the RNA interactions with ribosomal proteins on a secondary structure diagram (Figure 3). The protein interaction data used were originally obtained by

FIG. 3. Predicted secondary structures of E. coli 16S rRNA (9). Small numbers indicate sequence numbering, large numbers helix numbering according to Brosius et al. (1981) (7). A. Regions of the 16S rRNA of *E. coli* contacted by the ribosomal proteins S2 to S20 are highlighted. Proteins S1 and S21 are not included. Actual contacts as observed in the crystal structure of the 30S ribosomal subunit of *T. thermophilus* are shown with colored circles around the RNA residues in question. No distinction was made between backbone-only, base-only, and contacts to both backbone and base. B. Distribution of relative fluorescence hybridization intensities of 176 oligonucleotide probes targeting the 16S rRNA of *E. coli*. The different colors indicate different brightness classes (class I: red, through VI: black)

studying the crystal structure of the 30S ribosomal subunit of *T. thermophilus* (6). Based on a sequence alignment, the *T. thermophilus* data was transferred to a 16S rRNA secondary structure model for *E. coli* (Figure 3A) (23). The contacts between the ribosomal proteins and RNA are quite equally distributed throughout the 5', central, and 3' major domains. The 3' minor domain, comprising helix 49 and 50, has very few interactions with proteins (6). The only RNA helices in Figure 3A that have no protein interactions are helix 11, 15, and 38 (numbering according to Brosius et al. 1981 (7)). Nevertheless, probe binding is hindered on the 3' half of helix 11, as well as on the 5' half of helix 38 (brightness class V, Figure 3B). On the other hand, regions with many RNA-protein interactions, such as helix 17 or the 5' end of helix 23, were highly accessible. There is little correlation between the in situ accessibility of probe target regions (Figure 3B) and the extent of interaction of these sites with ribosomal proteins (Figure 3A).

Thus, the 3D model can not explain the differences in probe-conferred hybridization signals. This counterintuitive result may be explained by the fact that the flow cytometric quantification of probe-conferred fluorescence signals by Behrens et al. (2003) was done on paraformaldehyde (PFA) fixed cells (4). Upon treatment with PFA, ribosomes most probably undergo massive conformational changes, including protein denaturation. Formaldehyde is also able to form Schiff bases with the primary amino groups of adenine, guanine, and cytosine, thereby influencing RNA-RNA interactions. Interestingly, the native ribosomal subunits have been described as relatively inaccessible to oligonucleotide hybridization. Bogdanov et al. (1988) tried to identify rRNA regions located on the surface of ribosomal subunits by binding DNA oligonucleotides to the rRNA (5). Binding sites were identified by RNase H hydrolysis. Although the accessibility of the large enzyme RNase H to the bound DNA oligonucleotides might be hindered by the 3D-structure of the 30S subunit in

some cases, Bogdanov et al. (1988) could only identify two regions (positions 8 to 15, and 773 to 782) where oligonucleotides have unhindered access to rRNA in the native 30S ribosomal subunit (5). These regions were also accessible in our study (class II and III probes) (4). The low accessibility of native 30S subunits for oligonucleotide hybridization described by Bogdanov et al. (1988) is contradictory to the relatively high number of class I and II target sites (covering 37% of the whole 16S rRNA sequence) found by Behrens et al. (2003) (4, 5). We therefore conclude that fixation and hybridization must significantly increase probe accessibility to 16S rRNA target sites.

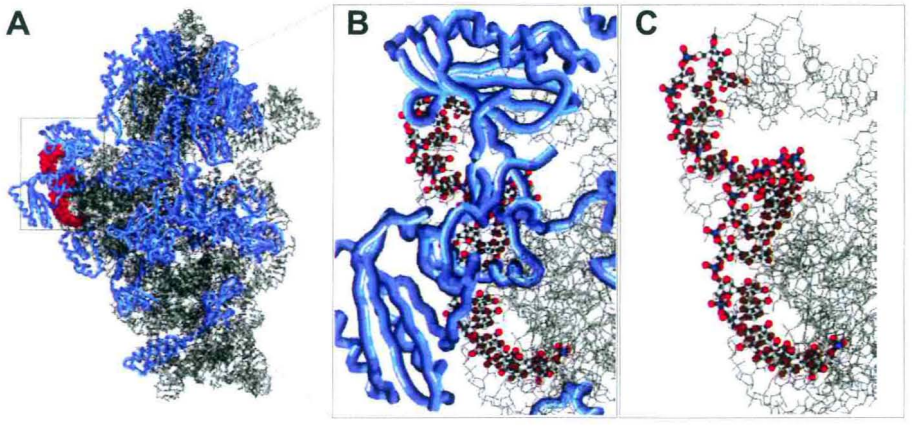

FIG. 4. Detailed view of the transition zone between the 5' end of helix 23 and 24 (helix numbering according to Brosius et al. (1981) (7)) within the 30S ribosomal subunit of *E. coli*. A. Overview of the whole 30S subunit with the region shown on large scale highlighted in red. Proteins are shown as blue tubes. B. Marked as ball and stick model is the target region of probe Eco 668 (position 668 to 685). Figure with ribosomal proteins shown as blue tubes C. The same as B but without ribosomal proteins.

Apart from the changes induced by our FISH protocol, oligonucleotide hybridization itself likely causes massive conformational changes within the ribosome. A 18mer oligonucleotide has a length of 55 Å (Figure 4). The double helix formed by an oligonucleotide of that size bound to its rRNA target has a length of more than 1.5 helix turns (Figure 4C). Taking into account that the 30S subunit has a width of roughly 70 Å, it is clear that hybridization of a 18mer oligonucleotide must result in

enormous distortions of the native ribosome structure. The strong conformational effect of oligonucleotide hybridization was suggested by a study of Fuchs et al. (2000) in which unlabeled helper oligonucleotides were successfully used to increase probe conferred fluorescence signals (12). Inaccessible target sites are likely opened up by conformational changes introduced by hybridization of helper probes (12). This also suggests that a significant proportion of site-specific hindrance may originate in RNA-RNA interactions.

Gutell and coworkers (2002) compared their comparative structure model with the high-resolution crystal structure of the 30S subunit of *T. thermosphilus* in terms of base-base and base-backbone interactions of the 16S rRNA molecule (15). They transformed all tertiary rRNA interactions of both models onto a secondary structure diagram of the 16S rRNA of *T. thermosphilus* (data not shown), and found that most of the intramolecular interactions are located in loop regions. Loop regions have higher sequence conservation than helix regions (23) and play an important role in stabilizing the tertiary fold of rRNA. These regions of complex tertiary RNA interactions cannot be linked to target sites of low probe accessibility in a denatured ribosome, confirming that differences in probe-conferred hybridization signals cannot be predicted from 3D models of the native 30S ribosomal subunit. Site-specific hindrance of probe binding on the level of RNA interactions seems to originate more in intra-helix base pairing than in helix-spanning tertiary rRNA interactions. Long, smooth, helical regions often show limited accessibility compared with short, irregular helices that are interrupted by unpaired nucleotide bulges (Figure 3B).

We performed a limited study to test the influence of different fixation protocols, storage of fixed cells, and SDS in the hybridization buffer on probe accessibility. We quantified the probe-conferred fluorescence intensity for ten Cy3-labeled oligonucleotides after hybridization to differently treated *E. coli* cells (Figure

5). With one exception (Eco645), where the fluorescence intensity is less than 50% for ethanol fixed cells compared to PFA treated cells, there is no clear difference in probe-mediated fluorescence between ethanol- and PFA-fixed of *E. coli* cells. Nor is there a significant difference between cells kept in a mixture of ethanol and 1xPBS and cells stored in 1xPBS buffer. Although ethanol fixation mainly works by dehydration, whereas PFA is able to interact covalently with primary amino groups, both methods seemed to lead to similar denatured states of the ribosome. It is hard to determine what the effect of SDS in the hybridization buffer is.

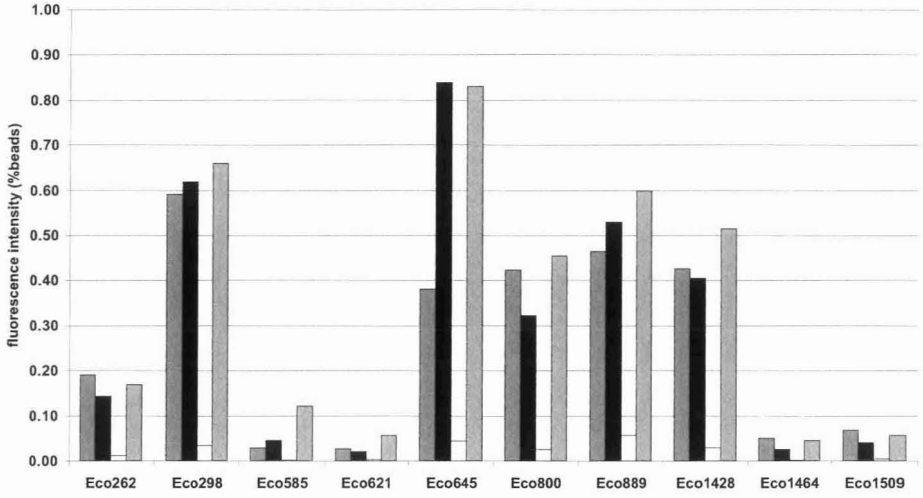

FIG. 5. Comparison of fluorescence intensities of Cy3-labeled oligonucleotides probes hybridized with and without SDS in the hybridization buffer to differently fixed *E. coli* cells. Fluorescence intensity is expressed as percentage of standard beads. Dark gray bars: Ethanol fixation, storage in ethanol/1xPBS mixture, standard hybridization with 0.01% SDS; black bars: PFA fixation, storage in ethanol/1xPBS mixture, standard hybridization with 0.01% SDS; white bars: PFA fixation, storage in ethanol/1xPBS mixture, hybridization without SDS; light gray bars: PFA fixation, storage in 1x PBS, standard hybridization with 0.01% SDS.

Cell wall permeability, protein removal, and/or ribosome folding might all be affected. To optimize FISH for the in situ identification of *Archaea* Burggraf et al. (1994) varied the SDS concentration in hybridization and washing solutions from 0.01 to 1% to achieve better probe penetration through the more rigid archaeal cell walls (8). Rajagopal and coworkers (2002) studied growth of *E. coli* in the presence of 10% SDS (21). They found elevated expression of the ATP-dependent proteases *clpP* and

clpB that enable the cell to get rid of denatured and aggregated proteins in the cytoplasm. Apparently, SDS in the growth medium caused protein misfolding in the cytoplasm. Ribosomal proteins, compared to other RNA-binding proteins, make fewer base-specific interactions and tend instead to interact through salt-bridges between positively charged residues on the protein and phosphate oxygen atoms on the RNA (6). Specific interactions based on shape and charge complementarity will most likely be interrupted in the presence of SDS. This is consistent with our observation that in situ accessibility of the 16S rRNA (4) does not match the rRNA-protein interaction data (6) (Figure 3).

Our conclusions from this and former studies for rational probe design are: (i) The 3D structure of the native small ribosomal subunit is not relevant to probe accessibility, since FISH is performed in a strongly denaturing environment, although the degree of denaturation can be modulated; (ii) the influence of protein-rRNA interactions on target site accessibility can, for the same reasons, generally be neglected; (iii) intra-helix, secondary base interactions are more important than tertiary rRNA-rRNA contacts; (iv) if possible, probes should not be targeted to long, smooth helical regions. However, these regions are among the most variable in the 16S rRNA and therefore of particular interest for the design of specific probes; (v) if targeting these regions is unavoidable, consider the use of helper probes (12).

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Nucleobase-Specific Quenching Effects on Fluorescence In Situ Hybridization with rRNA-Targeted Oligonucleotide Probes

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Manuscript in preparation

Nucleobase-Specific Quenching Effects on Fluorescence In Situ Hybridization with rRNA-Targeted Oligonucleotide Probes

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ABSTRACT

Oligonucleotide probes labeled with fluorescent dyes are used in a variety of in situ applications to detect specific DNA or RNA molecules. It has been described that fluorescence of some dyes is quenched upon probe hybridization in a sequence specific way. Here, we examine the relevance of nucleotide specific quenching for fluorescence in situ hybridization (FISH) to whole fixed cells since previous reports focused on hybridization reactions of fluorescently labeled oligonucleotides to their complementary sequences in solution. A statistical analysis of 113 carboxyfluorescein labeled oligonucleotide probes revealed that probe-conferred fluorescence upon FISH is not quenched but elevated by the presence of cytosine nucleobases at position 2, 3, and 5 close to the probe's 5' end. Guanine nucleotides at position six from the probe's 5' end significantly reduced probe fluorescence intensity upon hybridization. Probes quenched upon hybridization to purified RNA or 30S ribosomal subunits in solution were not quenched upon FISH. We were able to link the significant reduction in nucleotide-mediated quenching upon FISH to the fixation of cells with formaldehyde. The high protein concentration within cells may prevent quenching of probe fluorescence in situ.

INTRODUCTION

Fluorescently labeled, rRNA-targeted oligonucleotide probes are widely used in applied and environmental microbiology for the direct identification of individual cells (2, 9). It has been reported that the fluorescence of some commonly used fluorophores conjugated to oligodeoxyribonucleotides is quenched by interactions between dye and nucleobase (7, 10-12, 14-16, 19, 37). Dye-nucleotide interactions have been confirmed for acridine (14), stilbene (20-22), pyrene (23, 32, 39), oxazine (17, 29), rhodamines (30, 35, 38), coumarins (31), fluoresceins (8, 19, 25, 26, 34,

37), BODIPY derivatives (18, 34), ruthenium complexes (24), and methylene blue (3). Generally dyes that emit in the green and yellow wavelengths are more affected by nucleotide quenching than fluorophores that fluoresce in the blue and red wavelengths. Most of the dyes were quenched by guanine (3, 8, 26, 34, 38, 39). Guanine is the most oxidizable nucleobase and exhibits good electron donating properties (31, 33). The phenomenon of fluorescence quenching is believed to involve a photoinduced electron transfer mechanism between the dye and a nucleotide residue (11, 12, 14, 20, 22, 31, 34, 37). Therefore fluorescence quenching is dependent on the distance between a dye and a guanine.

Fluorophores that are conjugated to the end of single-stranded oligonucleotides can also be quenched upon hybridization to the complementary sequence. It has been reported that hybridization of oligonucleotides labeled with fluorescein at the 5' end to the complementary sequences resulted in a decrease in fluorescence intensity (8, 18, 19, 25, 26, 34). The quenching was attributed to the presence of a guanine in the complementary strand in close proximity to the dye. Torimura et al. reported an 86% quenching ratio for the 5'-fluorescein-labeled oligonucleotide 5'-CCCCCCCCCCTTTTTT upon hybridization to its complement (34). Analogous quenching effects were also described for oligonucleotides labeled with fluorescein at the 3' end (25, 26, 37).

The relevance of nucleotide quenching for fluorescence in situ hybridization (FISH) to whole fixed cells is unknown. Experimental data have hitherto not been systematically evaluated for the effect of nucleotide quenching on FISH with rRNA-targeted oligonucleotide probes. In this study, we therefore studied two forms of nucleotide quenching: (i) sequence-specific variations in probe fluorescence in solution monitored through the ratio of oligonucleotide and dye absorption maxima at 260 and 496 nm; (ii) the 'hybridization-effect', quenching of probe-conferred

fluorescence upon hybridization to its complementary sequence. We statistically analyzed 113 oligonucleotides monolabeled at their 5' end with carboxy-fluorescein for effects of their nucleotide sequence on the fluorescence in solution and upon hybridization to whole fixed *Escherichia coli* cells. Probe sequences and measured fluorescence intensities were adapted from a study of Fuchs et al. (1998) (13). Since nucleotide quenching is distance dependent we mainly focused on the 5' nucleotide adjacent to the label. A set of fifteen newly designed, carboxy-fluorescein labeled probes was used to study fluorescence quenching upon hybridization to purified RNA and isolated 30S ribosomal subunits in vitro. Probes significantly quenched upon hybridization in solution were subsequently used for in situ hybridization with whole fixed *E. coli* cells.

MATERIALS AND METHODS

Microorganisms and fixation. *Escherichia coli*, strain K12, DSM 30083^T (DSM=Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany) was grown as recommended by the strain collection. Cells were harvested in the exponential growth phase (OD_{600nm} ~ 0.5), washed once with 1xPBS (phosphate-buffered saline: 130 mM sodium chloride, 10 mM sodium phosphate buffer, pH 7.2) and fixed with 4% paraformaldehyde as described before (1). Isolated RNA was fixed with 4% formaldehyde for 16 h at 4°C. The fixed RNA was purified by phenol/choroform extraction and ethanol precipitation as described as described in the section 'Isolation of total RNA'.

Probe design, labeling, and quality control. The oligonucleotide probes were those reported in Fuchs et al. (1998) (13). A list containing all probes additionally designed in this study is shown in Table 1. All probes were fully complementary to 16S rRNA sequences of *E. coli*. Each probe was synthesized,

Table 1. Oligonucleotide probes used in this study.

Probe	Sequence 5' -> 3'	Label
Eco647	CTACGAGACTCAAGCTTG	5': 6-FAM
Eco648	TCTACGAGACTCAAGCTT	5': 6-FAM
Eco649	CTCTACGAGACTCAAGCT	5': 6-FAM
Eco650	CCTCTACGAGACTCAAGC	5': 6-FAM
Eco651	CCCTCTACGAGACTCAAG	5': 6-FAM
Eco652	CCCCTCTACGAGACTCAA	5': 6-FAM
Eco653	CCCCCTCTACGAGACTCA	5': 6-FAM
Eco654	CCCCCCTCTACGAGACTC	5': 6-FAM
Eco655	ACCCCCCTCTACGAGACT	5': 6-FAM
Eco656	TACCCCCCTCTACGAGAC	5': 6-FAM
Eco657	CTACCCCCCTCTACGAGA	5': 6-FAM
Eco658	TCTACCCCCCTCTACGAG	5': 6-FAM
Eco659	TTCTACCCCCCTCTACGA	5': 6-FAM
Eco660	ATTCTACCCCCCTCTACG	5': 6-FAM
Eco661	AATTCTACCCCCCTCTAC	5': 6-FAM
Eco654_4A	AAAACCCCCCTCTACGAGACTC	5': 6-FAM
Eco654_3	CCCCCCTCTACGAGACTC	3': 6-FAM

monolabeled at the 5' end with 6-FAM (6-carboxyfluorescein) in the last step of solid phase synthesis, and HPLC purified by ThermoHybaid Interactiva Division GmbH (Ulm, Germany). Aliquots of each probe were analyzed in a spectrophotometer (Beckmann DU530, München, Germany) as described by Behrens et al. (2003) (5).

Preparation of total RNA. Isolation of prokaryotic RNA was performed according to Oelmüller et al. (1990) (27). 25 ml cell suspension were harvested by 5 min centrifugation at 5000 rpm at 4°C in a bench top centrifuge. Cells were washed once in 1 ml ice-cold AE buffer (20 mM sodium acetate, pH 5.5, 1 mM disodium acetate). Cell pellets were frozen in liquid nitrogen and stored at –80°C until further usage. Then pellets were resuspended in 0.5 ml ice-cold AE buffer and immediately transferred to an 2 ml polystyrene tube containing a hot solution of 0.9 ml phenol-chloroform isoamylalcohol (25:24:1 vol) and 10 µl 25% (w/v) SDS. The tubes were shaken at 60°C for 5 min. Afterwards the solution was immediately chilled on ice to 0 - 4°C. The separation of organic and aqueous phases was done by centrifugation at 4°C for 5 min at 13000 rpm in a Biofuge fresco centrifuge (Heraeus Instruments). The

aqueous phase was supplemented with 2 M sodium acetate, pH 5.2, to a final concentration of 0.25 M and repeatedly extracted (1-2 times) with phenol-chloroform-isoamylalcohol (25:24:1 vol), until no interphase was visible any more. Nucleic acids were precipitated after adding 2.5 vol 96% ethanol by incubation for at least 2 h at –20°C. After precipitation nucleic acids were pelleted by centrifugation for 30 min at 13000 rpm at 4°C in a Biofuge fresco centrifuge (Heraeus Instruments). The pellet was washed once with 75% ethanol, centrifuged again for 15 min and dried 20 min at room temperature. Residual DNA was removed using the DNA-*free*™ DNase Treatment & Removal Reagents (Ambion, Inc.), following the manufacturers instructions. An additional phenol-chloroform-isoamylalcohol extraction was done followed by precipitation of the RNA in 96% ethanol. Finally, the RNA was resuspended in 60 µl dH_20 and concentration was determined spectrophotometrically by measuring the absorption at 260 and 280 nm of an 100fold dilution in dH_20. Aliquots of the RNA were stored at –80°C.

Preparation of small ribosomal subunits. Preparation of 30S ribosomal subunits was done as described by Rheinberger et al. (1988) (28).

Fluorescence *in situ* hybridization. Approximately $1x10^8$ fixed cells were hybridized in 100 µl buffer containing 0.9 M sodium chloride, 0.1% sodium dodecyl sulfate, 20 mM Tris-HCl (pH 8.4), and 2 µM of fluorescent probe at 46°C for 3 h (36). Subsequently, cells were pelleted by centrifugation for 3 min at 4,000 x g and resuspended in 100 µl hybridization buffer containing no probe. Washing of the cells was performed for 30 min at 46°C. Subsequently cells were again pelleted by centrifugation for 3 min at 4,000 x g. The pellet was resuspended in 50 µl of the above buffer and measured immediately.

Melting curves of oligonucleotides. The fluorescence intensity was measured for 100 nM fluorescent oligonucleotides in 20 mM Tris-HCl pH 8.4, 0.9 M

sodium chloride, 0.1% sodium dodecyl sulfate, and the concentration of total RNA or 30S ribosomal subunits used for duplex formation was 0.5 µM. Melting curves of fluorescent oligonucleotides and duplexes were measured on ABI PRISM 7700 in 50 µl of the above buffer using the following protocol: 25°C for 2 min, 95°C for 2 min, then decreasing the temperature to 25°C within 9 min, incubation at 25°C for 2 min, then increasing the temperature to 95°C in 9 min. (26). Melting curves on whole fixed cells were measured, after in situ hybridization with the following program: 25°C for 2 min, then increasing the temperature to 95°C within 20 min, 95°C for 2 min. For accurate comparison of the fluorescence of single-stranded oligonucleotides versus corresponding duplexes at room temperature, melting curves were normalized at 93-95°C. At this temperature the fluorescence of the single-stranded oligonucleotide probes became equal to the fluorescence of the corresponding duplex, because duplexes are completely melted. No matter whether total RNA, 30S ribosomal subunits, or whole fixed *E. coli* cells were used as hybridization targets the normalization procedure excludes variations in fluorescence readings between different wells and plates therefore permitting the comparison of fluorescence values between different experimental setups at 25°C.

RESULTS

A statistical analysis of sequence-specific variations in probe fluorescence in solution, monitored through the ratio of oligonucleotide and dye absorption maxima at 260 and 496 nm, is in preparation. Preliminary results have shown no direct correlation between the nucleotide sequence of the probe's 5' end and the quotient of the dye absorption maxima at 260 and 496 nm.

In 1998 Fuchs and coworkers quantified the fluorescence signals conferred by 171 carboxyfluorescein-labeled oligonucleotides targeting the 16S rRNA of *E. coli*

(13). The fluorescence signals of 10,000 cells hybridized with a single probe were recorded by a flow cytometer and signal intensities were interpreted as probe target site accessibility. We statistically reevaluated a subset of 113 of these probes (domains II and III) for sequence-specific effects on the probe-conferred fluorescence. Since nucleotide-based quenching depends on the distance between nucleobase and fluorophore the statistical analysis focused on the six nucleotides in close proximity to the labeled 5' end of the oligonucleotide (Figure 1, white rectangles). The influence of the probe target sequence (Figure 1, black rectangles) as well as of bases downstream of (3' to) the probe target sequence (Figure 1, gray rectangles) was also investigated.

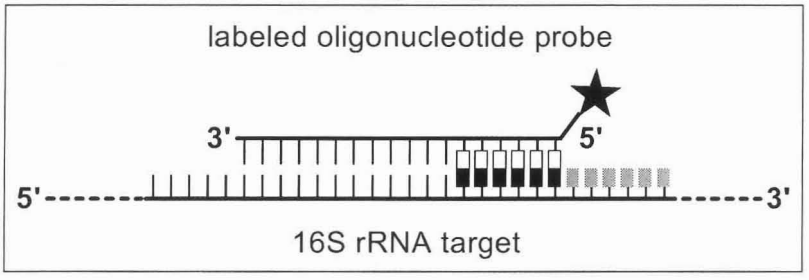

FIG. 1. Schematic representation of a labeled oligonucleotide probe binding to its complementary sequences on the 16S rRNA. The black star indicates a fluorescent dye conjugated to the 5' end of the probe via a short carbon linker. The six bases adjacent to the 5' end of the probe are highlighted by white boxes. The complementary bases on the target site are indicated by black rectangles. The six bases downstream of (3' to) the probe target site are shown as gray quadrangles. The probe is named after its 3' position on the 16S rRNA.

We applied an analysis of variance to determine the effect of each of the six nucleotide-positions adjacent to the oligonucleotide 5' end on the probe-conferred fluorescence upon hybridization to whole fixed *E. coli* cells (Table 2). Our statistical test revealed that the nucleotide composition in close proximity to the labeled 5' end clearly affects probe-conferred fluorescence. But the described effects are not homogeneous. Effects could only significantly been proven for position 2, 3, 5 and 6 (counted from the 5' end of the probe) indicated by P values < 0.05. The nucleobases directly at the 5' end (position 1) or on position 4 seem to have no significant

influence on the probe fluorescence upon hybridization ($P > 0.05$). On position 2, 3 and 5 cytosine has a significantly positive effect on the probe-conferred fluorescence. A negative influence on the probe fluorescence could only significantly be assigned to the presence of a guanine nucleotide at the sixth position from the labeled 5' end.

Table 2. Results of the statistical analysis of variance (ANOVA) on the effect of the nucleotide composition at the 5' end of carboxyfluorescein labeled oligonucleotides on the probe-conferred fluorescence upon FISH. All values are expressed in relation to Thymine. The 'intercept value' is the estimated fluorescence intensity when positions 1 to 6 are occupied by Thymine nucleotides. The 'Estimate values' describe the change in fluorescence intensity when other bases than Thymine occur at the corresponding position. Whether the effects of two bases are different from one another, can be read from their 'Confidence Limits'. If the 'Conficence Limits' overlap the difference between the bases is not significant. Do the 'Confidence Limits' comprise zero, the effects of the bases at these position are not significantly different from zero.

Parameter	Nt	Estimate	Error	T Value	Pr > |t|	95% Confidence Limits	
Intercept		10.99	3.68	2.98	0.0031	3.74	18.24
NtPos1	A	-3.53	2.24	-1.58	0.1154	-7.93	0.87
	C	-0.78	2.06	-0.38	0.7040	-4.84	3.27
	G	3.82	2.33	1.64	0.1023	0.77	8.41
	T	0.00	-	-	-	-	-
NtPos2	A	0.95	2.25	0.42	0.6727	-3.47	5.37
	C	7.07	2.03	3.48	0.0006	3.08	11.07
	G	3.20	2.07	1.54	0.1234	0.88	7.28
	T	0.00	-	-	-	-	-
NtPos3	A	-1.14	2.44	-0.47	0.6413	-5.94	3.66
	C	7.90	2.12	3.72	0.0002	3.72	12.08
	G	0.23	2.15	0.11	0.9141	-4.00	4.46
	T	0.00	-	-	-	-	-
NtPos4	A	1.53	2.53	0.60	0.5459	-3.45	6.51
	C	2.34	2.00	1.17	0.2428	-1.60	6.28
	G	0.61	2.21	0.27	0.7845	-3.75	4.96
	T	0.00	-	-	-	-	-
NtPos5	A	0.66	2.07	0.32	0.7488	-3.41	4.74
	C	5.62	2.00	2.81	0.0053	1.68	9.56
	G	-2.40	2.05	-1.17	0.2441	-6.45	1.65
	T	0.00	-	-	-	-	-
NtPos6	A	-0.01	2.21	-0.00	0.9976	-4.05	4.04
	C	-0.41	1.96	-0.21	0.8328	-4.26	3.44
	G	-7.06	2.21	-3.20	0.0015	-11.40	-2.72
	T	0.00	-	-	-	-	-

To experimentally determine the degree of nucleotide quenching for FISH we designed fifteen oligonucleotide probes complementary to helix 23 of the *E. coli* 16S rRNA (helix numbering according to Brosius et al. 1981 (6)). The probes Eco647 to Eco661 (Table 1) cover the target positions 647 to 678 comprising a region where six guanine nucleotides cluster between positions 666 and 671. Oligonucleotide binding to this region should result in quenching of the probe conferred-fluorescence if the

guanine nucleotides on the target strand will be brought in close proximity to the labeled 5' end of the probe during hybridization.

The oligonucleotides were hybridized to an excess of purified RNA, isolated small ribosomal subunits, or whole fixed *E. coli* cells. Melting curves were measured on an ABI PRISM 7700 and the normalized fluorescence at 25°C was calculated for single-stranded oligonucleotides and duplexes. Figure 2 shows the relative fluorescence signals measured for the single-stranded oligonucleotides and for duplexes formed upon hybridization to the different targets. We observed a decrease in fluorescence intensity for probes with increasing number of cytosine nucleotides at the labeled 5' end upon hybridization to purified RNA or 30S ribosomal subunits. For probe Eco654, with six cytosine nucleotides at the 5' end (Table 1), the reduction in fluorescence was 27% as compared to the fluorescence of the single stranded probe. For nearly all probes hybridized to whole fixed *E. coli* cells, no reduction in fluorescence upon duplex formation was measured. Actually, Eco654 and Eco661 showed a slight increase in fluorescence intensity upon hybridization to whole fixed cells compared to signals obtained for the single stranded oligonucleotides.

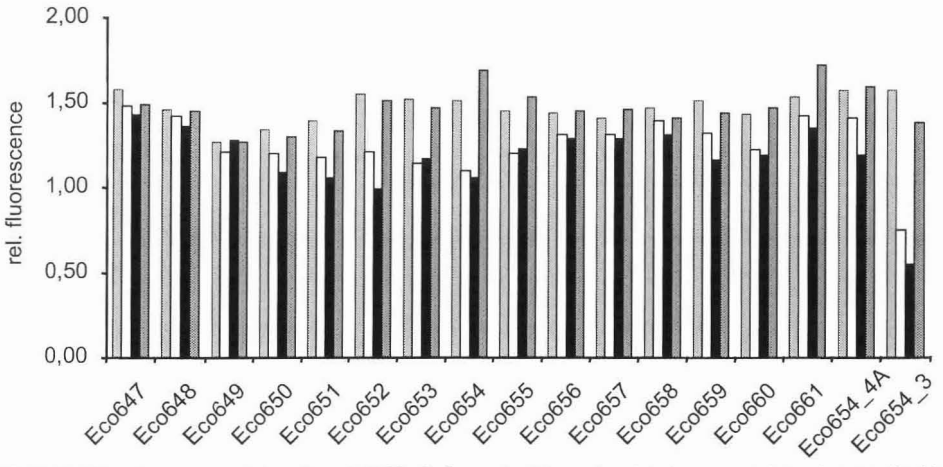

FIG. 2. Relative fluorescence intensities at 25°C of oligonucleotide probes labeled with carboxy-fluorescein. Light gray bars: 100 nM probe in hybridization buffer. White bars: probes hybridized to purified RNA. Black bars: probes hybridized to isolated 30S ribosomal subunits. Dark gray bars: probes hybridized to whole fixed *E. coli cells* (in situ hybridization).

Melting curves of Eco654, Eco654_4A, and Eco654_3. Probe Eco654 was hybridized to purified RNA, isolated 30S ribosomal subunits, and whole fixed cells. Melting curves of the three duplexes are presented in Figure 3. The decline in fluorescence with increasing temperature reflects the general effect of temperature-based fluorescence quenching (Figure 3 A). A decrease in fluorescence intensity upon hybridization has been observed, when purified RNA or small ribosomal subunits are used for duplex formation (Figure 3 B and C). For in situ hybridization with whole fixed *E. coli* cells a slight increase in fluorescence was recorded (Figure 3 D).

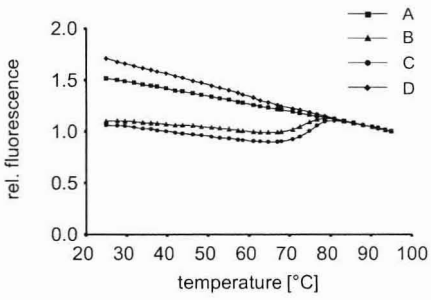

FIG. 3. Melting curves of the fluorescein-labeled oligonucleotide Eco654 hybridized to different targets. (A) 100 nM probe without target. (B) Probe hybridized to isolated RNA. (C) Probe hybridized to prepared 30S ribosomal subunits. (D) Probe hybridized to whole fixed *E. coli* cells (FISH).

Figure 4 shows melting curves of duplexes formed upon hybridization of probe Eco654, Eco654_4A, and Eco654_3 to isolated RNA. The reduction in fluorescence upon hybridization is expressed as percentage of the fluorescence intensity measured for the single-stranded oligonucleotide at 25°C (Figure 4A). The decrease in fluorescence for the 3'-labeled probe Eco654_3 was 50% (Figure 4 D). The fluorescence of the 5'-labeled oligonucleotide Eco654 was reduced by 27% as previously mentioned. Probe Eco654_4A carries a four-adenine nucleotide extension at the 5' end, separating dye and cytosine-rich 5' end of the original probe (Eco654)

by approximately 30 Å. The decrease in fluorescence at 25°C for probe Eco654_4A was only 7% upon hybridization (Figure 4 C).

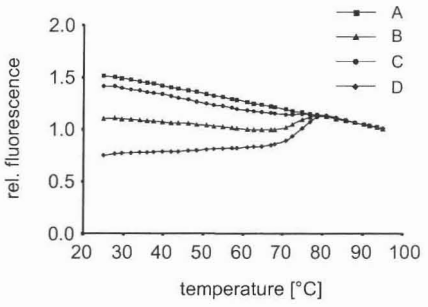

FIG. 4. Melting curves of carboxy-fluorescein-labeled oligonucleotides. (A) Single-stranded probe Eco654 labeled at the 5' end. (B) Probe Eco654 labeled at the 5' end upon hybridization to an excess of purified RNA. (C) Probe Eco654_4A labeled at the 5' end upon hybridization to an excess of isolated RNA. The probe was designed with four additional adenine nucleotides extending the regular sequence at the 5' end. (D) Probe Eco654_3 labeled at the 3' end upon hybridization to purified RNA.

We observed that probes that are strongly quenched upon hybridization in solution are not quenched during FISH, e.g. Eco654. To investigate the effects of whole fixed cells that might compensate for the observed fluorescence quenching in vitro, we recorded melting curves of probe Eco654 in the presence of a high protein concentration and upon hybridization to formaldehyde fixed, isolated RNA. In the presence of 50 µg bovine serum albumine (BSA) the quenching of probe Eco654 upon hybridization to purified RNA was decreased (Figure 5 C).

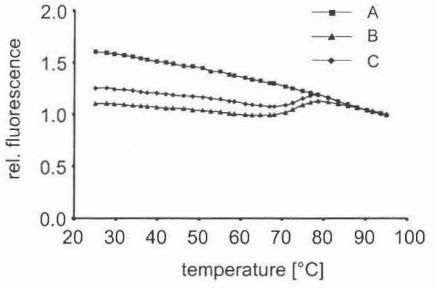

FIG. 5. Melting curves of the fluorescein-labeled oligonucleotide Eco654 hybridized to purified RNA in the presence and absence of an excess of bovine serum albunine (BSA). (A) 100 nM probe without target. (B) Probe hybridized to isolated RNA. (C) Probe hybridized to isolated RNA in the presence of 50 µg BSA.

The reduction in fluorescence compared to the single stranded probe at 25°C (Figure 5 A) was only 17%, whereas in the absence of BSA a fluorescence quenching of 27% (Figure 5 B) was observed.

Also formaldehyde fixation of purified RNA affected the probe conferred-fluorescence upon hybridization (Figure 6 C). In comparison to none hybridized probes the fluorescence intensity of probe Eco654 at 25°C was decreased by 23% upon hybridization to formaldehyde treated RNA. Together, the presence of high amounts of proteins and the formaldehyde fixation could prevent at least 50% of the nucleotide mediated quenching of probe Eco654 upon hybridization to purified RNA in solution.

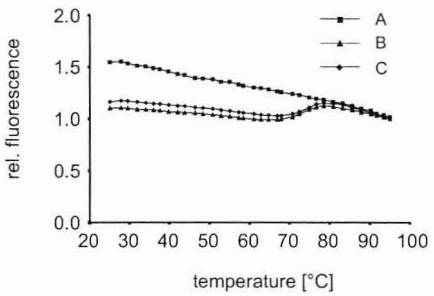

FIG. 6. Melting curves of the fluorescein-labeled oligonucleotide Eco654 hybridized to formaldehyde treated RNA. (A) 100 nM probe without target. (B) Probe hybridized to isolated RNA. (C) Probe hybridized to formaldehyde fixed RNA.

DISCUSSION

We statistically analyzed a set of 113 fluorescein-labeled oligonucleotide probes for indications of sequence-dependent effects on the probe-conferred fluorescence upon hybridization to whole fixed *E. coli* cells (13). We focused our attention on the sequence composition of the probe's 5' end in close proximity to the conjugated dye. The statistical test revealed that the probe-conferred fluorescence is directly dependent on the nucleobases present at position 2, 3 and 5 of the probe. But only the increase in probe fluorescence intensity by the effect of cytosine nucleotides at

these positions was significant. An elevated probe fluorescence mediated by the occurrence of cytosines at position 2, 3 and 5 might be due to the presence of guanines on the complementary strand. Nazarenko et al. (2002) have shown that probe-conferred fluorescence intensity can increase or decrease upon hybridization of the labeled strand to its complement depending on the sequence and position of the fluorophore (26). They describe that the increase in fluorescence upon hybridization depends on the presence of at least one guanine within the distance of 4 nucleotides close to the dye. For adenine at position 3 and guanine at position 5 a negative effect on the probe-conferred fluorescence was estimated. But the statistical analysis revealed with a high probability that these effects occurred just by chance. However, probe fluorescence is significantly quenched, if guanine is present six nucleotides from the probe's 5' end. To confirm the significance of the results obtained for the six nucleotides adjacent to the probes's 5' end the statistical analysis should be extended to probe positions 7 to 12 and 13 to 18.

The statistical analysis has shown that an effect of the probe sequence on the fluorescence of the conjugated dye exists. Nevertheless, the mathematical analysis of variance should be extended on the nucleotide composition of the probe target site and on the six positions immediately downstream of the probe target site (Figure 1) to obtain more information on the relevance of nucleotide-specific quenching for FISH. However, our experiments did not reveal evidence for quenching caused by the presence of guanine nucleotides immediately 3' to the probe target site. A series of six guanine nucleotides downstream of the probe Eco648 target site seemed to have no effect on the probe-conferred fluorescence. On the other hand, influences on the probe fluorescence resulting from differences in probe target site accessibility cannot be excluded for the used probe data set. Furthermore, the applied statistical

analysis does not consider the possibility of combined nucleobase effects of adjacent nucleotides on the probe fluorescence upon hybridization.

We intentionally designed fifteen carboxyfluorescein-labeled oligonucleotides targeting a region on the 16S rRNA of *E. coli* where six guanine nucleotides cluster between positions 666 and 671 (Table 1). Upon hybridization to purified RNA and isolated 30S ribosomal subunits we observed a decrease in probe-conferred fluorescence with increasing numbers of cytosines at the probe's 5' end (Figure 2). Probe Eco654, with six cytosine nucleotides at the six positions directly adjacent to the label carrying 5' end, was quenched up to 27% compared to the single stranded oligonucleotide at 25°C (Figure 3). Isolated 30S ribosomal subunits reacted like purified RNA and quenched probe fluorescence upon hybridization indicating that ribosomal proteins do not affect nucleotide quenching in solution. This is not astonishing since the presence of SDS in the hybridization buffer will probably disintegrate protein-protein and protein-RNA interactions based on shape or charge complementarity. This is contradictory to our findings that hybridization of probe Eco654 to purified RNA in the presence of 50 µg of bovine serum albumine resulted in a decreased quenching of 20% (Figure 5 C). Thus, high amounts of denatured proteins seem to partially prevent nucleotide quenching upon hybridization.

Probes quenched upon hybridization in solution were not quenched in FISH with whole fixed *E. coli* cells (Figure 2). For probe Eco654 that revealed the highest quenching ratio upon hybridization in solution, no decrease in probe fluorescence upon in situ hybridization was observed (Figure 3). On the contrary, a slight increase in probe-conferred fluorescence at 25°C compared to the single stranded oligonucleotide has been measured. This fact is supported by our statistical analysis of the nucleobase composition of probes in close proximity to the conjugated dye on their in situ conferred fluorescence. For the occurrence of cytosines at position 2, 3

and 5 from the probe's 5' end, an increase in probe fluorescence upon hybridization to whole fixed *E. coli* cells has been confirmed.

FISH is performed on paraformaldehyde (PFA) fixed cells. PFA is able to form Schiff bases (imines) with the primary amino groups of adenine, guanine, and cytosine. Reactions of paraformaldehyde with secondary amino groups resulting in the formation of enamines should also be possible. The excellent electron donating properties of nucleobases, responsible for the quenching of fluorescent dyes, will most likely be altered upon reaction with PFA. We have shown that the treatment of purified RNA with formaldehyde resulted in a decrease in quenching upon hybridization of probe Eco654 to its complement (Figure 6 C).

The influences of nucleotides brought in close proximity to the dye by tertiary folding of the rRNA target molecule might affect probe fluorescence as well. Torimura et al. (2001) confirmed that the microenvironmental location of a modified dye in the DNA helix is important to understand quenching phenomena based on photoinduced electron transfer in a DNA probe (34). The environment formed by the tertiary folding of the rRNA in situ might influence dye fluorescence in addition to the primary nucleotide sequence of the probe or its target site. Behrens and coworkers (2003) have shown that the native three-dimensional structure of the small ribosomal subunit is not a suitable model for predicting accessibility of probe target sites because fixation and hybridization conditions result in a denatured stage of the ribosome (4). Therefore, a 3D model of the 30S ribosomal subunit does not help to explain potential higher-order structure effects on dye signal intensity in situ.

The extent of fluorescence quenching upon hybridization is greatly dependent on the position of the fluorophore within the oligonucleotide sequence (26). The fluorescence intensity of probe Eco654_3, labeled with fluorescein at the 3' end, was only 50% of that of the single-stranded probe and therefore a quarter higher than the

fluorescence quenching measured for the same sequence carrying the label at the 5' end (probe Eco654; Figure 3 B and D). The differences in the measured quenching ratio for probe Eco654 and Eco654_3 might depend on the different linker groups used for 5' and 3' labeling. Whereas the 5' end was labeled with phosphor amidite derivatives of the fluorophore, 3' labeling was done with succinimidyl ester derivatives of the dye. Independently from the labeling site both probes were not quenched upon FISH.

Zahavy and coworkers (1999) reported that photoinduced electron transfer takes place over distances up to 40 Å (39). In fact, we were able to show that most of the fluorescence quenching of probe Eco654 could be prevented by simply adding four adenine nucleotides to the 5' end (Eco654_4A; Figure 4 C), although the length of the non-hybridizing four adenine nucleotide-linker corresponds to approximately only 12 Å. A preliminary study of Nazarenko et al. (2002) on the fluorescence polarization of fluorescein upon duplex formation gives reasons for the assumption that the mobility of the fluorophore in the quenched state is decreased (26). Using a longer linker to connect dye and end-standing nucleotide would result in an elevated freedom of movement (motion) of the dye molecule yielding higher fluorescence intensities.

The statistical analysis and experiments presented in this study have shown that the nucleotide composition in close proximity to the dye affects fluorescence intensity of carboxyfluorescein labeled oligonucleotide probes upon hybridization. However, nucleotide-based quenching of probe-conferred fluorescence could only be confirmed for hybridization reactions in solution with isolated nucleic acids. We were able to show, that formaldehyde fixation and high concentrations of intracellular proteins might compensate nucleotide quenching upon FISH. At the current state of the study that needs to be extended we can not generally exclude nucleotide

quenching for FISH, but we are gaining more and more evidence that the nucleotide sequence effects on the probe fluorescence upon FISH can be neglected.

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5

The Identification of Microorganisms by Fluorescence In Situ Hybridization

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The identification of microorganisms by fluorescence *in situ* hybridisation

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Fluorescence *in situ* hybridisation (FISH) with rRNA-targeted oligonucleotide probes facilitates the rapid and specific identification of individual microbial cells in their natural environments. Over the past year there have been a number of methodological developments in this area and new applications of FISH in microbial ecology and biotechnology have been reported.

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Abbreviations

DGGE	denaturing gradient gel electrophoresis
FISH	fluorescence *in situ* hybridisation
PCR	polymerase chain reaction
PNA	peptide nucleic acid
rRNA	ribosomal RNA
SRB	sulphate-reducing bacteria

Introduction

A method for the rapid and specific identification of individual microbial cells within their natural environments has been long awaited. Most microorganisms have very limited morphological detail, preventing the visual identification possible with higher animals and plants. Traditional cultivation methods are time-consuming and frequently only work for a minority of the bacterial species present in a sample. In recent years, molecular biological methods have extended our view to those microorganisms that have proved impossible to culture. Techniques based on the polymerase chain reaction (PCR) now facilitate the rapid and sensitive detection of bacteria independent of whether or not they can be cultured; however, these techniques provide only limited information on the number and spatial distribution of microorganisms. There was therefore a need for a microscopy technique similar to that of the famous Gram-staining method. The test needed to be as sensitive as the well-established immunofluorescence techniques [1], but instead of targeting antigens would be based on nucleic acids. The comparative analysis of homologous nucleic acid sequences, most notably of ribosomal RNA (rRNA) molecules and the genes encoding them, has over the past 25 years profoundly changed our view of microbial systematics [2]. Large databases of sequence information exist, and rRNA gene fragments are today routinely retrieved without prior cultivation. Since their first application as 'phylogenetic stains' in 1989 [3], fluorescently labelled, rRNA-targeted oligonucleotide probes have become a common tool for the direct, cultivation-independent identification of individual bacterial cells. Fluorescence *in situ* hybridisation (FISH) with rRNA-targeted oligonucleotide probes has been developed for the *in situ* identification of individual microbial cells and is now a well-established technique.

A detailed account of the development of FISH methods in microbial ecology has been published previously [4]. This review will focus on methodological improvements and applications of FISH in microbial ecology and biotechnology over the past year. Updated information on medical applications is available in a recent review by Moter *et al.* [5•]. More information on *in situ* nucleic acid amplification and flow cytometric analysis can be found elsewhere [6].

Methodological aspects of FISH

rRNAs are the main target molecules for FISH for several reasons: they can be found in all living organisms; they are relatively stable and occur in high copy numbers (usually several thousand per cell); and they include both variable and highly conserved sequence domains [4,7]. Signature sequences unique to a chosen group of microorganisms, ranging from whole phyla to individual species, can therefore be identified by comparative sequence analysis. Bacteria and archaea contain 5S, 16S, and 23S rRNAs with lengths of approximately 120, 1500 and 3000 nucleotides, respectively. In the vast majority of applications FISH probes target 16S rRNA. The public databases now include 16S rRNA sequences for most cultured microbial species, as well as numerous sequences directly retrieved from the environment [8••,9••]. Probes are designed using sequence information from these databases and program packages such as ARB [10•,11•].

A typical FISH protocol includes four steps: the fixation and permeabilisation of the sample; hybridisation; washing steps to remove unbound probe; and the detection of labelled cells by microscopy or flow cytometry. Detailed descriptions of this procedure, which can be completed within a few hours, are available elsewhere [12]. FISH is fully compatible with direct count methods [13,14].

The oligonucleotide probes used in FISH are generally between 15 and 30 nucleotides long and covalently linked at the 5′-end to a single fluorescent dye molecule. Common fluorophors include fluorescein, tetramethylrhodamine, Texas red and, increasingly, carbocyanine dyes like Cy3 or Cy5 [15]. The carbocyanine dyes have greatly increased the sensitivity of FISH [13], but further improvements are still needed. The microorganisms living in oligotrophic environments, such as the open ocean, are typically small with low

cellular rRNA content. The profound influence of cellular growth rate and nutritional status on cell detection by FISH has been described [4]. A recent study on this topic focused on *Rhodopseudomonas palustris* [16].

Polynucleotide probes consisting of nearly full-length 16S and/or 23S rRNA genes and each labelled with several fluorochrome molecules were shown to detect almost all cells present in marine bacterioplankton. However, probes of this size contain many conserved regions and are only able to discriminate between distantly related groups, such as the bacteria, crenarchaeota and euryarchaeota [17••]. Shorter polynucleotide probes that target a defined variable region of approximately 250 nucleotides of the 23S rRNA have been shown to allow differentiation among genera [18]. Oligonucleotide probes can be labelled at both the 5′- and 3′-end, and/or several probes applied simultaneously, thereby targeting each rRNA molecule with several fluorophores [19,20].

There have also been attempts to improve FISH detection by boosting cellular ribosome content before fixation. Samples are preincubated in a cocktail of substrate and antibiotics, which should in theory result in cell activation and rRNA synthesis without cell division. In drinking water [21], over 50% of all bacteria detected could be affiliated to the β-subclass of proteobacteria. Improved fluorescence signals were also reported for an oligotrophic cooling water system after pretreatment with glucose and chloramphenicol [22]. The problem with this approach is the inevitable selectivity of substrates and antibiotics.

Peptide nucleic acid (PNA) molecules are uncharged DNA analogues that bind to nucleic acids much more strongly than oligonucleotides because there is no electrostatic repulsion between the PNA probe and the negatively charged sugar–phosphate backbone of the target molecule [23,24]. Fluorescently labelled PNA molecules could substitute for oligonucleotides. First applications with marine picoplankton [25] and tap water samples [26], however, showed only small improvements. At this time, high prices and specificity problems are slowing the application of PNAs to FISH.

Low cellular rRNA content and a strong variation in the accessibility of 16S rRNA probe target sites has also been described [27]. Unlabelled oligonucleotides, so-called helpers, that bind adjacent to the probe target site were shown to increase target accessibility in *Escherichia coli* [28•]. It was suggested that the helpers open higher-order structures in the rRNA, which otherwise hinder probe binding. A globally abundant group of small actinobacteria could only be reliably detected when helpers were added to the hybridisation buffer [29•]. Accessibility studies were recently extended to the 23S rRNA molecule of *E. coli* [30•].

Applications

The first applications of FISH were in less diverse systems: for example, the identification of bacterial symbionts *in situ* or the identification of magnetotactic bacteria that could be physically removed from the environment before hybridisation. Over the past couple of years, more and more applications to complex samples, such as activated-sludge or soil, have been published. This trend continues.

Marine environments

Several studies have investigated the microbial community structure of marine sediments. Based on a FISH protocol initially applied to samples from intertidal mud flats in the North Sea [31], the sulphate-reducing bacteria (SRB) in a marine Arctic sediment (from Spitzbergen, Norway) were simultaneously studied by FISH and rRNA slot-blot hybridisation using group-specific and genus-specific 16S rRNA-targeted oligonucleotide probes [32•]. Both methods used for the quantification of bacterial populations showed comparable results.

FISH was also successfully used for the visualisation of a hypothetical microbial consortium that catalyse the anaerobic oxidation of methane in marine sediments [33••]. Recent data had suggested an involvement of archaea and SRB in this process [34,35]. Indeed, microscopic evidence was obtained for a structured consortium in which a core of archaea is surrounded by a layer of SRB.

Additional studies have investigated marine bacterioplankton. A strong discrepancy between cell counts using direct microscopy, which usually yields counts of around 10^9 cells ml^{-1}, and the number of colony forming units detected on agar plates has long been described. In a study on North Sea bacterioplankton [36•], the species isolated on defined oligotrophic medium were compared to the sequences identified in a 16S rDNA clone library. FISH subsequently showed that most of the cultured strains were not abundant in the North Sea, whereas dominant groups did not grow on the culture medium used. Similar findings were obtained by a new combination of flow cytometric analysis, flow sorting, and subsequent molecular analysis of sorted cells by FISH and denaturing gradient gel electrophoresis (DGGE) [37•]. Even during a short incubation, the community structure of marine bacterioplankton shifted from the natural high abundances of members of the *Cytophaga–Flavobacterium* cluster towards rapidly growing α and γ proteobacteria.

Several studies have used FISH together with microautoradiography, this combined approach allows *in situ* identification and provides information on substrate utilisation in complex microbial communities [38,39]. Evidence was presented that free-living planktonic marine archaea are involved in the heterotrophic uptake of dissolved amino acids from aerobic seawater [20]. A similar study was carried out on the consumption of low and high molecular weight dissolved organic matter by marine bacterioplankton. The study found that the role of aerobic heterotrophic bacteria in carbon cycling would be more accurately described by using three groups instead of the

single bacterial compartment currently used in biogeochemical models [40•]. Microautoradiography and FISH were also combined to investigate the carbon metabolism of uncultured freshwater bacteria of the genus *Achromatium* [41•].

Limnology

In the field of limnology, the chemocline of the meromictic Lake Cadagno has been thoroughly studied [42–44]. FISH was used to study bacterial diversity, community composition, and seasonal population dynamics [42,43] and for an *in situ* analysis of SRB related to *Desulfocapsa thiozymogenes* [44].

There have also been several studies of river systems. The microbial community of bacterial aggregates (river snow) from the river Elbe was characterised using a combination of traditional cultivation techniques and FISH [45•]. In the United States, FISH was used to monitor the bacteria and protozoa occurring along the Ogeechee river [46]. A study of aggregates in the river Weser, North Germany, combined radiotracer methods with microsensor techniques to measure bacterial production and respiration [47]. The growth efficiency of the microbial communities attached to the aggregates could therefore directly be determined. The communities were further characterised by FISH. Changes in bacterial production and respiration were correlated to concurrent changes in the species composition.

Wastewater treatment

In the past, FISH has played a major role in the identification of abundant microorganisms in wastewater treatment plants (e.g. [48]). Recent research has focused on assigning key processes to groups of microorganisms identified *in situ* by FISH. In this respect, members of the β2 group of proteobacteria have been linked to enhanced biological phosphate removal [49•]. Similarly, the correlation of the nitrifying activity of a fluidised-bed reactor and changes in the composition of the bacterial community were closely followed using a set of FISH probes [50]. Numerous other examples of the use of FISH in wastewater treatment have been reported. For instance, an industrial bioremediation system designed for the removal of phenolic compounds was shown by FISH to be dominated by members of the *Cytophaga–Flavobacterium* cluster and γ proteobacteria. Of these two groups, only the latter was positively correlated with phenol degradation [51]. In a trickling filter biofilm, a novel group of planctomycetales capable of the anaerobic oxidation of ammonium was detected by screening with a set of FISH probes [52•]. Quantitative FISH was used to examine the relationship between foaming and the concentration of mycolic-acid-containing actinomycetes in completely mixed activated-sludge plants [53]. Newly designed probes have been used for the specific identification of filamentous bacteria of Eikelboom type 021N [54••].

Microsensors allow chemical and physical gradients to be monitored on the microscopic scale. The benefits of combining FISH and microsensors for assigning activities to defined bacterial populations have been outlined before [55]. In a nitrifying bioreactor this approach allowed cell-specific activities to be assigned to *Nitrosospira* spp. and uncultured *Nitrospira*-like bacteria in the dense bioreactor aggregates for the first time [56].

FISH was recently applied to the study of microbial communities in various acid mine environments in context of the prevailing geochemical and mineralogical conditions [57]. The technique facilitated the *in situ* identification of new species of acidophilic archaea that is an important contributor to acid mine drainage [58].

Symbioses

Symbiotic microorganisms are often very difficult to obtain in pure culture. FISH therefore continues to be an important tool for the identification of plant- or animal-associated microorganisms; the technique also reveals the location of these microorganisms in the host. FISH was applied to the detection of intracellular bacteria in the buds of Scotch pine [59]. Likewise, the method provided evidence for the presence of nitrogen-fixing bacteria in sugarcane [60], and was used to study early root colonisation of sugar beet seedlings in inoculated and native soil [61]. FISH was also used to study the spatial arrangement of bacteria in sponge tissues [62]. Studies on gall-forming bacteria in marine algae [63], endosymbionts of acanthamoebae [64•], and the microbial gut flora of wood-eating termites [65] comprise just a few more examples of the many applications of FISH in this field.

New probes

Many publications have described the design, testing and application of new probes for the detection of a wide range of microorganisms: Gram-positive SRB of the *Desulfotomaculum* group [66]; the acidophilic *Acidiphilium* and *Thiobacillus* [67]; subgroups of the *Cytophaga-Flavobacterium* cluster [68]; methane-oxidising bacteria [69]; toxigenic bacteria associated with a dinoflagellate [70]; and the bacteria and archaea dwelling in hypersaline ponds of solar salterns [71,72•]. Probes are only as good as the database used for their design; therefore, there is a constant need for probe re-evaluation. For example, probe EUB338, routinely used to quantify members of the domain bacteria, was recently supplemented by two probes, EUB338-II and EUB338-III, which target groups of bacteria missed by EUB338 [73].

Conclusions

FISH using rRNA-targeted probes is the method of choice for all studies in which exact cell numbers and cellular locations need to be determined. The methodology is being continuously improved. So far, however, microscopic analysis by FISH has not been automated sufficiently to allow high sample throughput, which would be desirable in many ecological investigations [74]. Accurate quantification still remains a challenging task and each new study needs careful controls. Further method development is therefore needed with respect to FISH sensitivity and automation.

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Part III:

Appendix

A List of Oligonucleotide Probes

Sequences and relative fluorescence intensities of probes targeting the 16S rRNA of *Escherichia coli*

No.	Probe name	*E.coli* position[a] 5'	3'	Probe sequence (5'->3')	Relative probe fluorescence[b]	Brightness class[c]
1	Eco1	1	19	TGATCAAACTCTTCAATTT	0.68	II
2	Eco13	13	31	CAATCTGAGCCATGATCAA	0.58	III
3	Eco20	20	37	AGCGTTCAATCTGAGCCA	1.15	I
4	Eco32	32	47	GCCTGCCGCCAGCGTT	0.54	III
5	Eco38	38	54	GTGTTAGGCCTGCCGCC	0.55	III
6	Eco48	48	65	TCGACTTGCATGTGTTAG	0.54	III
7	Eco55	55	72	TTACCGTTCGACTTGCAT	0.74	II
8	Eco66	66	83	GCTGCTTCCTGTTACCGT	0.77	II
9	Eco73	73	90	GCAGCAAGCTGCTTCCTG	0.27	IV
10	Eco84	84	101	TCGTCAGCAAAGCAGCAA	0.18	V
11	Eco91	91	108	CCGCCACTCGTCAGCAAA	0.82	I
12	Eco102	102	118	ACTCACCCGTCCGCCAC	0.75	II
13	Eco109	109	126	CAGACATTACTCACCCGT	0.82	I
14	Eco119	119	136	GGCAGTTTCCCAGACATT	0.78	II
15	Eco127	127	144	CTCCATCAGGCAGTTTCC	0.73	II
16	Eco137	137	154	AGTTATCCCCCTCCATCA	0.71	II
17	Eco145	145	162	TTTCCAGTAGTTATCCCC	0.79	II
18	Eco155	155	172	TTAGCTACCGTTTCCAGT	0.99	I
19	Eco163	163	180	ATGCGGTATTAGCTACCG	0.32	IV
20	Eco173	173	190	TTGCGACGTTATGCGGTA	0.39	IV
21	Eco181	181	198	CTTTGGTCTTGCGACGTT	0.77	II
22	Eco191	191	209	AAGGTCCCCCTCTTTGGTC	0.75	II
23	Eco199	199	215	GGCCCTAAGGTCCCCCT	0.67	II
24	Eco210	210	226	CGATGGCAAGAGGCCCT	0.17	V
25	Eco216	216	233	GCACATCCGATGGCAAGA	0.29	IV
26	Eco227	227	243	TCCCATCTGGGCACATC	0.32	IV
27	Eco234	234	251	CTAGCTAATCCCATCTGG	0.63	II
28	Eco244	244	261	ACCCCACCTACTAGCTAA	0.56	III
29	Eco252	252	268	AGCCGTTACCCCACCTA	1.02	I
30	Eco262	262	279	TCGCCTAGGTGAGCCGTT	0.16	V
31	Eco269	269	285	GGATCGTCGCCTAGGTG	0.32	IV
32	Eco280	280	297	CAGACCAGCTAGGGATCG	0.48	III
33	Eco285	285	302	CCTCTCAGACCAGCTAGG	0.70	II

34	Eco298	298	315	TGTGGCTGGTCATCCTCT	0.82	I
35	Eco303	303	320	TCCAGTGTGGCTGGTCAT	0.52	III
36	Eco316	316	333	ACCGTGTCTCAGTTCCAG	0.54	III
37	Eco321	321	338	TCTGGACCGTGTCTCAGT	0.78	II
38	Eco334	334	350	CTCCCGTAGGAGTCTGG	0.48	III
39	Eco343	343	359	CACTGCTGCCTCCCGTA	0.73	II
40	Eco351	351	369	CAATATTCCCCACTGCTGC	0.66	II
41	Eco360	360	377	CCATTGTGCAATATTCCC	0.69	II
42	Eco370	370	386	GGCTTGCGCCCATTGTG	0.54	III
43	Eco378	378	394	CTGCATCAGGCTTGCGC	1.09	I
44	Eco387	387	403	GCGGCATGGCTGCATCA	0.40	IV
45	Eco395	395	412	TTCATACACGCGGCATGG	1.06	I
46	Eco404	404	421	AAGGCCTTCTTCATACAC	0.64	II
47	Eco413	413	429	TACAACCCGAAGGCCTTC	0.69	II
48	Eco422	422	439	AAAGTACTTTACAACCCG	0.66	II
49	Eco431	431	448	TCCCCGCTGAAAGTACTT	0.33	IV
50	Eco440	440	456	TCCCTTCCTCCCCGCTG	0.94	I
51	Eco449	449	467	ATTAACTTTACTCCCTTCC	0.47	III
52	Eco455	455	473	AAAGGTATTAACTTTACTC	0.01	VI
53	Eco468	468	486	AACGTCAATGAGCAAAGGT	0.03	VI
54	Eco474	474	491	CGGGTAACGTCAATGAGC	0.25	IV
55	Eco487	487	504	GGTGCTTCTTCTGCGGGT	0.72	II
56	Eco492	492	509	TAGCCGGTGCTTCTTCTG	0.63	II
57	Eco505	505	522	GCTGGCACGGAGTTAGCC	0.40	IV
58	Eco510	510	527	CGGCTGCTGGCACGGAGT	0.41	III
59	Eco523	523	540	CTCCGTATTACCGCGGCT	0.76	II
60	Eco528	528	545	GCACCCTCCGTATTACCG	0.49	III
61	Eco541	541	558	CCGATTAACGCTTGCACC	0.66	II
62	Eco548	548	566	CAGTAATTCCGATTAACGC	0.45	III
63	Eco559	559	576	GCTTTACGCCCAGTAATT	0.49	III
64	Eco567	567	584	CTGCGTGCGCTTTACGCC	0.73	II
65	Eco577	577	594	AACAAACCGCCTGCGTGC	0.78	II
66	Eco585	585	602	TCTGACTTAACAAACCGC	0.14	V
67	Eco595	595	613	GGGATTTCACATCTGACTT	0.10	V
68	Eco603	603	620	GAGCCCGGGGATTTCACA	0.19	V
69	Eco614	614	631	GTTCCCAGGTTGAGCCCG	0.34	IV
70	Eco621	621	638	AGATGCAGTTCCCAGGTT	0.04	VI
71	Eco627	627	644	AGTATCAGATGCAGTTCC	0.18	V
72	Eco632	632	649	TTGCCAGTATCAGATGCA	0.04	VI
73	Eco639	639	656	CTCAAGCTTGCCAGTATC	0.09	V
74	Eco645	645	662	ACGAGACTCAAGCTTGCC	0.81	I

75	Eco650	650	667	CCTCTACGAGACTCAAGC	0.36	IV
76	Eco657	657	674	CTACCCCCCTCTACGAGA	0.47	III
77	Eco665	665	682	CTGGAATTCTACCCCCCT	1.14	I
78	Eco668	668	685	CACCTGGAATTCTACCCC	0.82	I
79	Eco675	675	692	ACCGCTACACCTGGAATT	0.62	II
80	Eco681	681	698	CATTTCACCGCTACACCT	1.30	I
81	Eco686	686	703	CTACGCATTTCACCGCTA	0.67	II
82	Eco690	690	707	ATCTCTACGCATTTCACC	1.27	I
83	Eco693	693	710	CAGATCTCTACGCATTTC	0.44	III
84	Eco704	704	721	CGGTATTCCTCCAGATCT	0.75	II
85	Eco711	711	728	TCGCCACCGGTATTCCTC	0.65	II
86	Eco722	722	737	GGGCCGCCTTCGCCTC	0.64	II
87	Eco729	729	744	GTCCAGGGGGCCGCCT	0.25	IV
88	Eco738	738	755	CGTCAGTCTTCGTCCAGG	0.67	II
89	Eco745	745	762	ACCTGAGCGTCAGTCTTC	0.33	IV
90	Eco756	756	773	CACGCTTTCGCACCTGAG	0.53	III
91	Eco763	763	780	TGCTCCCCACGCTTTCGC	0.79	II
92	Eco774	774	791	CTAATCCTGTTTGCTCCC	0.57	III
93	Eco781	781	799	CAGGGTATCTAATCCTGTT	0.25	IV
94	Eco792	792	809	CGTGGACTACCAGGGTAT	0.35	IV
95	Eco800	800	817	GTTTACGGCGTGGACTAC	0.37	IV
96	Eco810	810	827	AGTCGACATCGTTTACGG	0.50	III
97	Eco818	818	835	AACCTCCAAGTCGTCATC	0.62	II
98	Eco828	828	845	TCAAGGGCACAACCTCCA	0.56	III
99	Eco836	836	852	CCACGCCTCAAGGGCAC	0.15	V
100	Eco846	846	862	GCTCCGGAAGCCACGCC	0.39	IV
101	Eco853	853	870	ACGCGTTAGCTCCGGAAG	0.21	IV
102	Eco863	863	880	GGTCGACTTAACGCGTTA	0.39	IV
103	Eco871	871	888	CCCCAGGCGGTCGACTTA	0.73	II
104	Eco881	881	897	GGCCGTACTCCCCAGGC	0.77	II
105	Eco889	889	906	TAACCTTGCGGCCGTACT	0.62	II
106	Eco898	898	916	ATTTGAGTTTTAACCTTGC	0.52	III
107	Eco907	907	925	CGTCAATTCATTTGAGTTT	1.01	I
108	Eco917	917	933	CGGGCCCCCGTCAATTC	0.60	III
109	Eco926	926	941	CGCTTGTGCGGGCCCC	0.53	III
110	Eco934	934	951	CATGCTCCACCGCTTGTG	0.88	I
111	Eco942	942	959	TTAAACCACATGCTCCAC	0.72	II
112	Eco952	952	969	TTGCATCGAATTAAACCA	0.25	IV
113	Eco960	960	977	TCTTCGCGTTGCATCGAA	0.46	III
114	Eco970	970	987	CAGGTAAGGTTCTTCGCG	0.47	III
115	Eco978	978	995	GTCAAGACCAGGTAAGGT	0.22	IV

116	Eco988	988	1005	TTCCGTGGATGTCAAGAC	0.26	IV
117	Eco996	996	1013	CTGAAAACTTCCGTGGAT	0.20	V
118	Eco1006	1006	1023	ATTCTCATCTCTGAAAAC	0.06	V
119	Eco1014	1014	1031	GAAGGCACATTCTCATCT	0.16	V
120	Eco1024	1024	1041	CACGGTTCCCGAAGGCAC	0.60	III
121	Eco1032	1032	1049	ACCTGTCTCACGGTTCCC	0.57	III
122	Eco1042	1042	1059	GCCATGCAGCACCTGTCT	0.60	III
123	Eco1050	1050	1067	TGACGACAGCCATGCAGC	0.35	IV
124	Eco1060	1060	1077	CAACACGAGCTGACGACA	0.15	V
125	Eco1068	1068	1085	ACATTTCACAACACGAGC	0.20	V
126	Eco1078	1078	1095	ACTTAACCCAACATTTCA	0.18	V
127	Eco1086	1086	1103	GTTGCGGGACTTAACCCA	0.27	IV
128	Eco1097	1097	1112	GTTGCGCTCGTTGCGGG	0.27	IV
129	Eco1104	1104	1121	AGGATAAGGGTTGCGCTC	0.38	IV
130	Eco1113	1113	1130	TGGCAACAAAGGATAAGG	0.03	VI
131	Eco1122	1122	1139	CCGGACCGCTGGCAACAA	0.27	IV
132	Eco1131	1131	1146	TTCCCGGCCGGACCGC	0.48	III
133	Eco1140	1140	1157	TCTCCTTTGAGTTCCCGG	0.77	II
134	Eco1147	1147	1165	ACTGGCAGTCTCCTTTGAG	0.19	V
135	Eco1158	1158	1175	CCAGTTTATCACTGGCAG	0.03	VI
136	Eco1166	1166	1183	ACCTTCCTCCAGTTTATC	0.32	IV
137	Eco1176	1176	1193	CGTCATCCCCACCTTCCT	0.84	I
138	Eco1184	1184	1201	TGACTTGACGTCATCCCC	0.12	V
139	Eco1194	1194	1211	AGGGCCATGATGACTTGA	0.25	IV
140	Eco1202	1202	1219	TGGTCGTAAGGGCCATGA	0.05	VI
141	Eco1212	1212	1229	TGTGTAGCCCTGGTCGTA	0.63	II
142	Eco1220	1220	1237	GTAGCACGTGTGTAGCCC	0.62	II
143	Eco1230	1230	1247	ATGCGCCATTGTAGCACG	0.36	IV
144	Eco1238	1238	1255	CTCTTTGTATGCGCCATT	0.68	II
145	Eco1248	1248	1265	GAGGTCGCTTCTCTTTGT	0.61	II
146	Eco1256	1256	1273	GCTCTCGCGAGGTCGCTT	0.58	III
147	Eco1266	1266	1283	AGGTCCGCTTGCTCTCGC	0.52	III
148	Eco1274	1274	1291	ACTTTATGAGGTCCGCTT	0.26	IV
149	Eco1284	1284	1301	ACTACGACGCACTTTATG	0.33	IV
150	Eco1292	1292	1309	CAATCCGGACTACGACGC	0.42	III
151	Eco1302	1302	1319	TTGCAGACTCCAATCCGG	0.56	III
152	Eco1310	1310	1327	GAGTCGAGTTGCAGACTC	0.02	VI
153	Eco1320	1320	1337	CGACTTCATGGAGTCGAG	0.01	VI
154	Eco1328	1328	1345	AGCGATTCCGACTTCATG	0.65	II
155	Eco1338	1338	1355	CACGATTACTAGCGATTC	0.16	V
156	Eco1346	1346	1363	TTCTGATCCACGATTACT	0.51	III

157	Eco1356	1356	1373	CACCGTGGCATTCTGATC	0.43	III
158	Eco1364	1364	1382	GAACGTATTCACCGTGGCA	0.56	III
159	Eco1374	1374	1391	AAGGCCCGGGAACGTATT	0.58	III
160	Eco1383	1383	1400	GGTGTGTACAAGGCCCGG	0.58	III
161	Eco1392	1392	1409	GTGACGGGCGGTGTGTAC	0.48	III
162	Eco1401	1401	1418	TCCCATGGTGTGACGGGC	0.64	II
163	Eco1410	1410	1427	GCAACCCACTCCCATGGT	0.52	III
164	Eco1419	1419	1436	ACTTCTTTTGCAACCCAC	0.46	III
165	Eco1428	1428	1445	AAGCTACCTACTTCTTTT	0.66	II
166	Eco1437	1437	1454	CCGAAGGTTAAGCTACCT	0.04	VI
167	Eco1446	1446	1463	AGCGCCCTCCCGAAGGTT	0.59	III
168	Eco1455	1455	1472	AAAGTGGTAAGCGCCCTC	0.69	II
169	Eco1464	1464	1481	ATGAATCACAAAGTGGTA	0.03	VI
170	Eco1473	1473	1490	ACCCCAGTCATGAATCAC	0.66	II
171	Eco1482	1482	1499	TACGACTTCACCCCAGTC	0.56	III
172	Eco1491	1491	1508	TTACCTTGTTACGACTTC	0.39	IV
173	Eco1500	1500	1517	CCCCTACGGTTACCTTGT	0.51	III
174	Eco1509	1509	1525	CGCAGGTTCCCCTACGG	0.13	V
175	Eco1518	1518	1535	GTGATCCAACCGCAGGTT	0.17	V
176	Eco1526	1526	1542	TAAGGAGGTGATCCAAC	0.29	IV

Sequences and relative fluorescence intensities of probes targeting the 16S rRNA of *Pirellula* sp. strain 1

No.	Probe name	*E.coli* position[a] 5'	3'	Probe sequence (5'->3')	Relative probe fluorescence[b]	Brightness class[c]
177	Pir1	1	19	GGATCAAACCCTTCAATTT	0.69	II
178	Pir20	20	37	AACGTTCATTCTGAGCCA	0.71	II
179	Pir38	38	54	GCCTAATCCATGCCGCC	0.62	II
180	Pir55	55	73	TTCTCGTGCGACTTGCAT	0.63	II
181	Pir72	72	89	TAGCAAGCTACTCTGCTT	0.09	V
182	Pir91	91	109	TTCGCCACTGTCCGCTTT	0.65	II
183	Pir109	109	126	CACACGTTACTCTCCCTT	0.69	II
184	Pir127	127	145	GTCTCGAGGGCACGTAAC	0.57	III
185	Pir146	146	162	TTTCCCGACGCTATCCCG	0.72	II
186	Pir163	163	180	ATCCGGTATTACTGCCAG	0.58	III
187	Pir181	181	198	CATTTGATCCGTAGATATT	0.52	III
188	Pir198	198	225	TCGAGCGGAATCTCACC	0.35	IV
189	Pir216	216	233	TCCAATCCTCGAGCGGAA	0.26	IV
190	Pir234	234	251	CAAGCTAATAGTATGCGG	0.52	III
191	Pir252	252	268	AGCCATTACCTCACCAA	0.36	IV
192	Pir269	269	285	CCATCGCAGCCTTGGTG	0.62	II
193	Pir285	285	302	GCTCTCACACCCGGTAAC	0.68	II
194	Pir303	303	319	CCAGTGAGCCGGGCCAT	0.67	II
195	Pir321	321	338	TCTGGGCAGTGTCTCAGT	0.73	II
196	Pir340	340	355	CTGCAGCCACCCGTAGGT	0.68	II
197	Pir351	351	369	CGAAGATTCTCGACTGC	0.37	IV
198	Pir370	370	386	GACTTTCGTCCATTGCC	0.59	III
199	Pir387	387	403	GCGGCATCGCTCGGTCA	0.67	II
200	Pir404	404	421	AGGGCCTTCATCCCGCAC	0.47	III
201	Pir422	422	439	ACAGCGGTTTACAACCCG	0.50	III
202	Pir439	439	455	GCATTTCCTAACAACTGA	0.60	III
203	Pir453	453	472	TGGATAACCACCATGCA	0.13	V
204	Pir474	474	491	GAAGATCGGTCAAACATG	0.12	V
205	Pir492	492	509	TAGCCCGTCCTTCCTCT	0.70	II
206	Pir510	510	527	CGGCTGCTGGCACGTACT	0.57	III
207	Pir528	528	545	GGACGGTACGTGTTACCG	0.49	III
208	Pir548	548	567	CAGTGATACCGAATAACGT	0.75	II
209	Pir567	567	584	CTACGAACGCTTTAAGCC	0.64	II
210	Pir585	585	602	TCTCACCTTCTAGGCCGC	0.48	III
211	Pir603	603	620	GAGCCGTGGGCTTTCACA	0.09	V

212	Pir621	621	638	AAACGCAATTCCACGGTT	0.08	V
213	Pir639	639	656	CTCAAGCCTAGTGGTTTG	0.04	VI
214	Pir657	657	674	CCCATCACCCCTGTCTTC	0.67	II
215	Pir675	675	692	ACCGCTCCACCATAAGTT	0.60	III
216	Pir693	693	711	TATGATATCAACGCATTTC	0.40	IV
217	Pir711	711	728	TCGCCACCGGTGTTCCCT	0.66	II
218	Pir729	729	744	ACCCAGTGATGCGCTT	0.51	III
219	Pir745	745	762	TCCTCAGCGTCAGAAAAG	0.54	III
220	Pir763	763	780	CGCTACCCTAGCTTTCGT	0.57	III
221	Pir781	781	798	GGGGTATCTAATCCCGTT	0.64	II
222	Pir800	800	817	GTTTACGGCTAGGACTAC	0.62	II
223	Pir818	818	835	CTCAGCCTAGTGCTCATC	0.24	IV
224	Pir836	836	851	GAGAGAATGTGGAAGTTC	0.05	VI
225	Pir853	853	870	ATGGTTTCCCTGCGGCCG	0.55	III
226	Pir871	871	888	CCCCAGGCGGAGCACTTA	0.69	II
227	Pir888	888	904	GCCTTGCGACCATACTC	0.66	II
228	Pir907	907	952	CGTCAATTCCTTTGAGTTT	0.69	II
229	Pir926	926	941	CGCTTGTGTGAGCCCC	0.61	II
230	Pir942	942	959	TTAAGCCACATCCTCCAC	0.58	III
231	Pir961	961	977	TCTTCGCGTAGCCTCGA	0.16	V
232	Pir978	978	995	GTCAAGTCTAGGATAAGGT	0.52	III
233	Pir996	996	1013	CATAGGGATTCTCAAGCAT	0.58	III
234	Pir1014	1014	1031	GAAGGGCACTCTCTACTTT	0.51	III
235	Pir1032	1032	1048	CCTGTGCAAGAGCTCCCC	0.76	II
236	Pir1050	1050	1067	TGACGACAGCCATGCAGC	0.65	II
237	Pir1068	1068	1085	ACATCTCACGACACGAGC	0.68	II
238	Pir1086	1086	1103	GTTAAGGGACTTAACCCG	0.04	VI
239	Pir1104	1104	1121	AAGATAAGGGTTTCGCTC	0.39	IV
240	Pir1122	1122	1139	CATTACCCGCTGGCAACTA	0.76	II
241	Pir1140	1140	1157	TCTCTTTAGAGTCCCCGG	0.48	III
242	Pir1156	1156	1173	GGTTTGACACCGGCAGTC	0.44	III
243	Pir1176	1176	1193	CGTCATCCCCACCTTCCT	0.69	II
244	Pir1194	1194	1211	AAGGCCATGAGGACTTGA	0.26	IV
245	Pir1212	1212	1229	TGTGCAGCCCTAGACATA	0.67	II
246	Pir1230	1230	1247	CCGTGCCATTGTAGGACG	0.17	V
247	Pir1248	1248	1265	GGTATTGCGTCCGTTTGT	0.62	II
248	Pir1266	1266	1283	GGATTTGCTCCACCTCGC	0.63	II
249	Pir1284	1284	1301	ACTGAGGCACGGTTTCTA	0.12	V
250	Pir1302	1302	1319	TTGCAGCCTGCAATCCGA	0.07	V
251	Pir1320	1320	1337	CGGCTTCATGCAGGCGAG	0.29	IV
252	Pir1338	1338	1355	TACGATTACTAGCGATTC	0.14	V

253	Pir1356	1356	1373	CACCGTAGTATGCTGACC	0.64	II
254	Pir1371	1371	1391	AAGGCTCAGGAACACATT	0.30	IV
255	Pir1392	1392	1409	TTGACGGGCGGTGTGTAC	0.63	II
256	Pir1410	1410	1427	CCTCCCAACTTTCGTGGC	0.79	II
257	Pir1428	1428	1445	AGCTTGGTGACTTCGGGC	0.59	III
258	Pir1446	1446	1461	CTGCCTCCCTTGCGGGTT	0.79	II
259	Pir1464	1464	1481	GTCGAGCTGACCTTCGGC	0.51	III
260	Pir1482	1482	1499	TACGACTTAGTCCCAATT	0.62	II
261	Pir1500	1500	1517	CCCCTACGGCTACCTTGT	0.53	III
262	Pir1518	1518	1535	GTGATCCAGCCGCAGGTT	0.54	III
263	Pir1525	1525	1542	AAAGGAGGTGATCCAGCC	0.24	IV
264	Pir1527	1527	1544	AGAAAGGAGGTGATCCAG	0.32	IV

Sequences and relative fluorescence intensities of probes targeting the 16S rRNA of *Metallosphaera sedula*

No.	Probe name	*E.coli* position[a] 5'	3'	Probe sequence (5'->3')	Relative probe fluorescence[b]	Brightness class[c]
265	Met1	1	18	GATCAACCGGAATTAGGG	0.00	VI
266	Met13	13	30	CGGGTCCGGCAGGATCAA	0.24	IV
267	Met20	20	37	AGCGATCGGGTCCGGCA	0.17	V
268	Met32	32	47	CCCTACCCCTATAGCGAT	0.63	II
269	Met38	38	53	GCTTAGCCCTACCCCTAT	0.12	V
270	Met55	55	80	AGAGCGTACGACTCCCAT	0.13	V
271	Met84	84	109	TCCGCCACGCCCTCTTC	0.19	V
272	Met109	109	126	CACGTGTTACTCAGCCGT	0.19	V
273	Met119	119	135	GCAGGTTAGCCACGTGTT	0.58	III
274	Met127	127	143	TCCCAAGGGCAGGTTAGC	0.07	V
275	Met137	137	154	GGTTATCCAGATCCCAAG	0.44	III
276	Met145	145	162	TTTCCCGGGGTTATCCAG	0.19	V
277	Met155	155	172	TTAGCCCCAGTTTCCCGG	0.90	I
278	Met163	163	180	GCTCCGGATTAGCCCCAG	0.14	V
279	Met173	173	183	CCCTTGCCCGCTCCGGA	0.90	I
280	Met181	181	190	TTCCAGATTCCCTTGCCC	0.10	V
281	Met183a	183	193	ATCATTCCAGATTCCCTT	0.36	IV
282	Met183b	183	193	CAAGAGATCATTCCAGATT	0.23	IV
283	Met191	191	198	CTTTTAGGCAAGAGATCA	0.03	VI
284	Met193a	193	193	GAGGCTTTTAGGCAAGAG	0.16	V
285	Met193b	193	207	AGCCGAGAGGCTTTTAGG	0.08	V
286	Met199	199	210	CGGGATCAGCCGAGAGG	0.07	V
287	Met203	203	215	CTCGACGGGATCAGCCGA	0.62	II
288	Met210	210	226	CTTGGGCGCCTCTCGAC	0.10	V
289	Met216	216	233	CCCCATCCTTGGGCGCCT	0.34	IV
290	Met227	227	243	TGGGCCGCAGCCCCATC	0.05	VI
291	Met234	234	250	AGCCTGATGGGCCGCAG	0.76	II
292	Met244	244	260	CTCCCCCAACAGCCTGA	0.32	IV
293	Met262	262	279	TCGGTTTGGGGGACCTTT	0.79	II
294	Met269	269	285	CCGTTATCGGTTTGGGG	0.76	II
295	Met280	280	297	CACGGCCCCTACCCGTTA	1.03	I
296	Met285	285	301	CTCCCACGGCCCCTACC	1.64	I
297	Met298	298	313	TGGGGGCTCCCGCTCC	0.97	I
298	Met303	303	319	CCCAACTGGGGGCTCCC	1.79	I
299	Met316	316	333	CCCTTGTCTCAGTGCCCA	0.30	IV

300	Met321	321	338	CTGGGCCCTTGTCTCAGT	1.09	I
301	Met334	334	349	CCCCGTAGGGCCTGGG	0.04	VI
302	Met343	343	359	GCCTGGTGCGCCCCGTA	0.74	II
303	Met351	351	366	GTTCCGCGCCTGGTGC	0.40	IV
304	Met370	370	386	CGGTTTCCCGCATTGGG	0.13	V
305	Met387	387	403	TAGGGGTAACGCCCTCA	0.13	V
306	Met395	395	420	CGAGGGCACTAGGGGTAA	0.07	V
307	Met421	421	445	TGGAGAAAAGCCCTCTTG	0.06	V
308	Met446	446	495	TCCTCCACCTTTCTGGAG	0.20	V
309	Met492	492	509	TGCCCCCCGCTTATTCCT	0.40	IV
310	Met505	505	522	GCTGACACCAGTCTTGCC	0.62	II
311	Met510	510	526	GGCGGCTGACACCAGTCT	0.13	V
312	Met523	523	540	GCTGGTATTACCGCGGCG	0.60	III
313	Met528	528	545	GCGGGGCTGGTATTACCG	0.09	V
314	Met541	541	557	TCCCGATCACTCGCGGG	0.78	II
315	Met548	548	565	AATAAACGTCCCGATCAC	0.14	V
316	Met559	559	576	GCTTTAAGCCCAATAAAC	0.32	IV
317	Met567	567	584	CTACGGGCGCTTTAAGCC	0.18	V
318	Met585	585	602	GGTGACTTTACAGGCCGG	0.01	VI
319	Met603	603	620	GAGCCCGGGTCTTTAAAC	0.02	VI
320	Met615	615	632	GTTCCCCGAGTTGAGCCC	0.72	II
321	Met621	621	638	ACCGCCGTTCCCCGAGTT	0.03	VI
322	Met627	627	644	AGTATCACCGCCGTTCCC	0.82	I
323	Met632	632	649	CTGTAAGTATCACCGCCG	0.59	III
324	Met639	639	656	CCCTAGCCTGTAAGTATC	0.02	VI
325	Met645	645	661	CCGCCCCCTAGCCTGTA	0.81	I
326	Met650	650	666	CTCTCCCGCCCCCTAGC	1.60	I
327	Met654	654	669	GACCTCTCCCGCCCCC	1.58	I
328	Met657	657	673	CTCCGACCTCTCCCGCC	0.44	III
329	Met661	661	678	AGTACCTCCGACCTCTCC	1.13	I
330	Met665	665	682	CGGGAGTACCTCCGACCT	0.68	II
331	Met668	668	685	CTCCGGGAGTACCTCCGA	0.44	III
332	Met675	675	691	CCCCTACTCCGGGAGTA	0.17	V
333	Met681	681	698	GATTTCGCCCCTACTCCG	2.26	I
334	Met686	686	703	CTGAGGATTTCGCCCCTA	1.43	I
335	Met690	690	707	GGATCTGAGGATTTCGCC	0.38	IV
336	Met693	693	709	CGGGATCTGAGGATTTC	0.02	VI
337	Met704	704	721	TGGTGGTCCTCCCGGGAT	1.62	I
338	Met711	711	727	CGCCACTGGTGGTCCTC	0.09	V
339	Met722	722	738	CCGGACGCTTTCGCCAC	1.16	I
340	Met729	729	745	GTTCTAGCCGGACGCTT	0.19	V

341	Met738	738	754	GTCGGGCGCGTTCTAGC	1.17	I
342	Met745	745	760	CTCACCGTCGGGCGCG	3.26	I
343	Met750	750	766	TCGCCCCTCACCGTCGG	1.18	I
344	Met756	756	772	CGGCTTTCGCCCCTCAC	1.15	I
345	Met763	763	779	GCTACCCCGGCTTTCGC	0.20	V
346	Met768	768	785	CTATTTGCTACCCCGGCT	0.68	II
347	Met774	774	791	CTAATCCTATTTGCTACC	0.46	III
348	Met781	781	799	TAGGGTATCTAATCCTATT	0.55	III
349	Met792	792	809	CCGGGACTACTAGGGTAT	0.10	V
350	Met800	800	817	GTTTACAGCCGGGACTAC	0.21	IV
351	Met810	810	827	AGCCTGCATCGTTTACAG	0.53	III
352	Met818	818	835	CGACACCTAGCCTGCATC	0.18	V
353	Met823	823	840	CTACGCGACACCTAGCCT	1.03	I
354	Met828	828	845	AAAGCCTACGCGACACCT	0.13	V
355	Met836	836	849	GCAGGCACAAAGCCTACG	0.42	III
356	Met841	841	854	ACCGCGCAGGCACAAAGC	0.45	III
357	Met846	846	857	GGCACCGCGCAGGCAC	0.01	VI
358	Met850	850	867	GTTTTCCTGCGGCACCGC	0.83	I
359	Met853	853	870	CCAGTTTTCCTGCGGCAC	0.23	IV
360	Met858	858	575	GCTTACCAGTTTTCCTGC	1.75	I
361	Met863	863	880	GGCGGGCTTACCAGTTTT	0.91	I
362	Met867	867	883	GGCGGCGGGCTTACCAG	1.18	I
363	Met871	871	887	CCCAGGCGGCGGGCTTA	0.23	IV
364	Met876	876	892	TACTCCCCAGGCGGCGG	3.12	I
365	Met881	881	897	GGCCGTACTCCCCAGGC	1.00	I
366	Met885	885	901	GGCCGTACTCCCCAGGC	0.14	V
367	Met889	889	906	CAGCCTTGCGGCCGTACT	1.21	I
368	Met898	898	915	TTTAAGTTTCAGCCTTGC	0.56	III
369	Met902	902	919	TTTAAGTTTCAGCCTTGC	0.16	V
370	Met907	907	925	CGCCAATTCCTTTAAGTTT	0.14	V
371	Met917	917	933	GTGCTCCCCCGCCAATTC	0.80	II
372	Met926	926	941	CCCTTGTGGTGCTCCCC	0.22	IV
373	Met934	934	950	AGGTTCCACCCCTTGTGG	0.78	II
374	Met942	942	958	TGAGCCGCAGGTTCCAC	0.08	V
375	Met952	952	969	TTGACTCCAATTGAGCCG	0.84	I
376	Met960	960	976	CCAGGCGTTGACTCCAA	0.07	V
377	Met970	970	987	CCGGTGAGATTCCAGGCG	0.63	II
378	Met978	978	1043	GTCTCCCCCGGTGAGAT	0.13	V
379	Met1043a	1043	1043	CTGGCCGTCATCCTGCG	0.23	IV
380	Met1043b	1043	1043	CTGGCAAGGTCGTTAGC	0.14	V
381	Met1043c	1043	1051	ACCTCCTCTCCGCGAGT	0.11	V

382	Met1043d	1043	1060	GGCCATGCACCTCCTCT	0.08	V
383	Met1060	1060	1076	CAACACGAGCTGGCGAC	0.12	V
384	Met1078	1078	1095	GACTTAACCGGACATTTC	0.06	V
385	Met1097	1097	1113	GTCTCGCTCGTTGCCGG	0.75	II
386	Met1114	1114	1131	TTACCAACTAGAAGTGGG	0.03	VI
387	Met1122	1122	1136	GAGAGACGGTTACCAACT	0.03	VI
388	Met1137	1137	1151	TAGTGTGGACCGTCTCCG	0.03	VI
389	Met1140	1140	1157	CCTTCTAGTGTGGACCGT	0.23	IV
390	Met1147	1147	1165	CACCGGCAGTCCTTCTAGT	0.04	VI
391	Met1166	1166	1183	CTCCTTCCTCCGGTTTAA	0.13	V
392	Met1184	1184	1200	TGACCTGCCGTGGCCCC	0.03	VI
393	Met1202	1202	1218	GGAAGTTTCGGGGCATG	0.10	V
394	Met1220	1220	1236	GTAACCCGCGTGCGGCC	0.10	V
395	Met1238	1238	1255	CCCGCTGTCCCTGCCAT	0.14	V
396	Met1248	1248	1264	GGGTCGGATCCCGCTGT	2.01	I
397	Met1256	1256	1272	CCCTCTCGGGGTCGGAT	1.63	I
398	Met1266	1266	1282	GATTGCCTTCCCCTCTC	0.86	I
399	Met1274	1274	1291	GGTTTGTGGGATTGCCTT	0.39	IV
400	Met1284	1284	1301	ACTGAGGCAGGGTTTGTG	0.09	V
401	Met1292	1292	1309	CGATCCCAACTGAGGCAG	0.61	II
402	Met1296	1296	1313	CCCTCGATCCCAACTGAG	0.79	II
403	Met1302	1302	1319	TTTCAGCCCTCGATCCCA	0.28	IV
404	Met1306	1306	1323	CGAGTTTCAGCCCTCGAT	0.54	III
405	Met1314	1314	1331	CACGAGGGCGAGTTTCAG	0.61	II
406	Met1320	1320	1336	TCGTTCACGAGGGCGAG	0.03	VI
407	Met1328	1328	1345	AGGGATTCCTCGTTCACG	0.36	IV
408	Met1338	1338	1355	CGCGGTTACTAGGGATTC	0.03	VI
409	Met1346	1345	1363	TGTTGACCCGCGGTTACT	0.14	V
410	Met1356	1356	1373	TCACCGCGGGTTGTTGAC	0.04	VI
411	Met1364	1364	1381	GACGTATTCACCGCGGGT	0.41	III
412	Met1374	1374	1391	AAGGAGCAGGGACGTATT	0.10	V
413	Met1383	1383	1400	GGTGTGTGCAAGGAGCAG	0.21	IV
414	Met1392	1392	1408	CGACGGGCGGTGTGTGC	0.14	V
415	Met1401	1401	1416	GGGTGGAGCGACGGGC	1.62	I
416	Met1410	1410	1426	CCCTCCACTCGGGTGGA	0.03	VI
417	Met1419	1419	1436	CCTCACTTCCCCTCCAC	2.03	I
418	Met1428	1428	1447	GGCAAGAGGCCTCACTT	0.05	VI
419	Met1444	1444	1463	CTCCCACCCCGAGGGG	0.07	V
420	Met1464	1464	1480	CGGAGGAGAAGCTCGAC	0.01	VI
421	Met1482	1482	1498	ACGACTTCTCCCCCCTC	0.28	IV
422	Met1500	1500	1516	CCCTACGGCTACCTTGT	0.17	V

423	Met1518	1518	1534	TGATCCAGCCGCAGGTT	0.06	V
424	Met1526	1526	1542	ATGTGAGGTGATCCAGC	0.06	V
425	Met1535	1535	1551	GGAGCACAAATGTGAGG	0.02	VI

Sequences and relative fluorescence intensities of probes targeting the 18S rRNA of *Saccharomyces cerevisiae*

No.	Probe name	*E.coli* position[a] 5'	3'	Probe sequence (5'->3')	Relative probe fluorescence[b]	Brightness class[c]
426	Sac6	6	23	GGCAGGATCAACCAGATA	0.75	II
427	Sac23	23	41	ACAAGCATATGACTACTG	0.73	II
428	Sac41	41	56	ATGGCTTAATCTTTGAGA	0.31	IV
429	Sac56	56	73	CTTATACTTAGACATGCA	0.31	IV
430	Sac73	73	103	GTTTCACTGTATAAATTGC	0.31	IV
431	Sac103	103	121	TTTAATGAGCCATTCGCAG	0.69	II
432	Sac121	121	138	CAAATAAACGATAACTGAT	0.32	IV
433	Sac138	138	146	ATGTAGTAAAGGAACTATC	0.12	V
434	Sac146	146	162	AATTACCACAGTTATACCA	0.61	III
435	Sac162	162	180	AGCATGTATTAGCTCTAGA	0.84	I
436	Sac180	180	191	CAAAGGGTCGAGATTTTAA	0.18	V
437	Sac191	191	193	TAATAAATACATCTCTTCC	0.55	III
438	Sac193a	193	193	AAGACATTGATTTTTTATCT	0.48	III
439	Sac193b	193	193	TGAATCATCAAAGAGTCCGA	0.61	III
440	Sac193c	193	202	GCGATTCGAAAAGTTATTAT	0.39	IV
441	Sac202	202	217	CGCCAGCACAAGGCCATG	0.54	III
442	Sac217	217	236	GAAATTTGAATGAACCATC	0.40	IV
443	Sac236	236	252	TCGAAAGTTGATAGGGCAG	0.77	II
444	Sac252	252	270	TAGGCCACTATCCTACCAT	0.54	III
445	Sac270	270	287	TACCCGTTGAAACCATGGT	0.80	II
446	Sac287	287	305	CGAACCCTTATTCCCCGTT	0.89	I
447	Sac305	305	322	GGCTCCCTCTCCGGAATC	1.03	I
448	Sac322	322	339	GTGGTAGCCGTTTCTCAG	0.55	III
449	Sac339	339	356	TGCTGCCTTCCTTGGATG	1.02	I
450	Sac356	356	373	TGGGTAATTTGCGCGCCT	0.90	I
451	Sac373	373	391	CCTCCCTGAATTAGGATT	1.11	I
452	Sac391	391	408	CGTTATTTATTGTCACTAC	0.91	I
453	Sac408	408	425	CCGAATGGGCCCTGTATC	0.09	V
454	Sac425	425	439	CATTCCAATTACAAGACC	0.09	V
455	Sac439	439	486	GGTATTTACATTGTACTC	0.76	II
456	Sac486	486	502	CAATTGTTCCTCGTTAAG	0.83	I
457	Sac502	502	518	GCACCAGACTTGCCCTCC	0.94	I
458	Sac518	518	535	AATTACCGCGGCTGCTGG	1.07	I
459	Sac535	535	552	TACGCTATTGGAGCTGGA	0.94	I
460	Sac552	552	570	ACTGCAACAACTTTAATAT	0.51	III

461	Sac570	570	587	TCAACTACGAGCTTTTTAA	0.54	III
462	Sac587	587	600	CCAACCGGGCCCAAAGTT	0.18	V
463	Sac600	600	628	CGAAAAAATCGGACCGGC	0.09	V
464	Sac628	628	644	GTTGGAAATCCAGTACAC	0.13	V
465	Sac644	644	651	CCAGAAGGAAAGGCCCCG	0.05	VI
466	Sac651a	651	651	CAAGGACTCAAGGTTAGC	0.07	V
467	Sac651b	651	651	TGGTTCGCCAAGAGCCAC	0.05	VI
468	Sac651c	651	651	TTTTCAAAGTAAAAGTCCT	0.08	V
469	Sac651d	651	651	GCTTTGAACACTCTAATTT	0.82	I
470	Sac651e	651	651	TATTCGAGCAATACGCCTG	0.99	I
471	Sac651f	651	651	CTATTATTCCATGCTAATAT	0.57	III
472	Sac651g	651	651	TAGAACCAAACGTCCTATTC	0.45	III
473	Sac651h	651	651	GTCCTAGAAACCAACAAAAT	0.11	V
474	Sac651i	651	659	CCTATTAATCATTACGATGG	0.48	III
475	Sac659	659	672	GATGCCCCCGACCGTCC	0.99	I
476	Sac672	672	689	TCTGACAATTGAATACTG	0.47	III
477	Sac689	689	706	AATCCAAGAATTTCACCT	0.33	IV
478	Sac706	706	724	AGTAGTTAGTCTTCAATAA	0.28	IV
479	Sac724	724	740	CTTGGCAAATGCTTTCGCA	0.64	II
480	Sac740	740	759	TTGATTAATGAAAACGTCC	0.22	IV
481	Sac759	759	778	ATCCCCTAACTTTCGTTCT	0.95	I
482	Sac778	778	796	GGTATCTGATCATCTTCGA	1.03	I
483	Sac796	796	814	TATGGTTAAGACTACGACG	0.80	II
484	Sac814	814	832	TCCCTAGTCGGCATAGTTT	1.23	I
485	Sac832	832	845	TAAAAAAACACCACCCGAT	0.15	V
486	Sac845	845	858	AGGTGCCGAGTGGGTCATT	0.48	III
487	Sac858	858	876	AGACTTTGATTTCTCGTAA	0.84	I
488	Sac876	876	891	ACTCCCCCCAGAACCCAAA	1.02	I
489	Sac891	891	909	TTTCAGCCTTGCGACCATA	0.40	IV
490	Sac909	909	927	TCCGTCAATTCCTTTAAGT	1.04	I
491	Sac927	927	943	ACTCCTGGTGGTGCCCTT	0.70	II
492	Sac943	943	961	ATTAAGCCGCAGGCTCCA	0.05	VI
493	Sac959	959	977	TCCCCGTGTTGAGTCAAA	0.55	III
494	Sac977	977	993	TCTGGACCTGGTGAGTTT	0.80	II
495	Sac993	993	1025	TGTCAATCCTTATTGTGT	0.73	II
496	Sac1025	1025	1037	AGAAAGAGCTCTCAATCT	0.43	III
497	Sac1037	1037	1050	CACCACCCACAAAATCAA	0.89	I
498	Sac1050	1050	1067	TAAGAACGGCCATGCACC	0.68	II
499	Sac1067	1067	1084	CAAATCACTCCACCAACT	0.79	II
500	Sac1084	1084	1101	TATCGCAATTAAGCAGAC	0.28	IV
501	Sac1092	1092	1109	TCGTTCGTTATCGCAATT	0.77	II

502	Sac1101	1101	1118	GTTAAGGTCTCGTTCGTT	0.90	I
503	Sac1118	1118	1133	GCACCACTATTTAGTAGG	0.15	V
504	Sac1124	1124	1138	CAAATGCTAGCACCACTA	0.08	V
505	Sac1133	1133	1142	ATAACCAGCAAATGCTAG	0.03	VI
506	Sac1138	1138	1151	TAAGAAGTGGATAACCAG	0.01	VI
507	Sac1142	1142	1158	GTCCCTCTAAGAAGTGGA	0.35	IV
508	Sac1153	1153	1170	TTGAAACCGATAGTCCCT	0.21	V
509	Sac1158	1158	1175	TCGGCTTGAAACCGATAG	0.07	V
510	Sac1171	1171	1188	CCTCAAACTTCCATCGGC	0.85	I
511	Sac1175	1175	1192	ATTGCCTCAAACTTCCAT	0.79	II
512	Sac1192	1192	1208	GGCATCACAGACCTGTTA	0.82	I
513	Sac1208	1208	1224	GGCCCAGAACGTCTAAGG	0.55	III
514	Sac1224	1224	1240	AGTGTAGCGCGCGTGCG	0.67	II
515	Sac1240	1240	1256	ACTCGCTGGCTCCGTCA	0.41	IV
516	Sac1256	1256	1269	TCTCGGCCAAGGTTAGA	0.07	V
517	Sac1269	1269	1284	CAAGATTACCAAGACCT	0.04	VI
518	Sac1284	1284	1300	GCACGACGGAGTTTCAC	0.08	V
519	Sac1300	1300	1316	CAATGCTCTATCCCCAG	0.75	II
520	Sac1316	1316	1334	GTTGAAGAGCAATAATTAC	0.06	VI
521	Sac1334	1334	1352	GCTTACTAGGAATTCCTCG	0.79	II
522	Sac1343	1343	1360	TGACTTGCGCTTACTAGG	0.53	III
523	Sac1352	1352	1370	CGCAAGCTGATGACTTGCG	0.03	VI
524	Sac1361	1361	1378	GTAATCAACGCAAGCTGA	0.22	IV
525	Sac1370	1370	1388	GGGCAGGGACGTAATCAAC	0.64	II
526	Sac1388	1388	1406	ACGGGCGGTGTGTACAAAG	0.67	II
527	Sac1406	1406	1423	ATTCAATCGGTACTAGCGA	0.47	III
528	Sac1423	1423	1443	CTGAGGCCTCACTAAGCCA	0.12	V
529	Sac1443	1443	1449	CCCTTCTCTAAGCAGATCC	0.09	V
530	Sac1449	1449	1453	CTCTGAGATGGAGTTGCCC	0.06	VI
531	Sac1453	1453	1470	GTTTGTCCAAATTCTCCGC	0.16	V
532	Sac1470	1470	1488	GTTCCTCTAAATGACCAAG	0.48	III
533	Sac1488	1488	1506	ACCTTGTTACGACTTTTAG	0.85	I
534	Sac1502	1502	1519	TTCACCTACGGAAACCTT	0.12	V
535	Sac1506	1506	1524	GCAGGTTCACCTACGGAAA	0.03	VI
536	Sac1520	1520	1537	TAATGATCCTTCCGCAGG	0.49	III
537	Sac1524	1524	1537	TAATGATCCTTCCG	0.18	V

[a] *Escherichia coli* positions according to the numbering of **Brosius, J., T. J. Dull, D. D. Sleeter, and H. F. Noller**. 1981. In Gene organiszation and primary structure of a ribosomal RNA operon from *Escherichia coli*. J. Mol. Biol. **148**: 107-127.

[b] Fluorescence intensities expressed as percentage of the mean of each probe data set. The calculated mean was set to 50%.

[c] Probes were grouped according to their relative fluorescence into six classes of brightness:

Brightness class	Relative fluorescence
I	>1.0 - 0.81
II	0.8 - 0.61
III	0.6 - 0.41
IV	0.4 - 0.21
V	0.2 - 0.06
VI	0.05 - 0

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