Journal of Microbiological Methods 111 (2015) 78–86

Contents lists available at ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

A simple separation method for downstream biochemical analysis of aquatic microbes

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ARTICLE INFO

Article history:
Received 8 September 2014
Received in revised form 29 January 2015
Accepted 30 January 2015
Available online 2 February 2015

Keywords:
Marine microbes
Sucrose density gradient separation
Biochemical fractionation
Anaplerotic pathways

ABSTRACT

In order to study the chemical composition of aquatic microbes it is necessary to obtain completely separated fractions of subpopulations. Size separation by filtration is usually unsuccessful because the smaller group of organisms contaminates the larger fractions due to being trapped on filter surfaces of nominally much larger pore sizes. Here we demonstrate that a simple sucrose density separation method allowed us to separate microorganisms of even subtle size differences and to determine their bulk biochemical composition (proteins, polysaccharides + nucleic acids, and lipids). Both autotrophs and heterotrophs (through anaplerotic pathways) were labeled with ^{14}C-bicarbonate for biochemical fractionation. We provided proof of concept that eukaryotic microbes could be cleanly separated from prokaryotes in cultures and in field samples, enabling detection of differences in their biochemical makeup. We explored methodological issues regarding separation mechanisms, fixation, and pre-concentration via tangential flow filtration of oligotrophic marine waters where abundances of microorganisms are comparably low. By selecting an appropriate centrifugal force, two processes (i.e., isopycnal and rate–zonal separation) can be exploited simultaneously resulting in finely-separated density fractions, which also resulted in size separation. Future applications of this method include exploration of the stoichiometric, biochemical and genetic differences among subpopulations of microbes in a wide variety of aquatic environments.

1. Introduction

Separation of microbial communities is important where clean fractions are needed for elemental, biochemical, or genetic analysis of each population comprising the community. Previously, analyses of elemental stoichiometry or biochemical composition had to be corrected by subtracting the influence of unwanted prokaryotes in fractions of eukaryotes, which can lead to errors (e.g., Véra et al., 2001; Chrzanowski et al., 2010). Complete separation is also desirable in studies with radioisotopes where microbial predators need to be separated from more numerous labeled prey items. Microorganism separation, however, can be challenging in the size range of 0.2–20 μm. Organisms of interest such as bacteria and protists cannot be effectively separated from each other by filtration because of clumping and other mechanisms of retention of organisms smaller than the pore size of the filter. Heterotrophic flagellates are ubiquitous in aquatic environments, feed directly on aquatic prokaryotes, play an important role in controlling prokaryote populations, and significantly impact nutrient regeneration efficiencies in aquatic systems (Fenchel, 1982; Goldman and Caron, 1985; Fernandes and Mesquita, 1999; Pernthaler, 2005; Massana, 2011). Separation of these flagellates from their prokaryotic prey is almost impossible with filters, and biochemical analysis has only been performed on clean protist samples in situations where they can be grown on dissolved organic media (assuming that the contribution from prokaryotes is negligible, Simonds et al., 2010). Other protists such as phytoplankton can be grown axenically (Clark et al., 2014) to determine their stoichiometry and biochemical composition but phytoplankton have different compositions when grown in the absence of bacteria (Danger et al., 2007; Morris et al., 2008).

The present study shows that a modified sucrose density gradient method (Brakke, 1951) can be used to effectively separate microbes based on size. A similar method, the cesium density gradient centrifugation is used frequently in molecular biology mainly to separate viruses (e.g., Brum et al., 2013), and ^{13}C-labeled nucleic acids in stable isotope probing experiments (e.g., Radajewski et al., 2000; Uhlik et al., 2009). The much simpler sucrose density centrifugation does not require ultracentrifugation. It has only been used in a few cases with marine microbes (e.g., Matsumoto et al., 2002) and not specifically for the purpose of separating eukaryotic from prokaryotic microbes, or for downstream biochemical analysis. Another potential separation method based on density of the organism (i.e., Percoll silica colloids, Amersham Biosciences, 2002, e.g., Putzer et al., 1991; Nishino et al.,

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http://dx.doi.org/10.1016/j.mimet.2015.01.025
0167-7012/

2003) was abandoned early in our study as it was less successful in separating the target organisms than the sucrose density gradient fractionation.

2. Material and methods

2.1. Experimental organisms

Organisms used in this study included *Escherichia coli* parental cells and minicells (Adler et al., 1967), a non-axenic diatom culture (*Thalassiosira weissflogii*), and two heterotrophic flagellates: *Diplonema papillatum* (formerly *Isonema papillatum*, Porter, 1973) and *Cafeteria roenbergensis* (Fenchel and Patterson, 1988). The flagellate cultures contained an abundance of prokaryotes that served as food. The separation techniques were also tested on environmental samples derived from two sources: the estuarine and eutrophic Lafayette River (Norfolk, VA), and Gulf Stream water off Hatteras (NC) that was maintained in an acid-washed translucent carboy for 5 months on the lab bench without any nutrient addition, and that can therefore be regarded as an oligotrophic culture.

2.2. Culture details

Minicell-producing cultures of *E. coli* (strain χ1488, CGSC#6556, Coli Genetic Stock Center, Yale University) were transformed with a green fluorescent protein (GFP) vector (Bochdansky and Clouse, in press). The new strain is available at the CGSC under "GFP-minicells" #14165. The GFP-minicell cultures were grown in Luria–Bertani broth to late exponential phase at 37 °C. The culture was subsequently transferred into two 50 mL polypropylene Falcon tubes (Becton, Dickinson and Company) and centrifuged at 15,000 *g* for 15 min at 2 °C. The supernatant was decanted and the pellet resuspended in 10 mL of 0.2 μm filtered artificial seawater (ASW). The cells were centrifuged and resuspended two more times. The final suspension containing both minicells and parental cells was aliquoted into 1 mL subsamples and stored at −80 °C. *T. weissflogii* was grown in f/2 medium (Guillard and Ryther, 1962). Cultures of *D. papillatum* were grown in an enriched *Isonema* medium (American Type Culture Collection medium # 1728). Stock cultures of *C. roenbergensis* were kept in rice grain cultures at ca. 7 °C in the dark. Working cultures of *C. roenbergensis* were grown in 2% of full strength BD Difco™ marine broth in 200 mL flasks. Samples from the eutrophic Lafayette River and from the oligotrophic Gulf Stream off Hatteras Island (approximately 50 km off the coast) containing a diverse range of autotrophic and heterotrophic eukaryotes were collected with an acid-washed bucket from the surface.

2.3. Sucrose density gradient fractionation

Stock solutions of 50% sucrose were diluted with Buffered Saline Gelatine (BSG), which consisted of ultrapure water, 0.85% NaCl, 0.03% KH_2PO_4, 0.06% Na_2HPO_4, and 0.01% gelatine (Kemp et al., 1993) to create the spectrum of sucrose concentrations (15–50%). The solutions were chilled before initial use and refrigerated during storage as well to ensure consistent viscosity of the solutions between trials. The gradient was created in 15 mL conical polypropylene Falcon tubes by slowly dispensing the sucrose solutions with an automatic pipette (Kemp et al., 1993). Volumes of each layer were as follows: 0.5 mL of 50% solution, 0.5 mL 45%, 0.5 mL 40%, 0.5 mL 35%, 0.75 mL 30%, 1 mL 27.5%, 1 mL 25%, 1 mL 20%, 1 mL 15%; and 0.5 mL of formaldehyde-fixed sample (2% fin. conc.) layered on top. Initial trials with unfixed samples of *D. papillatum* cultures showed that most eukaryotic cells were destroyed during centrifugation, thus only fixed samples were subsequently used. A weaker sucrose gradient (a gradient from 15% to 35%) was tested initially but was not as effective and resulted in pelleting at the bottom of the tube, therefore a stronger gradient (15%–50%) was established. After pipetting, individual density layers were visible as bands of different refractive indices within the tube. The sample was then centrifuged at 4000 *g* for 30 min at 4 °C to create a smooth gradient and to settle the organisms in the sample. For biochemical fractionation, sucrose separations were scaled up to 50 mL Falcon tubes (BD) while using sample volumes of 2.5 mL (i.e., multiplying all volumes including fraction volumes by 5).

After centrifugation, a needle was pushed through the bottom of the tube while the tube was clamped in place. The cap of the tube was loosened, the needle was removed, and a set number of drops was counted into acid cleaned and muffled borosilicate glass tubes (9 mL) placed in a rack. The number of fractions collected varied slightly from trial to trial based on the size of the hole, but at least 20 fractions were taken during each trial. The initial volume of the gradient plus sample (7.25 mL) was divided by the number of fractions collected to calculate the volume of each fraction. Each fraction consisted of 10 drops for small-volume runs, or 2 mL for large-volume runs. Each fraction was filtered through a 0.2 μm polycarbonate filter, stained with one drop of Vectashield with DAPI and stored at −20 °C until cells were enumerated using an epifluorescence microscope.

2.4. Radiolabeling with ^{14}C sodium bicarbonate

To label heterotrophic cells with $H^{14}CO_3$, we exploited the fact that all heterotrophic organisms incorporate some CO_2 into organic metabolites as heterotrophic growth requires the presence of some CO_2 (Wood and Werkman, 1936; Hartman et al., 1972; Perez and Matin, 1982; Roslev et al., 2004; Miltner et al., 2005; Alonso-Sáez et al., 2010). This heterotrophic carbon fixation (anaplerotic metabolism) is facilitated by carboxylases working to replenish the tricarboxylic acid cycle (e.g., Feisthauer et al., 2008; Hanson et al., 2012). For instance, it is estimated that 3 to 8% of aquatic prokaryotes' carbon demand is satisfied by these anaplerotic reactions (Romanenko, 1964). This process has also been described for eukaryotic microbes such as fungi (Schinner et al., 1982), flagellates (Peak and Peak, 1980), and many higher organisms. Sodium bicarbonate was preferred over other tracers labeled with ^{14}C such as leucine because CO_2 was expected to be incorporated more evenly across biochemical fractions, whereas the amino acid leucine would have preferentially been incorporated into the protein fraction. The radiolabeled trials were performed with *D. papillatum* cultures. Forty microcuries of ^{14}C sodium bicarbonate (Perkin-Elmer) was added to roughly 11.5 mL of *D. papillatum* culture in a Nunclon™ 40 mL culture container immediately after the culture was inoculated with the stock culture of *D. papillatum* (see above). We assumed that after several generations, isotopic equilibrium would be established among all fractions (i.e., that the ^{14}C:^{12}C ratio was the same in all biochemical fractions, Nielsen and Olsen, 1989). The cultures were grown for one week, over which the cultures grew into a densely packed mass of cells. Separation was then performed with the high-volume (50 mL) sucrose density gradient method (see above).

Seventeen to 18 fractions were collected in each sucrose density gradient. Of these, 4 fractions previously determined to be closest to each of the prokaryote and eukaryote cell peaks were used for biochemical fractionation. Fractions 1–4 (most dense and first to come out of the gradient tube) contained the largest proportions of *D. papillatum* cells relative to prokaryotes, and fractions 8–11 contained the largest number of prokaryotes. In addition, fractions 8–11 were filtered through 3 μm polycarbonate filters to remove remaining *D. papillatum* cells if there were any left. Each fraction was diluted slightly with 0.5 mL of 0.2 μm filtered ASW to increase the total volume to 2.5 mL each; the increased volume reducing the effects of pipetting errors. To determine the total level of ^{14}C before biochemical fractionation, a subsample of 0.5 mL was filtered onto a 2.5 cm GF/F glass microfiber filter, placed in a 5 mL scintillation vial with 4 mL of scintillation cocktail, and counted using a Perkin-Elmer Tricarb model 3110TR liquid scintillation counter. A second subfraction of 1 mL was filtered onto a 2.5 cm GF/F glass microfiber filter and stored at −20 °C until biochemical fractionation. A third sub-