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Characterisation of endobacterial communities in ectomycorrhizas by DNA- and RNA-based molecular methods

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Abstract

The diversity of endobacteria associated with ectomycorrhizas of *Suillus variegatus* and *Tomentellopsis submollis*, in two Corsican pine (*Pinus nigra*) stands was analysed by cultivation-dependent and cultivation-independent molecular methods. Denaturing gradient gel electrophoresis (DGGE) analysis revealed the cultivable endobacterial communities associated with *S. variegatus* were similar within the same stand. The most abundant cultivable bacterial species belonged to the genera *Pseudomonas* and *Burkholderia*. Cultivation-independent molecular analysis indicated that the structure of the endobacterial communities in ectomycorrhizas was consistent across all samples regardless of ECM fungal species or the pine stand from which the samples were collected. However, comparison between rDNA- and rRNA-derived DGGE gels showed that metabolically active endobacterial species were not always detected in rDNA-based profiles. Clone libraries constructed from rRNA molecules indicated that *Pseudomonas* and *Burkholderia* spp. were metabolically active bacteria. As some of the most abundant cultivable bacteria, including *Bacillus*/*Paenibacillus* spp., were not detected in cultivation-independent DGGE profiles, a combination of cultivation-dependent and -independent approaches provided a more complete assessment of the diversity of endobacteria associated with ectomycorrhizas.

Keywords: Endobacteria; 16S rRNA; Metabolically active bacteria; Ectomycorrhiza

1. Introduction

Ectomycorrhizas are ubiquitous in temperate forest ecosystems and over 5000 species of ectomycorrhizal (ECM) fungi are found worldwide (Smith and Read, 1997). Ectomycorrhizas are mutualistic associations between host plants and certain basidiomycete and ascomycete fungi. It is known that ECM fungi can improve nutrient acquisition by host plants, partly through the activity of external hyphae (Perez-Moreno and Read, 2001). In return, 10–20% of current photosynthate is allocated to the ECM fungus (Smith and Read, 1997). Ectomycorrhizas are clearly important components of the nutrient and carbon cycles in forest ecosystems.

Bacteria that internally colonise plant tissues without causing disease symptoms are known as endophytic bacteria (Wilson, 1995). Endophytic bacteria have been observed in a wide range of different woody plant species and tissues, including Scots pine buds (Pirttilä et al., 2000) and spruce roots (Shishido et al., 1999). It has been suggested that they may be important in improving host plant growth (Bent and Chanway, 1998) or host defence against pathogens (Barka et al., 2002). Like endophytic bacteria in plants, endobacteria residing in ectomycorrhizas have been detected using cultivation-dependent methods either through direct isolation from surface-sterilised ECM root tips (Poole et al., 2001; Izumi et al., 2006) or through physical extraction and subsequent isolation of the bacteria from the inner tissues of ectomycorrhizas (Paul, 2002). Endobacteria in this context are defined as those bacteria that exist within the fungal or host compartments of the mycorrhiza, or conceivably within the cells of either of the symbionts.

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Some bacteria found in association with ectomycorrhizas have been called "Mycorrhiza Helper Bacteria" (MHB) (Frey-Klett and Garbaye, 2005) as they have been shown to stimulate the colonisation of tree roots by ECM fungi. For example, certain strains of *Bacillus subtilis* and *Pseudomonas fluorescens* stimulate the colonisation of Douglas fir roots by *Laccaria bicolor* (Duponnois and Garbaye, 1991) and a *Paenibacillus* spp. promotes infection of *Pinus sylvestris* roots by *Lactarius rufus* (Poole et al., 2001). Additionally, certain *Suillus* spp., which form distinctive tuberculate ectomycorrhizas (Agerer, 1987), harbour nitrogen-fixing *Bacillus* spp., giving rise to speculation that nitrogen can be provided to the host plant by these bacteria (Li et al., 1992). These interesting findings have relied on cultivation-dependent methods for the isolation and identification of bacterial species. However, we know that cultivating only detects a very small proportion of the species present in environmental bacterial communities (Amann et al., 1995).

The use of cultivation-independent molecular methods has identified a previously unreported bacterium (*Cytophaga-Flexibacter-Bacteroides*) in association with the mycelium of the ECM fungus *Tuber borchii* (Barbieri et al., 2000), and rarely cultivated alpha proteobacteria in the sporocarps of the same species (Barbieri et al., 2005). Cultivation-independent approaches clearly have the potential to identify novel bacterial species not detected using a cultivation-based approach, but these approaches are yet to be used for identifying endobacteria in ectomycorrhizas. Additionally, RNA-based cultivation-independent methods are likely to yield more useful information on viable and metabolically active members of microbial communities in situ than DNA-based methods, due to the fact that rRNA synthesis and bacterial cell growth are closely related (Wagner, 1994). With this in mind, the aims of this study were (i) to investigate whether direct DNA extraction from ectomycorrhizas detects novel endobacteria that are undetectable using cultivation-based approaches, and (ii) to determine which endobacteria are metabolically active by comparing DNA- and RNA-derived community profiles.

2. Materials and methods

2.1. Collection, morphotyping and surface sterilisation of ECM root tips

ECM root tips were collected from beneath decaying logs in two Corsican pine (*Pinus nigra* var. *maritima* (Ait.) Melville) stands in Culbin forest, NE Scotland (57°38′N, 03°43′W) in late April 2004. The stands were established on sandy soil with a weakly developed iron podsol (Gauld, 1981) approximately 50 years ago. The productivity of the trees in the two stands differed as a result of fertilisation with trees in the 'productive' site having a mean height of 20.5 m compared with 6.6 m in the 'non-productive' stand ($n = 10$). Ten discrete root fragments, each ~30 cm in length and bearing many ectomycorrhizas, were taken randomly from the organic layer underneath different trees in the productive and non-productive stands. After washing to remove soil particles from the root surface, ECM root tips were sorted, classified and grouped into morphotypes using the approach of Agerer (1987). Two distinctive, and very different, ECM morphotypes characteristic of suilloid and tomentelloid fungi were selected for further study. Suilloid and tomentelloid ECM fungal species were selected for this study as they are common in boreal forests (Wu et al., 2000; Kõljalg et al., 2002) and suilloid ectomycorrhizas, in particular, are known to associate with potential nitrogen fixing bacteria (Li et al., 1992).

ECM root tips were kept on moist filter paper, at 4 °C, prior to surface sterilisation. They were rinsed with sterilised water to remove soil particles before they were immersed in 30% hydrogen peroxide for 0 and 2 min. The hydrogen peroxide solution was then removed and the ECM root tips were rinsed four times with sterilised water. The final rinse water was collected to check sterility.

2.2. Identification of ECM fungi

DNA was extracted from two to three representative ECM root tips or tubercules of each morphotype as described by Gardes and Bruns (1993). The rDNA internal transcribed spacer (ITS) region was amplified using the primers ITS1F and ITS4B (Gardes and Bruns, 1993). The 25 µl reaction mix contained 0.5 µl of extracted DNA, 25 pmol of primers, 1x of TITANIUM *Taq* DNA polymerase (BD Biosciences Clontech, Palo Alto, USA), 250 µM of each dNTP, 1x reaction buffer (BD Biosciences Clontech, Palo Alto, USA) and 1 mM $MgCl_2$. Cycling parameters consisted of one cycle of 94 °C for 10 min; followed by 39 cycles of 94 °C for 30 s, 55 °C for 30 s and 74 °C for 30 s; with a final primer-extension of 74 °C for 10 min. The amplified ITS PCR products were subjected to RFLP analysis using the restriction enzyme *Taq*I to confirm that all ECM tips within each morphotype were colonised by the same ECM fungal species. Two ITS PCR products from each morphotype were purified using the Qiaquick PCR purification kit (Qiagen, Crawley, UK) and then sequenced using the primers ITS1F and ITS4B. Sequence reactions were carried out using the Big Dye Terminator v.1.1 Cycle Sequencing Kit on an ABI PRISM 310 Genetic Analyser (Applied Biosystems, Foster City, USA). Raw sequences were aligned and consensus sequences were produced using Sequencher software (version 3.0 Gene Codes Corporation, Ann Arbor, USA). Comparisons were made with reference sequences of the EMBL Nucleotide Sequence Database (Cochrane et al., 2006) using the FASTA algorithm (Pearson and Lipman, 1988) and with sequences obtained from sporocarp collections made from the same forest.