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Marine Chemistry 92 (2004) 295–306

MARINE CHEMISTRY

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Phylogenetically specific separation of rRNA from prokaryotes for isotopic analysis

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Received 4 November 2003; received in revised form 12 April 2004; accepted 30 June 2004
Available online 22 October 2004

Abstract

A wealth of genetic sequence information is available publicly and can be used to support investigations in organic geochemistry. Here, we present a new method to separate ribosomal RNA in order to use this rRNA as a “biomarker” for molecular isotopic studies. The primary goal is to obtain pure fractions that reflect selected phylogenetic groups. We demonstrate the ability to separate rRNA of a target organism from RNA representing a mixture of species. In this approach, an oligonucleotide probe containing a poly-d(GGGT) tail is hybridized to RNA in solution. Simultaneously, an aliquot of oligo-dT paramagnetic beads is hybridized to an oligonucleotide made of poly-d(CCCA) with a poly-dA tail. The two solutions are combined and a high-affinity GCAT complex is formed. The magnetic beads are captured and re-suspended in fresh solution. Careful determination of the melting temperatures of all three hybrids permits melting of the captured rRNA while leaving the majority of the oligonucleotides bound to the beads. Authenticity of the captured product is determined by reverse-transcriptase (RT)-PCR on a sub-sample; the remainder is washed, re-suspended in H_2O, and saved for subsequent isotopic analysis.
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Keywords: Analytical techniques; Carbon isotopes; Nucleic acids; RNA

1. Introduction

1.1. Background

More than a decade has passed since the first carbon isotopic measurements were made on samples of RNA obtained from a natural system. Coffin et al. (1990) recognized that the $\delta^{13}C$ values of total RNA extracted from a coastal salt marsh could be used to trace the sources of carbon utilized by the active biological community. The RNA extracted from these samples primarily derived from the microbial population (>90% of the total RNA), and the measured isotopic values reflected the mixture of carbon from terrestrial, marine, and estuarine sour-

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doi:10.1016/j.marchem.2004.06.032

ces. In prior work, Coffin et al. (1989) had measured the $\delta^{13}C$ values of total bacterial biomass collected using careful, size-based filtration. The major complication associated with both of these approaches is the need to verify the purity of the sample. RNA potentially is contaminated with co-precipitating humic substances, while filtered cells may be contaminated with small, non-bacterial particles. Subsequently, an improved method was developed to eliminate humic material from extracts of total RNA (Moran et al., 1993). However, later work done in the Gulf of Mexico (Kelley et al., 1998) revealed other problems: over the course of 1 year, the carbon isotopic composition of the mixed prokaryotic community varied between −33‰ and −18‰. Inability to identify the reason for this wide range in values limited the interpretation of the results. Values of $\delta^{13}C$ obtained from total cells or total RNA integrate the processes occurring within complex microbial consortia.

Several new techniques now are in the early stages of development. Each is designed to satisfy the same primary goal, which is to link specific metabolic pathways of carbon assimilation with the responsible microbial species or group. Some of these approaches are not based on analysis of nucleic acids. Incubation of environmental samples with natural or ^{13}C-labeled substrates, followed by isotopic analysis of the extracted bacterial fatty acids, has met with some success (Pelz et al., 1997; Boschker et al., 1998). However, the information value is limited, due to the variable intracellular fractionation between fatty acids and total biomass (e.g., Blair et al., 1985; Schouten et al., 1998; Hayes, 2001). Additionally, within the microbial consortia found in marine and coastal ecosystems, the diversity of fatty acids is much less than the presumed complexity of the population. Neither concentration distributions (e.g., Perry et al., 1979; Wakeham and Beier, 1991; Haddad et al., 1992) nor isotopic values (e.g., Gong and Hollander, 1997) can be linked directly to particular species. Only in the case of unique lipid biomarkers such as archaeol (Hinrichs et al., 1999) can an organism be linked to a specific process.

Nucleic acids have the potential to solve the problem of organism specificity, if sequence information and isotopic values can be obtained from the same sample. This was shown by Orphan et al. (2001), who coupled fluorescence in situ hybridization (FISH) with secondary ion mass spectrometry (SIMS) to measure the isotopic gradient across an aggregate of cells involved in the anaerobic oxidation of methane. FISH–SIMS has great potential to link particular species with their isotopic compositions. However, at present, the isotopic precision of this method is relatively poor (isotopic differences of <1–2‰ cannot be measured), and the accuracy is difficult to evaluate. The SIMS beam size, at 10–15 μm, is larger than most individual cells. It is necessary also to separate the cells from other carbon-containing particulate matter in the sample; many bacteria remain adhered to sediments and thus might not be amenable to FISH–SIMS. Finally, sample preparation and analysis is laborious and not easily amenable to automation and rapid throughput.

The method of Radajewski et al. (2000, 2002) combines isotope-labeling experiments with subsequent extraction of the labeled fraction of the nucleic acids for sequencing. In this approach, termed “stable isotope probing”, a ^{13}C-labeled substrate is incubated with a natural sample under approximately in situ conditions. Total DNA is extracted from the sample, and the fraction of DNA containing the ^{13}C label is isolated by ultracentrifugation, relying on the change in density acquired from the isotopic label for separation. Sequencing of both labeled and unlabeled DNA provides insight into which species within the total population are capable of metabolizing the substrate. This approach is very valuable and will have numerous applications. However, manipulation of samples during incubation experiments (classic “bottle effects”) is always a concern, and here the incubation period also becomes a critical factor. The ideal incubation must be calibrated to detect incorporation of the label into the primary consumer and stopped before the label migrates via trophic-level interactions; the method will always be biased against species with slower rates of metabolism.

Therefore, there are opportunities still to be gained from the separation and isotopic analysis of rRNA from environmental samples. This approach, called “RNA capture”, could be used to understand the natural isotopic distribution within microbial consortia. It also is amenable to incubation experiments. The analytical requirements are: (i) hybridization conditions that inhibit degradation of the RNA, (ii) the

ability to separate RNA from different microbial groups or species, (iii) validation of the authenticity of the captured sequence, (iv) minimal contamination from non-RNA carbon in the final sample, (v) practical sizes for the original samples, and (vi) adequate yield for isotopic analysis.

An example of an RNA capture method recently was published by MacGregor et al. (2002). Although the approach presented here is similar, significant differences are reflected in each of our steps (i–vi) above. Ultimately, the major difference is the size of the final sample, which determines the scaling of the experiments. The isotopic data presented by MacGregor et al. (2002) were obtained from bacterial cultures and were measured using an elemental analyzer coupled to an isotope ratio mass spectrometer (EA-irMS); the typical minimum sample size of 1 μmol of carbon limits the extent to which this approach can be applied practically to environmental samples. Recent work by Sessions et al. (2002) has improved the moving wire interface developed by Brand and Dobberstein (1996). The current threshold for this "nano-combustion" interface to attain 0.3‰ precision is ~3 nmol of carbon (3 nmol C), or 1‰ precision at 1.5 nmol C. The details of this instrument that allows isotopic measurements to be made precisely and accurately on very small samples are not addressed here, but it serves as the guide for the method that is presented and for the discussion that follows.

2. Methods

2.1. Materials

Sera-Mag™ paramagnetic, oligo-dT particles (10 mg/ml; binding capacity 340 pmol/mg) were stored at 4 °C in 0.05% sodium azide to inhibit microbial growth. The modifier oligonucleotide (CA-clamp; 5′-CCCACCCACCCACCCACCCCCCCCAAAAAAA-AAAAAAAAAAAAA-3′) and hybridization probes (ARC915-GGGT: 5′-GTGCTCCCCCGCCAAT-TCCTCCCCCGGGTGGGTGGGTGGGTGGG-3′; and ARC915-Long: 5′-GTGCTCCCCCGCCAATT-CCTTTAAGTTTC-IntSpacer18-GGGTGGGTG-GGTGGGTGGG -3′) were synthesized by Integrated DNA Technologies and re-hydrated from lyophilized powder to 0.5 nmol/μl in sterile H_2O. Hybridization buffer was purchased from Ambion® (100 ml) and sterile-filtered through a 0.2 μm Pall-Gelman Acrodisc HT-Tuffryn membrane syringe filter into aliquots of 2 ml. A fresh tube of sterile buffer was used for each experiment.

Primers for RT-PCR were synthesized by Integrated DNA Technologies and re-hydrated from lyophilized powder to 1 nmol/μl in sterile H_2O (stock solutions) and diluted further to 20 pmol/μl (working solutions). Reverse transcriptase (RT: eAMV, 20 units/μl, Sigma) was diluted 1:40 and stored in working aliquots at −80°C. PCR reactions were performed using JumpStart® REDTaq® ReadyMix (Sigma), also stored in aliquots at −80°C.

2.2. Modification of oligo-dT beads

Fifty microliters of beads were transferred to a 200-μl PCR tube and captured on the wall of the tube using a Dynal® magnetic particle separator. Beads were washed twice with 50 μl of sterile hybridization buffer and re-suspended in 100 μl of buffer with 2 μl of CA-clamp oligonucleotides. Hybridization was performed in an Eppendorf Mastercycler Gradient Thermocycler using a touch-down program: 80°C (2 min), 75°C (2 min), 73°C (2 min), 71°C (2 min), . . ., 57°C (2 min) for a total of 22 min. Beads were captured on the wall of the tube and unbound CA-clamp was aspirated by pipetting. Two washes were performed with fresh hybridization buffer at 57°C.

2.3. Melting temperature of CA-clamp

The melting temperature of the CA-clamp-oligo-dT hybrids was determined by addition of 100 μl fresh hybridization buffer at 30°C. Beads were captured on the wall of the tube for 1 min using the magnetic particle separator, and the hybridization buffer was transferred to a 100 μl capacity quartz mini-cell; UV absorbances at 260nm (Abs_{max}) and 320nm (background) were measured on a Beckman-Coulter DU640 UV–VIS spectrophotometer. Buffer was returned to the tube, beads re-suspended, and the temperature was increased. After 4 min, beads were re-captured and the absorbance measurements were repeated. Melting was performed at 5°C increments

up to 85°C, which was defined as 100% melted to normalize the absorbance values from replicate experiments.

2.4. Probe binding capacity

A dilute working solution of probe ARC915-d(GGGT) was prepared in hybridization buffer (5 pmol/μl). Aliquots of modified beads were suspended in 100 μl of hybridization buffer, and probe was added incrementally. After addition of each aliquot, samples were incubated for 4 min at 30°C, the beads were captured, and absorbance of the solution was measured. Probe was added to >2× the expected binding capacity of the beads. Binding capacity for the hybridization probe ARC915-Long was established in separate experiments and found to be ≥ the bead capacity for ARC915-d(GGGT).

2.5. Melting temperature of probe complex

An aliquot of beads saturated with ARC915-d(GGGT) probe was suspended in 100 μl of fresh hybridization buffer at 30°C. Incremental melting was done identically as it was for the melting of the CA-clamp-oligo-dT bead complex, and UV absorbances were measured every 5°C.

2.6. Extraction of RNA

Pelleted cells from pure cultures of *Halobacterium salinarium* (ATCC strain 33171, grown on Medium 217+25% NaCl), *Saccharomyces cerevisiae* (ATCC strain 287, grown on Difco® YM broth), and *Escherichia coli* (ATCC strain JM109, grown on Difco® LB broth) were lysed in a mixture of 9 ml of 50 mM NaOAc (pH 5.2), 1 ml of 5% SDS, and 1 mg of Proteinase K (Life Technologies) at 65°C for 30 min. The aqueous phase was extracted with 10 ml of acidic phenol, followed by 10 ml of phenol/chloroform/isoamyl alcohol (25:24:1), followed by 10 ml of 100% chloroform. Nucleic acids were precipitated out of the aqueous phase by mixing 1:1 with isopropanol plus 200 μl of 5M NaCl, incubating at −80°C for >1 h, and centrifuging at 12,000×*g* for 10 min. Pellets were washed 3× with 70% EtOH in H_2O, and nucleic acids were re-suspended in Nanopure H_2O. Visual inspection by gel electrophoresis confirmed that the samples were not only enriched in RNA but also contained some DNA. These extracts have been stored at −80°C without visible degradation for >3years.

All environmental samples were extracted in RNAWiz® (Ambion) according to manufacturer instructions. Samples of mollusk and vestimentiferan tissue, including *Riftia pachyptila*, *Solemya velum*, *Calyptogena pacifica*, and *Bathymodiolus thermophilus*, were obtained from K.T. Scott and C. Cavanaugh of Harvard University. The sample of *Axinella mexicana* was obtained from laboratory archives. Marsh sediments were cored by hand in Woods Hole, MA, USA, and frozen on dry ice for transport to the laboratory; sediment from the Eel River fan was obtained by T. Eglinton and D. Montlucon and stored on N_2(l). Water-column particulate matter from Lake Mishwam, Woburn, MA, USA, was obtained by peristaltic pump and collection onto 0.2 μm polycarbonate filters. Filters were stored on dry ice. Yields of RNA from all extractions were quantified by UV–VIS spectrophotometry as above; data are reported in Table 1.

2.7. rRNA capture

For optimization of capture conditions, 2 μg of RNA from *H. salinarium* was hybridized to probe ARC915-Long in 50 μl of hybridization buffer containing 25% formamide. For competitive hybridization experiments, ~1 μg of RNA from *E. coli* and ≤1 μg of RNA from *S. cerevisiae* were added to ~1 μg of RNA from *H. salinarium* to achieve a final mixture of ≤60 pg totRNA/μl. The temperature program was 72°C (7 min), hold 44°C (overnight). Hybridization temperature was optimized using the gradient function of the Eppendorf Mastercycler; hybridization time was not optimized, as long incubations did not induce degradation of the RNA. RNA hybrids were captured for 1 h with 0.5 mg of CA-modified beads pre-equilibrated to 44°C in 50 μl of hybridization buffer. The unbound fraction was removed following capture of the beads, and samples were washed in 100 μl of buffer (without formamide) at successively increasing temperatures: 44, 48, 52, 56, 60, 62, 64 and 80°C. Aliquots of 20 μl were saved for RT-PCR, and the remainder of each fraction was precipitated in isopropanol+10 μl of 5M NaCl.

Table 1
Total extractable RNA content of typical samples

Samples	Yield	For 50μg	(fg/cell)[a]
Culture			
E. coli, late log phase	20 μg/ml	2.5 ml	56
Water			
Stratified Lake (Mishwam; summer; 0.5 m)	1 μg/ml	50 l	0.2
Stratified Lake (Mishwam; summer; 1.6 m)	0.3 μg/ml	170 l	0.04
Stratified Lake (Mishwam; summer; 2.6 m)	0.4 μg/ml	120 l	0.04
Tissue			
R. pachyptila (trophosome)	3300 μg/g	15 mg	
C. pacifica (gill)	3100 μg/g	16 mg	
S. velum (whole)	1800±360 μg/g	28±6 mg	
B. thermophilus (gill)	1300 μg/g	40 mg	
A. mexicana (whole)	530 μg/g	94 mg	
Sediment			
Marsh sediment (Woodneck; summer; surface)[b]	41±14 μg/g	1.4±0.7 g	8±3
Pelagic sediment (Eel River; surface)	20 μg/g	2.4 g	4
Marsh sediment (Wild Harbor; late winter; deep)[c]	4.1±0.73 μg/g	13±2.2 g	0.8±0.1

[a] Cellular RNA content scales with metabolic rate, requiring proportionally more cells in slow-growth environments. RNA content for *E. coli* from Madigan et al. (1997); 5×10^9 cells/g assumed for sediments; actual counts used for water samples (M. Fisher, personal communication).

[b] n=10.

[c] n=4.

2.8. RT-PCR

From the 20% aliquots of recovered rRNA, a series of RT-PCR reactions was compared to pure RNA from *H. salinarium* at known concentration. Two microliters of each recovered sample, 20 μl of sterile H_2O, 1 μl forward primer (1100F, 5′-GGCAAC-GAGCGMGACCC-3′; Lane, 1991), 1 μl reverse primer (1390Ra, 5′-GACGGGCGGTGWGTRCAA-3′; Lane, 1991), and 1 μl eAMV-RT working solutions were added to 25 μl REDTaq ReadyMix. Negative controls were no template, no eAMV-RT, and totRNA from *E. coli*. The RT reaction was carried out for 15 min at 48°C; 25 cycles of PCR were performed: denature 90°C (15 s), anneal 69.8°C (30 s), extension 72°C (60 s).

To amplify the RNA from *E. coli* and *S. cerevisiae* recovered during competitive hybridizations, 2 μl of each recovered sample, 20 μl of sterile H_2O, 1 μl forward and reverse primers (*S. cerevisiae*: 1179F, 5′- AATTTGACTCAACACGGG-3′ and 1438R, 5′-GGGCATCACAG ACCTGTTAT-3′, Hendriks et al., 1989; *E. coli*: 27F, 5′-AGAGTTTGATCCTGGCT-CAG-3′ and 1100R, 5′- AGGGTTGCGCTCGTTG - 3′ F, Lane, 1991), and 1 μl eAMV-RT working solutions were added to 25 μl REDTaq ReadyMix. Negative controls were no template, no eAMV-RT, and totRNA from *H. salinarium*. The RT reaction was carried out for 15 min at 48°C; 25 cycles of PCR were performed: denature 90°C (15 s), anneal (30 s), extension 72°C (60 s).

3. Results

3.1. RNA capture design

We originally attempted to develop a protocol based on hybridization of RNA to a biotinylated oligonucleotide, followed by capture using streptavidin-coated beads (i.e., similar to MacGregor et al., 2002). However, yields of RNA obtained by this approach were low and unreliable, and process blanks were high (Pearson et al., 2002). Subsequently, streptavidin beads were abandoned in favor of a method employing magnetic beads coated with poly-dT oligonucleotides (Fig. 1).

One option for RNA capture was to design a single hybridization probe containing a poly-dA tail; this probe would bind to 16S rRNA at one end, and the poly-dA tail would bind directly to the oligo-dT beads. However, since addition of oligo-dT beads to a solution of total RNA extracted from an environmental sample could result in competitive binding with eukaryotic mRNA, this approach was abandoned. We instead modified the oligo-dT sequences using the "CA-clamp" (Fig. 1) to mask the mRNA binding capacity of the beads and create a unique tail of poly-d(CCCA) that was not complementary to

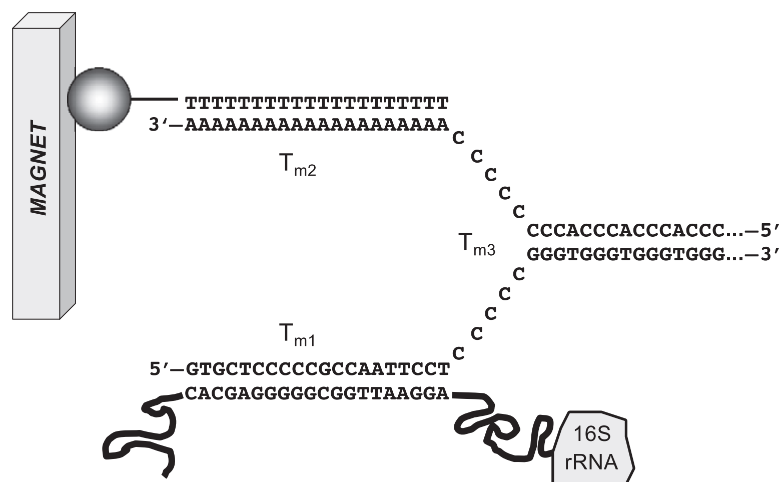

Fig. 1. Cartoon of the rRNA-hybrid complex. Target rRNA is bound to a complementary oligonucleotide probe that contains a long tail of sequence poly-d(GGGT). This RNA-probe hybrid attaches to paramagnetic particles that are modified to poly-d(CCCA). Hybrid lengths and compositions are designed to adhere to the requirement $T_{m1}<T_{m2}<T_{m3}$. Captured rRNA is separated from non-target RNA by attraction to an external magnet, followed by re-suspension in fresh buffer.

naturally occurring sequences. The rRNA capture probe was synthesized with a poly-d(GGGT) tail to hybridize uniquely with the modified beads.

All three hybrid sequences in the rRNA-probe-bead complex were designed to satisfy the requirement: $T_{m1}<T_{m2}<T_{m3}$. The following results show how this design was optimized: specifically, it permits reverse melting and recovery of rRNA, while minimizing contamination of the final sample by oligonucleotides. Although the present example applies to the Domain level only (Woese et al., 1990), the same approach will be applied to the development of future probes and could, in theory, be adapted to any degree of phylogenetic specificity.

3.2. Modification of oligo-dT beads

Experiments to monitor the binding of the CA-clamp oligonucleotide to the oligo-dT beads indicated that at constant temperature, complete reaction occurred within 10 min (not shown). To maximize the formation of complete hybrids of highest possible melting temperature (21 successive A–T bonds), a touchdown hybridization (Don et al., 1991) was implemented. "Touchdown" first was developed to increase the specificity of primer-product amplification in the polymerase chain reaction (PCR; Mullis et al., 1986). Here, it is used to maximize the hybridization of free oligo-dT units to the poly-dA section of the CA-clamp. This minimizes the availability of sites that could bind eukaryotic mRNA and cause contamination of samples. It also assures that during the release of the captured 16S rRNA, minimal oligonucleotide will separate from the beads.

3.3. Melting temperature of the CA-clamp

The stability of the modified bead complex was tested by measuring incremental melting over a range of temperatures (Fig. 2). The data are shown as the percentage of oligonucelotide that remains bound to the beads; the yield at 85°C is assumed to reflect 100% melting. Temperatures >85°C may promote degradation or dissociation of the oligo-dT chain and were avoided. The average and standard deviation of three replicate samples is shown. During characterization of probes for FISH, the melting temperature (T_m) typically is reported as the temperature of 50% binding (Stahl and Amann, 1991). Here, the temperature is 72°C; however, to minimize release of oligonucleotides into samples of captured 16S rRNA, we define the T_m for capture-oligos to be at ≥75% bound, or 65°C. This temperature is 8°C above the final temperature used during touchdown hybridization of the CA-clamp to the beads. To improve retention of the probe at 65°C, the touchdown

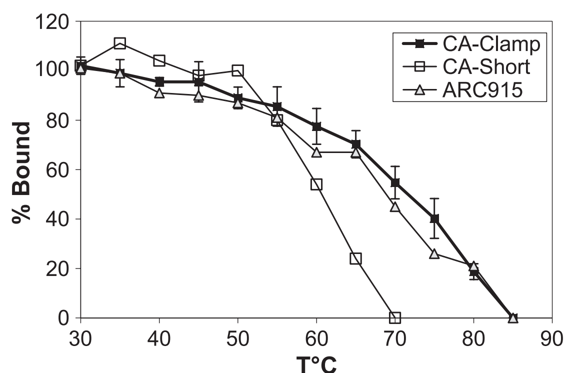

Fig. 2. Melting experiments to determine the temperature of dissociation of the bead-oligonucleotide complexes. Solid squares show the melting profile of the long (poly-dA_{21}) CA-clamp oligonucleotide, open squares show the melting profile of a short (poly-dA_{15}) CA-clamp oligonucleotide, and triangles show the melting profile of the long (poly-dA_{21}) CA-clamp oligonucleotide after binding to probe ARC915-d(GGGT).

program may be modified to end at a higher temperature.

Also shown in Fig. 2 is the melting curve for a CA-clamp oligonucleotide designed with a poly-dA tail having only 15 dA nucleotides (CA-Short). The melting temperature is correspondingly lower, illustrating the importance of long hybrids to achieve stability of the complex.

3.4. Probe binding capacity

To determine how much of the ARC915-d(GGGT) hybridization probe could be captured by the modified beads, samples of modified beads were titrated with a dilute solution of probe (Fig. 3). Here, the dashed line shows the predicted increase in UV absorbance of the hybridization solution if no binding occurs between the probe and the modified beads. The solid line shows the predicted result, based on the binding capacity of the beads as reported by the manufacturer. The data represent the average and standard deviation of three replicate experiments. Although there is some variability between samples, in general, the data are consistent with the expected levels of binding. This indicates that there is minimal change in the binding properties of the beads associated with modification from the original oligo-dT form to the poly-d(CCCA) derivative. Calibration of the binding capacity of the beads is critical in order to avoid incomplete capture of rRNA-hybrids during RNA capture experiments.

3.5. Melting temperature of probe complex

To establish that the melting temperature of the bead-probe complex (T_{m3}) was ≥ the melting temperature of the CA-clamp modifier (T_{m2}), an aliquot of beads that had been saturated with probe ARC915-d(GGGT) was subjected to an identical melting protocol. These data also are shown in Fig. 1. The melting profile is comparable to what was observed for dissociation of the hybrid formed between the oligo-dT beads and the CA-clamp; if there is an additional contribution from dissociation of the ARC915-d(GGGT) probe, it is minimal. A high melting temperature for T_{m3} is expected due to the high GC-content (75%) of the hybrid formed between the poly-d(GGGT) tail and the poly-d(CCCA) modifier.

3.6. rRNA capture

The selective hybridization and capture of 16S rRNA from *H. salinarium* is shown in Fig. 4. The unhybridized material (U) represents excess RNA that was not captured onto the beads. The lane marked 44 °C shows the RNA recovered in an initial wash performed at the temperature of hybridization; this wash removes the remainder of the unhybridized mixture. Subsequent washes were performed at

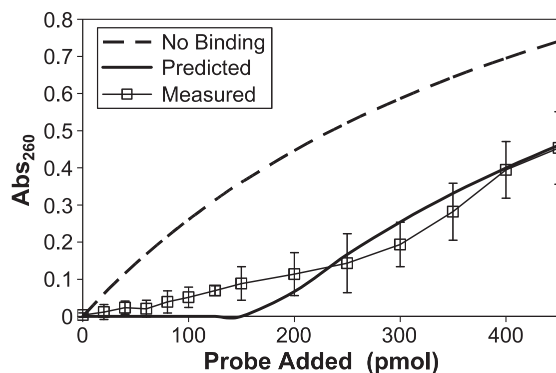

Fig. 3. Titration of 0.5 mg of modified beads with probe ARC915-d(GGGT). Predicted binding capacity of the beads is 340 pmol/mg. If no binding occurred between probe and modified beads, the absorbance would follow the dashed line. Data from triplicate experiments are shown with squares.

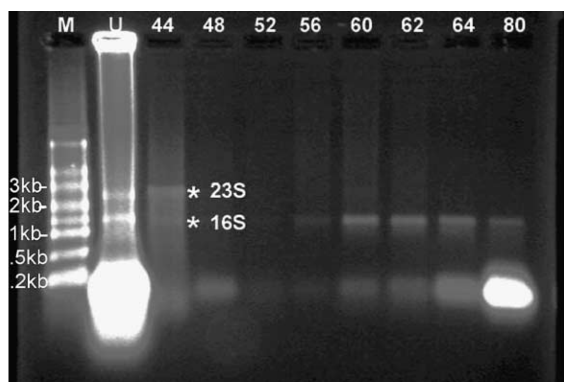

Fig. 4. Capture of 16S rRNA from *H. salinarium*, with subsequent elution from the beads. M: molecular size markers; U: unhybridized RNA; numbers: temperature (°C) of elution of captured products.

incrementally increasing temperatures designed to destabilize imperfect hybrids. Very little material eluted at 48 and 52°C, indicating that non-specific hybridization was minimal. The majority of the hybridized 16S rRNA molecule eluted between 60 and 64°C, while the majority of the oligonucleotides used for capture eluted at 80°C.

Based on quantification of the recovered RNA by UV–VIS spectrophotometry, the RNA yield from lanes 60–64°C is approximately 5% of the total amount of RNA added to the original mixture, or ~100 ng. We estimate that intact 16S rRNA molecules represent no more than 20% of the original sample by mass; therefore, this represents an estimated capture efficiency of ~25%. Certainly, this result could be improved with further refinement of the hybridization protocol; however, a yield of 100 ng of RNA is equivalent to approximately 35 ng of carbon. This is adequate for a minimal isotopic analysis: one measurement of $\delta^{13}C$ could be performed to a precision of ±0.3‰. A more desirable analysis of five replicates would be achieved by using ≥10 μg of total RNA and scaling the initial reaction mixture proportionately. In further discussion, it will be assumed that the starting sample size contains a minimum of 10 μg of RNA.

The low efficiency of hybridization may be related to a number of factors. The total concentration of RNA in the mixture may affect the mobility of the probe and therefore limit its accessibility to the target binding site. Conversely, the efficiency may be related entirely to the ratio of probe/target during each hybridization. Preliminary work suggests that the former is more likely to be the limiting variable. Under the current protocol, the probe is added in a ratio of nearly 200:1 with respect to 16S rRNA targets. A small increase in the total amount of RNA added to the reaction mixture affects the outcome of the hybridization reaction (not shown); yet, probe is still present in great excess under these circumstances.

3.7. RT-PCR

Reverse-transcriptase (RT) transcribes RNA into a DNA template suitable for amplification by primers for PCR. This approach provides a control method for the evaluation of the purity of captured RNA samples. Here, a set of RT-PCR experiments demonstrated the selectivity of the hybridization and capture protocol. Capture probe ARC915-Long was allowed to hybridize competitively to a sample containing mixed RNA representing pure cultures of the three domains: Eukaryotes (*S. cerevisiae*), Bacteria (*E. coli*), and Archaea (*H. salinarium*). The material that was recovered at each temperature of elution was examined by RT-PCR to check for non-specific hybridization. In the absence of such cross-hybridization reactions, the 16S rRNA recovered at temperatures 60–64°C should amplify only with PCR primers specific for Archaea. Similarly, the RNA of *E. coli* and *S. cerevisiae* should remain in the lanes reflecting lower temperatures of elution (U-48°C).

For RT-PCR, primers were chosen that would generate small DNA transcripts upon amplification (with the exception of *E. coli*); small transcripts are necessary for the future adaptation of this validation assay to quantitative PCR (Q-PCR). Fig. 5 shows the results obtained for reverse transcription and amplification of the RNA from each of the three Domains. For each set of primers, amplification of 1% of each of the temperature-resolved fractions was compared to amplification of 1 ng of pure RNA of the authentic target. Positive results were obtained for the amplification of RNA from *H. salinarium* (Fig. 5a). The captured 16S rRNA that was recovered at temperatures 60–64°C was amplified by archaeal-specific primers. There also is leftover, excess RNA in lanes U and 44°C; this reflects the overall inefficiency of the hybridization and capture

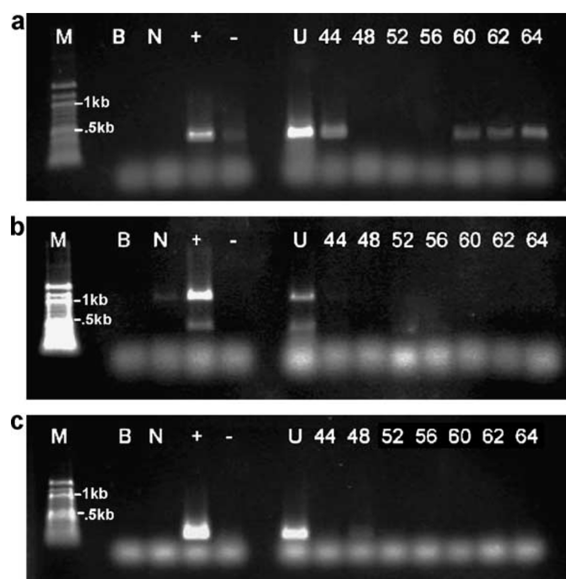

Fig. 5. Detection of captured 16S rRNA by RT-PCR: (a) amplification of *H. salinarium* using primers 1100F/1390Ra; (b) amplification of *E. coli* using primers 27F/1100R; (c) amplification of *S. cerevisiae* using primers 1179F/1438R. M: molecular size markers; B: no transcript control; N: no reverse transcriptase control; +: 1 ng of target RNA; −: 1 ng non-target RNA; U: unhybridized RNA; numbers: 1% of recovered RNA at each temperature (°C) of elution. Non-target negative controls (−) were *E. coli* in (a) and *H. salinarium* in (b) and (c).

process. For the other two domains, the vast majority of the RNA remained in the unhybridized fraction (U). In Fig. 5b, RNA from *E. coli* is seen in lane U while it is faintly visible in lane 44°C, representing the initial bead-washing step. For *S. cerevisiae* (Fig. 5c), a very small amount of amplicon also can be detected in the 48°C fraction. In general, however, the extent of cross-hybridization is negligible. The 16S rRNA of *H. salinarium* that was captured and recovered in lanes 60–64°C appears to be free from contamination by rRNA of Bacteria and Eukaryotes. The recovered RNA would be suitable for isotopic analysis.

During future application of the RNA capture method to complex, natural samples, this validation assay may be more easily systematized using quantitative PCR (Q-PCR). Since PCR is an exponential process, Q-PCR provides a moderately sensitive means to compare the ratios of target and non-target products (Suzuki et al., 2000); the presence of cross-hybridized products at the <1% (ideal) or >10% (contaminated) levels would be distinguished by Q-PCR.

4. Discussion

4.1. Analytical criteria

Our approach to RNA capture was designed to satisfy the six analytical criteria mentioned previously (i–vi). The first, inhibition of degradation, does not pose a significant problem as long as care is taken to stabilize the RNA and to minimize contamination of samples and reagents by RNase (Chomczynski, 1992). The second criterion, purification of rRNA, is the focus of this paper. Here, we used short, complementary oligonucleotide probes (20–29 nucleotides; Amann et al., 1995) for direct hybridization to 16S rRNA. Greater specificity at the genus or species level, as well as increased efficiency of hybridization, could be obtained by the use of polyribonucleotide probes of ~100 nucleotides (e.g., DeLong et al., 1999; Pernthaler et al., 2002); such improvements could increase the ability of RNA capture to recover minor species from environmental samples. However, long probes would complicate the order of melting of the hybrid complex and could result in the significant release of probe along with eluted RNA. In all cases, significant cross-hybridization of non-target species is a legitimate concern. It will remain essential to satisfy analytical requirement (iii) by confirming the authenticity of the captured sequences using RT-PCR.

To obtain accurate isotopic values (criterion iv), the RNA sample must contain very little carbon from non-RNA sources. Further clean-up of the captured RNA is required to achieve a low level of background contamination. It is beyond the scope of this paper to discuss the minimization of carbon contamination in detail. Residual oligonucleotides and organic reagents can be removed through a careful combination of washing, re-precipitation, and/or the use of commercial spin columns (A. Pearson, unpublished data). The process blank for the entire RNA capture procedure typically is 0.2–0.3 nmol C, based on replicate analyses of blanks and on multiple dilution series (Pearson et al., 2002). Assuming an instrumental precision of ±0.3‰ (Sessions et al., 2002), the blank-

corrected $\delta^{13}C$ value can be determined to within 1‰ for a 1.5-nmol sample and to within 0.6‰ for a 5-nmol sample (single analysis, 95% confidence limits);[1] replicate analyses can reduce the sample standard deviation to the extent that at n=5, the $\delta^{13}C$ value reported for the sample approaches the measurement precision of ±0.3‰.

4.2. Sizes of environmental samples

The amount of material required for analysis (criteria v–vi) is controlled by the analytical throughput, the biomass density, and the proportion of the total biomass that represents the species or group of interest. Assuming that the objective is to recover RNA from multiple organisms from within the same bulk sample, we predict that the reaction should be scaled by a factor of ($nfeb^{-1}$) (10 μg), where n is the number of groups or species, f is the fractional abundance of that group, e is the efficiency of the capture probe, b is the total extractable RNA, and 10 μg is the analytical requirement for replicate isotopic analyses and RT-PCR. Information from FISH or other quantitative methods could be used to assist in the design of experiments.

Total RNA yield was determined experimentally for several typical classes of environmental samples (Table 1). If we further assume that the minimum requirement for a multiple-component experiment is 50 μg of RNA, the necessary amount of bulk sample varies widely. From samples of pure biomass, including bacterial cultures or whole tissues, enough RNA can be obtained from milligrams of starting material. In systems with fewer cells and slower rates of growth, the sample size is correspondingly higher. In all cases, however, the sample sizes are modest and suggest that complex experimental design will be possible. The primary factor that remains to be tested is the applicability of RNA capture to species that are present in low relative abundance or that have slow growth rates (fewer ribosomes). In the competitive hybridization experiment conducted here, 33% of the total RNA was from the target organism, *H. salinarium*. Based on relative intensity of gel-stained bands, only ~20% of the original *H. salinarium* total RNA consisted of 16S molecules; therefore, RNA targets in this experiment made up only (0.33×0.20)=0.066, or 7% of the total mixture. The minimum allowable concentration of target must be less than 7%, but currently we do not know the practical limit.

4.3. Applications

The interpretation of $\delta^{13}C$ values obtained for samples of pure RNA with a natural abundance of ^{13}C presumes that the isotopic fractionation between RNA and biomass is known. Early work on *E. coli*, a heterotrophic species, suggested that RNA is similar isotopically to whole cells (Blair et al., 1985). Our preliminary work on *H. salinarium* and *S. cerevisiae*, as well as *E. coli*, is consistent with this result (0–1.5‰ enrichment in ^{13}C relative to cells). As yet, there is no information about the fractionation expressed by autotrophic organisms using any of the different pathways of CO_2 fixation; nor is there any information about the fractionation expressed by methanotrophs. However, since about half of the carbon in RNA is contained within the ribose sugar, the $\delta^{13}C$ value of RNA may track the $\delta^{13}C$ value of the carbohydrate fraction, regardless of metabolic pathway. Further work is needed to confirm this principle across a broad spectrum of metabolic pathways.

There are numerous immediate applications of RNA capture to the study of environmental problems. An often-cited estimate is that less than 1% of all prokaryotes identified in the natural environment can be cultivated by conventional methods (Pace, 1997). The inability to culture the vast majority of "species" means that we have little conception of the functional metabolic activity of most microbes in their natural habitats. RNA capture followed by isotopic analysis will provide a new approach to investigate the ecological roles of uncultivable microorganisms.

There are many questions that can be addressed through isotopic analysis of species-specific RNA. It may be possible to use the $\delta^{13}C$ value of RNA to determine the pathway of carbon fixation expressed by uncultivable species that are suspected to be autotrophs. The fractionation between carbon substrate and biomass ($\varepsilon=\delta^{13}C_{substrate}-\delta^{13}C_{biomass}$) differs with enzymatic pathway (e.g., Roeske and O'Leary,

[1] Calculated using Eq. (5.18) from the monograph, "Practice and principles of isotopic measurements in organic geochemistry", J.M. Hayes (2002). Assumptions: $\delta_b-\delta_T\leq5$‰, $\sigma_{nb}=\sigma_{nT}$=0.05 nmol C.

1984; Gelwicks et al., 1989; Van der Meer et al., 2001). Analysis of pure cultures can provide values of ε for different metabolic pathways, and if the isotopic composition of the substrate is known, the $\delta^{13}C_{RNA}$ value measured for an uncultivated species may be used to infer its metabolic pathway of carbon fixation.

Finally, there are many opportunities to use the RNA capture approach in conjunction with experiments that incorporate ^{13}C-labeled substrates. Similar to the “stable isotope probing” method of Radajewski et al. (2000, 2002), labeling experiments could be used to identify the organisms for which a particular ^{13}C-labeled substrate is the preferred source of carbon.

5. Conclusions

Our practical approach to RNA capture confirms that rRNA molecules can be separated from complex mixtures and used as phylogenetically specific biomarkers. In the future, these techniques will be adapted to the study of isotopic fractionation within complex microbial ecosystems. The high biomass density and metabolic complexity of environments, such as microbial mats, may allow application of RNA capture on a very fine scale. It also may be possible to use robotic sample manipulation to achieve a high throughput by processing samples in a 96-well plate format. Large data sets could be generated and used to solve models of the carbon isotopic dynamics of metabolically interdependent consortia.

In addition, the method is not limited to analysis of the stable isotopes of carbon. Although the measurements will be more challenging analytically, the nucleic acids are also rich in nitrogen and hydrogen. Both of these elements play an important role in the recycling of nutrients and energy within microbial systems, and values of $\delta^{15}N$ and/or δD would help answer important questions about the role of each element. Finally, with the development of new methods for continuous flow radiocarbon (^{14}C) analysis by accelerator mass spectrometry (Schneider et al., submitted for publication), it may be possible to obtain organism-specific ^{14}C values. This would also permit information to be obtained about the age of the carbon substrates utilized by microorganisms in the environment.

Acknowledgements

The authors are grateful for the tremendous amount of valuable advice given by E. DeLong. K.T. Scott and C. Cavanaugh provided samples of bivalves and vestimentiferans. M. Fisher assisted with samples from Lake Mishwam. This work was supported by a WHOI-RCRC Grant to A.P., J.M.H., and K. Edwards; by NASA Astrobiology Award NCC2-1053 to J.M.H.; and by NSF Grant EAR-0311937 to A.P. This is WHOI contribution #11141.

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