Detection and Capturing of ^{14}C Radioactively-Labeled Small Subunit rRNA from Mixed Microbial Communities of a Microbial Mat Using Magnetic Beads

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Abstract Carbon cycling in the hypersaline microbial mats from Chiprana Lake, Spain is primarily dependent on phototrophic microorganisms with the ability to fix CO_2 into organics that can be further utilized by aerobic as well as anaerobic heterotrophic bacteria. Here, mat pieces were incubated in seawater amended with ^{14}C sodium bicarbonate and the incorporation of the radiocarbon in the small subunit ribosomal RNA (SSU rRNA) of mat organisms was followed using scintillation counter and autoradiography. Different domains of SSU rRNA were separated from the total RNA by means of streptavidin-coated magnetic beads and biotin-labeled oligonucleotide probes. The ^{14}C label was detected in isolated RNA by both scintillation counter and autoradiography, however the latter technique was less sensitive. Using scintillation counter, the radiolabel incorporation increased with time with a maximum rate of 0.18 Bq ng^{-1} detected after 25 days. The bacterial SSU rRNA could be captured using the magnetic beads, however the hybridization efficiency was around 20%. The captured RNA was radioactively labeled, which could be mainly due to the fixation of radiocarbon by phototrophic organisms. In conclusion, the incubation of microbial mats in the presence of radiolabeled bicarbonate leads to the incorporation of the ^{14}C label into RNA molecules through photosynthesis and this label can be detected using scintillation counter. The used approach could be useful in studying the fate of fixed carbon and its uptake by other microorganisms in complex microbial mats, particularly when species-specific probes

are used and the hybridization efficiency and RNA yield are further optimized.

Keywords Cyanobacterial mats · Radiocarbon · Magnetic capture beads · Oligonucleotide probes · 16S rRNA

Introduction

Microbial mats are composed of physiologically different groups with oxygenic (i.e., cyanobacteria) and anoxygenic phototrophs (sulfur-oxidizing bacteria and chloroflexi) capable of converting CO_2 into organic carbon [1]. As a result, low molecular weight compounds are excreted directly in the mats and can be utilized by aerobic as well as anaerobic heterotrophic bacteria [2]. Different types of dissolved organic compounds are produced in the dark under anoxic conditions by fermenting storage compounds like glycogen [1, 3–5]. These compounds as well as cyanobacterial extrapolymeric substances (EPS) and the organics released after cell death and lysis can become available to heterotrophic bacteria. Microbial mats have also been shown to grow and degrade petroleum compounds [6]. It is a challenge to identify which microorganism(s) in this complex microbial community utilize which of the numerous autochthonous and allochthonous organics within the mats. It is hypothesized that the microorganisms that are involved in the uptake of a ^{14}C labeled compound will eventually incorporate this label into their nucleic acids, which could eventually facilitate their identification.

Several techniques based on stable and radioactive isotopes were designed and have successfully enabled the identification of specific bacterial populations that are actively involved in the uptake of a certain substrate. For

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example, stable isotope probing (SIP) technique involved the separation of heavy DNA (i.e. ^{13}C or ^{15}N labeled) from natural samples by density-gradient centrifugation after incubation with isotope-labeled substrate [7, 8], followed by sequencing and identification of key microorganisms involved in the assimilation of this substrate. The technique provided more sensitive results when performed on RNA or polar-lipid-derived fatty acids [9–11]. The isotope measurements in single microbial cells became possible with the help of isotopic measurement using high resolution ion microprobe, a technique known as NanoSIMS [12]. The coupling of this technique with halogen in situ hybridization (HISH–SIMS) allowed the simultaneous phylogenetic identification and quantification of metabolic activities in single bacterial cells in the environment [13]. Unlike stable isotopes, the use of radioactive isotopes was very limited although they are more sensitive, most likely because of the difficulty to handle them and the strict requirements of special precautions as well as the high cost of radioactively labeled compounds. The microorganisms feeding on certain radioactively labeled substrates could, however, be identified by MAR–FISH technique which combines fluorescent in situ hybridization with microautoradiography [14]. So far, little evidence is provided that the incubation of microorganisms in the presence of radioactively labeled substance leads to the labeling of their nucleic acids, although the ^{14}C labeling of fatty acids by soil microorganisms has been shown [15].

Here, the incorporation of ^{14}C in the SSU rRNA extracted from a microbial mat after incubation in ^{14}C sodium bicarbonate for 26 days was followed using a scintillation counter and autoradiography. Different domains of SSU rRNA were isolated from the mixed labeled RNA using magnetic bead capture and specific oligonucleotide probes [16].

Materials and Methods

Experimental Setup

Mat samples originated from a permanent hypersaline inland lake of Western Europe, ‘La Salada de Chiprana’, northeastern Spain were used in this study [17]. Mat cores (six cores, each 1 cm in diameter) were embedded in 5 ml 1.5% agarose in 6 separate glass tubes (Fig. 1). The agarose was prepared in artificial seawater with a salinity adjusted to 7‰, to mimic the environment where the mats were collected from. After cooling, the agarose solution was poured carefully around an upright mat core, and left at room temperature till it solidified. 5 ml seawater amended with 200 kBq ^{14}C labeled sodium bicarbonate was added. The experiment was run for 26 days and samples were collected after 1, 3, 6, 10, 19 and 26 days by sacrificing one tube each time.

RNA Isolation

RNA was extracted from ca. 5 g of the mat cores obtained from the above experiment by bead beating, phenol–chloroform extraction, and ethanol precipitation [18]. Bead beating was done with an MSK–Zellhomogenisator (B. Braun Biotech International, Melsungen, Germany), using a 70 ml capacity stainless-steel chamber, for 40 s at 4,000 reciprocations per minute. The final RNA pellet was resuspended in 50 μl RNase-free ddH_2O . The total extract was incubated with 1 μl DNase enzyme for 1 h at 37°C to remove traces of DNA. If needed, RNA was further purified with the RNeasy Plant Mini Kit (QIAGEN GmbH, Hilden, Germany). All solutions were prepared with diethylpyrocarbonate (DEPC)-treated distilled, deionized water (ddH_2O) [18]. RNA extracts were separated by

Fig. 1 Photograph showing (a) a laminated cyanobacterial mats and (b) the experimental setup with a mat piece embedded in 1.5% agarose and incubated in ^{14}C amended seawater

electrophoresis on polyacrylamide gels [19] and nucleic acid bands were visualized by ethidium bromide staining. The concentrations of RNA were measured using Nanodrop (Thermo-Scientific, Germany).

Detection of ^{14}C Incorporation in RNA

Gel bands corresponding to 16S rRNA were cut from the gel and placed in 30 μl RNase-free ddH_2O in an Eppendorf tube for two days. The eluted RNA was transferred to a scintillation counter vial containing 5 ml of scintillation cocktail (Packard Ultima Gold XR). The samples were counted on a Canberra-Packard 2400 TR liquid scintillation counter. For autoradiography, the RNA was first transferred from the polyacrylamide gels to a nylon membrane by blotting. The gels were first equilibrated for 30 min in $0.5\times$ TBE buffer (890 mM Tris-HCL, 890 mM boric acid, 20 mM EDTA) and then placed on top of a nylon membrane between two stacks of filter papers (Whatman 3MM). The electrotransfer was performed at 400 mA for 1 h. The membrane was transferred to a denaturing solution (0.4 M NaOH, 0.6 M NaCl) and then washed twice for 10 min in $2.5\times$ SSC (0.37 M NaCl and 0.037 M sodium citrate). The membrane was then exposed for 45 s to 302 nm UV light to cross link the nucleic acid fragments to the membrane. The membrane was then exposed to X-ray film in a dark room and the film was then developed and observed for any signals.

Magnetic Bead Capture of rRNA

The ^{14}C labeled RNA isolated from the mat samples (see above) was mixed with a hybridization buffer ($5\times$ SSC, 0.1% N-laurylsarcosine, 0.1% NaCl, 0.02% SDS) in 100 ml final volume. The mixture was incubated for 10 min at 70°C and 30 min at room temperature [20]. To this mixture, biotin labeled oligonucleotide probes (Interactiva, Ulm, Germany) were added individually to separate aliquots and the stringency of hybridization for each probe was adjusted by changing formamide concentration. The probes used were: Uni 1390 (GACGGGCGGTGTGTACAA), specific for all SSU rRNA [21]; Bact 338 (GCTG CCTCCCGTAGGAGT), targeting bacteria [22]; Euk 1379 (TAGAAAGGGCAGGGA), targeting Eukarya [23] and Arc 915 (GTGCTCCCCCGCCAATTCCT), targeting Archaea [24]. The hybridization mixture was incubated overnight at room temperature on a Dynal Sample Mixer 159.02 (Dynal Inc., Long Island, NY) at approximately 10 rpm. Dynabeads M-280 Streptavidin (Deutsche Dynal GmbH, Hamburg, Germany) were washed three times in an Eppendorf tube with an equal volume of $0.5\times$ SSC ($20\times$ SSC: 3 M NaCl, 0.3 M sodium citrate). The beads were then resuspended in 0.1% blocking reagent (Roche

Molecular Biochemicals, Mannheim, Germany) solution in $0.5\times$ SSC, aliquoted into reaction tubes, and incubated for 1 h on an end-over-end mixer. The beads were collected on the tube side using a magnet and the blocking mixture was removed by pipetting. Afterwards, the hybridization mixture (containing the probe and RNA) was added and incubated at room temperature for 2 h with mixing. The probe-hybridized RNA was captured using the magnetic beads and the RNA was then eluted in RNase-free ddH_2O at 90°C for 3 min. A magnet was used to separate the magnetic beads from the eluate. The eluted RNA was checked for the ^{14}C radioactivity using scintillation counter. RNA was concentrated by precipitation with one volume of isopropanol and 0.5 vol 7.5 M ammonium acetate, washed once with three volumes of 70% ethanol, and resuspended in RNase-free ddH_2O . Aliquots of each eluted RNA sample were separated by electrophoresis on two-phase (3.3%/10%) polyacrylamide gels [19]. Nucleic acid bands were visualized by ethidium bromide staining.

Results and Discussion

This study tested ^{14}C labeling of RNA of mat microorganisms after incubation in the presence of ^{14}C -bicarbonate and the possibility to use group-specific oligonucleotide probes to capture the labeled RNA. RNA molecule was chosen for this purpose because it can be labeled more quickly than DNA and does not require the cells to multiply [11]. Total nucleic acids, including low molecular weight (LMW) DNA and ribosomal RNA, were readily extracted from the studied mats (Fig. 2, left panel). Different RNA extraction protocols were shown to result in different qualities and quantities of RNA, however the used protocol worked well on similar samples [19]. Most LMW DNA was already degraded after 1 h of incubation in the presence of DNase enzyme. The concentrations of ribosomal RNA after DNA digestion was in the range of $15\text{--}25\text{ }\mu\text{g ml}^{-1}$ as measured using Nanodrop. The 16S rRNA was eluted out of the gel by placing the excised band in 30 μl PCR water and the concentration of eluted 16S rRNA was between $2\text{--}5\text{ }\mu\text{g ml}^{-1}$. The detection of ^{14}C radioactivity incorporated into 16S rRNA was possible using scintillation counter. ^{14}C was incorporated at a rate of $0.04\text{--}0.18\text{ Bq ng}^{-1}$ of 16S rRNA (Fig. 2, right panel). The detected radioactivity at day 19 and 26 were higher than at earlier days with a maximum of 0.18 Bq ng^{-1} after 26 days of incubation (Fig. 2, right panel). ^{14}C was also detected by autoradiography, however this method was less sensitive than scintillation counting. Faint bands of the radioactively labeled nucleic acids were only visible after 2–3 weeks (Fig. 3). Only one band appeared labeled on the X-ray film while the other not, indicating the detection

Fig. 2 Total extracts of nucleic acids before DNase treatment (*left*) and ^{14}C radioactivity incorporation rate at different time intervals of the incubation (*right*)

limitation of the autoradiography technique. Previous studies have shown that liquid scintillation counting and autoradiography yielded similar results, nevertheless scintillation counting was less laborious, faster and allowed the analysis of a large number of samples [25, 26]. This is because the determination of radioisotope is performed automatically by a liquid scintillation counter.

The universal and domain-level probes were hybridized with RNA isolated from the cyanobacterial mats after

incubation with ^{14}C labeled bicarbonate and the 16S rRNA was detectably captured using magnetic beads (Fig. 4a). Hybridization was observed with bacterial and universal probes (Bact 338 and Uni 1390, respectively) but not with archaeal and eukaryotic probes (Arc 915 and Euk 1379, respectively; Fig. 4b). The use of the same archaeal and eukaryotic probes under the same hybridization conditions was previously shown to yield detectable hybridization products [10]. Therefore, the failure to detect the RNA of Archaea and eukaryotes in our mats indicates that they are either very low in abundance or they are not playing a role in the carbon cycling in these mats. Diatoms were indeed observed by direct microscopy in these mats but at a much lower abundance than cyanobacteria [17]. The low detectability of archaeal and eukaryotic RNA could also be due to the low efficiency of hybridization. Comparison of hybridized to unhybridized RNA obtained from the mats suggests that the hybridization efficiency was around 20%. This efficiency was slightly improved by allowing the biotin-labeled probes to hybridize with the RNA overnight at room temperature (ca. 20°C). The addition of extra probe or increasing temperature did not result in a higher yield. Therefore, optimization of hybridization conditions is still required, especially when working with environmental samples and if maximum rRNA recovery is needed. A previous study showed that hybridization efficiency of rRNA from sediments and pure-cultures using the same probes was comparable, although humic acids co-extracted with the environmental rRNA and the degradation of rRNA during extraction might have had an effect [16]. Small amounts of humic acids had an inhibitory effect in some cases but some higher concentrations enhanced recovery in others depending on the probe used. Hybridization generally decreased with increasing amounts of degraded RNA [16]. This suggests that magnetic bead capture can't be used quantitatively with natural samples.

Fig. 3 The separation of 16S and 23S rRNA from total nucleic acid extracts, obtained from a cyanobacterial mat before and after incubation in the presence of ^{14}C labeled sodium bicarbonate on polyacrylamide gels (**a**, **b**) and after exposure to X-ray films (**c**, **d**). Note that faint bands were visible on the X-ray film suggesting their radiolabeling

Fig. 4 A scheme showing the principle of RNA capture using streptavidin-coated magnetic beads and biotin-labeled oligonucleotide probes (a). Polyacrylamide gel electrophoretic separation of specific rRNAs from a cyanobacterial mat after hybridization using oligonucleotide probes on magnetic beads (b). Note that hybridization was only possible with the bacterial and universal probes

The captured bacterial RNA, after washing out of the beads by heating, was still found to be labeled with ^{14}C . This labeled RNA belongs most likely to prokaryotic phototrophic microorganisms that fixed ^{14}C through the process of photosynthesis and possibly to heterotrophic bacteria that could have fed on radioactively labeled exudates. These phototrophs could include, in addition to cyanobacteria, chloroflexi as well as sulfur-oxidizing bacteria, which were shown abundant in these mats [17]. At this stage, it is not possible to identify exactly which bacteria have incorporated the label as the probe captures all bacterial rRNA, regardless of the presence or absence of the label. So far there is no technique available that can be used to separate radioactively labeled from non-labeled nucleic acids. One way around this problem is to use probes that target a specific bacterial group or preferably individual bacterial species. In this case, if the captured rRNA is labeled, this would mean that this bacterium has been involved in the assimilation of the labeled compound. For this purpose, relatively large amounts of RNA are required for reliable detection of the radiolabel, especially that PCR amplification cannot be used as it results in the loss of the radiolabel. Since RNA concentrations in many environmental samples are low [19], the only alternative is to perform large scale RNA extractions, which have become now possible with the availability of bead beaters and freezer mills that are capable of handling 10–50 g of samples.

In conclusion, prokaryotic phototrophic microorganisms in microbial mats have the ability to fix radiocarbon through the process of photosynthesis and incorporate the label into their RNA and possibly the RNA of bacteria that grow on labeled exudates. Magnetic capture of the rRNAs using specific oligonucleotide probes and subsequent checking if the captured RNA is radioactively labeled could supply useful information on the bacteria involved in

the uptake of the labeled substrate. Unlike MAR-FISH, which is a microscopic method detecting the incorporated radiolabel in bacterial biomass, this approach is easier, less time consuming and does not require expensive tools. The technique can be ideally used in defined bacterial cell mixtures but more difficult in natural samples. This technique could be further improved by increasing hybridization efficiency and yields of RNA.

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