Journal of Microbiological Methods 115 (2015) 112–120

Contents lists available at ScienceDirect

Journal of Microbiological Methods

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

Microbial rRNA:rDNA gene ratios may be unexpectedly low due to extracellular DNA preservation in soils

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ARTICLE INFO

Article history:
Received 26 February 2015
Received in revised form 30 May 2015
Accepted 30 May 2015
Available online 6 June 2015

Keywords:
Activity
eDNA
iDNA
Oligotrophy
Preservation

ABSTRACT

We tested a method of estimating the activity of detectable individual bacterial and archaeal OTUs within a community by calculating ratios of absolute 16S rRNA to rDNA copy numbers. We investigated phylogenetically coherent patterns of activity among soil prokaryotes in non-growing soil communities. 'Activity ratios' were calculated for bacteria and archaea in soil sampled from a tropical rainforest and temperate agricultural field and incubated for one year at two levels of moisture availability and with and without carbon additions. Prior to calculating activity ratios, we corrected the relative abundances of OTUs to account for multiple copies of the 16S gene per genome. Although necessary to ensure accurate activity ratios, this correction did not change our interpretation of differences in microbial community composition across treatments. Activity ratios in this study were lower than those previously published (0.0003–210, logarithmic mean = 0.24), suggesting significant extracellular DNA preservation. After controlling for the influence of individual incubation jars, significant differences in activity ratios between all members of each phylum were observed. Planctomycetes and Firmicutes had the highest activity ratios and Crenarchaeota had the lowest activity overall. Our results suggest that greater caution should be taken in interpreting soil microbial community data derived from extracted DNA. Indirect extraction methods may be useful in ensuring that microbes identified from extracellular DNA are not erroneously interpreted as components of an active microbial community.

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1. Introduction

High-throughput sequencing of microbial DNA has become increasingly common in environmental microbial ecology over the last decade (Caporaso et al., 2011). These techniques are increasingly being used to explore the uncultured majority of microbes in soils and sediments, and apply macro-ecological theories to the microbiome (Fierer et al., 2012). For example, some studies have proposed that ecological niches and environmental life-strategies of environmental microorganisms may be coherent at high taxonomic levels (i.e., phyla) (Fierer et al., 2007; Philippot et al., 2010), and broadly divisible into "copiotrophs" and "oligotrophs". Theorized copiotrophs (i.e., β-Proteobacteria, Firmicutes, and Bacteroidetes) make up a greater proportion of microbial communities in soils with higher carbon (C) availability (Cleveland et al., 2007), and their relative abundance increases rapidly when exposed to readily available C sources (Goldfarb et al., 2011). Conversely, theorized oligotrophs (i.e., Acidobacteria and Verrucomicrobia) are more abundant in low C systems (Fierer et al., 2007), and do not increase in population after the addition of available C (Goldfarb et al., 2011). This copiotroph–oligotroph dichotomy has become an increasingly accepted conceptual framework in microbial ecology, supported by the identification of defining genomic traits of a model copiotroph and oligotroph (Lauro et al., 2009), and the high abundance of genes related to high carbon affinity and desiccation resistance in selected Acidobacteria genomes (Ward et al., 2009).

However, studies investigating phylogenetic conservation of ecological functions in environmental microbes tend to have two limitations. The first applies chiefly to studies investigating trophic strategy. Many experiments showing a lack of increased growth in response to the addition of labile C feature incubation times from days (Goldfarb et al., 2011) up to a month (Fierer et al., 2007). Some dormant bacteria can stochastically awaken and form a rapidly growing population after months or even years of inactivity (Buerger et al., 2012). A lack of response to one specific stimulant (labile C) over a period of less than a year may not be useful as experimental evidence of an oligotrophic survival strategy. The second limitation applies more generally to studies that rely on sequencing and quantifying 16S rRNA genes (rDNA) rather than 16S rRNA itself. Characterizing rDNA does not necessarily reflect the active community, only the potential for activity (Lennon and Jones, 2011; DeAngelis and Firestone, 2012), because it includes living, dormant, and non-viable organisms.

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http://dx.doi.org/10.1016/j.mimet.2015.05.027
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Studies have reported that microbial β-diversity can differ significantly between rDNA- and rRNA-based soil communities (Angel et al., 2013; Baldrian et al., 2012; DeAngelis and Firestone, 2012). This may in part be due to high-quality extracellular DNA (eDNA) preserved in soil and sediment, either sorbed to clay minerals (Ogram et al., 1988; Pietramellara et al., 2009) or as a component of biofilms (Bockelmann et al., 2006; Alawi et al., 2014). Additionally the number of 16S gene copies is variable between different organisms' genomes, ranging from one to fifteen copies in bacteria and archaea (Kembel et al., 2012). Not considering multiple rDNA copies could cause overestimation of relative abundance of some OTUs, thus affecting β-diversity results. Although 16S copy number correction has been shown to be useful in studies of nematodes (Darby et al., 2013) the tools needed to correct 16S rDNA for bacteria and archaea are relatively new (Kembel et al., 2012; Langille et al., 2013) and the use of these tools is not widely published in the study of soil microbial communities.

The study reported here uses a culture-free specific activity method (Kemp et al., 1993) to identify active members of microbial communities in incubated soils. We included sequencing and quantification of both rDNA and rRNA to estimate 'activity ratios' for soil bacteria and archaea. Bacterial pure culture studies have shown that, in many (but not all) cases, active cells contain more ribosomes than those that are less active, resulting in a higher ratio of rRNA to rDNA (Blazewicz et al., 2013). Calculating this ratio in a mixed community requires quantification of both 16S rDNA and rRNA, followed by multiplication of absolute quantities by the relative abundance of a group as determined by sequencing. The accuracy of this ratio depends upon correcting the 16S rDNA for number of copies per genome, and on sufficient sequencing depth to reflect true α- and β-diversities of sequenced communities (Caporaso et al., 2011). In theory, the resulting 'activity ratio' represents the average number of ribosomes present in all cells for a given OTU. This can then be used as a measurement of the general protein production capacity of individual taxa, including those that are difficult to culture or whose specific functions are currently unknown (Blazewicz et al., 2013). Similar methods have been in use for decades, but have been applied either in observational studies (Brettar et al., 2011; Campbell and Kirchman, 2012) or pure cultures (Kemp et al., 1993; Muttray et al., 2001). To the authors' knowledge, no study has yet applied these techniques to entire soil communities in experimental microcosms.

Soil rRNA and rDNA was extracted from soils taken from two geographically distinct areas and incubated at two levels of moisture availability, and with or without added C for one year. We tested three hypotheses: 1) Microbial community composition as determined by rRNA sequencing will be more similar across treatments compared to composition determined from rDNA sequencing. 2) Correcting for 16S gene copy number will make a statistically significant difference in a DNA-based measure of microbial community β-diversity. 3) Putative copiotrophic and oligotrophic groups will be distinguished by the magnitude and variability of their activity ratios in each of six treatment groups: activity ratios of copiotrophic groups will be lower and highly variable between treatments; those of oligotrophic groups will be higher and more constant. As these incubations will result in universally oligotrophic microenvironments after one year, oligotrophic organisms should have higher activity ratios than copiotrophs. Furthermore, copiotrotrophs should respond to the addition of labile carbon, while oligotrophs should not.

2. Materials and methods

2.1. Soil descriptions

Soils were sampled from two geographically distinct sites. A forested slope in the El Yunque National Forest in Rio Grande, Puerto Rico (18° 19′ 54.57″ N, 65° 46′ 28.12″ W), hereafter referred to as the 'Rio Grande' soil, was sampled in February 2011. Soil was taken from a moderately steep, north-facing convex contour, and could be classified as either the Yunque or Los Guineos series, both of which are very-fine, kaolinitic, isothermic Humic Hapludox (Soil Survey Staff, 2010). The second soil was sampled in late July 2011, from a cornfield near the USDA-ARS Integrated Cropping Research Laboratory in Brookings, South Dakota, USA (44° 20′ 27.94″ N, 96° 47′ 17.31″ W), hereafter the 'Brookings' soil. Soil in the area is mapped as a Kranzburg-Brookings complex. Given the slightly alkaline pH measured in these soil samples, the soil likely more closely resembled the Kranzburg series, classified as fine-silty, mixed, superactive, frigid Calcic Hapludolls (Soil Survey Staff, 2010). The Rio Grande and Brookings soils were sampled to depths of 30 cm and 15 cm, respectively. A thin layer of organic material (O horizon) was scraped away from the surface of the Rio Grande soil prior to sampling. Approximately 48 h passed between sampling at both sites and storage at the USDA-ARS Beltsville Agricultural research Center in Beltsville, Maryland, USA. Soils were stored at 4 °C until laboratory analysis and incubation.

2.2. Incubation conditions

Soils from each site were bulked and passed through a 4 mm sieve before assignment to one of three incubation treatments for each soil, with three replicates for each treatment. Soils were incubated for one year in glass jars at 25 °C with the following adjustments: (1) −0.25 MPa water potential with the addition of 0.005 g of dried, powdered *Zea mays* shoot (C/N = 40.1) per gram of dry soil, (2) −0.25 MPa water potential, and (3) −2.5 MPa water potential. These levels were chosen to represent surface soil conditions expected in summer months in 1) a temperate agricultural soil with one season's corn crop plowed under to a depth of 15 cm, 2) a temperate agricultural soil, and 3) a desert soil or an agricultural soil during a drought year. *Z. mays* was harvested from a USDA agricultural research plot the previous summer. Moisture was measured weekly, and corrected if needed by pipetting water onto soil surfaces, without mixing. Moisture corrections were made gravimetrically based on soil water potential vs. volumetric water curves. These were constructed using a WP4 Dewpoint Potentiometer (Decagon Devices, Pullman, WA). A small hole was drilled through the top of each lid and covered with filter paper to allow continuous gas exchange with the atmosphere.

Following incubation, 1-g subsamples of each replicate soil treatment were taken from approximately 5 cm depths within the jars (without mixing) and placed into sterile 10-mL plastic tubes containing 1-mL of LifeGuard solution (MoBio Laboratories, Carlsbad, CA). Preserved soils were stored at −20 °C until extraction.

2.3. Soil chemical and physical analyses

Physical and chemical tests were performed on representative samples from each soil. The pH of each soil was measured in a 2:1 suspension of 0.01 M $CaCl_2$ using an ion-selective probe. Textures were measured using the hydrometer method of particle size analysis (Orr and Gee, 2002) (Appendix S1, Supplementary material).

Total C and N were measured on representative samples of pre- and post-incubation soil treatments using dry combustion with a LECO CHN 2000 analyzer (LECO Corporation, Lakeville, MI) (Appendix S2, Supplementary material). As the Brookings soil appeared to contain carbonates, samples of this soil were subjected to acid fumigation (Harris et al., 2001) prior to measurement.

2.4. Processing ribosomal RNA and DNA

2.4.1. Simultaneous extraction of rRNA and rDNA

At the beginning of the experiment DNA was extracted from each replicate incubation jar using a MoBio Powersoil DNA isolation kit (MoBio Laboratories, Carlsbad, CA). DNA was stored at −20 °C for one year. Following the one-year incubation DNA and rRNA were extracted

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