APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Dec. 1996, p. 4504–4513 Vol. 62, No. 12
0099-2240/96/$04.00+0

Characterization of Universal Small-Subunit rRNA Hybridization Probes for Quantitative Molecular Microbial Ecology Studies

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Received 8 July 1996/Accepted 18 September 1996

Universal oligonucleotide hybridization probes targeting the small-subunit rRNA are commonly used to quantify total microbial representation in environmental samples. Universal probes also serve to normalize results obtained with probes targeting specific phylogenetic groups of microorganisms. In this study, six universal probes were evaluated for stability of probe-target duplexes by using rRNA from nine organisms representing the three domains of *Bacteria*, *Archaea*, and *Eucarya*. Domain-specific variations in dissociation temperatures were observed for all probes. This could lead to a significant bias when these probes are used to quantify microbial populations in environmental samples. We suggest lowering the posthybridization wash stringency for two of the universal probes (S-*-Univ-1390-a-A-18 and S-*-Univ-1392-a-A-15) examined. These two probes were evaluated with traditional and modified hybridization conditions to characterize defined mixtures of rRNAs extracted from pure cultures and rRNA samples obtained from anaerobic digester samples. Probe S-*-Univ-1390-a-A-18 provided excellent estimations of domain-level community composition of these samples and is recommended for future use in microbial ecology studies.

Nucleic acid-based techniques are used widely to characterize microbial communities in environmental samples. The foundation for many of these techniques lies in comparative sequencing of homologous biopolymers to infer phylogenetic relationships (37, 39). Because of their universal distribution, high conservation, and absence of interspecies transfer, rRNAs are often considered the most useful biopolymers for such comparative analyses (13, 35, 36). In particular, the small-subunit (SSU) rRNA has been used most commonly to construct phylogenetic trees (e.g., see reference 38). In turn, these trees serve as a basis for the design of rRNA-targeted oligonucleotide hybridization probes with different levels of specificity (e.g., universal or domain-, family-, or species-specific probes). Since the mid-1980s, hundreds of oligonucleotide probes have been designed (for reviews, see references 4 and 23) and used to identify and quantify populations in a number of environments (35).

The microbial characterization of environmental samples using rRNA-targeted probes involves specific duplex formation between the probe and target rRNA present in nucleic acid extracts or in fixed cells (whole-cell hybridization). To achieve specificity, experimental conditions must be optimized. An important parameter in this context is the melting temperature (T_m). For an oligonucleotide, the T_m is defined as the equilibrium temperature at which half of the probe-target duplexes are dissociated (31, 34). The T_m for an oligonucleotide is concentration dependent, since association and dissociation reactions are intermolecular, but time independent, since the T_m is defined for equilibrium (34). The dissociation temperature (T_d) or duplex retention temperature, defined as the temperature at which 50% of the duplex remains intact during a specified washing period (34), does not correspond to equilibrium conditions and may therefore be different from the T_m. The T_d is an important experimental parameter which can be used to distinguish between oligomer duplexes with and without mismatches and thus to evaluate probe specificity. We routinely perform posthybridization washes at the T_d determined for perfect duplexes (no mismatches between oligonucleotide and target). At this wash temperature, duplexes with one or more mismatches generally are 100% disassociated, which results in the desired specificity.

The T_d is determined by (i) the number of hydrogen bonds between complementary bases; (ii) the degree of base stacking, which involves interactions between the electron systems of the adjacent bases in the same chain; and (iii) interactions between negatively charged phosphate groups and positive ions present in solution (34). A variety of factors can affect these forces and thus impact duplex stability and the T_d. These factors include duplex structure (28); chain content and chain sequence (34); chain length (33, 34); the number, types, and positions of mismatches (10, 20, 21, 31, 34); the presence of terminal unpaired bases (dangling ends) (8, 22); and hybridization and wash conditions (31, 34). A number of empirical equations have been derived to estimate the T_d for oligonucleotide probes (31, 34). These empirical equations are useful during the process of probe design. However, since they do not incorporate all of the factors affecting duplex stability, we recommend performing experimental T_d studies for each new probe to determine the actual T_d for a given set of hybridization conditions (26, 31). This is especially important when probes are used to discriminate between targets that differ in only a few nucleotides or for probes designed to target a wide range of organisms (universal probes).

Universal probes are used to quantify the amount of rRNA contributed by all populations present in a sample. They serve as a basis for normalizing results obtained with phylogenetic group-specific probes: the amount of taxon-specific SSU rRNA (determined with a specific probe) is often expressed as a percentage of the total SSU rRNA recovered (determined with a universal probe) (e.g., see references 7, 11, 18, 24, 27, and

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29). Ideally, universal probes should match perfectly with their target sites in the SSU rRNA of all organisms. In addition, a universal probe should provide a similar hybridization response for all organisms, thus eliminating bias during the normalization step. A probe should be of sufficient length (≥15 nucleotides for commonly used hybridization conditions [26]) to avoid a T_d lower than room temperature. Since completely conserved universal sequences of sufficient length are not present in the SSU rRNA because of interdomain sequence divergence, degenerate sites have been included during the design of universal probes. Consequently, the use of probe mixtures and use of base analogs (e.g., inosine) are commonly employed design strategies. Several universal oligonucleotide probes targeting the SSU rRNA have been designed, and some of them have been extensively used in microbial ecology research (e.g., see references 5, 11, 18, 24, and 29), despite the lack of comprehensive probe characterization studies. In this study, we present probe-target duplex stability studies for newly designed and previously described universal probes and discuss the consequences of our results for quantitative microbial characterizations of complex environmental samples.

MATERIALS AND METHODS

Oligonucleotide probe syntheses and labeling. Six universal probes (Table 1) were synthesized with a DNA synthesizer (Applied Biosystems, Foster City, Calif.), purified by high-performance liquid chromatography, and 5′ end labeled with ^{32}P by using T4 polynucleotide kinase (Promega Corp., Madison, Wis.) and [γ-^{32}P]ATP (ICN Radiochemicals, Irvine, Calif.) (26). The CHECK_PROBE software provided by the Ribosomal Database Project (RDP) (15) was used to determine possible mismatches between these probes and their target SSU rRNAs.

Nucleic acid extraction and PAGE. RNAs from nine pure cultures (Table 1) were extracted by a low-pH hot-phenol extraction procedure (32). The quality of the nucleic acid extract was evaluated by 3.3%–10% (wt/vol) discontinuous polyacrylamide gel electrophoresis (PAGE) (1a). PAGE was also used to quantify the rRNA concentrations in *Methanobacterium bryantii* and *Saccharomyces cerevisiae* nucleic acid extracts. The concentration of high-quality *Escherichia coli* rRNA was estimated by UV spectrophotometry, assuming that 1 mg of RNA per ml corresponds to 20 optical density units at a wavelength of 260 nm. *M. bryantii* and *S. cerevisiae* RNA samples were run on a PAGE gel together with a dilution series of *E. coli* rRNA. After electrophoresis, the gel was stained with ethidium bromide, a digitized image of the gel was obtained using a BioVideo-PC Computer Imaging System (BioImaging Technologies, Brookfield, Wis.), and the fluorescent intensities of the SSU rRNA bands were quantified to determine the SSU rRNA concentrations for *M. bryantii* and *S. cerevisiae* relative to the estimated concentration of *E. coli* SSU rRNA (23).

Optimization of wash temperature. Denatured RNA samples (50 ng) were applied by slot blotting to Magna Charge nylon membranes (Micron Separation Inc., Westborough, Mass.) and were hybridized as previously described (26). After hybridization, the membranes were washed with 50 ml of wash solution containing 1% sodium dodecyl sulfate and 1× SSC (0.15 M NaCl and 0.015 M sodium citrate) for 1 h at 40°C. This wash step was repeated once. Subsequently, the membranes were cut into individual slots. Each membrane (consisting of one hybridized slot) was washed in a scintillation vial containing 3 ml of wash solution for 10 min at 30°C. Then, the membrane was washed in 3 ml of fresh wash solution for another 10 min at 35°C. This procedure was repeated 13 times at increasing temperatures (40, 43, 46, 49, 52, 55, 58, 61, 64, 67, 70, 75, and 80°C). Scintillation cocktail (3 ml) (Ecoscint; National Diagnostics, Manville, N.J.) was then added to each scintillation vial, and liquid scintillation counting was conducted to quantify the radioactivity in the wash solution. The amount of probe that remained attached to the membrane after the final wash at 80°C was also quantified by liquid scintillation counting. Each experiment was performed in duplicate.

For probes S-*-Univ-1390-a-A-18 and S-*-Univ-1392-a-A-15 (probe nomenclature has been standardized according to the oligonucleotide probe database [1]), an alternative method (replicate-slot T_d method) was also used to evaluate duplex stability. The RNAs used in this study were extracted from pure cultures of *E. coli*, *M. bryantii*, and *S. cerevisiae*. After the hybridized membranes were washed twice at 40°C, they were cut into individual slots and were washed for 30 min at 16 different temperatures (one slot at each temperature) (26). Each experiment was performed in triplicate.

Quantification of domains in environmental samples. Probes S-*-Univ-1390-a-A-18 and S-*-Univ-1392-a-A-15, and domain-specific probes for *Archaea* (S-D-Arch-0915-a-A-20 [3]), *Bacteria* (S-D-Bact-0338-a-A-18 [2]), and *Eucarya* (S-D-Euca-0502-a-A-16 [2]), were used in hybridizations with defined mixtures of RNAs extracted from pure cultures (synthetic samples) and RNAs extracted from environmental samples (anaerobic digester samples [27]). The synthetic samples were prepared by combining three pure-culture (*E. coli*, *M. bryantii*, and *S. cerevisiae*) RNA solutions for which the SSU rRNA concentrations had been determined by PAGE combined with quantification of fluorescence intensities of SSU rRNA bands (described above).

Denatured RNA samples and dilution series of pure-culture (*E. coli*, *M. bryantii*, and *S. cerevisiae*) RNA samples were applied in triplicate to Magna Charge nylon membranes (26). These samples included the synthetic samples, RNA extracted from anaerobic digester samples, and RNA extracted from pure cultures of *E. coli*, *M. bryantii*, and *S. cerevisiae*. For each pure-culture RNA dilution series, five membranes were prepared. Of these five membranes, one was hybridized with the respective domain-specific probe, two were hybridized with probe S-*-Univ-1390-a-A-18, and the final two were hybridized with probe S-*-Univ-1392-a-A-15 (26). After hybridization, each membrane was washed twice with 50 ml of wash solution at 40°C for 1 h. Then, the membranes were washed for another 30 min at the following temperatures: 55 and 44°C for probe S-*-Univ-1390-a-A-18, 47 and 40°C for probe S-*-Univ-1392-a-A-15, 55°C for probe S-D-Bact-0338-a-A-18, 58°C for probe S-D-Arch-0915-a-A-20, and 52°C for probe S-D-Euca-0502-a-A-16.

After washing, the membranes were air dried and exposed to Storage Phosphor screens (Molecular Dynamics, Sunnyvale, Calif.). Subsequently, the exposed screens were scanned with a PhosphorImager (Molecular Dynamics) and the image was quantified with the software package ImageQuant (Molecular Dynamics). Standard curves were constructed for each membrane with the dilution series of pure culture RNAs (24). These standard curves were then used to determine the concentrations of SSU rRNAs in the samples.

RESULTS AND DISCUSSION

Evaluation of probe target groups. Nine organisms were chosen to represent the three domains of *Bacteria*, *Archaea*, and *Eucarya* (Table 1). One gram-positive (*Clostridium innocuum*) and two gram-negative (*E. coli* and *Desulfovibrio desulfuricans*) microorganisms were selected to represent the bacterial domain, methanogens from three different orders (*M. bryantii* from the order *Methanobacteriales*, *Methanosarcina acetivorans* from the order *Methanosarcinales*, and *Methanococcus voltae* from the order *Methanococcales*) were chosen to represent the *Archaea*, and three phylogenetically diverse eukaryotes (*S. cerevisiae*, *Dictyostelium discoideum*, and *Zea mays*) were selected to represent the *Eucarya*. In addition to phylogenetic considerations, SSU rRNA sequence availability was an important consideration during the selection of the organisms. Each one of the selected organisms has a nearly complete SSU rRNA sequence available and shares the same target sequence variation of the majority of species in the respective domains (Table 1).

Evaluation of RNA quality. It is widely recognized that RNA is susceptible to degradation by RNase (30). In addition, it appears that some regions in the rRNAs are more sensitive to RNase degradation than others, which may have important consequences for quantitative hybridizations (23). Therefore, it is important to work with intact rRNA when performing quantitative hybridization. During various points in this study, PAGE analyses were used to evaluate the quality of the pure-culture and environmental nucleic acid samples. None of the RNA extracts showed any signs of degradation (data not reported).

Evaluation of probe-target duplex stability. The amount of probe eluted during a 10-min wash at increasing temperatures was determined by measuring the radioactivity of the wash solutions. Likewise, the amount of probe that remained on each membrane after completion of all washes was determined. The average background radioactivity was subtracted from each value. The total amount of radioactivity detected (i.e., the cumulative addition of radioactivity eluted at different temperatures, including the radioactivity that remained on the membrane) was considered to be proportional to the total amount of probe hybridized to the rRNA on the membrane. A normalized wash curve was constructed by using the percentage of the total probe that had eluted at each temperature in

TABLE 1. Universal SSU rRNA probes, target sequences for the nine organisms included in this study, and experimental T_ds

Probe[a] and organism	Probe or target sequence[b]	Domain-specific T_d[c] (°C) (ΔT_d[d])	ΔT_d (°C)[e]	% of mismatches[f]			
				0	1	≥2	N
S-*-Univ-1390-a-A-18	3′ AAC**A**TGTGTGGCGGGCAG 5′						
E. coli	1390 UCCCGGGCCUUG**U**ACACACCGCCCGUCACACCAUGG			77	8	0.5	14
D. desulfuricans	1397 UCCCGGGCCUUG**U**ACACACCGCCCGUCACACCACGA	56.5 (1.7)					
C. innocuum	1390 UCUCGGGCCUUG**U**ACACACCGCCCGUCAAACCAUGG						
M. bryantii	1340 CCCUGCUCCUUG**C**ACACACCGCCCGUCACGCCACCC			0	82	0	18
M. acetivorans	1329 CCCUGCUCCUUG**C**ACACACCGCCCGUCAAACCACCC	51.8 (3.2)	7.6				
M. voltae	1327 CCCUGCUCCUUG**C**ACACACCGCCCGUCACACCAUCC						
S. cerevisiae	1625 CCCUGCCCUUUG**U**ACACACCGCCCGUCGCUAGUACC			94	2	0.5	2.5
D. discoideum	1703 CCCUGCCCUUUG**U**ACACACCGCCCGUCGCUCCUACC	57.4 (1.0)					
Z. mays	1634 CCCUGCCCUUUG**U**ACACACCGCCCGUCGCUCCUACC						
S-*-Univ-1392-a-A-15[g]	3′ C**R**TGTGTGGCGGGCA 5′						
E. coli	1392 CCCGGGCCUUG**U**ACACACCGCCCGUCACACCAUGG			78	7	0	13
D. desulfuricans	1399 CCCGGGCCUUG**U**ACACACCGCCCGUCACACCACGA	46.0 (0.7)					
C. innocuum	1392 CUCGGGCCUUG**U**ACACACCGCCCGUCAAACCAUGG						
M. bryantii	1342 CCUGCUCCUUG**C**ACACACCGCCCGUCACGCCACCC			84.5	0	0	15.5
M. acetivorans	1331 CCUGCUCCUUG**C**ACACACCGCCCGUCAAACCACCC	53.6 (3.6)	9.5				
M. voltae	1329 CCUGCUCCUUG**C**ACACACCGCCCGUCACACCAUCC						
S. cerevisiae	1627 CCUGCCCUUUG**U**ACACACCGCCCGUCGCUAGUACC			87	1	4	5
D. discoideum	1705 CCUGCCCUUUG**U**ACACACCGCCCGUCGCUCCUACC	47.1 (0.3)					
Z. mays	1636 CCUGCCCUUUG**U**ACACACCGCCCGUCGCUCCUACC						
S-*-Univ-1392-b-A-15	3′ C**D**TGTGTGGCGGGCA 5′						
E. coli	1392 CCCGGGCCUUG**U**ACACACCGCCCGUCACACCAUGG			78	7	0	13
D. desulfuricans	1399 CCCGGGCCUUG**U**ACACACCGCCCGUCACACCACGA	44.4 (1.2)					
C. innocuum	1392 CUCGGGCCUUG**U**ACACACCGCCCGUCAAACCAUGG						
M. bryantii	1342 CCUGCUCCUUG**C**ACACACCGCCCGUCACGCCACCC			88	0	0	12
M. acetivorans	1331 CCUGCUCCUUG**C**ACACACCGCCCGUCAAACCACCC	49.8 (3.5)	7.5				
M. voltae	1329 CCUGCUCCUUG**C**ACACACCGCCCGUCACACCAUCC						
S. cerevisiae	1627 CCUGCCCUUUG**U**ACACACCGCCCGUCGCUAGUACC			87	1	4	5
D. discoideum	1705 CCUGCCCUUUG**U**ACACACCGCCCGUCGCUCCUACC	45.5 (0.3)					
Z. mays	1636 CCUGCCCUUUG**U**ACACACCGCCCGUCGCUCCUACC						
S-*-Univ-1392-c-A-15	3′ C**A**TGTGTGGCGGGCA 5′						
E. coli	1392 CCCGGGCCUUG**U**ACACACCGCCCGUCACACCAUGG			78	7	0	13
D. desulfuricans	1399 CCCGGGCCUUG**U**ACACACCGCCCGUCACACCACGA	48.6 (1.6)					
C. innocuum	1392 CUCGGGCCUUG**U**ACACACCGCCCGUCAAACCAUGG						
M. bryantii	1342 CCUGCUCCUUG**C**ACACACCGCCCGUCACGCCACCC			1	87.5	0	11.5
M. acetivorans	1331 CCUGCUCCUUG**C**ACACACCGCCCGUCAAACCACCC	47.1 (4.1)	5.5				
M. voltae	1329 CCUGCUCCUUG**C**ACACACCGCCCGUCACACCAUCC						
S. cerevisiae	1627 CCUGCCCUUUG**U**ACACACCGCCCGUCGCUAGUACC			87	1	4	5
D. discoideum	1705 CCUGCCCUUUG**U**ACACACCGCCCGUCGCUCCUACC	49.7 (0.4)					
Z. mays	1636 CCUGCCCUUUG**U**ACACACCGCCCGUCGCUCCUACC						
S-*-Univ-0519-a-A-18[g]	3′ GTCG**K**CGGCGCCATTA**W**G 5′						
E. coli	519 ACUCCGUGCCAGC**A**GCCGCGGUAAU**A**CGGAGGGUGC			78	7	0.5	13.5
D. desulfuricans	526 ACUCCGUGCCAGC**A**GCCGCGGUAAU**A**CGGAGGGUGC	52.5 (1.4)					
C. innocuum	532 ACUACGUGCCAG**YA**GCCGCGGUAAU**A**CGUAGGUGGC						
M. bryantii	462 GACCGGUGCCAGC**C**GCCGCGGUAA**CA**CCGGCAGCUC			49	39	4	7
M. acetivorans	459 GACCGGUGCCAGC**C**GCCGCGGUAA**CA**CCGGCGGCCC	48.3 (2.8)	6.0				
M. voltae	451 GUUCGGUGCCAGC**A**GCCGCGGUAA**CA**CCGACGGCCC						
S. cerevisiae	566 GUCUGGUGCCAGC**A**GCCGCGGUAAU**U**CCAGCUCCAA			82	8	1	5
D. discoideum	559 GUCUGGUGCCAGC**R**GCCGCGGUAAU**U**CCAGCUCCAA	51.6 (0.4)					
Z. mays	570 GUCUGGUGCCAGC**A**GCCGCGGUAAU**U**CCAGCUCCAA						

Continued on following page

TABLE 1—*Continued*

Probe[a] and organism	Probe or target sequence[b]	Domain-specific T_d[c] (°C) (ΔT_d[d])	ΔT_d (°C)[e]	% of mismatches[f]			
				0	1	≥2	N
S-*-Univ-0915-a-A-16	3′ TCCTTAACYGCCCCCG 5′						
E. coli	915 UAAAACUCAAA**U**GAAUUG**A**CGGGGGCCCGCACAAGC			48	30	7	13
D. desulfuricans	919 UGAAACUCAAAG**A**AAUUG**A**CGGGGGCCCGCACAAGC	55.4 (5.4)					
C. innocuum	920 NGAAACUCAAAGGAAUUG**A**CGGGGGCCCGCACAAGC						
M. bryantii	863 UGAAACUUAAAGGAAUUG**G**CGGGGG**A**GCACCACAAC			0	95	3	2
M. acetivorans	860 UGAAACUUAAAGGAAUUG**G**CGGGGG**A**GCACAACAAC	51.7 (1.1)	12.1				
M. voltae	851 UGAAACUUAAAGGAAUUG**G**CGGGGG**A**GCACCACAAC						
S. cerevisiae	1136 UGAAACUUAAAGGAAUUG**A**CGG**AA**G**G**GCACCACUAG			0	0	98	1
D. discoideum	1134 UGAAACUUAAAGGAAUUG**A**CGG**AA**G**G**GCACACAAUG	45.7 (1.3)					
Z. mays	1144 UGAAACUUAAAGGAAUUG**A**CGG**AA**G**G**GCACCACCAG						

[a] Probe nomenclature has been standardized according to the oligonucleotide probe database (1).
[b] Obtained from RDP (15); R, A or G; D, I; W, A or T; K, T or G; Y, T or C.
[c] Average of experimental T_ds observed for the three organisms in the same domain.
[d] Range of experimental T_ds observed for the three organisms in the same domain.
[e] Range of experimental T_ds observed for the nine organisms.
[f] Percentage of SSU rRNA sequences available in RDP that have 0, 1, or ≥2 mismatches with the probe. N indicates the percentage of SSU rRNA sequences available in RDP that have at least one uncertain nucleotide in the target site.
[g] First reported in reference 19.

a cumulative addition, to infer the total normalized radioactivity that would elute at each temperature. The resulting wash curves (averages of duplicate experiments) are shown in Fig. 1, and the domain-specific T_ds (averages from the three organisms within each domain) obtained from these curves are listed in Table 1.

Probe S-*-Univ-1390-a-A-18 perfectly matches the SSU rRNA of the *Bacteria* and *Eucarya* organisms included in this study. However, the experimental average T_d of the *Eucarya* is 0.9°C higher than that of the *Bacteria* (Fig. 1a and Table 1). The three *Archaea* organisms have one internal A:C mismatch with this probe, which resulted in a lower average T_d (4.7 and 5.6°C lower than the average T_ds of the *Bacteria* and *Eucarya*, respectively). This experimentally observed reduction in T_d is consistent with expectations: a 1% mismatch generally reduces the T_d of oligonucleotide probes by 1 to 1.5°C (31), and one base accounts for 5.6% of the total number of bases in probe S-*-Univ-1390-a-A-18. Therefore, one mismatch is expected to reduce the T_d by 5.6 to 8.4°C.

Probe S-*-Univ-1392-a-A-15 consists of a mixture of two oligomers differing from each other at the second base from the 3′ end of the probe. The probe mixture was synthesized by adding an equimolar mixture of A and G during the corresponding synthesis step. The oligomers with an A perfectly match the *Bacteria* and *Eucarya*, while the oligomers with a G match the *Archaea*. Therefore, there is one additional G:C pair when the probe hybridizes with SSU rRNA from the *Archaea* compared with those from the other two domains. According to the empirical equation used to estimate T_ds for oligonucleotide probes (31), one additional G:C pair increases the T_d by 2.7°C. However, our experimental results indicate a difference of approximately 7°C (Fig. 1b and Table 1). Since this result was obtained with a probe mixture, it is difficult to compare this value with the estimate obtained with the empirical equation. This is because both oligomers in the probe mixture contribute to duplex formation, but their relative contributions to the overall hybridization signal are difficult to determine experimentally (see below).

Probe S-*-Univ-1392-b-A-15 targets the same site as probe S-*-Univ-1392-a-A-15 but contains the base analog inosine (I) as its second base from the 3′ end instead of G or A. By using this base analog in the degenerate position, probe S-*-Univ-1392-b-A-15 forms a perfect match with the target sequences of all organisms included in this study. For the *Bacteria* and *Eucarya*, the I pairs with a U; and for the *Archaea*, the I pairs with a C. However, even though I is often considered a universal base, the stability of I-containing base pairs is not constant. For example, it has been reported that the degree of stability of I-containing base pairs is highly sequence dependent and that sequence-independent stabilities approximately decrease as follows: I:C>I:A>I:T≥I:G>I:U (12, 16). The results of our study are in agreement with these previous reports. The experimental T_d for the *Archaea* averages 49.8°C, which is 4.3 and 5.4°C higher than the average T_d for the *Eucarya* and *Bacteria*, respectively (Fig. 1c and Table 1). Thus, the I:C base pair (*Archaea*) contributes more to duplex stability than the I:U base pair (*Eucarya* and *Bacteria*).

Probe S-*-Univ-1392-c-A-15 targets the same sequence as probes S-*-Univ-1392-a-A-15 and S-*-Univ-1392-b-A-15. It perfectly matches the SSU rRNA of *Bacteria* and *Eucarya* and has one internal A-C mismatch with archaeal SSU rRNA. Because of this mismatch, the average experimental T_d for the *Archaea* is reduced by 1.5 and 2.6°C compared with the average T_d of the *Bacteria* and *Eucarya*, respectively (Fig. 1d and Table 1). One base accounts for 6.7% of the total number of bases in this probe, and, assuming that a 1% mismatch reduces the T_d by 1 to 1.5°C (31), one mismatch is expected to reduce the T_d by 6.7 to 10°C. This estimate is much higher than the experimentally determined value. This discrepancy may be explained because the mismatch site is close to the end of the duplex, which typically results in weak destabilization (31).

Hybridizations of probes S-*-Univ-1392-a-A-15 and S-*-Univ-1392-c-A-15 with rRNA from the *Bacteria* and *Eucarya* result in the formation of identical duplexes of target and perfectly matching oligomers. However, for both *Bacteria* and *Eucarya*, the average experimental T_ds for the mixed probe (S-*-Univ-1392-a-A-15) are 2.6°C lower than those for probe S-*-Univ-1392-c-A-15 (Table 1). As discussed above, when probe S-*-Univ-1392-a-A-15 is used, both oligomers in the probe mixture contribute to duplex formation. During posthybridization wash steps, the oligomer with one mismatch is eluted first at lower temperatures, while the perfectly matching

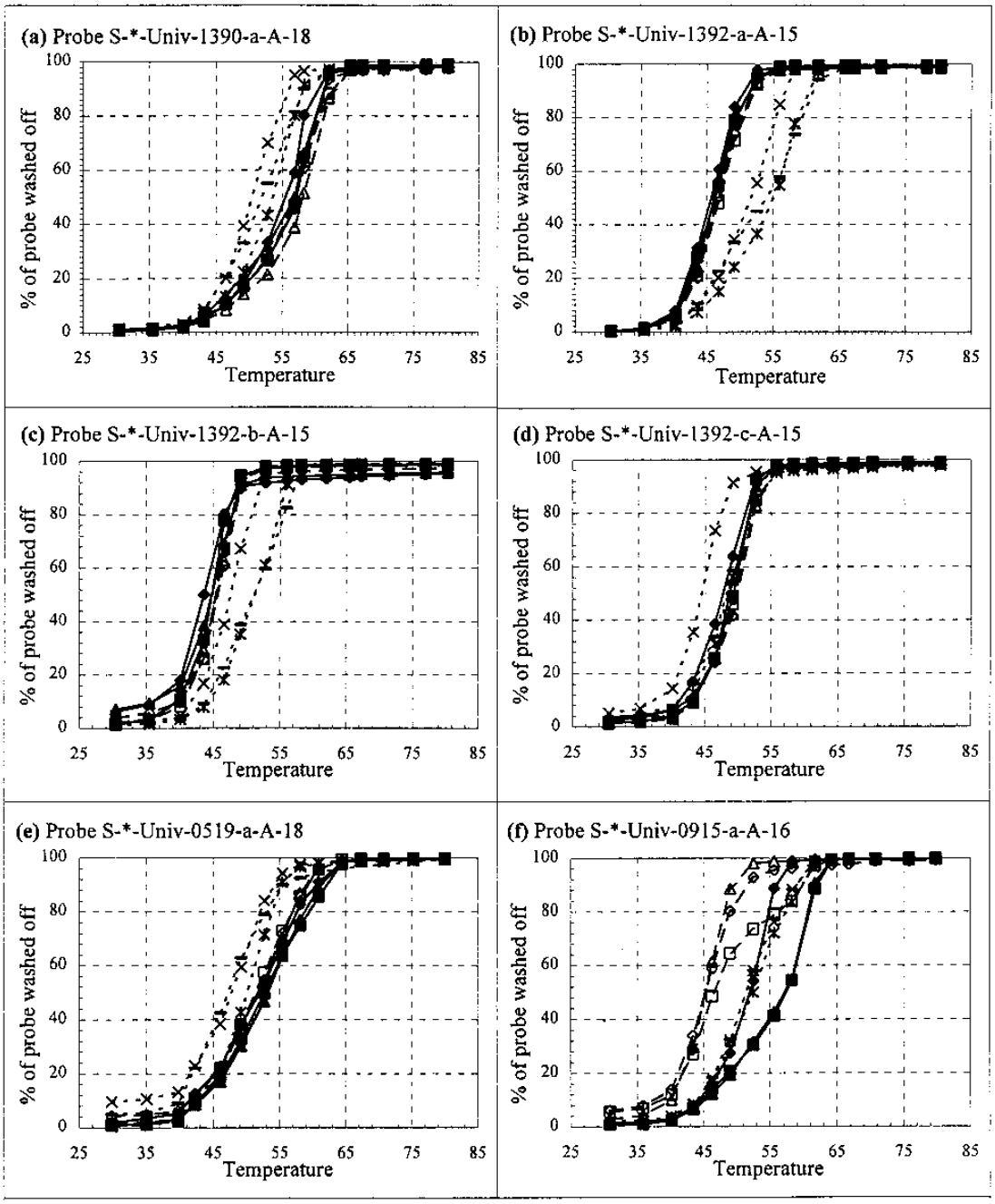

FIG. 1. Normalized oligonucleotide T_d curves obtained by the elution method. ◆, *E. coli*; ■, *D. sulfuricans*; ▲, *C. innocuum*; ×, *M. bryantii*; ∗, *M. acetivorans*; —, *M. voltae*; △, *D. discoideum*; ◇, *S. cerevisiae*; □, *Z. mays.*

oligomer is washed off at higher temperatures. As a consequence, the overall T_d curve for probe S-*-Univ-1392-a-A-15 is shifted to the left compared with the T_d curve for probe S-*-Univ-1392-c-A-15.

Probe S-*-Univ-0519-a-A-18 consists of a mixture of four oligomers. The degenerate sites are the second base from the 5′ end (A or T) and the fifth base from the 3′ end (T or G). These four oligomers target all *Bacteria* and *Eucarya* organisms included in this study but have one internal A:C mismatch with the *Archaea*. The average experimental T_d is 52.5°C for the *Bacteria* and 51.6°C for the *Eucarya* (Fig. 1e and Table 1). This domain-specific difference in T_d is difficult to interpret, since the relative contribution of each of the four oligomers in the probe mixture to the overall hybridization response is unknown. Because of the A:C mismatch, the average experimental T_d for the *Archaea* (48.3°C) is significantly lower than the T_d for the *Bacteria* and *Eucarya.*

Probe S-*-Univ-0915-a-A-16 consists of a mixture of two oligomers: the eighth position from the 5′ end of the probe contains a T or a C. This probe perfectly matches with *C. innocuum* and has one mismatch with *E. coli* at the second base (C:U) and with *D. desulfuricans* at the third base (C:A) from the 3′ end of the probe. The experimental T_ds for the three species of *Bacteria* are 51.8°C for *E. coli* and 57.2°C for *D.*

TABLE 2. Experimental T_ds of the probes targeting the 1400 region of the SSU rRNA for the three *Archaea* species

Probe	T_d (°C) for:			ΔT_d[a] (°C)
	M. bryantii	*M. acetivorans*	*M. voltae*	
S-*-Univ-1390-a-A-18	50.3	53.4	51.8	3.1
S-*-Univ-1392-a-A-15	51.5	55.0	54.1	3.5
S-*-Univ-1392-b-A-15	47.4	50.9	50.9	3.5
S-*-Univ-1392-c-A-15	44.5	48.6	48.2	4.1

[a] ΔT_d = maximum T_d − minimum T_d.

desulfuricans and *C. innocuum. D. desulfuricans* has the same experimental T_d as *C. innocuum* even though *D. desulfuricans* has one mismatch. This inconsistency suggests that the *D. desulfuricans* strain used in this study (ATCC 27774) in fact does not have a mismatch with probe S-*-Univ-0915-a-A-16. For the 15 *Desulfovibrio* species for which (partial) SSU rRNA sequences are available in RDP (15), 9 do not have sequence data in the 915 region, 3 have the same sequence as the one provided in Table 1 (including *D. desulfuricans* ATCC 27774), and 3 do not have a mismatch with probe S-*-Univ-0915-a-A-16. Resequencing of this SSU rRNA region would be necessary to resolve this ambiguity. The three *Eucarya* organisms have one G:G mismatch at the 5′ end and two C:A mismatches at the third and fourth positions from the 5′ end of the probe. As a consequence, the average experimental T_d is significantly lower than the average T_ds for the other two domains (Fig. 1f and Table 1). The *Archaea* organisms have one terminal G:A mismatch at the 5′ end of the probe. As a result of this mismatch, the melting curve for the *Archaea* is similar to the one for *E. coli*, and the average experimental T_d is 51.7°C.

For all six probes evaluated, we demonstrated that organisms within the same domain, sharing the same target sequence, can have different T_ds and melting curves. The four probes that target the 1400 region of the SSU rRNA demonstrated excellent behavior for *Bacteria* and *Eucarya*. For these four probes, the range of T_ds for the three *Bacteria* organisms is lower than or equal to 2°C (average, 1.4 ± 0.6°C). For the three *Eucarya* organisms, the range of T_ds is lower than or equal to 1°C (average, 0.6 ± 0.3°C). Even though these differences are small, they are significant since the reproducibility of our experiments was excellent (below). Duplicate melting curves were almost identical, and differences between experimental T_ds for the three *Bacteria* and three *Eucarya* organisms were always less than 0.5°C (average, 0.22 ± 0.11°C). In contrast, the probes in the 1400 region demonstrate a much larger range in T_d for the *Archaea*. The difference in experimental T_ds is as high as 4.1°C for the three *Archaea* organisms (Table 1). As shown in Table 2, which compares the experimental T_ds for the three *Archaea* organisms, *M. acetivorans* has the highest T_d and *M. bryantii* has the lowest T_d for all four probes targeting the 1400 region. This consistent behavior may be caused by differences in sequence outside the probe region and differences in secondary and higher-order structure among the SSU rRNAs of *Archaea*. Similarly, the observation that the *Eucarya* organisms have T_ds for probes in the 1400 region that are consistently about 1°C higher than those for the *Bacteria* (Table 1), despite identical target sequences, may be due to domain-specific sequence and structure variations.

Validity of the elution T_d method. The reproducibility of the T_d experiment, evaluated by constructing two melting curves for each organism for each probe (during the same hybridization experiment), was excellent. The difference between experimental T_ds for duplicates was less than 1°C in all cases and less than 0.5°C (average, 0.22 ± 0.11°C) in most cases.

The reproducibility of the elution T_d method was also evaluated by performing two independent hybridization experiments for probe S-*-Univ-1390-a-A-18 using four rRNA samples. The resulting T_ds were compared with the T_ds obtained in the first hybridization (Table 3). The second experiment resulted in T_ds for all organisms slightly higher than those obtained from the first experiment. For species with a perfect match, the largest difference in T_d observed was 0.7°C. For *M. voltae*, which has one mismatch with probe S-*-Univ-1390-a-A-18, the difference between the two experimental T_ds was 1.8°C. These small differences between T_ds obtained from two separate hybridizations are likely due to slightly different hybridization conditions (e.g., probe concentration).

The final wash step for the elution T_d method is performed in a manner different from that of the posthybridization wash step used during routine quantitative hybridizations. In the elution T_d method, the membrane is washed for 10 min at one temperature and is subsequently transferred to the next wash solutions at higher temperatures. During routine hybridizations, the membrane is washed at an experimentally determined wash temperature for 30 min. We chose to evaluate duplex stability using the elution T_d method because of the greater amount of information provided by this method compared with the more commonly employed T_d method (replicate-slot T_d method) (e.g., see reference 26). The main motivation for our choice is that the elution T_d method does not suffer from variation due to slot blotting or membrane variability and thus results in a much smoother melting curve. However, since the T_d does not correspond to equilibrium conditions, the length of the wash time can have an impact on the T_d. It was estimated that the T_d determined with a 10-min wash (elution T_d method) should be 1 to 2°C higher than the T_d determined with a 30-min wash (replicate-slot T_d method) by using an empirical equation provided by Tijssen (34). For probes S-*-Univ-1390-a-A-18 and S-*-Univ-1392-a-A-15, the T_ds were also determined by the replicate-slot T_d method. The resulting T_d curves are shown in Fig. 2. Table 4 compares the experimental T_ds for the two different methods. As expected, the experimental values determined by the elution method were slightly higher than those obtained with a 30-min wash (replicate-slot method). Nevertheless, because of the advantages of the elution method, we prefer to use this method to evaluate the duplex stability and melting behavior of newly designed probes and to select a posthybridization wash temperature about 1 to 2°C lower than the experimentally obtained T_d. Subsequently, it is important to evaluate the specificity of newly designed probes experimentally at the selected hybridization and wash conditions using an appropriate set of reference rRNAs (23).

Characterization of environmental samples. Our duplex stability studies show that all probes examined have variable T_ds for rRNAs from different domains. This could result in signif-

TABLE 3. Comparison of results obtained with two independent elution T_d experiments for probe S-*-Univ-1390-a-A-18

Species	T_d (°C) for expt:	
	1	2
E. coli	55.4	55.6
D. desulfuricans	57.1	57.8
M. voltae	51.8	53.7
D. discoideum	58.1	58.6

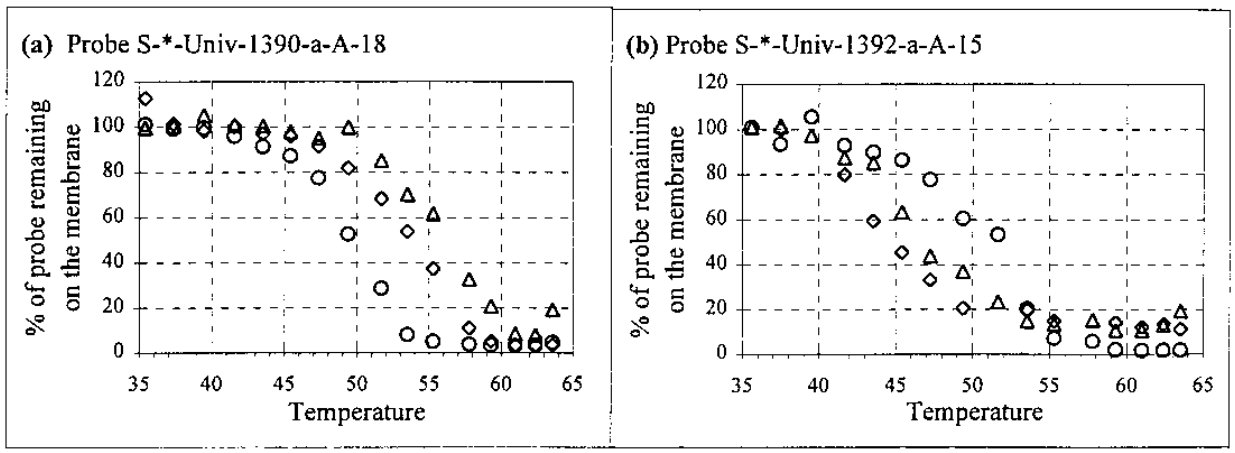

FIG. 2. Normalized oligonucleotide T_d curves obtained by the replicate-slot method. ◇, *E. coli*; ○, *M. bryantii*; △, *S. cerevisiae.*

icant bias when these probes are used to characterize environmental samples. For example, if probe S-*-Univ-1390-a-A-18 is used for hybridization with a wash temperature of 55°C (approximate T_d for *Bacteria* and *Eucarya*), Fig. 1a shows that the amount of probe that remains on the membrane ranges from 15 to 69% for the various organisms. Similarly, if probe S-*-Univ-1392-a-A-15 is used at its commonly employed wash temperature of 47°C (32), the amount of probe that remains hybridized varies from 37 to 85% (Fig. 1b). This domain-specific bias decreases significantly when the posthybridization wash stringency is reduced (by decreasing the wash temperature) (Fig. 1). The reduction in bias appears most significant for probe S-*-Univ-1390-a-A-18: the various melting curves for this probe are converging at a higher temperature than those for the other probes. Consequently, probe S-*-Univ-1390-a-A-18 was selected to characterize a number of synthetic and environmental samples at the domain level and to evaluate the effect of a reduction of wash stringency on quantitative hybridizations. Since probe S-*-Univ-1392-a-A-15 has been commonly used to normalize specific probe results (e.g., see references 7, 11, 18, 24, and 25), we also included this probe in our domain-level characterization.

If the posthybridization wash temperature for probe S-*-Univ-1390-a-A-18 is reduced from 55 to 44°C, Fig. 1a predicts that the range of domain-specific bias changes from 54% (69% minus 15%) to 6% (95% minus 89%). An issue of concern related to a reduced wash temperature is that the probes may hybridize to multiple sites in the SSU rRNAs or to other nucleic acids present in the samples. Thus, to prevent nonspecific binding to nontarget sites, the wash stringency should be reduced only to a limited extent. We determined experimentally that lowering the posthybridization wash temperature to 44°C did not result in significant levels of nonspecific binding (1b).

Figure 3 shows the response obtained after hybridizing similar amounts of *E. coli*, *M. bryantii*, and *S. cerevisiae* rRNA with probes S-*-Univ-1390-a-A-18 and S-*-Univ-1392-a-A-15 for two posthybridization wash stringencies. As predicted from our duplex stability studies (Fig. 1), the high-stringency washes resulted in an underestimation and overestimation of the archaeal response for probes S-*-Univ-1390-a-A-18 and S-*-Univ-1392-a-A-15, respectively. For probe S-*-Univ-1390-a-A-18, a reduction in wash stringency to 44°C significantly reduced the bias, resulting in a similar hybridization response for the three domains (Fig. 3). For probe S-*-Univ-1392-a-A-15, only a minor improvement was observed after reducing the wash stringency to 40°C. We believe that this lack of improvement in hybridization response is related to the properties of probe S-*-Univ-1392-a-A-15. This probe consists of a mixture of two different oligomers (Table 1). During the low-stringency hybridization step, both oligomers bind to the target site of the SSU rRNA for all three domains. Thus, assuming that probe S-*-Univ-1392-a-A-15 consists of an equimolar mixture of two oligomers, approximately 50% of the SSU rRNAs form duplexes with oligomers that perfectly match the target site, while the other 50% of the SSU rRNAs form duplexes with oligomers that have one mismatch. If we assume that a 1% mismatch lowers the T_d by 1.5°C, the T_d for a duplex with one

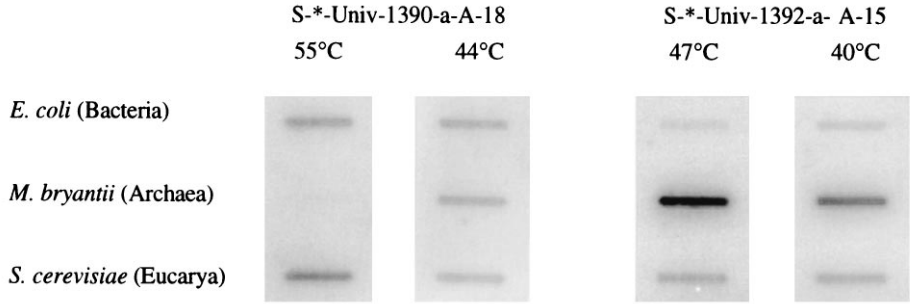

FIG. 3. Hybridization response with probes S-*-Univ-1390-a-A-18 and S-*-Univ-1392-a-A-15 to rRNA from pure cultures of *E. coli* (4.32×10^{-3} pmol per slot), *M. bryantii* (4.32×10^{-3} pmol per slot), and *S. cerevisiae* (3.83×10^{-3} pmol per slot). Two posthybridization wash stringencies were used for each probe.

TABLE 4. Comparison of experimental T_ds obtained by the elution method and replicate-slot method

Probe	Species	Exptl T_d (°C) by:	
		Elution method	Replicate-slot method
S-*-Univ-1390-a-A-18	*E. coli*	55.4	53.9
	M. bryantii	50.3	50.1
	S. cerevisiae	57.1	55.8
S-*-Univ-1392-a-A-15	*E. coli*	45.6	44.7
	M. bryantii	51.5	50.5
	S. cerevisiae	47.0	46.0

mismatch would be approximately 10°C lower for this probe. For a wash temperature of 40°C, about 95% of the oligomers that perfectly match the target site would remain bound (Fig. 1d) (probe S-*-Univ-1392-c-A-15 consists of one oligomer that perfectly matches the *Bacteria* and *Eucarya*). Oligomers that have one mismatch with the archaeal SSU rRNA would have a T_d of approximately 47°C (from Fig. 1d), and a large fraction of these oligomers would remain bound during a posthybridization wash at 40°C. Oligomers that have one mismatch with bacterial and eucaryal SSU rRNAs would have T_ds of approximately 39°C (49°C [from Fig. 1d] minus 10°C) and 40°C (50°C [from Fig. 1d] minus 10°C), respectively. Consequently, approximately 50% of the oligomers bound to eucaryal SSU rRNAs would be removed during a wash at 40°C, and an even larger percentage of the oligomers bound to bacterial SSU rRNAs would be eluted at this wash temperature. This analysis explains the overestimation of archaeal rRNA with probe S-*-Univ-1392-a-A-15, despite a low-stringency wash (40°C) (Fig. 3). In order to reduce the difference in response for the three domains, the wash temperature should be reduced further. However, even at lower wash stringencies, it will remain difficult to predict the behavior of this probe mixture quantitatively.

Even though the above analysis illustrates the impact of different wash stringencies for probes S-*-Univ-1390-a-A-18 and S-*-Univ-1392-a-A-15, we designed a more rigorous experiment to illustrate the results presented in Fig. 3 quantitatively. Different amounts of pure-culture *E. coli*, *M. bryantii*, and *S. cerevisiae* RNA were combined in samples A, B, and C (Table 5). In addition, two nucleic acid extracts obtained from environmental samples (samples D and E) were included in this experiment. We used probes for the *Archaea*, *Bacteria*, and *Eucarya* to determine the concentrations of rRNA associated with the three domains in these samples. As in the previous experiment (Fig. 3), we used hybridization results obtained with probes S-*-Univ-1390-a-A-18 and S-*-Univ-1392-a-A-15 at two different posthybridization wash stringencies to normalize the results obtained with the domain-specific probes. For samples A and B, the use of probe S-*-Univ-1390-a-A-18 combined with a wash at 55°C overestimated the relative levels of *Bacteria* and *Eucarya* and underestimated the relative concentration of *Archaea* (Table 5). This bias was significantly reduced when the wash stringency was decreased to 44°C. The use of probe S-*-Univ-1392-a-A-15 at a wash stringency of 47°C greatly overestimated the relative archaeal rRNA concentration and underestimated the relative bacterial levels in samples A and B. Although the bias is reduced when the wash temperature is decreased to 40°C, the experimental values are still very different from the true values. For sample C, which consisted of bacterial and eucaryal rRNA only, the use of probe S-*-Univ-1390-a-A-18 resulted in good experimental estimates of relative domain concentrations for both wash stringencies (Table 5). In addition, our results indicate that the use of probe S-*-Univ-1392-a-A-15 with a low-stringency wash may be acceptable for samples in which no *Archaea* organisms are present.

Samples D and E consisted of RNA extracts obtained from full-scale sewage sludge anaerobic digesters (27). Both digesters were performing well when sampled, as determined by metabolic activity assays and chemical analyses of sludge samples and biogas (27). Therefore, we expected a significant population of methanogens to be present in these digesters. We used the hybridization approach that provided the best estimate for samples A, B, and C (use of probe S-*-Univ-1390-a-A-18 with a low-stringency wash) to estimate the relative domain levels in samples D and E. Table 5 shows that relative archaeal (methanogen) rRNA levels were 11.5% ± 1.6% and 6.7% ± 0.8% for samples D and E, respectively. Other experimental conditions resulted in a significant bias: the use of probe S-*-Univ-1390-a-A-18 with a high-stringency wash re-

TABLE 5. Domain-level characterization of synthetic and environmental samples with probes S-*-Univ-1390-a-A-18 and S-*-Univ-1392-a-A-15 with two different posthybridization wash stringencies

Sample	Domain	% of total SSU rRNA (mean ± SD) with:			
		S-*-Univ-1390-a-A-18 at:		S-*-Univ-1392-a-A-15 at:	
		55°C	44°C	47°	40°C
A (75.8% *E. coli* rRNA, 18.6% *M. bryantii* rRNA, and 5.6% *S. cerevisiae* rRNA)	*Bacteria*	90.3 ± 4.1	73.5 ± 10.4	7.6 ± 1.0	21.2 ± 2.8
	Archaea	1.9 ± 0.5	25.5 ± 2.4	101.5 ± 11.5	91.5 ± 9.6
	Eucarya	11.3 ± 3.2	5.6 ± 1.2	5.0 ± 1.0	5.9 ± 1.2
B (62.9% *E. coli* rRNA, 9.3% *M. bryantii* rRNA, and 27.9% *S. cerevisiae* rRNA)	*Bacteria*	65.2 ± 0.3	64.6 ± 9.6	10.3 ± 1.5	23.6 ± 3.6
	Archaea	0.8 ± 0.3	15.8 ± 2.7	81.3 ± 14.4	69.5 ± 12.8
	Eucarya	40.6 ± 5.8	27.5 ± 3.0	34.5 ± 4.9	33.8 ± 2.8
C (94.0% *E. coli* rRNA, 0% *M. bryantii* rRNA, and 6.0% *S. cerevisiae* rRNA)	*Bacteria*	96.3 ± 14.0	94.8 ± 13.3	72.3 ± 17.7	98.7 ± 25.0
	Archaea	0.01 ± 0.03	0.12 ± 0.61	4.4 ± 21.9	1.4 ± 7.1
	Eucarya	11.4 ± 2.7	7.3 ± 1.5	39.2 ± 7.3	17.7 ± 4.4
D (rRNA extracted from anaerobic digester sample [Addison, Ill.] [27])	*Bacteria*	63.8 ± 8.7	75.4 ± 10.6	13.7 ± 1.8	21.9 ± 3.0
	Archaea	0.4 ± 0.1	11.5 ± 1.6	51.2 ± 2.9	36.8 ± 2.6
	Eucarya	0.5 ± 0.4	0.4 ± 0.3	0.4 ± 0.3	0.6 ± 0.4
E (rRNA extracted from anaerobic digester sample [Aurora, Ill.] [27])	*Bacteria*	85.4 ± 8.0	88.7 ± 7.9	21.8 ± 2.0	32.3 ± 3.2
	Archaea	0.2 ± 0.1	6.7 ± 0.8	45.7 ± 3.0	26.7 ± 2.0
	Eucarya	0.6 ± 0.3	0.5 ± 0.3	0.6 ± 0.4	0.8 ± 0.4

sulted in an underestimation of relative methanogen levels, while probe S-*-Univ-1392-a-A-15 for both wash conditions greatly overestimated methanogen rRNA concentrations.

Consequences for probe design. This study reveals a number of issues that should be considered when designing oligonucleotide probes for quantitative hybridization studies. First, our results underscore the importance of reducing the number of degenerate positions in oligonucleotide probes. If degeneracy cannot be avoided, both hydrogen bonds and base stacking effects should be considered. A probe should be designed such that the stabilities of different duplexes are as close as possible to each other. The use of probe mixtures and the base analog I are the most commonly employed design strategies for degeneracy problems. Our results indicate unexpected complications when probe mixtures are used in probe design. These problems are further exacerbated if the two oligomers in the degenerate probe mixture form duplexes with different stabilities (e.g., due to different number of hydrogen bonds). The results with probe S-*-Univ-1392-b-A-15, which includes I, confirm other findings (6, 12, 16) that the incorporation of only one I can have significant effects on the range of observed T_ds. The incorporation of I in probe S-*-Univ-1392-b-A-15 resulted in a range of T_ds as large as 7.5°C.

The second and perhaps more significant finding is the observed difference in T_ds for species that have the same target sequence. The effect of higher-order structure and neighboring sequences may be more important than anticipated. Therefore, the sequence close to the target sequence should be taken into consideration during the process of probe design, because the sequence of the adjacent unpaired regions can affect the stability of the duplex. Similar neighboring sequences are more likely to have similar stacking energies and thus result in similar stabilities and T_ds. In addition, higher-order structure ideally should be taken into account during probe design. However, the limited availability of comparative higher-order structure data does not make this feasible at this time.

The observed differences in T_ds for species which have the same target sequence is especially of concern for quantitative hybridization studies. However, the differences between T_ds are usually more pronounced when comparing organisms from different domains than for comparisons within the same domain. Thus, even though this effect should be evaluated carefully for each probe, it can be expected that it may not be as important for more specific probes, because species with closer phylogenetic relationships should have more similar rRNA structures.

An additional probe design strategy would be to use the large-subunit (LSU) rRNA instead of the SSU rRNA for the design of universal probes. Because of the greater information content available in the LSU rRNA, it may contain a greater number of universal or near-universal target regions. Databases of LSU rRNAs, however, are relatively small compared with SSU rRNA databases (15), and their use remains relatively limited. One LSU rRNA universal probe has recently been published (4), but characterization studies were not provided.

In conclusion, this study identified problems for quantitative hybridizations with universal oligonucleotide probes which have been used extensively in molecular microbial ecology studies. Using a systematic characterization of hybridization parameters, we were able to improve the precision of quantitative hybridization results for a probe targeting the 1400 region of the SSU rRNA substantially. Our findings underscore the importance of rigorous probe characterization studies for all newly designed probes. Even though the recommendations provided in this study have resulted in a significant reduction of the bias introduced by previous work, further universal probe design efforts are desirable. For example, a detailed evaluation of conserved sites in the SSU rRNA outside the 1400 region (e.g., see reference 9) would be beneficial. It is also possible to use other base analogs for degenerate positions. Recently, a number of new universal base analogs have been proposed (14, 17). We anticipate that some of these base analogs will play an important role in future universal (and specific) oligonucleotide probe design efforts.

ACKNOWLEDGMENTS

We are thankful to Matt Kane (Laboratory of Molecular Systematics, Smithsonian Institution) for preliminary characterization of probes S-*-Univ-1390-a-A-18 and S-*-Univ-1392-c-A-15.

This research was supported by grants from the U.S. National Science Foundation (BES 9410476) and the University of Illinois Research Board.

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