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SHORT NOTE

Bacterial communities associated with tuberculate ectomycorrhizae of *Rhizopogon* spp.

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Abstract We have previously reported the design of a new PCR primer pair that allows amplification of a broad range of eubacterial 16S rDNA sequences from ectomycorrhizae (ECM) without co-amplification of plastid or mitochondrial sequences. Here, we report using a similar primer combination to generate three small 16S rDNA libraries from tuberculate ECM of *Rhizopogon* spp., two from *R. vinicolor* ECM (libraries Rvi18 and Rvi24) and one from *R. vesiculosus* ECM (library Rve13). At the class level, libraries were dominated by sequences from the *Alphaproteobacteria*, *Gammaproteobacteria*, and *Acidobacteria*, with some *Sphingobacteria*, *Actinobacteria*, *Planctomycetacia*, and *Verrucomicrobiae* present as well. Based on the parsimony test implemented in TreeClimber, libraries Rvi18 and Rvi24 were significantly different from Rve13 at the α=0.05 level, while they were only borderline significantly different from each other (p=0.07). Differences between Rvi and Rve libraries were primarily due to differences in the number of *Alphaproteobacteria* sequences and specifically sequences from the *Rhizobiales*, which were more common in the Rve13 library. It is currently unknown what drives these differences between eubacterial communities. Amplification success for eubacterial 16S rDNA sequences was generally low in this study indicating low abundance of bacteria on tuberculate ECM. Attempts to amplify nitrogenase reductase (*nifH*) sequences were unsuccessful.

Keywords Tuberculate ectomycorrhizae · *Rhizopogon* · Bacteria · PCR · Library comparison

Introduction

Bacteria are known to affect the functioning of ectomycorrhizal symbioses in many ways including establishment, mobilization of minerals, nitrogen fixation, and antagonism of pathogens (for recent review, see Frey-Klett et al. 2007). However, much of what we know about bacteria associated with ectomycorrhizae is based on culturable bacteria, which in some environments account for less than 1% of all bacteria (Hugenholtz et al. 1998). Relatively few attempts have been made to characterize bacterial communities associated with ECM as a whole using culture-independent techniques (but see Mogge et al. 2000; Bertaux et al. 2005; Burke et al. 2008). PCR-based analyses of bacterial communities associated with ECM have been hampered by the fact that ECM contain ample amounts of plant genomic DNA and that priming sites for general eubacterial 16S rDNA primers tend to be conserved in plant plastid and mitochondrial sequences; the latter is not the case for fungal mitochondrial sequences, because fungal mitochondria are extremely A+T rich and fast evolving (Bruns and Szaro 1992). Co-amplification of plastid and mitochondrial DNA from plant roots when using general eubacterial 16S rDNA primers has been described before (Chelius and Triplett 2001; Sakai et al. 2004; Green and Minz 2005). In a preliminary study, we have found as many as 70% and 20% of 16S rDNA sequences from ECM to be related to plant chloroplast and mitochondrial sequences, respectively, and only one out of ten sequences originating from a bacterium (Kretzer and Bai, unpublished data). A number of methods have been proposed to alleviate the problem including

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suicide polymerase endonuclease restriction (Green and Minz 2005) and use of PCR primers capable to discriminate against plastid DNA combined with separation of mitochondrial sequences by size (Chelius and Triplett 2001; Sakai et al. 2004). We have recently described a primer pair, 41f and 1223r, capable of amplifying a wide range of eubacterial sequences while at the same time discriminating against both plant plastid and mitochondrial sequences, thereby eliminating the need for size separation of mitochondrial sequences (Burke et al. 2008). Here, we use a similar primer pair, R1n and 1223r, to characterize eubacterial communities from tuberculate ECM. The bacterial 16S rDNA primer R1n was originally adapted from Weidner et al. (1996), who did not specifically design their primers to discriminate against organellar DNAs; however, our sequence alignments predicted that their forward primer R1n had a 3′ G:A mismatch to mitochondrial 16S rDNA sequences of most higher plants. Primer 41f used in Burke et al. (2008) is a later modification of R1n with the same 3′ annealing site but a lower annealing temperature that more closely matches that of our reverse primer 1223r. The reverse primer 1223r was designed to have a 3′ A:G mismatch to most chloroplast 16S rDNA sequences except certain algal groups and a 3′ A:C mismatch to mitochondrial 16S rDNA sequences of most higher plants (for details on primer design, see Burke et al. 2008).

We were interested in testing our new primers to characterize eubacterial communities associated with tuberculate ECM of Douglas-fir (*Pseudotsuga menziesii*). Tuberculate ECM consist of dense clusters of ECM roots that are encased in a sheath of fungal hyphae. The mycobiont in tuberculate ECM of Douglas-fir was originally identified as *Rhizopogon vinicolor* (Zak 1971), but was later shown to be a species complex of two sister species both forming tuberculate ECM on Douglas-fir and tentatively referred to as *R. vinicolor* and *R. vesiculosus*, respectively (Kretzer et al. 2003). Tuberculate ECM of Douglas-fir seemed like ideal first study objects, because they are fairly large providing ample material for DNA extraction. In addition, tuberculate ECM have often been hypothesized to provide a microaerophilic environment suitable for nitrogen fixation (Li et al. 1992; de Boer et al. 2005). Indeed, two nitrogen fixing bacteria have been isolated from tuberculate ECM of Douglas-fir, an *Azospirillum* sp., (Li and Hung 1987) and a *Bacillus* sp. (Li et al. 1992). The current study had the following objectives: (1) test the effectiveness of PCR primer pair R1n and 1223r for culture-independent analysis of eubacterial communities associated with ECM; (2) provide a culture-independent description of bacterial communities associated with tuberculate ECM of Douglas-fir; (3) assess if previously isolated nitrogen fixing bacteria are major or minor components of that community; and (4) assess differences between eubacterial communities from different tuberculate ECM.

Materials and methods

Tuberculate ECM of *R. vinicolor* and *R. vesiculosus* were collected in June of 2006 from plot MP3 described in Kretzer et al. (2005). ECM were briefly surface rinsed to remove adhering soil and coarse woody debris and examined under a dissecting microscope to make sure that they were not in a decaying state. Samples were freeze dried the same day that they were collected. Genomic DNA was extracted from approximately 100 mg ECM using the FastDNA® Kit (Q-Biogene) following the manufacturer's instructions except that the cell lysis solution was replaced with filtered (0.2 μm pore size) and autoclaved CTAB buffer (100 mM Tris, 20 mM EDTA, 1.4 M NaCl, 2% CTAB, pH 8.0) and that a chloroform extraction step was included after removal of the cell debris.

Although there are subtle morphological differences between tuberculate ECM of *R. vinicolor* and *R. vesiculosus*, fungal species identification was verified by restriction fragment length polymorphism (RFLP) analysis of the internal transcribed spacer (ITS) region as described in Kretzer et al. (2003) except that PCR primer ITS4 was substituted with ITS4B (Gardes and Bruns 1993) resulting in slightly larger fragment sizes. Eubacterial 16S rDNA was amplified using primers R1n (GCTCAGATTGAACGCTG GCG) and 1223r (CCATTGTAGTACGTGTGTA) in a PCR containing 1× polymerase buffer, 5% DMSO, 2 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 μM of each primer, 2.5 U of Taq DNA polymerase (Promega), and 2 μl of template DNA in a 50 μl reaction. Cycling conditions were 5 min at 94°C followed by 35 cycles of 45 s at 94°C, 30 s at 45°C, 2 min at 72°C, and a final extension of 10 min at 72°C. PCR amplification of nitrogenase reductase (*nifH*) sequences followed the nested protocol of Widmer et al. (1999) with minor modifications; DNA extracted from a clover nodule was used as positive control. The negative control for all PCR was an extraction blank (DNA extraction without added ECM tissue); for nested PCR, first round product from the extraction blank was carried over as negative control.

Since eubacterial 16S rDNA amplifications were generally weak, if positive at all, multiple 50 μl reactions (up to 10) were pooled for purification and cloning. PCR products were purified using the QIAquick PCR Purification Kit from Qiagen and cloned using either the PCR Cloning Kit from Qiagen (libraries Rve13 and Rvi24) or the pGEM-T Easy Kit from Promega (library Rvi18). White colonies were screened whether or not they contained inserts of the approximately expected size using a standard PCR assay with primers M13 forward and M13 reverse. Only library Rvi18 was found to be

heavily contaminated with approximately 50-bp-long sequences that in a similar case had been found to represent primer dimers (data not shown). A second Rvi18 library was therefore generated after further gel purification of PCR products using the MinElute Kit from Qiagen. Plasmids with approximately expected insert sized were purified using either the Wizard® Plus SV Minipreps DNA Purification System from Promega or the QIAprep Spin Miniprep Kit from Qiagen. Purified plasmids were submitted to the Cornell University Life Sciences Core Laboratories Center for sequencing with Big Dye Terminator chemistry and an Applied Biosystems automated 3730 DNA analyzer.

Sixteen S rDNA sequences were assembled and edited using either Sequence Navigator (Applied Biosystems Inc.) or Sequencher 4.7 (Gene Codes Corporation) and have been submitted to GenBank under accession numbers EU826779–EU826874. Assignment of sequences to taxonomic groups was achieved using the Naïve Bayesian Classifier of Wang et al. (2007) available online via the Ribosomal Database Project (RDP) at http://rdp.cme.msu.edu/. The Classifier uses a bootstrap procedure to estimate "confidence" values for individual taxonomic placements. Bootstrap values below 95%, however, have been shown to grossly overestimate classification accuracy (Wang et al. 2007), and a 95% bootstrap support threshold was therefore used for all analyses. Although we have found in previous work that the RDP Classifier effectively identifies chloroplast-derived sequences, we have not found it to effectively identify plant mitochondrial sequences (unpublished observations). The absence of organellar sequences from our sequence libraries was therefore further verified by checking the top 20 matches obtained with the RDP Sequence Match tool as well as NCBI Blast search.

Sequence libraries were compared using both the RDP Library Compare tool, which assesses differences in representation of individual taxonomic groups (Wang et al. 2007), and TreeClimber, which uses a phylogenetic framework to provide an overall assessment of differences in sequence representation (Schloss and Handelsman 2006). Input trees for TreeClimber were generated using ClustalX v. 1.8 with default parameters for sequence alignment and PAUP* version 4.0b (10) to construct both unweighted parsimony trees with ten random sequence additions and neighbor-joining trees using uncorrected *p* distances.

Results

DNA was extracted from 20 independently collected tuberculate ECM samples. All samples amplified readily with fungal ITS primers 1F and 4B with the exception of one that amplified only faintly, but most amplified very strongly. However, only four samples amplified weakly with eubacterial 16S rDNA primers R1n and 1223r, and 16S rDNA libraries were generated from three of them. In two of those samples, the mycobiont was identified as *R. vinicolor* (samples Rvi18 and Rvi24) and in one as *R. vesiculosus* (sample Rve13). Thirty two 16S rDNA sequences were obtained from each library varying in length from 917 to 1,177 bp. Consistent with our expectations for primer specificity, all sequences were assigned to the domain Bacteria with 100% bootstrap support, and no mitochondrial or chloroplast-derived sequences were identified. Eighty one sequences (84%) could be assigned to a bacterial class using the RDP Classifier with a 95% bootstrap support threshold, while only 38 (40%) could be assigned to a genus. For full classification of all sequences, consult Table 1 of the Electronic supplementary material. Libraries were found to be dominated by sequences from the *Alphaproteobacteria*, *Gammaproteobacteria*, and *Acidobacteria*, with some *Sphingobacteria*, *Actinobacteria*, *Planctomycetacia*, and *Verrucomicrobiae* present as well (Fig. 1). Sequences that could be classified to genus level included the genera *Acidocella* (two sequences), *Bradyrhizobium* (eight sequences), *Hyphomicrobium* (one sequence), *Isosphaera* (one sequence), *Labrys* (one sequence), *Mesorhizobium* (one sequence), *Niastella* (one sequence), *Pseudomonas* (three sequences), *Rhodoplanes* (one sequence), and two *Acidobacteriaceae* genera identified as Gp1 (17 sequences) and Gp3 (two sequences) that we were unable to find additional information on. With the exception of three *Pseudomonas* and two Gp3 sequences that occurred exclusively in library Rvi24, genera that were detected more than once generally occurred in more than one library. Sequences belonging to the genera *Azospirillum* or *Bacillus* that were previously isolated as nitrogen fixers from tuberculate ECM of Douglas-fir (Li and Hung 1987; Li et al. 1992) were not detected in this study.

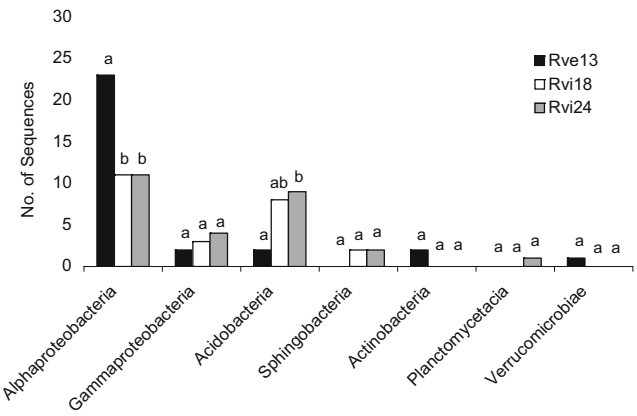

Fig. 1 Number of sequences in the three libraries assigned to different bacterial classes by the RDP Classifier (95% bootstrap support threshold). Different letters above bars indicate significant differences in numbers of sequences according to the RDP Library Compare tool (α=0.05)

Currently available algorithms for library comparisons allow only for pairwise comparisons between two libraries. We used TreeClimber to test if overall differences in sequence composition between libraries are significant or likely due to random chance. Libraries Rvi18 and Rvi24 were significantly different from Rve13 at the α=0.05 level, while they were only borderline significantly different from each other (p=0.07). These results were independent of whether the input trees were parsimony or neighbor-joining trees. Using the RDP Library Compare tool with a 95% bootstrap support threshold for classification of included sequences, it was found that differences between Rvi and Rve libraries were primarily due to differences in the number of *Alphaproteobacteria* sequences and specifically sequences from the *Rhizobiales*, which were significantly more common in the Rve13 library (α=0.05). Significant differences also existed between libraries Rve13 and Rvi18 in the representation of the taxa *Bradyrhizobiaceae*, *Bradyrhizobium*, and *Acetobacteraceae* and between libraries Rve13 and Rvi24 in the representation of *Acidobacteria*.

First round PCR amplification of *nifH* sequences produced weak bands and unspecific smears, which is consistent with what has been reported by Widmer et al. (1999). A second, nested amplification occasionally produced bands of expected size, but amplification was never above background level as determined by stringent negative controls described under "Materials and methods". Cloning and sequencing of these PCR products was therefore not pursued.

Discussion

PCR amplification success for bacterial 16S rDNA sequences was low in this study. That is consistent with microscopic studies of Douglas-fir tuberculate ECM by Massicotte et al. (1992) who found bacteria primarily associated with the surface of tuberculate ECM, and since tuberculate ECM of Douglas-fir have a low surface-to-volume ratio, the amount of bacterial template DNA present is likely to be low. In addition, we lightly surface washed tuberculate ECM in this study to remove adhering soil and coarse woody debris, but washing without sterilizing agents is not expected to eliminate bacteria from the surface of ECM (Izumi et al. 2006a). Despite low amplification success, three small 16S rDNA libraries were established and the representation of bacterial phyla in them was fairly typical of soil microbial communities (Hugenholtz et al. 1998). It would therefore be interesting in future studies to compare communities from tuberculate ECM to bulk soil and to verify that recovered sequences are truly associated with ECM rather than contaminants from the soil using fluorescent in situ hybridization (Amann et al. 1995; Bertaux et al. 2003, 2005).

Sequence composition of 16S rDNA libraries was significantly different between *R. vinicolor* and *R. vesiculosus* tuberculate ECM according to the parsimony test implemented in TreeClimber, and those differences were largely driven by different representation of *Alphaproteobacteria* and more specifically *Rhizobiales* as revealed by the RDP Library Compare tool. Interestingly, the two libraries obtained from *R. vinicolor* were not significantly or only borderline significantly different from each other. Although the trend for greater similarity among libraries from conspecific ECM fungi in our data is interesting, the small number of libraries does not allow any conclusions on whether ECM fungal species identity has a significant impact on bacterial community composition. In an associated study, we have used terminal restriction fragment length polymorphism analysis of 16S rDNA amplicons from non-tuberculate ECM of Douglas-fir, and have not detected any significant effect of fungal species identity on community profiles (Burke et al. 2008). Similarly, Izumi et al. (2007, 2008) failed to see differences in bacterial ECM communities from pines due to fungal species identity based on denaturing gradient gel electrophoresis of 16S rDNA sequences. It should be stated that all cited studies were conducted in the field and that field sampling of ECM introduces many other variables such as soil heterogeneity, humidity, host health and age, ECM developmental stage, etc. that should make it difficult to detect effects of ECM fungal species on bacterial community composition. In contrast to ECM fungi, arbuscular mycorrhizal fungi were recently found to associate with lineage-specific microbial communities, which might be due to the more standardized conditions of pot cultures and the enrichment effect of long-term cultivation in pot cultures (Rillig et al. 2006).

Using primers and a nested PCR protocol developed by Zani et al. (2000), Izumi et al. (2006b) have been able to amplify *nifH* sequences from tuberculate ECM of *Suillus variegatus*, but our attempts at amplifying *nifH* sequences were unsuccessful. While this may simply be the result of using different ECM or different PCR primers and conditions, which are known to affect amplification of *nifH* sequences (Diallo et al. 2008), we also had problems with contaminations of nested *nifH* amplifications. Although weak amplifications were occasionally obtained in our study, they were never above background levels as determined by our unusually stringent negative controls (see "Materials and methods"), and others have reported contamination of *nifH* amplifications as well (Zehr et at. 2003; Goto et al. 2005). Our inability to amplify *nifH*-like sequences does not imply that nitrogen-fixing bacteria are not present in the mycorrhizosphere of Douglas-fir tuberculate ECM, but they are below our detection limits. In fact, a number of 16S rDNA sequences were clearly placed in

the genus *Bradyrhizobium*, which is well known for its nitrogen-fixing capabilities. However, we did not find *Azospirillum* sp. or *Bacillus* sp. that were previously isolated from tuberculate ECM of Douglas-fir based on their nitrogen-fixing capabilities (Li and Hung 1987; Li et al. 1992) in our 16S rDNA libraries.

ECM with tuberculate morphology have been observed on both angiosperms (Dell et al. 1990; Haug et al. 1991) and gymnosperms (Randall and Grand 1986; Massicotte et al. 1992; Paul et al. 2006). Mycobionts have so far been identified primarily from gymnosperm hosts and have been found to be either *Suillus* spp. or *Rhizopogon* spp. (Zak 1971; Randall and Grand 1986; Kretzer et al. 2003). Recently, the mycobiont from a tuberculate ECM of *Quercus* sp. has been identified as an unknown Boletaceae sp. (Smith and Pfister 2008). Based on our current understanding of *Suillus* and *Rhizopogon* phylogenies (Kretzer et al. 1996; Grubisha et al. 2002) and in the light of the new findings by Smith and Pfister (2008), it must be concluded that the tuberculate morphology has evolved multiple times independently. It is tantalizing that the selective advantage driving this parallel evolution remains obscure. As stated earlier, it has been hypothesized that tuberculate ECM might provide a microaerophilic environment suitable for nitrogen fixation (Li et al. 1992; de Boer et al. 2005). In agreement with that hypothesis, nitrogen-fixing bacteria have been isolated from tuberculate ECM (Li and Hung 1987; Li et al. 1992) and *nifH* sequences have been amplified from tuberculate ECM of *Suillus variegatus* (Izumi et al. 2006b). While nitrogen-fixing bacteria thus seem to be associated with various tuberculate ECM, it is questionable whether nitrogen fixation is the primary adaptive function of the tuberculate morphology. For one, nitrogen-fixing bacteria and *nifH* sequences have been found associated with non-tuberculate ECM as well (Li and Hung 1987; Izumi et al. 2006b). Furthermore, Massicotte et al. (1992) observed bacteria primarily on the surface of Douglas-fir tuberculate ECM, which runs contrary to the idea that they might exploit the microaerophilic environment created by the dense clusters of respiring ECM tips for nitrogen fixation. Lastly, our low PCR amplification success suggests that tuberculate ECM of Douglas-fir are only sparsely colonized by bacteria of any kind making it unlikely in our opinion that symbiotic associations with bacteria are their primary adaptive function. Alternative hypotheses that have been suggested include protection from water stress and parasites (Zak 1971; Li et al. 1992). It is furthermore also possible that different selection pressures have driven the evolution of tuberculate morphologies in different lineages.

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