Diverse Bacteria Inhabit Living Hyphae of Phylogenetically Diverse Fungal Endophytes

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Both the establishment and outcomes of plant-fungus symbioses can be influenced by abiotic factors, the interplay of fungal and plant genotypes, and additional microbes associated with fungal mycelia. Recently, bacterial endosymbionts were documented in soilborne Glomeromycota and Mucoromycotina and in at least one species each of mycorrhizal Basidiomycota and Ascomycota. Here we show for the first time that phylogenetically diverse endohyphal bacteria occur in living hyphae of diverse foliar endophytes, including representatives of four classes of Ascomycota. We examined 414 isolates of endophytic fungi, isolated from photosynthetic tissues of six species of cupressaceous trees in five biogeographic provinces, for endohyphal bacteria using microscopy and molecular techniques. Viable bacteria were observed within living hyphae of endophytic Pezizomycetes, Dothideomycetes, Eurotiomycetes, and Sordariomycetes from all tree species and biotic regions surveyed. A focus on 29 fungus/bacterium associations revealed that bacterial and fungal phylogenies were incongruent with each other and with taxonomic relationships of host plants. Overall, eight families and 15 distinct genotypes of endohyphal bacteria were recovered; most were members of the Proteobacteria, but a small number of Bacillaceae also were found, including one that appears to occur as an endophyte of plants. Frequent loss of bacteria following subculturing suggests a facultative association. Our study recovered distinct lineages of endohyphal bacteria relative to previous studies, is the first to document their occurrence in foliar endophytes representing four of the most species-rich classes of fungi, and highlights for the first time their diversity and phylogenetic relationships with regard both to the endophytes they inhabit and the plants in which these endophyte-bacterium symbiota occur.

Traits related to the establishment and outcome of plant-fungus symbioses can reflect not only abiotic conditions and the unique interactions of particular fungal and plant genotypes (49, 50, 56, 59, 62, 67) but also additional microbes that interact intimately with fungal mycelia (4, 12, 42). For example, mycorrhizosphere-associated actinomycetes release volatile compounds that influence spore germination in the arbuscular mycorrhizal (AM) fungus Gigaspora margarita (Glomeromycota) (14). Levy et al. (34) describe Burkholderia spp. that colonize spores and hyphae of the AM fungus Gigaspora decipiens and are associated with decreased spore germination. Diverse “helper” bacteria have been implicated in promoting hyphal growth and the establishment of ectomycorrhizal symbioses (23, 26, 57, 70). Minardi et al. (43) found that a consortium of ectosymbiotic bacteria limited the ability of the pathogen Fusarium oxysporum to infect and cause vascular wilts in lettuce, with virulence restored to the pathogen when ectosymbionts were removed.

In addition to interacting with environmental and ectosymbiotic bacteria, some plant-associated fungi harbor bacteria within their hyphae (first noted as “bacteria-like organisms” of unknown function) (38). These bacteria, best known from living hyphae of several species of the Glomeromycota and Mucoromycotina, can alter fungal interactions with host plants in diverse ways (see references 12, 31, and 51). For example, the vertically transmitted bacterium “Candidatus Glomeribacter gigasporarum” colonizes spores and hyphae of the AM fungus Gigaspora gigasporarum (9, 10). Removal of the bacterial partner from the fungal spores suppresses fungal growth and development, altering the morphology of the fungal cell wall, vacuoles, and lipid bodies (37). In turn, the discovery of phosphate-solubilizing bacteria within Glomus mossea spores (44), coupled with the recovery of a P-transporter operon in Burkholderia sp. from Gigaspora margarita (54), suggests a competitive role in phosphate acquisition and transport by these bacteria within the AM symbiosis. Within the Mucoromycotina, Partida-Martinez and Hertweck (51) reported that a soilborne plant pathogen, Rhizopus microsporus, harbors endosymbiotic Burkholderia that produces a phytotoxin (rhizoxin) responsible for the pathogenicity of the fungus.

These examples, coupled with the discovery of bacteria within hyphae of the ectomycorrhizal Dikarya (Tuber borchii; Ascomycota; Laccaria bicolor and Piriformospora indica; Basidiomycota) (5–8, 58), suggest that the capacity to harbor endohyphal bacteria is widespread among fungi. To date, however, endocellular bacteria have been recovered only from fungi that occur in the soil and rhizosphere (12, 31). Here we report for the first time that phylogenetically diverse bacteria occur within living hyphae of foliar endophytic fungi, including members of four classes of filamentous Ascomycota. We use a combination of light and fluorescence microscopy to visualize bacterial infections within living hyphae of represen-
Figure 1. Endophyte growth and development. (A), (B), and (C) represent photographs from pure culture. (A) NJ1 grown on 2% MEA, showing hyphal growth after 1 week (scale bar, 5 mm). (B) NJ2 grown on 2% MEA, showing mycelial growth and sporulation after 2 weeks (scale bar, 20 mm). (C) NJ3 grown on 2% MEA, showing mycelial growth and sporulation after 3 weeks (scale bar, 20 mm). (D) Phenotypic differentiation of NJ1 and NJ2 at the genus level, showing morphological differences in colony morphology, aerial mycelium, and sporulation characteristics. (E) Gene tree showing the molecular phylogeny of NJ1 and NJ2 based on ITS sequences. The tree was constructed using the Maximum Likelihood method and is rooted with an outgroup species from the family Sclerotiniaceae. (F) Gene tree showing the molecular phylogeny of NJ1 and NJ2 based on 5.8S sequences. The tree was constructed using the Maximum Likelihood method and is rooted with an outgroup species from the family Sclerotiniaceae. (G) Gene tree showing the molecular phylogeny of NJ1 and NJ2 based on RPB2 sequences. The tree was constructed using the Maximum Likelihood method and is rooted with an outgroup species from the family Sclerotiniaceae.

**Legend:** ITS = internal transcribed spacer; RPB2 = RNA polymerase subunit B2.
cycling parameters were as described above, except that annealing temperatures were 58°C (10F and 1507R) or 55°C (27F and 1429R). SYBR green I stain (Molecular Probes, Invitrogen) was used to detect DNA bands on 1.5% agarose gels. Positive PCR products were cleaned, quantified, and normalized at the University of Arizona Genomic Analysis and Technology Core facility (GATC), followed by bidirectional sequencing with PCR primers (5 μM) using an Applied Biosystems 3730XL DNA analyzer. PCR products of insufficient concentration for sequencing were cloned using Agilent Technologies StrataClone cloning kits by following the manufacturer’s instructions. Water was used in place of template for negative controls. Positive clones were amplified and sequenced using the primers M13F and M13R.

The software applications Phred and Phrap (18, 19) were used to call bases and assemble bidirectional reads into contiguous consensus sequences, with automation provided by ChromaSeq (39), implemented in Mesquite, v. 2.6 (40). Base calls were verified by inspection of chromatograms in the Sequencher (v. 4.5) software program (Gene Codes Corp., Ann Arbor, MI). ITS rDNA and partial LSU rDNA sequence data and bacterial 16S rRNA gene sequences have been submitted to GenBank (see below) or were published previously by the authors (25).

Diversity and taxonomic placement of endophytes. Previous studies have used 90 to 97% ITS rDNA sequence similarity to estimate species boundaries for environmental samples of fungi (97% [O’Brien et al. (48)], 90% [Hoffman and Arnold (28)], and 95% [Arnold et al. (11)]). Empirical estimates of percent sequence divergence between sister species for four endophyte-containing ascomycetous genera (Botryosphaeria, Colletotrichum, Mycosphaerella, and Xylella) indicated that groups based on 95% ITS rDNA sequence similarity conservatively estimate species (65). We used Sequencher v. 4.5 (Gene Codes Corp., Ann Arbor, MI) to assemble ITS data. For the requirement of maintaining a 20% multiple alignment minimum overlap; see Arnold et al. [3]) to estimate fungal operational taxonomic units (OTU) at 95% ITS rDNA sequence similarity and bacterial OTU at 97% 16S sequence similarity (61).

Taxonomic placement for fungi at the level of order and above was estimated by BLAST comparisons with the curated ITS rDNA database for fungi maintained by the Alabama Fungal Metagenomics Project (FMP) (http://www. fungus.mex.ars.usda.gov) and the ITS4 analysis (see below). Bacterial taxonomy was estimated by BLAST comparisons with GenBank and the Ribosomal Database Project (RDP) (release 9) classifier program (Naive Bayesian rRNA Classifier, version 2.0, July 2007) (15, 68), combined with phylogenetic analyses (see below). All statistical analyses were performed in JMP 7.0 software program (SAS Institute Inc. Cary, NC).

Live/Dead stain. Live/Dead stain was used to confirm the presence and viability of endophyphal bacteria for fungal isolates identified as containing bacteria on the basis of light microscopy and PCR (above). We treated fresh mycelia in sterile water with 2 μl of Molecular Probes Live/Dead fluorescent stain for 15 min and prepared slides with Vectashield HardSet 1400 medium (Vector Laboratories, Inc.) to prevent photobleaching. A Leica DM4000B microscope with a Luminera camera and 100-W mercury arc lamp was used for fluorescent imaging with a GFP filter set, 3500 emissive plus 4000 excitative. Mycelium was mounted on gelatin-coated glass slides using Mowiol solution and examined with an Olympus BX61 microscope with a mercury arc lamp or a Leica DMI 6000 confocal system equipped with a 543-nm laser (63× oil; 1,024 × 1,024 format; z-axis acquisition; line average = 1; frame average = 4). Leica software, LAS-AF v. 1.8.2, was used to capture images.

Phylogenetic analyses. Based on positive 16S rRNA gene PCR results and the lack of any extrahyphal bacteria in our axenic cultures, endophyphal bacteria were observed in 75 of 414 fungal isolates. Twenty-one infected isolates, representing a broad diversity of the Pezizomycotina, and 49 isolates in which infections were never observed and which represented a broad array of ITS rDNA genotype groups were sequenced for 1,200 bp of LSU rDNA, following the method of Arnold et al. (3) (primers LROR or LR3R and LR7; protocols as described above).

These 70 sequences were integrated into core alignments of 157 representative Ascomycota, partitioned by class into Sordariomycetes, Dothideomycetes, and Eurotiomycetes (26) using the Mesquite software program, v. 2.6 (40). Details of taxon sampling are given in Table 2 and also in Table S1 in the supplemental material. Because our sample of Pezizomycotina contained only one isolate with strong evidence of endophyphal bacteria, we did not address phylogenetic relationships of pezizomycotina endophytes here.

Alignment for each class, based on LSU rDNA secondary structure defined by Saccharomycodes cernuusiae (13), was performed using ClustalW with manual adjustment of the “fit gaps” parameter to include all ungapped aligned columns. Phylograms were constructed using a 20% multiple alignment minimum overlap for each matrix details given in Table S2 in the supplemental material. Alignments are available online at http://arnoldlab.net/algnaligns.html.

Phylogenetic relationships for members of each class were inferred using parsimony and Bayesian Metropolis-coupled Markov chain Monte Carlo (MCMCMC) analyses. For the former, the five sets of 200 heuristic searches with random stepwise addition and tree bisection-reconnection (TBR) were implemented using the program PAUP* 4b10, which incorporates the “ratchet” method (28, 47, 60) in PAUP* 4b10 (64). The “filter trees” command in PAUP* 4b10 was used to select all shortest trees, which were used to assemble strict consensus. Bootstrap support was determined using 1,000 replicates and the default settings for nonparametric neighbor-joining (NJ) analysis as implemented in PAUP* 4b10. Cladues with >70% bootstrap support were considered strongly supported. Details of parsimony searches are given in Table S2 in the supplemental material.

For Bayesian analyses, the Modeltest 3.7 software program (53) was used to select the appropriate model of evolution; in each case, GTR+I+G was selected using the AIC information criterion. Analyses were executed in MrBayes 3.1.2 (30) for four runs of up to 6 million generations each, initiated with random trees, four chains, and sampling every 1,000th generation. Likelihoods converged to a steady state and for each replicate tree, premature convergence was discarded as burn-in. Cades with >95% posterior probability values were considered to have significant support.

Twenty-nine bacterial 16S DNA sequences, derived from total genomic DNA extractions of fungal endophytes with no evidence of extrahyphal bacteria (Table 2), and seven 16S sequences from bacterial endophytes obtained directly from plant material (Table 3) were incorporated into a core alignment of 30 sequences of named bacterial taxa obtained from the Ribosomal Database Project 9 website (Cole et al. [15]; http://rdp.cme.msu.edu) using Seqmatch (type strains, isolates, ≥1,200 bp, good quality; ca. 1,400 bp, together with eight additional sequences from GenBank that represented endosymbionts of fungi isolated in previous studies (see Table S3 in the supplemental material) (9–11, 52). Data were aligned using the NAST server and screened for chimeric se- quences (minimum sequence length = 300 bp) (http://greengenes.lbl.gov/) (17). Ambiguous regions were excluded, resulting in a matrix of 6,300 characters (http://arnoldlab.net/alignments.html).

For the bacterial data set, a heuristic search using maximum parsimony with random stepwise additions and TBR branch swapping was implemented in PAUP* 4b10, resulting in 167 optimal trees of 3,686 steps. Support was assessed using a nonparametric neighbor-joining bootstrap (1,000 replicates). Bayesian MCMCMC analysis was implemented in MrBayes v. 3.1.2 for 2.5 million genera- tions, initiated with random trees, four chains, and sampling every 1,000th tree, using GTR+I+G based on evaluation in Modeltest 3.7 (53). After elimination of the first 1,400 trees as burn-in, the remaining 1,100 trees were used to infer a majority-rule consensus. These results were complemented by phylogenetic inference in the ARB software program, v. 7.12.07 (36), using the ASML maxi-
<table>
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<th>Fungal class</th>
<th>16S genotype</th>
<th>Top 16S BLAST, GenBank accession</th>
<th>Bacterial lineage (based on RDP classifier)</th>
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Bacterial taxonomic classifications are based on BLAST results and the Ribosomal Database Project (RDP) classifier program (Naive Bayesian rRNA Classifier, version 2.0, July 2007) (68). Fifteen distinct 16S rRNA gene OTU, based on 97% sequence similarity, are designated in groups A to R. Asterisks indicate one genotype found also as a bacterial endophyte in plant tissue, such that its status as only an endosymbiont of fungi is not clear (genotype A; see Table 3). Fungal identifications were based on comparisons with the FMP curated database in March 2009 and are approximate at the genus and species levels but strongly supported at the class level based on phylogenetic analyses (Fig. 3 to 5).

**RESULTS**

Cultivable endophytes were isolated from healthy foliage of all plant species and from every study site (Table 1), yielding 414 isolates from 4,560 tissue segments. ITS rDNA data for all isolates were assembled at 95% sequence similarity to yield 113 OTU (putative species; Fisher's alpha = 51.2). Representatives of 5 classes and approximately 10 orders, 13 families, and 28 genera were recovered. The most common orders differed among host species and sites, but the entire data set comprised a high frequency of Pezizales (Pezizomycetes), Capnodiales, Pleosporales, Botryosphaeriales, Dothideales (Dothideomycetes), Helotiales (Leotiomycetes), and Xylariales (Sordariomycetes) (see Table S4 in the supplemental material). Host specificity, diversity, and geographic distributions of these fungi will be addressed in a forthcoming paper (M. T. Hoffman and A. E. Arnold, unpublished data).

**Endohyphal bacteria.** Endohyphal bacteria initially were observed in 75 of 414 endophyte isolates (18%; determined by positive PCR results using 16S primers and no evidence of FISH). Bacterial cells were detected in 75 of 414 endophyte isolates (18%) by PCR amplification of 16S rRNA genes, and in situ hybridization (FISH) for isolates HM046622 to HM046627 and HM11772 to HM117749. Additional LSU rDNA sequence data have been submitted to GenBank under accession numbers HM117750 to HM117756.

**Phylogenetic inferences and taxonomic placement.** Phylogenetic analyses confirmed BLAST-level taxonomy at the class level for fungal endophytes in the Dothideomycetes, Eurotiomycetes, and Sordariomycetes (see Fig. 2 to 4). No pattern regarding the structure of endophyte lineages as a function of
host plant taxonomy was evident (i.e., the sister relationship of Cupressus and Juniperus and their sister relationship to Platy-
cladus are not reflected in the phylogenetic relationships of their fungal associates). Isolates containing bacterial associates were spread broadly across endophyte-containing clades in each class, indicating a phylogenetically widespread capacity to harbor bacteria.

Taxonomic placement of endohyphal bacteria initially was assessed using BLAST comparisons in GenBank, typically yielding matches to unidentified or nameless environmental samples. The RDP classifier (68) placed these bacteria in two phyla, the Proteobacteria (including the Alphaproteobacteria, Betaproteobacteria, and Gammaproteobacteria) and Firmicutes. Overall, we recovered five orders and 10 families, putatively identified as Sphingomonadaceae (Alphaproteobacteria), Burkholderiaceae, Comamonadaceae, and Oxalobacteraceae (Betaproteobacteria), Moraxellaceae, Xanthomonadaceae, Pasteurel-
laceae, and Enterobacteriaceae (Gammaproteobacteria) and Bacillaceae and Paenibacillaceae (Firmicutes) (Table 2).

Based on 97% 16S rRNA gene identity, these bacteria represented 15 OTU. Of these, nine were found only once. Three of the remaining six OTU were found in fungi from multiple genera of Cupressaceae, four were found in fungi from more than one biogeographic region, and all were found in fungi representing multiple genera. Four genotypes each were found in fungi representing different classes of Pezizomycotina (gen-

otypes A, B, C, and E) (Table 2).

Proteobacteria were observed in association with the Do-
thideomycetes, Eurotiomycetes, and Sordariomycetes and in endophytes from all three plant genera representing hosts in Arizona and North Carolina. Firmicutes were found in the Eurotiomycetes and one member of the Pezizomycetes. Nom-

<table>
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<tr>
<th>Isolate</th>
<th>Plant species</th>
<th>Location</th>
<th>16S genotype</th>
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</table>

a Isolates, collection sites, 16S rRNA gene OTU based on 97% sequence similarity when compared among themselves and with the endohyphal bacteria listed in Table 2, top RDP classifier matches and accession information, and bacterial lineages are given.

b Site abbreviations are spelled out in Table 1.

FIG. 1. Fluorescent in situ hybridization (FISH) microscopy showing hyphae of two isolates of endophytic fungi harboring endohyphal bacteria. Panel A (isolate 9084b; Dothideomycetes) shows the TAMRA fluorophore at the site of internal structure in hyphal cells. Panel B (isolate 9143; Sordariomycetes) shows the TAMRA fluorophore with the DAPI counterstain (blue), highlighting the fungal nuclear and mitochondrial DNA in addition to bacteria (yellow/green). Scale bar, 10 μm (A) or 25 μm (B).
FIG. 2. Phylogenetic relationships of fungal endophytes with representative Dothideomycetes based on a majority rule consensus tree from Bayesian analysis. Branch support values indicate parsimony bootstrap proportions (≥70%; before slash) and Bayesian posterior probabilities (≥95%; after slash). Taxon labels in bold indicate endophytes isolated in this study and screened for bacterial symbionts. Isolate names are followed by the plant species and collection site. Endophytes in which endohyphal bacteria were observed indicate the GenBank accession number for the top 16S BLAST match, taxonomic placement, and 16S rRNA gene genotype group. The open circle (isolate 6731) represents an infected endophyte from another study (1).
FIG. 3. Phylogenetic relationships of endophytes with representative Eurotiomycetes based on a majority-rule consensus tree from Bayesian analysis of 25 LSU rDNA sequences. Branch support values indicate parsimony bootstrap proportions (≥70%; before slash) and Bayesian posterior probabilities (≥95%; after slash). Taxon labels in bold indicate endophytes isolated in this study and screened for bacterial symbionts. Isolate names are followed by the plant species and collection site. Endophytes in which endohyphal bacteria were observed indicate the GenBank accession number for the top 16S BLAST match, taxonomic placement, and 16S rRNA gene genotype group. The open circle (isolate 4466) represents an infected endophyte from another study (1).
**FIG. 4.** Phylogenetic relationships of endophytes with representative Sordariomycetes based on a majority rule consensus tree from Bayesian analysis of 95 LSU rDNA sequences. Branch support values indicate parsimony bootstrap proportions (≥70%; before slash) and Bayesian posterior probabilities (≥95%; after slash). Taxon labels in bold indicate endophytes isolated in this study and screened for bacterial symbionts. Isolate names are followed by the plant species and collection site. Endophytes in which endohyphal bacteria were observed indicate the GenBank accession number for the top 16S BLAST match, taxonomic placement, and 16S rRNA gene genotype group. The open circle (isolate 6722) represents an infected endophyte from another study (1).
inal logistic analysis provided no evidence for a significant effect of host genus (*Cupressus*, *Juniperus*, or *Platycladus*), region (Arizona versus North Carolina), or fungal class on the incidence of major bacterial groups (*Alpha*-, *Beta*-, and *Gammaproteobacteria*, *Firmicutes*; *P* > 0.05 for all effects). Phylogenetic relationships of endohyphal bacteria from fungal endophytes were not congruent with those of the endophytes they inhabited (Fig. 2 to 5), nor with relationships among host plants (data not shown). The bacterial lineages recovered here were distinct from those recognized previously as endosymbionts of fungi (Fig. 5).

Comparison of bacterial endophytes and endohyphal bacteria. Only one bacterial genotype was represented both among bacterial endophytes (isolated from surface-sterilized plant tissue only) and putative endohyphal bacteria (genotype A, *Bacillales*; Tables 2 and 3). This genotype was isolated directly from foliage of *Juniperus osteosperma* (Chuska Mountains) and *Cupressus arizonica* and *Juniperus deppeana* (Sky Islands) and was amplified from genomic DNA from a fungal culture recovered from *J. deppeana* (Sky Islands). Two additional endophytes recovered here were members of the *Bacillaceae*, representing genotypes that never were found as endohyphal bacteria (genotypes H and J) (Tables 2 and 3). One bacterial endophyte represented the *Enterobacteriaceae* (putative *Erwinia*). It was not found as a bacterial endosymbiont within fungal hyphae.

FIG. 5. Phylogenetic relationships of 29 endohyphal bacteria, 7 bacterial endophytes (paired circles), and 38 named taxa based on Bayesian analysis of 16S ribosomal RNA gene sequences. Branch support values indicate parsimony bootstrap proportions (≥70%; before slash) and Bayesian posterior probabilities (≥95%; after slash). Branches in bold indicate ≥70% neighbor-joining bootstrap proportions. Named bacterial sequences and GenBank accession numbers are listed in Table S3 in the supplemental material; genotype groups for fungi are indicated by letters. Previously described bacterial endosymbionts of Glomeromycota and Mucoromycotina (9, 10, 51) are marked with solid squares.
DISCUSSION

Recent studies have highlighted the potential of endosymbiotic microbes, including bacteria and viruses, to shape the ecological roles of fungi (see, e.g., references 16, 42, and 51). Endohyphal bacteria have been found previously in living hyphae of plant-associated Glomeromycota, Mucoromycota, and up to three taxa of mycorrhizal Dickarya. Our study is the first to compare their phylogenetic relationships with diverse fungal hosts and the first to document their occurrence in ascomycetous endophytes of foliage representing four of the most species-rich classes of Ascomycota (Pezizomycetes, Sordariomycetes, Dothideomycetes, and Eurotiomycetes), which together include numerous plant pathogens, saprotrophs, and pathogens and parasites of animals (30). Coupled with previous studies demonstrating the presence of bacteria in living mycelia of mycorrhizal or soilborne fungi (6, 7, 9, 51), our data provide strong evidence that the ability of fungi to harbor endohyphal bacteria is phylogenetically widespread.

Our survey data show that endohyphal bacterium-endophyte associations occur in regions with divergent biogeographic histories and markedly different environmental conditions (e.g., diverse, arid-land montane systems versus piedmont forest in southeastern North America) and in fungi associated with three genera of plants. Screening of a small number of isolates obtained in previous studies (Fig. 2 to 4) confirmed that endohyphal bacteria occur in endophytes from additional biogeographic provinces and host plant lineages: infections were observed in endophytes isolated from the Pinaceae in boreal forest (endophyte 4466 from *Picea mariana* in Canada) and two families of angiosperms from a tropical forest (endophyte 6722 from *Faramea occidentalis*, Rubiaceae, and endophyte 6731 from *Swartzia simplex*, Fabaceae, recovered at Barro Colorado Island, Panama). Because infections either were lost or became more difficult to detect following subculturing and because of potential limitations of our microscopy, screening methods, and primer selection, our results likely underestimate the incidence of endohyphal bacteria in these plant-associated fungi.

Examination with Live/Dead stain provided evidence that bacteria within fungal hyphae were viable and showed that such bacteria occur within living fungal tissues. FISH confirmed these results by diagnosing the presence of bacteria using 16S rRNA gene-specific tags (Fig. 1). In turn, our phylogenetic inferences show that endohyphal bacteria include a greater phylogenetic diversity—both within and beyond the *Proteobacteria*—than was previously known (23, 32, 52) (Fig. 5).

In contrast to previous studies, which have focused primarily on bacteria associated with a particular fungal species or closely related group of species, we addressed the diversity of bacteria associated with a phylogenetically broad sample of ecologically similar fungi. Our analyses provide no evidence of cocladogenesis: fungal and bacterial trees were obviously incongruent, and neither matched the phylogenetic relationships among the plant species surveyed here. Our phylogenetic results are consistent with the hypothesis of horizontal transmission both for fungal endophytes, as expected (1, 28), and for bacterial symbionts (Fig. 2 to 5).

Using cultivation media infused with antibiotics, we have found that many of these fungi can be cured of their endosymbionts (Hoffman and Arnold, unpublished data), and we currently are investigating methods to reinfect cured mycelia. These attempts will shed light on our hypotheses regarding the facultative nature of these associations, their transmission modes, and their costs and benefits with regard to the inhabited fungi. Notably, some endophytes were unable to grow when transferred to media containing antibiotics (Hoffman and Arnold, unpublished data), suggesting that the relationship may be more intimate or tend toward greater obligacy in some pairs of fungi/bacteria than others. For one isolate (9143), we have successfully isolated the bacterial partner from the fungus and confirmed its identity using 16S rRNA gene sequencing (27a). Attempts to reinfect the endophyte with this bacterium have been ineffective to date.

Discovery of the diversity and ecological roles of fungal endophytes encompasses a trove of future research that will be important for understanding plant ecology and evolution. So too does elucidation of the incidence, diversity, and ecological importance of their endohyphal bacteria, which appear to be common but previously overlooked inhabitants of plant-symbiotic fungi. Evidence from a variety of studies (reviewed in references 12 and 32) indicates that endohyphal bacteria have the capacity to alter the outcome of plant-fungus interactions in a phylogenetically diverse array of symbioses. Experimental assessment of such effects will provide key evidence for understanding the degree to which bacterial associates influence the nature of endophytic symbioses. In turn, ancestral-state reconstructions and further sampling of both endohyphal bacteria (of fungi) and bacterial endophytes (of plants) will elucidate whether bacteria have transitioned from endophytic to endophytophyl lifestyles (or the reverse) or whether they have followed a different and yet-to-be-identified evolutionary trajectory.

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