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Isolation of a Substantial Proportion of Forest Soil Bacterial Communities Detected via Pyrotag Sequencing

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We isolated 1,264 bacterial strains from forest soils previously surveyed via pyrosequencing of rRNA gene amplicons. Conventional culturing techniques recovered a substantial proportion of the community, with isolates representing 22% of 98,557 total pyrotags. Growth characteristics of isolates indicated that ecological traits were associated with relative abundances of corresponding pyrotag operational taxonomic units.

Forest soil microbial communities provide critical ecosystem services (1–3) and have essential roles in forest productivity and climatic effects. For decades, large field studies, such as the Long-Term Soil Productivity Study (LTSP), have examined factors important to sustainable forest management (4). More recently, with molecular microbial ecology approaches, field studies have begun to examine the relationships between soil microbial community composition and other elements of forest systems (5–7). These studies have yielded important advances, but a major emerging limitation is our poor understanding of the functional roles of microbial populations identified by molecular analysis. Moreover, it has become clear that the operational taxonomic units (OTUs) defined in culture-independent studies contain a surprisingly large amount of fine-scale phylogenetic diversity (8), but the functional and ecological boundaries that differentiate these microdiverse clusters into cohesive populations remain largely unexplored outside marine systems.

Hartmann et al. (7) extensively characterized bacterial and fungal communities in forest soils from LTSP sites in two biogeoclimatic zones in British Columbia, Canada, via high-throughput pyrosequencing of rRNA gene and internally transcribed spacer amplicons (pyrotag sequencing). That study found that soil compaction and organic matter removal during tree harvesting had long-term effects on the composition of soil communities. Thus, the functional relevance of affected populations in these communities is important to understand.

Physiological studies with pure cultures of microorganisms remain the main avenue for characterizing the functions of those organisms. It is generally asserted that only a minor fraction of soil microbial communities can currently be cultivated, thus limiting our ability to functionally understand these communities. Estimates of the culturability of soil bacteria range from 0.1% to 5.2% of viable bacterial cells (9–12). Recent developments in cultivation techniques yielded much greater proportions of viable cells, including previously uncultivated taxa (9–17). However, total numbers of culturable cells do not describe what proportion of the diversity of a bacterial community is culturable. Few studies, and none investigating forest soil communities, have compared the identities of the organisms that can be isolated to those of organisms detected by extensive culture-independent surveys. To that end, our understanding of the culturability of bacterial communities in forest soils remains incomplete. In this study, we used a range of standard media to isolate bacteria from forest soil communities previously characterized by pyrotag sequencing (7). Based on rRNA gene sequences of the isolates, we compared the taxa isolated to the composition of the corresponding pyrotag libraries.

Four composite soil samples, from both organic and mineral soil horizons at two LTSP sites in British Columbia, were used as inocula for cultivation. These were a subset of samples from six LTSP sites previously analyzed by pyrotag sequencing (7). The samples were stored at −80°C prior to both this cultivation study and the previous pyrotag study, which was a logistical necessity to ensure that samples most closely resembled the community *in situ* and that the same community was analyzed in both studies. Microbial cells were removed from soil using a vigorous extraction procedure. Dilutions of cell extracts were plated on eight conventional agar-based media incubated at 25°C. Media and incubation conditions were intended to maximize the recovered taxonomic diversity and select for organisms with a range of niches. Established medium recipes were used, except the pHs of the modified Burk's medium and soil extract medium were adjusted to 6.0 to be within the pH range of forest soils used in this study, from 4.5 to 6.0. Over 37 days of incubation, a collection of 1,264 bacterial strains was obtained (see Tables S1 and S2 in the supplemental material). Isolates were selected on the basis of colony morphology to maximize diversity and minimize redundancy in the culture collection. Taxonomic identity and purity of the isolates were determined by amplifying and Sanger sequencing the V1 to V3 regions of their 16S rRNA genes. We note that soil processing prior to cultivation, particularly storage at −80°C, likely altered the proportions of recovered taxa, and additional strains might have been recovered from fresh soil. Accordingly, none of the conclusions drawn in this study are based on comparisons between the proportional abundances of taxa in the isolate collection

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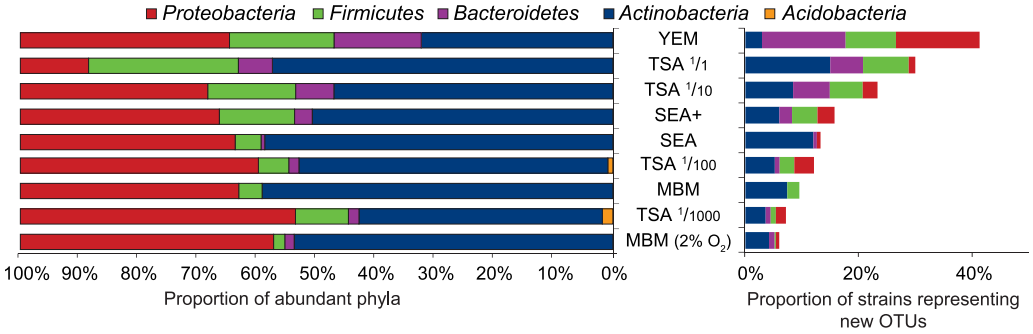

FIG 1 Relative abundances of major phyla isolated and proportions of new OTUs isolated on each medium. Media are arranged in descending order of nutrient richness. New OTUs are ones not previously detected by pyrotag analysis. YEM, yeast extract-mannitol; TSA $^{1}/_{1}$, full-strength Trypticase soy agar; TSA $^{1}/_{10}$, 10-fold dilution of TSA; SEA+, soil extract agar supplemented with 100-fold-diluted Trypticase soy; SEA, soil extract agar; TSA $^{1}/_{100}$, 100-fold dilution of TSA; MBM, modified Burk's medium; TSA $^{1}/_{1000}$, 1,000-fold dilution of TSA; MBM (2% O_2), modified Burk's medium incubated under 2% O_2.

and those in the pyrotag data set. Details of materials and methods are provided in the supplemental material.

Taxa representing a substantial portion of the bacterial community, based on culture-independent pyrotag analysis, were isolated using conventional methods and essentially standard media. Isolate OTUs clustered at a Levenshtein distance of 7 (≈97% identity) with 1.4% of the total pyrotag OTUs. However, because many of the isolate OTUs corresponded to abundant pyrotag OTUs, the culture collection collectively represented 22% of the total of 98,557 pyrotags in the combined data set from the corresponding soil samples (see Table S3 in the supplemental material). There were substantial differences in the proportions of major phyla isolated on the different media (Fig. 1). Notably, *Acidobacteria* were obtained only on nutrient-poor media and *Proteobacteria* and *Actinobacteria* were the most readily cultured phyla on all media. Many OTUs required dilute media for growth, including the six most abundant OTUs obtained (OTUs 1, 4, 11, 23, 29, and 40). UniFrac analysis of the strain libraries from the four samples, based on the presence or absence of isolate OTUs, indicated that soil horizon had a greater influence on community composition than did site (see Fig. S1 in the supplemental material). This evaluation of community similarity based on isolate library composition was consistent with previous evaluations based on pyrotag analysis (7).

Isolates represented both abundant and rare pyrotag OTUs (Fig. 2), including 14% of all pyrotags from OTUs of greater than 0.10% relative abundance (see Fig. S2 in the supplemental material). Of the eight OTUs with a relative abundance of ≥1.0%, two were cultured. Of the 122 OTUs with a relative abundance of ≥0.10%, 18 were isolated. Among the abundant OTUs isolated, 13 strains corresponded to the dominant pyrotag OTU affiliated with *Bradyrhizobium* and 272 isolates corresponded to OTUs with a relative abundance of ≥0.10%, including three isolates from the phylum *Acidobacteria*. Many of these abundant and taxonomically interesting isolates were subcultured without difficulty, so it appears that most isolated strains can be maintained in pure culture. Since abundant taxa are likely of functional importance to their communities, such an isolate library creates the opportunity to study key community members through genomic and physiological investigations. Subsequently, these findings demonstrate that culturing has the potential to substantially advance our understanding of the ecology of these forest soils, and microbial systems in general.

Microdiverse clusters within individual pyrotag OTUs were defined as sequences which cluster together at a Levenshtein distance of 3 (≈99% identity). Isolates almost invariably represented the most abundant microdiverse clusters within pyrotag OTUs (Fig. 2). A rare exception to this trend was the most abundant microdiverse cluster within OTU165, affiliated with the *Nocardiaceae*. Generally, there appears to be no difference in the relative culturability of microdiverse clusters within individual OTUs, suggesting that culturability is a trait conserved among higher taxonomic levels.

Many isolates were obtained that were not detected by the previous pyrotag analysis. Ninety-six isolate OTUs (representing 14.5% of all isolates) did not cluster with pyrotag OTUs (see Table S3 in the supplemental material). These new OTUs were affiliated predominantly with families that include sporeformers, the *Actinomycetales* and *Bacillales*, but they were also associated with the *Flavobacteriales*, *Sphingobacteriales*, *Rhizobiales*, *Xanthomonadales*, *Burkholderiales*, *Enterobacteriales*, *Pseudomonadales*, and

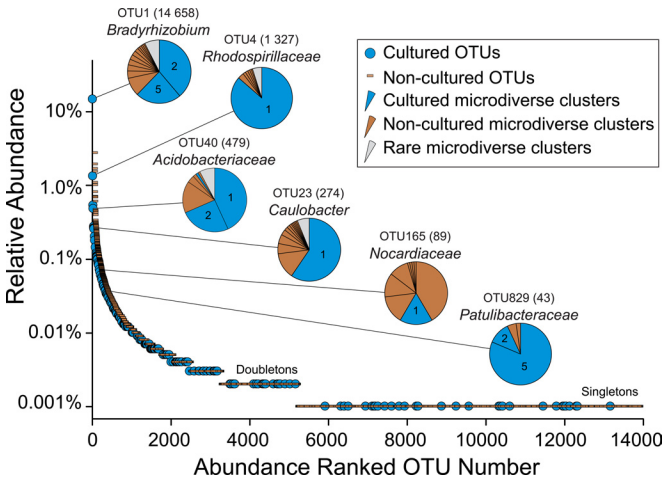

FIG 2 Comparison of 289 isolate OTUs versus 13,860 previously determined pyrotag OTUs. Numbers in parentheses are pyrotags in selected OTUs. Pie charts show relative numbers of pyrotag OTUs in microdiverse clusters within selected pyrotag OTUs. The lowest confident taxonomic classification of each selected OTU, defined by Hartmann et al. (7), is displayed above each pie chart. Numbers in pie charts are numbers of isolates representing each microdiverse cluster. Rare microdiverse clusters are those with less than 0.3% relative abundance.

Sphingomonadales. The new OTUs grew faster (on average, they were isolated 4 days earlier from colonies twice as large) and grew on richer media than the isolates represented by pyrotag OTUs (Fig. 1). Conversely, abundant pyrotag OTUs (≥0.1% relative abundance) were more frequently isolated after extended incubation on dilute growth media using dilute inocula (see Fig. S3 in the supplemental material). In particular, dilute growth media incubated under an atmosphere with reduced oxygen tension yielded both the fewest new OTUs and the most abundant pyrotag OTUs. These observations collectively suggest that the efficiency of recovery of abundant community members through cultivation increases as the incubation conditions decrease in nutrient availability and favor slower growth. Furthermore, they suggest that the taxa most easily isolated from forest soils on nutrient-rich media are "weedy," rare community members that are easily cultivable under nutrient-rich conditions, as observed previously (18), while abundant taxa are typically slow-growing members adapted to competition under oligotrophic conditions.

This study is the first of its kind to show that standard media and culturing techniques have the potential to isolate abundant members of forest soil bacterial communities, as evaluated through high-throughput sequencing of rRNA gene amplicons. Moreover, it provides guidance on how to obtain such isolates more effectively. It is also the first to suggest that culturability among microdiverse clusters within OTUs in forest soils is likely most strongly influenced by relative abundance. Together, the results presented here have important implications for the field of microbial ecology, because they show that conventional cultivation techniques and subsequent culture-based studies have the potential to characterize the physiology and ecology of abundant community members.

Nucleotide sequence accession numbers. The isolate 16S rRNA sequences were deposited in GenBank; accession numbers range from KC255500 to KC256763. GenBank sequence identifiers and accession numbers corresponding to each isolate are listed in detail in Table S1 in the supplemental material.

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REFERENCES

1. **Van Der Heijden MGA, Bardgett RD, Van Straalen NM.** 2008. The unseen majority: soil microbes as drivers of plant diversity and productivity in terrestrial ecosystems. Ecol. Lett. **11**:296–310.
2. **Doran JW, Zeiss MR.** 2000. Soil health and sustainability: managing the biotic component of soil quality. Appl. Soil Ecol. **15**:3–11.
3. **Staley JT, Konopka A.** 1985. Measurement of in situ activities of nonphotosynthetic microorganisms in aquatic and terrestrial habitats. Annu. Rev. Microbiol. **39**:321–346.
4. **Powers RF.** 2006. Long-Term Soil Productivity: genesis of the concept and principles behind the program. Can. J. For. Res. **36**:519–528.
5. **Baldrian P, Kolarik M, Stursova M, Kopecky J, Valaskova V, Vetrovsky T.** 2012. Active and total microbial communities in forest soil are largely different and highly stratified during decomposition. ISME J. **6**:248–258.
6. **Eilers KG, Debenport S, Anderson S, Fierer N.** 2012. Digging deeper to find unique microbial communities: the strong effect of depth on the structure of bacterial and archaeal communities in soil. Soil Biol. Biochem. **50**:58–65.
7. **Hartmann M, Howes CG, VanInsberghe D, Yu H, Bachar D, Christen R, Nilsson RH, Hallam SJ, Mohn WW.** 2012. Significant and persistent impact of timber harvesting on soil microbial communities in northern coniferous forests. ISME J. **6**:2199–2218.
8. **Acinas SG, Klepac-Ceraj V, Hunt DE, Pharino C, Ceraj I, Distel DL, Polz MF.** 2004. Fine-scale phylogenetic architecture of a complex bacterial community. Nature **430**:551–554.
9. **Davis KER, Joseph SJ, Janssen PH.** 2005. Effects of growth medium, inoculum size, and incubation time on culturability and isolation of soil bacteria. Appl. Environ. Microbiol. **71**:826–834.
10. **Janssen PH, Yates PS, Grinton BE, Taylor PM, Sait M.** 2002. Improved culturability of soil bacteria and isolation in pure culture of novel members of the divisions *Acidobacteria*, *Actinobacteria*, *Proteobacteria*, and *Verrucomicrobia*. Appl. Environ. Microbiol. **68**:2391–2396.
11. **Sait M, Hugenholtz P, Janssen PH.** 2002. Cultivation of globally distributed soil bacteria from phylogenetic lineages previously only detected in cultivation-independent surveys. Environ. Microbiol. **4**:654–666.
12. **Stewart EJ.** 2012. Growing unculturable bacteria. J. Bacteriol. **194**:4151–4160.
13. **Alain K, Querellou J.** 2009. Cultivating the uncultured: limits, advances and future challenges. Extremophiles **13**:583–594.
14. **Bruns A, Nübel U, Cypionka H, Overmann J.** 2003. Effect of signal compounds and incubation conditions on the culturability of freshwater bacterioplankton. Appl. Environ. Microbiol. **69**:1980–1989.
15. **George IF, Hartmann M, Liles MR, Agathos SN.** 2011. Recovery of as-yet-uncultured soil *Acidobacteria* on dilute solid media. Appl. Environ. Microbiol. **77**:8184–8188.
16. **Keller M, Zengler K.** 2004. Tapping into microbial diversity. Nat. Rev. Microbiol. **2**:141–150.
17. **Zengler K, Toledo G, Rappé M, Elkins J, Mathur EJ, Short JM, Keller M.** 2002. Cultivating the uncultured. Proc. Natl. Acad. Sci. U. S. A. **99**:15681.
18. **Shade A, Hogan CS, Klimowicz AK, Linske M, McManus PS, Handelsman J.** 2012. Culturing captures members of the soil rare biosphere. Environ. Microbiol. **14**:2247–2252.