The concentration of heterotrophic growth substrates in culture medium is a crucial determinant of the culturability of subsurface soil microbes

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Abstract

Soil microbial communities have crucial roles in soil fertility and biogeochemistry. While surface soils have been relatively well characterized, we know far less about the microbiology of oligotrophic shallow subsurface soils that often contain microbial taxa that are distinct from surface soils. We hypothesized that the concentration of heterotrophic growth substrates in microbiological growth medium is a critical determinant of the ability to culture subsurface soil microbes. To test this, we conducted dilution-to-extinction experiments with microbial cells separated from subsurface soils collected in a mid-elevation conifer forest in Arizona, USA. We diluted cells into two defined growth media with identical concentrations of inorganic nutrients and vitamins but contained a 100-fold difference in the availability of an amino acid and organic carbon mixture. Across both media formulations, we isolated a total of 133 pure cultures. The concentration of heterotrophic growth substrate in the medium facilitated the isolation of taxonomically distinct microbes. We isolated representative cultures of the most abundant member in the soil community (*Bradyrhizobium* spp.) and representatives of five of the top 10 most abundant *Actinobacteria* phylotypes, including *Nocardioides* spp., *Mycobacterium* spp., and several other phylogenetically divergent lineages. Flow cytometric analysis of the cultures showed that SYBR green-stained cells isolated on medium with reduced heterotrophic substrate concentrations had significantly lower nucleic-acid fluorescence than those isolated on medium with higher substrate concentrations. These results show that medium substrate concentration can facilitate the isolation of certain microbial lineages and that dilution-to-extinction is an effective method to isolate abundant soil microbes.

Importance

Isolating environmental microbes and studying their physiology under controlled conditions is an essential aspect of understanding their ecology. In this study, we use modified high-throughput cultivation methods to isolate subsurface soil microbes. Subsurface ecosystems are typically nutrient-poor environments that harbor diverse microbial communities—the vast majority of which are uncultured. We show that a critical component of whether a microbe is

culturable from subsurface soils is the concentration of growth substrates in the culture medium. Our results offer new insight into technical approaches and growth medium design that can be used to access the vast uncultured diversity of soil microbes.

Introduction:

Soil microbial communities are tremendously diverse and mediate crucial aspects of plant fertility, biogeochemistry, pollutant mitigation, and carbon sequestration (1–4). While the diversity and community composition of surface soils have been relatively well described, we know far less about the microbes inhabiting deeper soils, despite their key role in soil formation and mineralization of key plant nutrients. In contrast to surface soils that are typically rich in plant-derived compounds, subsurface soils are often oligotrophic and characterized by lower amounts of organic carbon, mineralizable nitrogen, and phosphorus (5–8). The temperature and soil moisture of subsurface soils also vary less over time than surface environments (9). As depth from the surface increases, the diversity of subsurface microbial communities decreases and the dissimilarity between the subsurface microbial community composition increases relative to surface microbial communities (10, 11). Moreover, the microbial taxa that are abundant in subsurface environments are generally absent from microbial culture and genome databases (10). Thus, we do not understand fundamental biological aspects of a major fraction of subsurface soil microbes (10).

Part of the challenge in culturing and studying subsurface microbes is that many of these uncultured lineages likely require low nutrient concentrations for optimal growth. These microbes are termed 'oligotrophs' and are paradoxical to what we understand about most cultivated microbes that tend to grow best when nutrient availability is high. Although oligotrophs dominate most free-living microbial ecosystems (12), the concept of oligotrophy is enigmatic: there is no consensus on what defines oligotrophic metabolism. Several general traits of oligotrophic microbes have emerged from studies of aquatic oligotrophs. For example, oligotrophs are small, slowly growing cells (13–16). The genomes of marine oligotrophs tend to be streamlined often coding for fewer copies of the rRNA gene operon and transcriptional regulators than microbes capable of robust growth at high nutrient availability, suggesting oligotrophs lack the ability to sense and rapidly respond to varying environmental conditions (12, 14, 17). Instead, genomic inventories in oligotrophs point toward a reliance on broad-specificity, high-affinity transport functions and relatively constitutive gene expression (15, 18–20). Because oligotrophs do not attain high yields on low nutrient media, traditional methods of quantifying microbial growth are ineffective (for example, optical density measurements and plating on solid media) (16, 21, 22).

While the physiology of aquatic oligotrophs have been fairly well studied, far fewer studies have focused on the physiology of oligotrophic soil microbes. Although many soil cultivation approaches rely on the use of low nutrient media (23–26), we lack a fundamental understanding about why these media facilitate the isolation of distinct taxa and often produce higher culturable counts than nutrient-rich media. We hypothesized that the concentration of heterotrophic growth substrates in a growth medium would constrain the taxa that grow on a defined artificial medium. We tested this by extracting cells from oligotrophic subsurface soils and inoculating high-throughput dilution-to-extinction experiments on a defined medium with differing amounts of heterotrophic substrates. We

isolated several bacteria representing abundant microbial community members. In these experiments, the substrate content significantly influenced which taxa grew in the laboratory. Moreover, we provide evidence that cells isolated on low nutrient medium may contain reduced nucleic acid content relative to those isolated on higher nutrient medium.

Results:

We collected shallow subsurface soil (55 cm) from the Oracle Ridge field site in a mid-elevation conifer forest that is part of the Santa Catalina Mountains Critical Zone Observatory in Arizona, USA. We modified high-throughput dilution-to-extinction approaches designed for aquatic microbes (21, 27) to culture shallow subsurface soil microbes from these samples (Fig. 1). The primary modification to existing protocols was to add a cell-separation step to detach inoculum cells from mineral soils prior to diluting them to extinction. We diluted the inoculum to an average of 5 cells well^{-1} into deep-well polytetrafluoroethylene 96-well plates containing a defined Artificial Subterranean Medium (ASM), having either low or high concentrations of heterotrophic growth substrates (ASM-low and ASM-high, respectively). The ASM-low and ASM-high media contained identical inorganic mineral and vitamin amendments but a 100-fold difference in the concentration of organic carbon and amino acids (Supplementary Table 1). We prepared triplicate 96-well plates for each growth medium formulation. These dilution-to-extinction experiments were screened for growth with flow cytometry after ≈4 weeks of incubation and again after ≈11 weeks of incubation. Wells displaying growth were sub-cultured into larger volumes and subsequently identified by 16S rRNA gene sequencing.

Across both medium types, a total of 214 wells (119 for ASM-low and 95 for ASM-high) exceeded our growth threshold (1.0×10^4 cells ml^{-1}) after 11 weeks of incubation. We successfully propagated 182 (85%) of the cultures from microtiter plates to polycarbonate flasks containing fresh medium. Of the cultures that successfully propagated, we confirmed that 73% (133 cultures) were pure cultures by amplifying and sequencing full-length 16S rRNA gene sequences. The remaining 49 cultures were either mixed (16S rRNA genes did not assemble due to base ambiguities) or did not amplify under several amplification conditions. We defined microbial culturability as the ratio of cells capable of being isolated in a given condition to the total number of cells initially diluted into a cultivation chamber (28). By the end of the experiment, we approached ≈20% culturability across both medium formulations (Fig. 2). In general, microbial culturability was higher for ASM-low than for ASM-high, but this effect was only significant after 4 weeks of incubation (Fig. 2; Wilcoxon rank-sum test $P \leq 0.05$ at 4 weeks).

Cultures isolated on ASM-high were taxonomically distinct (at a genus level) from those isolated on ASM-low (Kruskall-Wallis rank sum, $P=1.28 \times 10^{-5}$). All pure cultures grown on both ASM-low and ASM-high belonged to one of two bacterial phyla: Actinobacteria (110 cultures; 83% of the pure cultures) or Proteobacteria (23 cultures; 17% of the pure cultures) (Fig. 3). The Proteobacterial cultures were exclusively Alphaproteobacteria, 57% of which were isolated on ASM-low medium and the remainder (43%) were isolated on ASM-high (Fig. 3 and Supplementary Fig. 1). Cultures that classified as *Bradyrhizobium* spp. were the most frequent alphaproteobacterial isolates (13 isolates), seven of which were isolated on ASM-low medium. Cultures classified as *Reyranella* spp. and *Nordella* spp. were also isolated on both ASM-high

and ASM-low medium. The remaining five alphaproteobacterial cultures were taxonomically distinct—three were isolated on ASM-low and two on ASM-high.

The taxonomy of the actinobacterial cultures was split among three classes: Actinobacteria (107 cultures), Thermoleophilia (2 cultures), and Acidimicrobiia (1 culture). Of these Actinobacteria, 65 (59%) were isolated on ASM-low, and 45 (41%) on ASM-high. The actinobacterial cultures were numerically dominated by two genera that were differentially isolated on ASM-low and ASM-high: *Nocardioides* and *Mycobacterium*. *Nocardioides* spp. (46 cultures) were exclusively isolated on ASM-low medium (Fig 3 and Supplemental Fig. 2). Other cultures that were isolated on ASM-low included those classified as *Arthrobacter* (3 cultures), *Marmoricola* (2 cultures), *Nakamurella* (2 cultures), *Aeromicrobium* (1 culture), *Blastococcus* (1 culture), and *Patulibacter* (1 culture) (Fig 3 and Supplemental Fig 2). While cultures classified as *Mycobacterium* sp. were generally isolated on ASM-high (38 cultures), we did isolate seven mycobacterial cultures on ASM-low medium—five of which form a phylogenetically distinct cluster from those isolated on ASM-high (Fig. 3 and Supplementary Fig. 2). Other actinobacterial cultures isolated on ASM-high included *Jatrophihabitans* (4 cultures), *Conexibacter* (1 culture), and *Amycolatopsis* (1 culture).

Interestingly, we isolated what are likely the first members of two novel actinobacterial lineages on ASM-low. The first such culture—Microtrichales sp. str. AZCC_0197—belongs to the Microtrichales order of the Acidimicrobiia class. The best 16S rRNA gene sequence match to an existing isolate is 93.4% identity to *Aquihabitans daechunggensis* str. G128, but str. AZCC_0197 more closely matched numerous 16S rRNA gene sequences from environmental clones of uncultured Acidimicrobiia. The second lineage—Frankiales sp. strains AZCC_0102 and AZCC_0072—classified as members of the Frankiales order of the Actinobacteria class with best matches of <97% nucleotide identity to existing Frankiales isolates (29).

Several of the cultures we isolated are representative of abundant members of the subsurface soil microbial community at Oracle Ridge. We matched the 16S rRNA gene sequences from our cultures to the operational taxonomic units (OTUs) present in the Oracle Ridge soil sample collected at 55 cm. The cultures isolated from these 55 cm subsamples are representative of 13 OTUs (Fig. 4) that comprise a total of 8.8% of the total amplifiable microbial community in subsamples collected at the same time and depth. For example, our *Bradyrhizobium* isolates match a single *Bradyrhizobium* OTU that was the most abundant OTU at 55 cm (relative abundance of 5.7%) (Fig. 4). Similarly, we isolated representatives of most of the abundant Actinobacteria (Fig. 4) including: two *Mycobacterium* OTUs (the 11th and 17th most abundant OTUs overall, and the most abundant and sixth most abundant actinobacterial OTUs, respectively); *Nocardioides* (the 13th most abundant OTU overall, and third most abundant actinobacterial OTU), and two *Arthrobacter* OTUs (16th and 1,271st most abundant OTU overall, and the fifth and 143rd most abundant actinobacterial OTUs). The other Actinobacteria cultured in these experiments represent rarer species in bulk soils. The 16S rRNA gene sequences from several of our pure cultures did not match any of the 16S rRNA gene amplicon OTUs amplified from these soils, including *Nakamurella* (2 cultures), *Nocardioides* (5 cultures), *Mycobacterium* (1 culture), *Jatrophihabitans* (1 culture), *Patulibacter* (1 culture), *Conexibacter* (1 culture), *Rhizobiales* sp. "GAS113" (1 culture), *Reyranella* (1 culture), and *Microtrichales* sp. str. AZCC_0197.

Cultures isolated on ASM-low exhibited significantly lower mean nucleic acid fluorescence than those isolated on ASM-high (Fig. 5; Kruskal-Wallis Rank sum $P=6.3 \times 10^{-7}$). The overall mean fluorescence was not significantly different across phyla (Kruskal-Wallis Rank sum $P=0.647$) but was significant across assignable genera (Kruskal-Wallis Rank sum $P=3.0 \times 10^{-6}$). Moreover, the mean nucleic acid fluorescence values within a given genera were generally similar (Supplementary Fig. 3). For example, *Mycobacterium* isolates had relatively high nucleic acid fluorescence, regardless of which medium they were isolated on (Supplementary Fig. 3). In contrast, *Nocardioides* (ASM-low) and *Jatrophihabitans* (ASM-high) displayed relatively low nucleic acid fluorescence. Interestingly, when cultured on ASM-high, we observed a clear nucleic acid fluorescence dichotomy across the *Bradyrhizobium* isolates (Supplementary Fig. 3).

Discussion:

We designed a proof-of-concept workflow to determine the feasibility of high-throughput dilution-to-extinction cultivation for the isolation of soil microbes. The method was conceptually based on an improved high-throughput culturing workflow that was designed to isolate oligotrophic marine microbes in inert plastic microtiter plates using automated flow cytometry to detect microbial growth rapidly and at relatively low cell densities (21). However, unlike aquatic samples, microbial cells in soils are heterogeneously dispersed within, or attached to, a complex matrix comprised of non-cellular organic matter and minerals. The inherent complexity of the soil matrix complicates accurate enumeration of viable cells—especially of small oligotrophic microbes—because mineral and organic matter can interfere with flow cytometry. To circumvent these issues, we separated cells by gently shaking soils in a cell extraction buffer containing a dispersing agent and a nonionic surfactant. Cells were separated from this slurry by density gradient centrifugation (Fig. 1). This procedure allows cells to be 'floated' on top of a solution of Nycodenz while allowing minerals to pellet through the Nycodenz (30).

We expected 168 pure cultures across all experiments, based on the proportion of cultivation chambers displaying measurable growth (214 chambers) and the number of chambers inoculated (576 chambers). The number of pure cultures we obtained (133) was within 38% of this value. However, this result does not account for the cultures that were initially scored as positive for growth but did not successfully subculture. Some of the cultures that failed to propagate from microtiter plates to larger volumes might have been false positives, where flow cytometer instrument noise or well-to-well carryover was mistaken for a low-density culture. The mean culturability we observed for a given experiment (1.4%-11%; Fig. 2) is comparable to dilution-to-extinction cultivation studies of marine microbes, which report 0.5%-14.3% culturability (27). Similar to previous observations for soil microbes (25), we observed the culturability increased by lengthening the incubation time (Fig. 2).

The concentration of heterotrophic growth substrates in the isolation medium was a determinant of the ability to isolate certain microbial lineages (Fig. 3). Carbon type and availability are crucial for heterotrophic soil microbes since carbon acts as both a source of electrons for respiration and carbon for biomass. To accommodate this requirement, many common microbial growth medium formulations for heterotrophic microbes supply diverse growth substrates (yeast extract or casein digests, for example), usually at concentrations much higher than are normally available *in situ*. Two key assumptions made with these

219 common media formulations are that: 1) microbes will use only the relevant constituents and
220 any remaining compounds will have minor or no effect on microbial growth; and 2) microbes
221 grow optimally when nutrient availability is much greater than their half saturation constant
222 (31). While many microbes have the capacity to grow on these commonly used complex, high-
223 nutrient formulations, environmental nucleic acid data informs us that the vast majority of
224 Earth's microbes cannot (32).
225 Numerous studies have demonstrated that dilute growth media is superior to substrate
226 rich growth media for the isolation of novel soil microbes (25, 26, 33, 34). However, the
227 physiological explanation as to why low-nutrient media facilitates the growth of diverse
228 microbes, or high nutrient concentrations inhibit the growth of some taxa, are unclear. One
229 possible explanation for these concentration-dependent effects may be that growth medium
230 formulations applied at high concentrations contain high amounts of inhibitory substances—
231 substances that are reduced to non-inhibitory levels in dilute medium formulations. For
232 example, a key amino acid transporter in *Chlamydia trachomatis* can be blocked by non-
233 essential amino acids, preventing the transport of required amino acids, resulting in growth
234 inhibition (35). A similar phenomenon was demonstrated in the marine oligotroph '*Candidatus*
235 Pelagibacter ubique', where alanine was conditionally required for cell division but abolished
236 growth at higher concentrations (36). Furthermore, reactive oxygen species can be produced
237 during the autoclaving of nutrient-rich medium that either directly inhibit growth or combine
238 with organics in the medium to form inhibitory compounds (37, 38). Finally, growth inhibition
239 may be the result of misbalanced regulation of growth or accumulation of nutrient storage
240 structures (poly-β-hydroxybutyrate, for example) ultimately leading to cell lysis (39). A better
241 understanding of the mechanism(s) that enable growth on low nutrient medium—or prevent
242 growth on high-nutrient medium—may help us design better strategies for isolating
243 uncultivated lineages. Critically, having a collection of cultures that were isolated on medium
244 with identical constituents applied at different concentrations is a first step towards an
245 experimental method capable of addressing these questions.
246 While several of the taxa we isolated were abundant microbial members of the shallow
247 subsurface microbial community (Fig. 4), other isolates were rare or not identified in the
248 cultivation-independent soil microbial community at all. Although the cultivation of microbial
249 phylotypes that are not observed in cultivation-independent analyses has been observed (40),
250 dilution-to-extinction favors the cultivation of abundant microbes in a given sample (28), such
251 that the appearance of rarer taxa or taxa that were not observed in the original sample is
252 unusual. There are several possible explanations for this observation. First, the absence of a
253 particular taxon in a soil microbiome analysis may be the result of insufficient sequencing
254 depth (40). Similarly, the 'universal' primers used in the soil microbiome analysis (41) may have
255 been biased against some of the divergent lineages we obtained in pure culture, resulting in
256 either underrepresentation of these phylotypes in the original community or no amplification
257 at all. Finally, the taxa isolated in these experiments may have had similar buoyancies and
258 migrated to similar locations in the density gradient during cell extraction effectively skewing
259 the proportions of microbes that were diluted into microtiter plate wells. At this point, we do
260 not have sufficient evidence pointing toward one of these scenarios over the other.
261 Additionally, as is true in any microbial cultivation experiment, there are many taxa that we did
262 not isolate. In particular, these soils contained high relative abundances of Verrucomicrobia

263 related to '*Ca*. Udaeobacter copiosus' (42), and Acidobacteria (Subgroup 6), which belong to
264 highly sought-after lineages of uncultured microbes (22). The reasons for not culturing these
265 (and other) lineages are numerous but may be the result of inappropriate medium composition
266 (43, 44), toxic compounds in the cell separation constituents, long doubling times (> 6 days),
267 or dormancy (reviewed in (45)).
268 Similar to aquatic oligotrophs (15, 46), we provide evidence that soil oligotrophs contain
269 reduced nucleic acid content relative to other soil microbes that prefer higher nutrient
270 concentrations (Fig. 5). Depending on the taxa in question and the effective population size,
271 microbial genome reduction can be driven by genetic drift or streamlining selection (reviewed
272 in (47)). Genome streamlining is strongly linked with oligotrophy in free-living aquatic microbes
273 as a mechanism to reduce the overhead cost of replication in periodically nutrient-limited
274 environments (reviewed in (14)). However, direct evidence for streamlined genomes in
275 terrestrial oligotrophs has been elusive. Metagenome-assembled genomes suggest that some
276 lineages of abundant and ubiquitous uncultured Verrucomicrobia may contain reduced
277 genome sizes (42), and a more recent study showed that fire-affected warm soils selected for
278 groups of microbes with significantly smaller genomes than cooler soils (48). However, there
279 are few definitive ways to identify these taxa as oligotrophs short of culturing them. The
280 appearance of reduced nucleic acid content in cultures isolated on ASM-low is also an
281 indication that genome reduction may be a successful life strategy for soil oligotrophs. We
282 speculate that "streamlined" genome sizes in soil oligotrophs may be larger than those
283 observed in marine habitats, since success in the complex soil matrix necessitates a greater
284 variety of gene function to succeed (14).
285 We were surprised to identify taxa with such distinct growth substrate concentration
286 preferences from highly oligotrophic soils (0.5% organic carbon). For example, while the
287 environmental distribution of *Nocardioides* sp.—which were isolated on ASM-low—indicate a
288 preference for deeper, more oligotrophic soils (Fig. 4), we also isolated numerous
289 *Mycobacterium* spp. from the same samples. The majority of these *Mycobacterium* isolates
290 were isolated on ASM-high medium and did not show a clear preference for subsurface soils
291 (Fig. 4). Interestingly, we observed that the *Nocardioides* sp. appeared sooner (within 4 weeks)
292 in the dilution-to-extinction screens, and *Mycobacterium* sp. growth was only apparent
293 between 4 and 11 weeks of incubation. These observations suggest that the oligotrophs from
294 these soil samples may grow relatively rapidly compared to the microbes requiring higher
295 nutrient concentrations. This finding is contrary to the perception that oligotrophic microbes
296 grow more slowly than those microbes capable of growth on higher nutrient concentrations
297 (16), suggesting that oligotrophy in shallow subsurface soils may be characterized by traits that
298 are distinct from those described for aquatic microbes.
299 The development of cultivation techniques emphasizing the high-throughput and sensitive
300 detection of microbial growth on low-nutrient medium has revolutionized the field of aquatic
301 microbial ecology in that these methods enable the cultivation of microbes that were
302 previously inaccessible using standard techniques (21, 27, 49–51). Here we show that similar
303 principles facilitate the cultivation of abundant soil oligotrophs. Moreover, we demonstrate
304 that in addition to considering the nutritional composition of a given growth medium, another
305 obstacle to culturing some lineages is the concentration of growth substrates in the growth
306 medium. Although we do not yet understand the mechanism of substrate-induced growth

inhibition, there is evidence that this phenomenon is widespread and results in the inability to culture some microbial lineages in the laboratory (12). Future mechanistic studies aimed at deciphering the physiology of substrate-induced growth inhibition will likely lead to new cultivation approaches that will allow us to isolate abundant free-living oligotrophic microbes.

Materials and Methods:

Soil source: Fresh soil samples were collected from a soil pit on August 16, 2017 at the Oracle Ridge site in the Catalina Jemez Critical Zone Observatory (coordinates: 32.45 N, -110.74 W, elevation 2,103 m). We collected ~300 g subsamples from 0-5 cm, 10 cm, 20 cm, 30 cm, 40 cm and 55 cm depths. We analyzed the microbial community composition at each depth (see '*Soil microbial community analysis,*' below) and conducted cultivation experiments from the 55 cm soil sample. Soils were kept cool with ice packs for <4 h while in transit to the laboratory. At the laboratory, the soils were sieved to 2 mm and kept at 4°C for 50 days at which point cells were separated from mineral soil.

Soil microbial community analysis: Replicate 1.0 g subsamples were weighed into MoBio PowerSoil DNA extraction kits and extracted according to the manufacturer's instructions. We amplified 16S rRNA gene fragments using 515F-Y (5'-TATGGTAATTGTGTGYCAGCMGCCGCGGTAA) and 926R (5'-AGTCAGTCAGGGCCGYCAATTCMTTTRAGT) (52). PCR products were purified using the QIAquick PCR purification kit (Qiagen, Germantown, MD) per manufacturer's specification. Cleaned products were quantified using Tecan fluorometric methods (Tecan Group, Mannedorf, Switzerland), normalized, and pooled for Illumina MiSeq sequencing using custom sequencing primers and the MiSeq Reagent v2 500 cycle Kit (Roche, Branford, CT) following the manufacturer's protocols. Operational taxonomic units (OTUs) were generated *de novo* from raw Illumina sequence reads using the UPARSE pipeline (53). Paired-end reads were trimmed of adapter sequences, barcodes and primers prior to assembly. Sequences of low-quality, and singletons were discarded, and the remaining sequences were subjected to a dereplication step and abundances were determined. Chimera filtering of the sequences was completed during clustering while taxonomy was assigned to the OTUs with mothur (54) using version 123 of the SILVA 16S ribosomal RNA database (55) as the reference. OTUs and corresponding taxonomy assignment tables were generated and used in subsequent analyses.

Cell separation: Cells were separated from sieved soils using density gradient centrifugation with Nycodenz. Briefly, we added 0.5 g wet soil to 44.8 ml of cell extraction buffer (137.5 mM NaCl, 26.78 mM tetrasodium pyrophosphate, and 0.27% (v/v) Tween 80). The soil-buffer slurry was vortexed for 30 seconds and shaken horizontally on a platform shaker for 2 hours at 4°C. We layered 15 ml aliquots of this soil-buffer slurry over 10.0 ml of 80% (w/v) Nycodenz solution in 50 mM tetrasodium pyrophosphate. We used 50 ml Nalgene Oak Ridge high-speed polycarbonate centrifuge tubes for density gradient centrifugations. Tubes containing the soil-buffer solution with Nycodenz were centrifuged at 17,000 × *g* for 30 minutes at 16°C. We extracted 3 × 0.5 mL aliquots from the resulting density gradient at a location of ~25 mm above the bottom of the tube (coincident with the approximate level of the top of the Nycodenz solution) to sterile microcentrifuge tubes containing 1.0 ml 137.5 mM NaCl. The microcentrifuge tubes containing Nycodenz/NaCl were vortexed and centrifuged for 20 min at

351 17,000 × *g*. The resulting faint cell pellets were resuspended in 137.5 mM NaCl, pooled and
352 stored at 4°C.
353
354 *Dilution-to-extinction:* An aliquot of cells extracted from the Nycodenz gradient were fixed
355 with 1.75% (final v/v) formaldehyde and stained with SYBR Green I (final stain concentration
356 was a 1:4,000 dilution of commercial stock) for 3.5 h at room temperature in the dark. Cells
357 were enumerated using a Millipore Guava flow cytometer, as described elsewhere (36). We
358 diluted cells into Artificial Subterranean Medium (ASM) -high or ASM-low nutrient medium
359 (Supplementary Table 1) to a density of 5 cells mL^{-1} and aliquoted 1.0 ml of the dilute cell
360 suspension into the wells of 2 ml polytetrafluoroethylene 96-well microtiter plates (Cowie
361 Technology, New Castle, DE) so that on average each well contains 5 cells. Plates were covered
362 with plastic lids that allow air circulation and incubated at 16°C in the dark. We screened the
363 dilution-to-extinction plates for growth by fixing (1.75% formaldehyde) and staining (1:4,000
364 dilution of commercial SYBR Green I stock) aliquots for 18h in the dark at room temperature
365 and counting by flow cytometry (EMD-Millipore Guava EasyCyte), as described previously (21).
366 We screened plates for growth at 4 and 11 weeks after inoculation. Positive cultures were
367 defined as cultures that exceeded 1.0×10^4 cells ml^{-1}.
368
369 *Culturability estimates and estimates of the number of pure cultures:* Culturability estimates
370 were determined by the equation: $V = -\ln(1-p)/X$, where V is the estimated culturability, p is the
371 proportion of inoculated cultivation chambers that displayed measurable growth (number of
372 chambers positive for growth ÷ total number of chambers inoculated), and X is the number of
373 cells added to each cultivation chamber (28). The number of pure cultures (u) was estimated as
374 follows: $u=-n(1-p)\ln(1-p)$, where n is the number of inoculated growth chambers (28).
375
376 *Culture transfer and storage:* We subcultured positive growth chambers into 25 ml of the
377 respective growth medium (ASM-high or ASM-low) in acid-washed, sterile polycarbonate
378 flasks and incubated them at 16°C. At the time of transfer, we assigned cultures a unique
379 Arizona Culture Collection (AZCC) number. Flasks were monitored for growth every other
380 week for 2 months. Flasks displaying growth within two months were cryopreserved in 10%
381 glycerol and stored at -80°C. If no growth appeared within two months, the cultures were
382 discarded and the assigned AZCC number was retired.
383
384 *Mean fluorescence calculations:* We calculated the mean fluorescence of each culture from
385 the subcultures grown in 25 ml volumes 12-15 weeks after inoculating. Culture aliquots were
386 fixed and stained for 15-18 h as described above in 'Dilution-to-extinction.' We manually gated
387 histograms of the intensity of SYBR Green I fluorescence (in arbitrary units) and extracted the
388 mean fluorescence of the gated peak for each culture.
389
390 *Culture identification*: Cultures were identified by full length 16S rRNA gene sequencing.
391 Briefly, we filtered 5-10 ml of cell biomass from 25 mL cultures on to 0.2 µm-pore size Supor
392 filters and extracted DNA using a Qiagen PowerSoil DNA extraction kit following the
393 manufacturer's instructions. We amplified full-length 16S rRNA genes from the resulting DNA
394 using the 27F-1492R primer set (27F: 5'-AGAGTTTGATCMTGGCTCAG-3'; 1492R: 5'-

ACCTTGTTACGACTT-3′ (56)). The reaction mix consisted of Promega's GoTaq HotStart 2x PCR master mix with final concentrations of 0.4 µM 27F and 0.4µM 1492R primers, and 1-11.5 µl of template DNA, in a total reaction volume of 25 µL. The thermocycling profile was 1 × 94°C for 10 min followed by 36 cycles of: 94°C for 45 s, 50°C for 90 s, 72°C for 90 s; and a single 72°C extension for 10 min. The resulting amplicons were cleaned and Sanger sequenced from either the 27F or 1492R primer by Eurofins Genomics (Louisville, KY, USA) using their standard techniques. Sequences were curated using 4Peaks (57) and Geneious Prime v2019.0.1 (58). Reads were trimmed and assembled using the moderate setting in Geneious. Those reads that failed to assemble with these metrics were considered "mixed" cultures and not analyzed further.

Culture taxonomy and determination of taxonomic differences across growth medium formulations: High-quality full length 16S rRNA gene sequences from the cultures were used to assign taxonomy and reconstruct phylogenetic relationships. We assigned taxonomy to all assembled 16S rRNA gene sequences using the SILVA database SINA aligner v128 (59). A Shapiro-Wilk test of normality was conducted in base R (60) on the distribution of taxa recovered from both media types. After concluding the data were non-parametric, we performed a Kruskal-Wallis test in R (Taxa ~ Media Type).

Taxonomic selection for phylogenetic reconstruction: To reconstruct a phylogeny of full length 16S rRNA genes, our culture sequences were compared to NCBI's Microbial Genomes and environmental sequences using web-blast (61). The top five hits for each sequence, from either NCBI database, were chosen based on the highest percent coverage and lowest e-value score. *E. coli* K-12 was used as the outgroup of the Alphaproteobacterial phylogeny, and *Bacillus subtilis* was used as the outgroup for the Actinobacterial tree. These sequences aligned with MAFFT (62) with turn checking enabled to conform sequence orientation to the outgroup. The alignment was then trimmed using TrimAl (63) with the automated1 setting to optimize sequence trimming for maximum likelihood phylogenetic analyses. We reconstructed phylogenetic relationships from this trimmed alignment in the CIPRES Gateway (64). Maximum-Likelihood (ML) trees were constructed using IQ-TREE with 10,000 ultrafast bootstrap trees and Bayesian Information Criterion to select the best fit nucleic acid substitution model (65, 66). After an initial round of ML trees, sequence alignments were heuristically curated with IQ-TREE to eliminate duplicitous sequences. Finalized ML trees were then imported into the ARB environment (67), where any duplicate sequences from our AZCC cultures were added to the ML trees through ARB's quick add parsimony function. Final trees were visualized with FigTree (68).

Environmental contextualization of AZCC isolates: We matched the AZCC isolate full length 16S rRNA gene sequences against a database of the clustered OTUs from the shallow soil depth profile samples (see *'soil microbial community analysis,'* above) using the usearch_global command (69) at a stringency of ≥97% identity, in both strand orientations, with maxaccepts=1 and maxrejects=0.

Data Availability: Full length Sanger Sequenced 16S PCR product sequences are available on NCBI GenBank, accession numbers: MK875836 - MK875967. Illumina data are available on the NCBI SRA, accession numbers: SRR9172130-SRR9172198.

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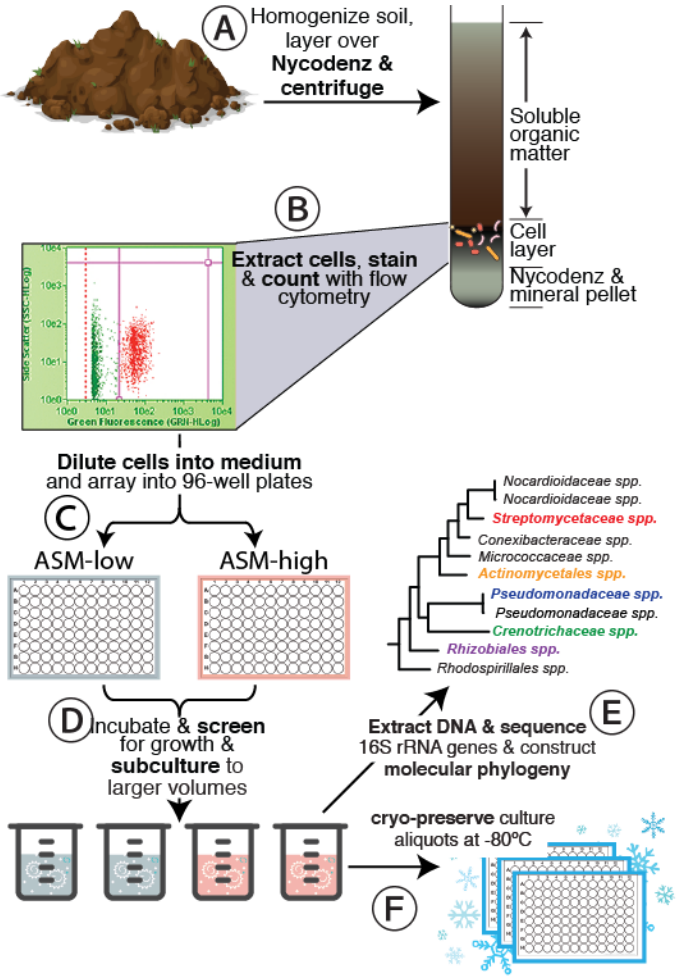

Figure 1: Dilution-to-extinction workflow. Soils are collected and brought to the lab where they are homogenized in cell extraction buffer, layered over a Nycodenz solution, and centrifuged (A). The cell layer is extracted from the Nycodenz solution and counted with flow cytometry (B). Counted cells are diluted into growth medium in 96-well microtiter plates to an average density of 5 cells well^{-1} (C). After incubation, the 96-well microtiter plates are screened for growth with flow cytometry, and wells displaying growth are subcultured into larger volumes (D). After incubating the subcultures, flasks displaying growth are identified by 16S rRNA gene sequencing and molecular phylogeny (E). Aliquots of these identified subcultures are cryopreserved at -80°C (F).

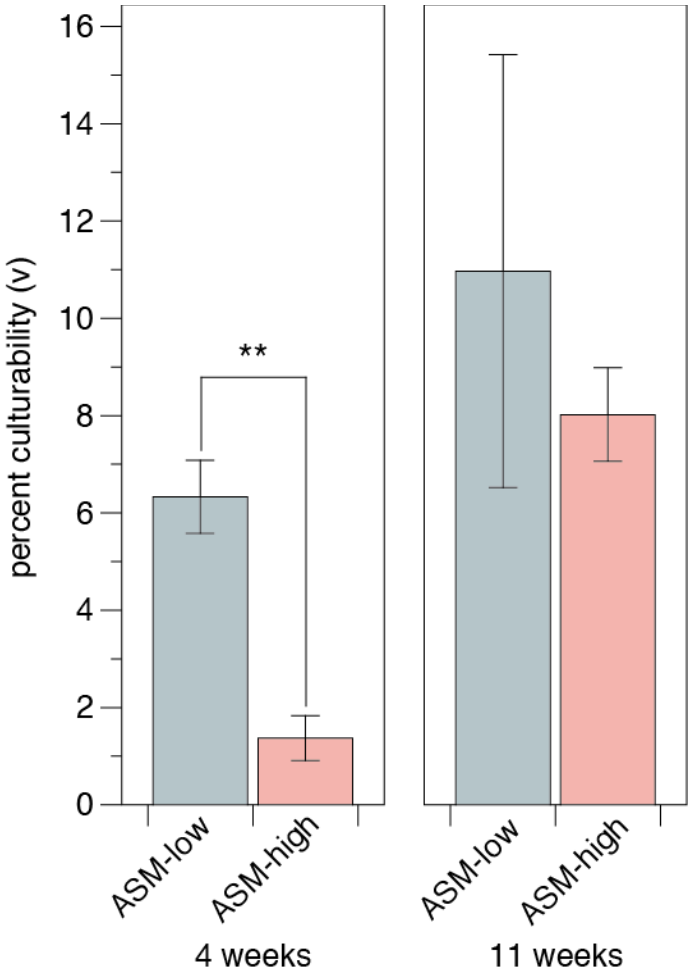

Figure 2: Microbial percent culturability (v) is greater on ASM-low than on ASM-high. Bar heights are the mean culturability ± standard deviation in 96 well microtiter plates (n=3) as calculated from the initial cell inoculum and the proportion of wells positive for growth. Double asterisks indicate Wilcoxon rank sum test P values ≤ 0.05.

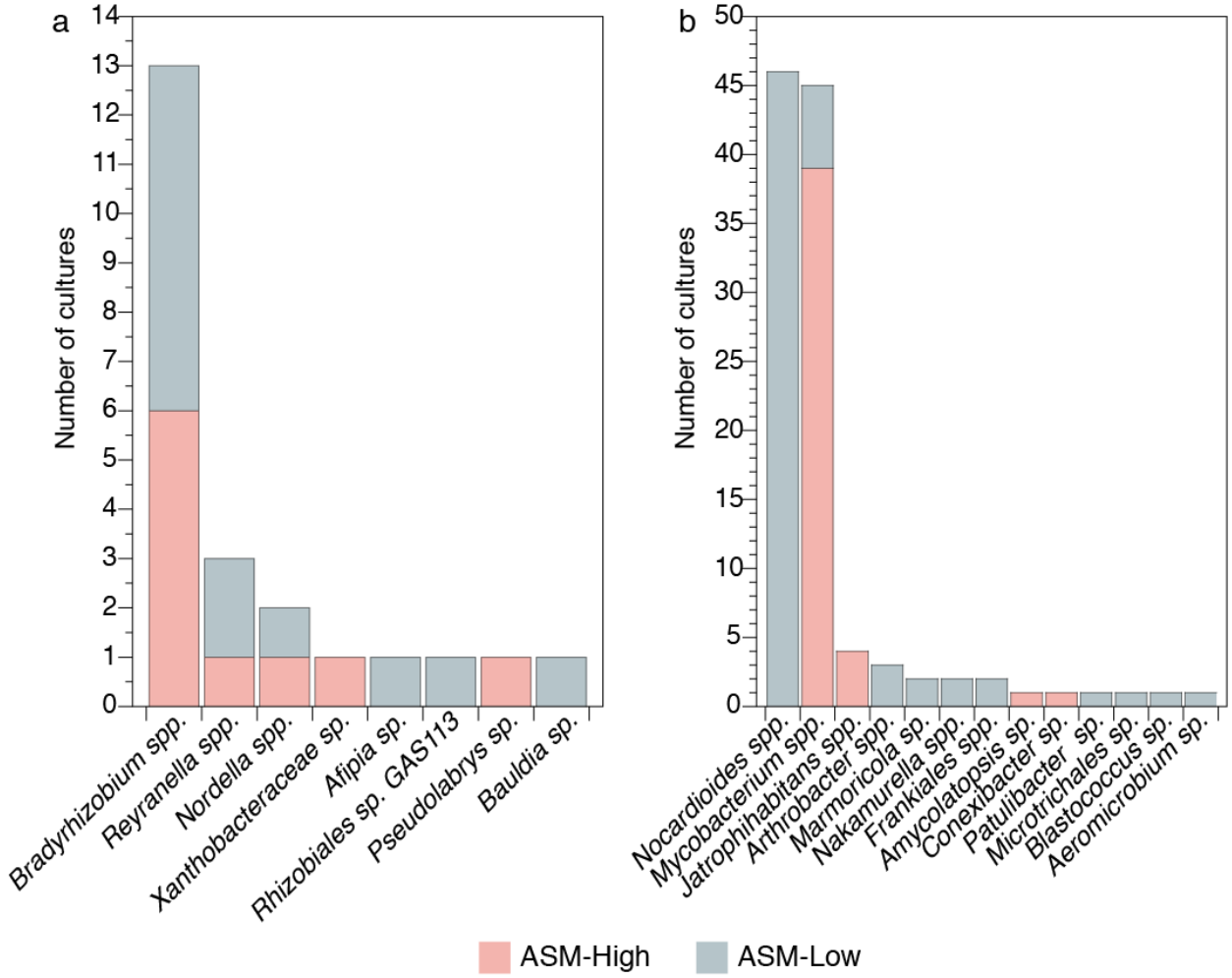

Figure 3: ASM-low and ASM-high cultured distinct alphaproteobacterial (a) and actinobacterial (b) microbial taxa. Bar heights are the number of cultures obtained for each taxon and are colored by the medium type on which they were isolated.

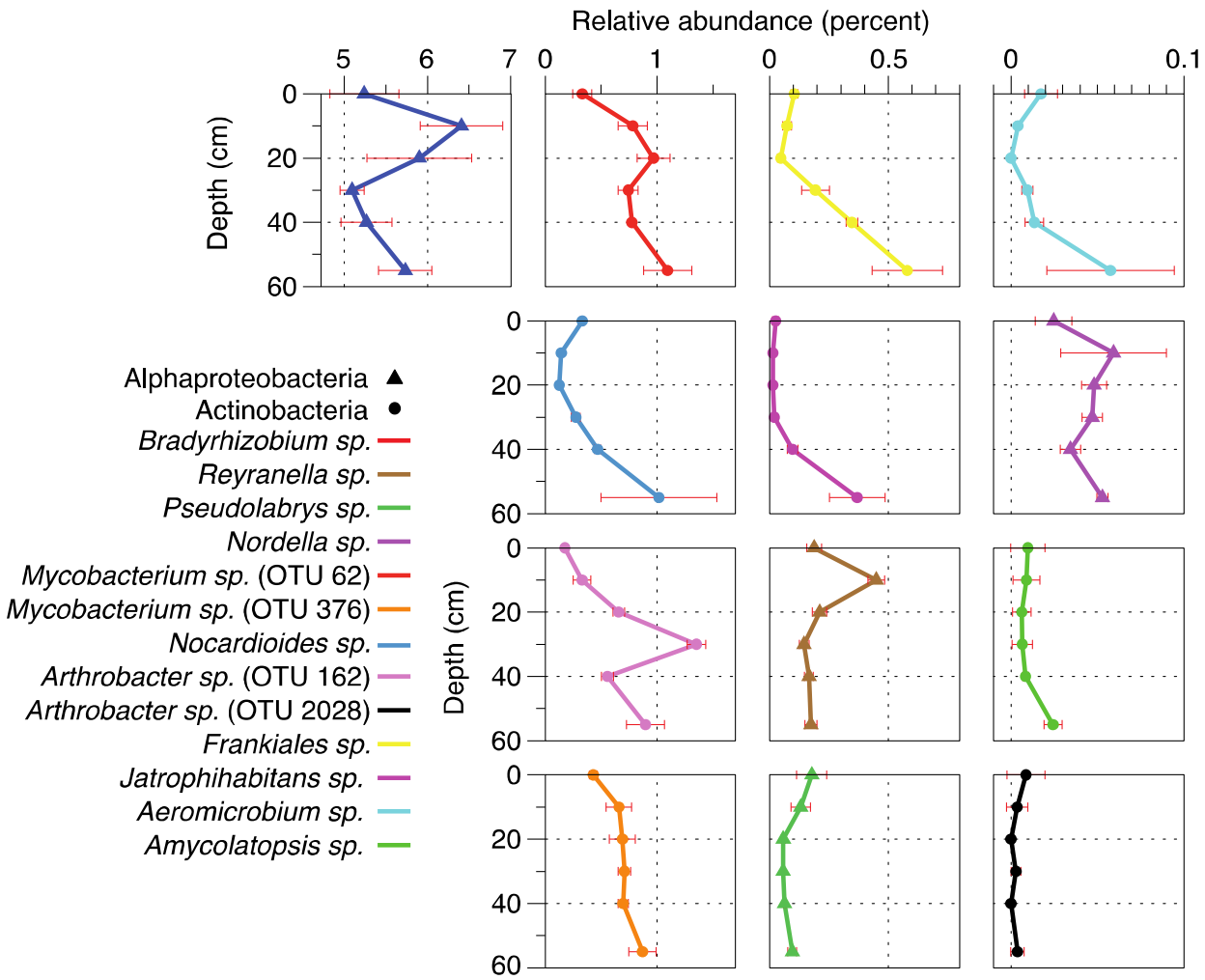

Figure 4: The cultures isolated in this study are representative of several abundant soil lineages that show dynamic depth distributions in Oracle Ridge soils. Points are the mean relative abundances ± standard deviation (n=3) of 16S rRNA gene sequence OTUs that matched cultured isolates at ≥97% identity. Error bars that are not visible are located behind the symbol. *Mycobacterium* and *Arthrobacter* cultures best-matched more than one OTU in the cultivation-independent surveys. The best-matching OTU number is designated in parentheses.

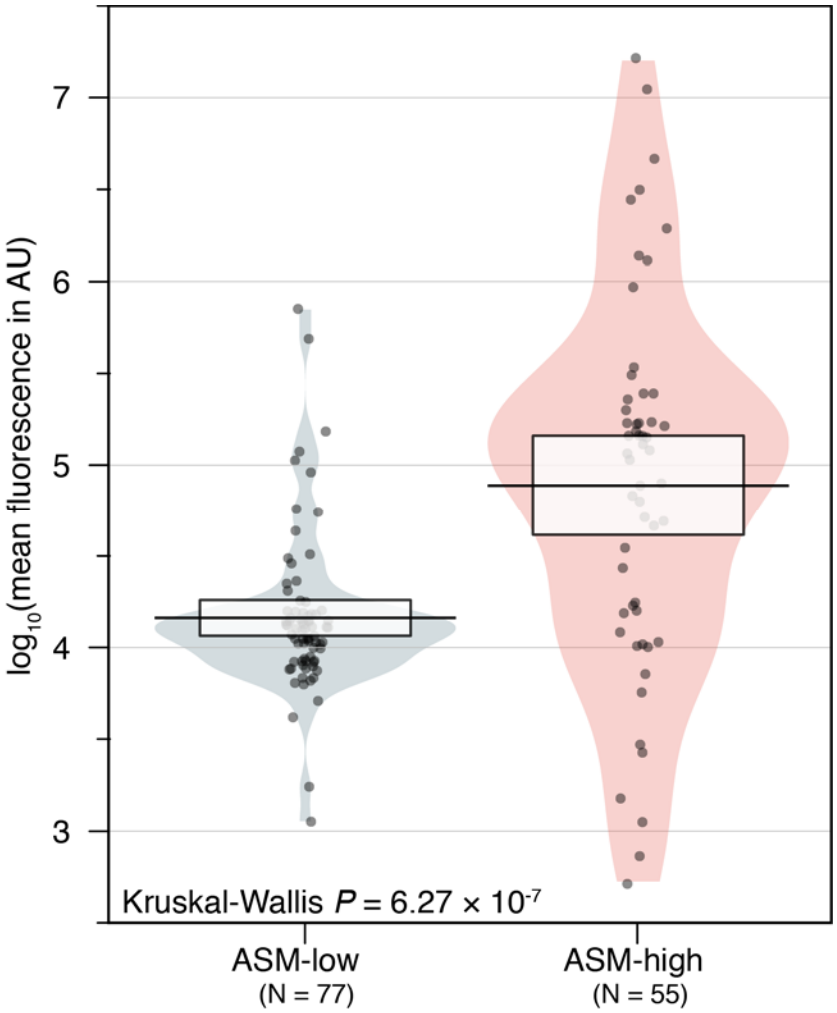

Figure 5: The mean nucleic acid fluorescence of taxa isolated on ASM-low was significantly lower than for those microbes isolated on ASM-high. Points are the mean $\log_{10}$ fluorescence (in arbitrary units [AU]) of fixed and SYBR green I-stained stationary phase cultures. The mean fluorescence value was obtained from manually-gated histogram plots of fluorescence within the Guava EasyCyte software. Only those cultures that were defined as pure cultures are plotted. The horizontal line in each plot is the mean fluorescence value and the box surrounding the mean is a 95% confidence interval. Shading illustrates the relative distribution of fluorescence values within each medium type.