SEPARATION OF Viable Microbes FROM SOIL MATRICES

by

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ABSTRACT

The majority of soil microbial biodiversity is uncultured in the laboratory. In part, the inability to cultivate many microbial lineages may be because these uncultured microbes are oligotrophic and do not grow on typical growth media. While there are several microbial cultivation methods designed for microbes inhabiting oligotrophic marine environments, these methods are challenging to implement in soil. This thesis describes the optimization of a Nycodenz™ density-gradient centrifugation method to separate viable cells from soil that are suitable for cultivation approaches that target oligotrophic microbes. Here we show that microbial cells are present but differentially distributed throughout the density gradient and that the distribution of these cells in the density gradient is soil-specific. This suggests that density-gradient centrifugation might be useful to enrich for specific microbial populations. To test this hypothesis in future experiments, we will quantify the types and relative abundances or microbial taxa from individual fractions to determine if different microbial taxa localize at different fractions on the density gradient after centrifugation.
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INTRODUCTION

Microbes are globally abundant and ubiquitous in soils, where they play crucial roles in Earth’s biogeochemistry, soil fertility, and contaminant remediation. These soil microbial communities are tremendously diverse with tens of thousands of unique phylotypes in a single gram of soil [1]. Our ability to infer specific environmental roles for individual microbial taxa is hindered by our inability to grow and study the majority of this microbial diversity under defined laboratory conditions. A recent DNA-based molecular study showed that although soil microbial communities are diverse, only ~2% of the total microbial taxa detected in soils account for almost half of 16S RNA gene sequences recovered from soil microbial communities worldwide [2]. Of these abundant and ubiquitous microbes, ~82% have no representative genome sequence match at ≥97% 16S rRNA gene sequence identity. This disparity between genome sequences and cultivation is related to the high overall microbial diversity of soil habitats and difficulty of growing microorganisms in the laboratory.

One possible explanation for why a large portion of this microbial biodiversity is recalcitrant to cultivation attempts is that many uncultivated lineages may be so-called ‘oligotrophs.’ Oligotrophy refers to the paradoxical physiology of microbes that grow optimally when nutrient availability is low. Oligotrophs dominate non-host-associated microbial environments globally [3]. These cells tend to be small, slow growing, and have unusual or combinatorial nutrient requirements [4–6]. In many cases, oligotrophic microbes are inhibited by high carbon content in routine microbial growth media, possibly because of competition with other microbes [7, 8]. Another explanation is that oligotrophs may rely on metabolic processes that do not adapt well when taken from a low-nutrient environment (most environments are low-nutrient) and are placed into a high-nutrient environment [8]. For example, the ubiquitous marine
oligotroph, ‘Candidatus Pelagibacter ubique,’ does not grow on typical growth media and is inhibited by trace amounts of common microbial growth medium formulations [9]. Repression by nutrient amendment is also observed in natural ecosystems. For example, high nitrogen and phosphorus levels shift bacterial communities to favor copiotrophic over oligotrophic taxa [10]. Because of this, low-nutrient growth media should be used for oligotrophic cultivation.

Researchers have since developed new oligotrophic cultivation methods in an attempt to expand the knowledge of cultured microbial biodiversity, most frequently in marine systems. While marine bacteria are prevalent in planktonic biomass, the culturability of these microorganisms rarely exceeds 0.01% [11–13]. Dilution-to-extinction methods were developed to increase the culturability of oligotrophs. Culturability is defined as the number of bacteria in a sample that can be cultivated in the laboratory [14]. The dilution-to-extinction methods are based on isolating cultures in small volumes of low-nutrient media and favors the most abundant microbes in a sample, rather than the most nutrient-tolerant [13]. The substrate concentrations of the media are typically three orders of magnitude lower than common laboratory media formulations [15]. Dilution-to-extinction methods have been effective in expanding the diversity of culturable marine bacteria [16].

Connon and Giovannoni increased the throughput and sensitivity of the dilution-to-extinction procedures [13, 15–18]. For marine microbes, cell inocula are counted and diluted into unamended seawater to a known concentration, typically between 1 to 5 cells per growth chamber. The concept of dilution-to-extinction culturing is that a single cell will germinate in each growth chamber to establish a pure culture. The growth chambers are incubated and screened for growth. The growth chambers are screened for growth with sensitive flow cytometry which can low density cell populations [19]. Such methods have resulted in a
culturability values that are 1.4 - 120 times higher than traditional agar plating of the same samples [16]. Another cultivation method that has also proven useful for oligotrophic communities is using different concentrations of diluted nutrient broth. The seawater inocula are diluted to several hundred cells per mL and then transferred into fresh media for incubation. However, this method still results in some isolates not showing significant growth, and some of the viable cells can be attributed to fragmentation instead of growth [8]. Fragmentation occurs when an organism splits into fragments, creating clones of itself. This results in low genetic diversity, and these populations are vulnerable to changing environments.

Although dilution-to-extinction culturing has resulted in successful culturing of previously uncultured bacteria, these approaches are challenging to implement for soil microbes [20]. The first step in dilution-to-extinction approaches is to accurately count the microbial inocula (Fig. 1). Direct counts can be obtained through microscopy or flow cytometry. In contrast to aquatic systems, accurate direct counts of microbes are challenging to obtain from soil because of interferences by the soil matric (organic and mineral constituents) [21]. While dilution-to-extinction methods have proven successful for certain soil microorganisms such as ammonia-oxidizing bacteria, these methods include typical growth media nutrients [22, 23]. Dilution-to-extinction methods for soil bacteria should include not only low-nutrient growth media but also a preceding soil separation step.
One way to circumvent the difficulty of obtaining direct counts from soil is to separate microbial cells from the soil particles before preparing direct counts. There are two steps in the soil microbial extraction process: dispersion and separation. The dispersion step uses physical or chemical means to dislodge any bacteria that are encompassed by soil aggregates [20]. Sieving, sedimentation, and densitometry are the possible separation methods. These separation methods separate the cells from the soil matrix. It is vital to separate viable cells from soil in order to culture organisms of interest, and densitometry is a very effective way to do so. Densitometry separates cells by their density—during centrifugation cells migrate through a density gradient, until they reach a density that matches their own. Nycodenz is an effective non-toxic density gradient medium that can be used to separate viable microbial cells from soil. Nycodenz is less viscous than other gradient mediums such as metrizamide and sucrose solutions, and it is relatively easy to remove from samples in comparison to other gradient media [24].
Here we optimize the Nycodenz density gradient method to extract viable cells from assorted soil samples. Our hypothesis is that different soil microbial taxa will partition at different locations in the density gradient, owing to differences in cell density. By deducing the specific location of individual microbial populations in the density gradient, we can extract specific fractions that are enriched in microbial populations for culturing.

**METHODS**

### 2.1 SOIL SOURCES

Soils were collected on Mount Bigelow located within the Santa Catalina Critical Zone Observatory just northeast of Tucson, AZ, USA (32.415 °N, -110.724 °W). We dug a 60 cm pit and collected soil horizontally into sterile plastic bags from depths of 0-5 cm and 50 cm on May 5, 2018. Soils were sieved to 2 mm and stored at 4°C until cell separation.

### 2.2 EXPERIMENTAL DESIGN

1. Blend soil in cell extraction buffer with immersion blender
2. Carefully layer soil-buffer slurry over Nycodenz solution in replicate (n=3) tubes
3. Centrifuge tubes
4. Collect 48 × 0.5 mL fractions from each tube
5. Wash cells obtained in each fraction
6. Stain and count cells in the washed fractions by flow cytometry

### 2.3 NYCODENZ PROTOCOL

We measured buffer and Nycodenz amounts by mass instead of volumes to obtain more consistency across replicate samples. We prepared the soil slurry by combining 0.5 g soil in a
cell extraction buffer consisting of 40 g saline solution plus 4.8 g of TSP-Tween solution. We blended the soil-buffer solution with an immersion blender for two minutes on ice. After blending, samples were allowed to settle for five minutes on ice. We layered 15 g aliquots of the soil-buffer solution over 14.3 g of the Nycodenz solution in sterile 50 ml polycarbonate centrifuge tubes (Fig. 2a) and centrifuged the tubes at 17,000 × g for 2 h at 16°C. A JA-17 rotor was used in a Beckman Coulter Avanti J-25 centrifuge. The resulting tube contained a large supernatant fraction and a pellet (Fig. 2b).

Figure 2: Appearance of Nycodenz density gradient tubes before (a) and after (b) centrifugation. The supernatant contains the separated viable cells which will be used for further analysis and culturing.
2.4 FRACTIONATION PROTOCOL

We removed tubes carefully from the centrifuge to minimize disrupting the density gradient and transferred 500 µL fractions of the Nycodenz density gradient—from the top of the tube—to 1500 µL microcentrifuge tubes containing 1 mL of the TSP-50 buffer. A total of 48 × 500 µL fractions were collected from each replicate. These fractions were vortexed and centrifuged 17,000 × g for 25 minutes. We removed the tubes, extracted the supernatant completely (without disrupting the pellet), and resuspended the cell pellet in 800 µL of 0.9% saline solution.

2.5 FLOW CYTOMETRY OF GRADIENT FRACTIONS

We partitioned the cells resuspended in saline for flow cytometry and microbial community analysis. For flow cytometry, we fixed 200 µL fractions with 0.9% formaldehyde and 2.4% SYBR Green I in filtered TE. Stained fractions were incubated overnight (~12-18 h) in the dark at room temperature. We ran the samples on the Guava with the following PMT voltages: SSC 999V, GRN 716V, YLW 250V, and RED 250V.

RESULTS AND DISCUSSION

Microbial cells were present throughout the Nycodenz density gradient for both surface and subsurface soil samples (Fig. 3). The surface soil fractions have a higher cell density than their subsurface soil counterparts. This indicates that there are either more microbial cells located in the surface soil than in the subsurface soil or that cells in surface soil horizons are more amenable to separation from the soil matrix than subsurface soils. For both soil depths, the cell densities are below the limit of detection from fractions 22 to 24 in the density gradient (Fig. 3). A possible explanation for this could be that the interface between the soil slurry and Nycodenz
occurs at that mark, but further research will need to be conducted to determine the characteristics of these fractions. Additionally, the surface samples have a lower fluorescence than the subsurface samples (Fig. 4). This may indicate a propensity for cells extracted from surface soils to have a lower nucleic acid content than subsurface soils, suggesting they are potentially smaller cells.

Figure 3: Distribution of Nycodenz-separated cells from surface and subsurface soils collected at Mount Bigelow. Each point is the mean cell density (n=3). Error bars show the standard deviation of the three replicates of each fraction.
Most density centrifugation protocols collect the faint whitish band directly above the Nycodenz after centrifugation [25–28]. Here we illustrate that high cell densities are found across a wide span of the Nycodenz gradient and that the distribution of cells in the Nycodenz gradient is soil-specific (Fig. 3). Thus, our results provide an extensive analysis of cells separated by density-gradient centrifugation. If cells of different taxa have can be differentiated by density, this optimized method could help researchers target specific microbial communities. If researchers know the relative density of an organism of interest, they can maximize their chances...
of targeting that specific organism. By fractionating samples throughout the density gradient, a more inclusive community analysis can be conducted. The application of this optimized Nycodenz density gradient centrifugation method could potentially increase the ability to isolate and culture previously uncultured bacteria such as the candidate phyla radiation.

The candidate phyla radiation is a recently announced expansion of the tree of life. “Candidate phyla” implies that this group lacks isolated representatives [29]. In other words, none of the microorganisms that are included in this phyla have been isolated and cultivated yet. This new branch represents more than 15% of all bacterial diversity and is hypothesized to contain more than 70 different phyla [30]. Culturing the candidate phyla would result in valuable information regarding the tree of life [29-32]. Optimizing the Nycodenz density-gradient centrifugation protocol could aid in cultivating these heretofore uncultivated and elusive microorganisms.

Future research is needed to determine taxonomic differences throughout the observed density gradient stratification. Three other soil sampling locations within the Santa Catalina Critical Zone Observatory that may be of interest are Marshall Gulch, Oracle Ridge, and Biosphere 2. The next step of this project is to conduct the optimized soil cell separation protocol on surface and subsurface soils from these locations to analyze the differences in microbial community between soils with different characteristics. These sampling locations include Marshall Gulch, Oracle Ridge, and Biosphere 2. After the optimized protocol has been used to separate the cells from those soil samples, DNA extractions, 16S rRNA gene amplicon sequencing, and PCR will be conducted to identify the microbial species present in each fraction. Additionally, community analysis for both depths at all four sites will be conducted to compare microbial taxa between soils.
REFERENCES


APPENDICES

5.1 REAGENTS

1) **TSP-tween**: 250 mM tetrasodium pyrophosphate (TSP) and 1.3% Tween 80

2) **TSP-50**: 50 mM TSP, filter sterilized through 0.2 µm filter

3) **Nycodenz**: 0.974 mM Nycodenz in TSP-50

4) **Sterile saline**: 0.154 mM NaCl in water, filter sterilized through 0.2 µm filter

5) **TE (Tris-EDTA buffer)**: Add 1 mL of 1M Tris-Cl and 0.2 mL 0.5M EDTA to 98.8 mL of distilled water. Filter sterilize using 0.2-µm-filtered.

5.2 SOIL SOURCE INFORMATION

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<th>Location</th>
<th>Depth</th>
<th>Longitude/Latitude</th>
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<th>Date fractionated</th>
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