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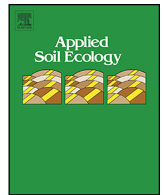

Arbuscular mycorrhizal hyphae in grassland select for a diverse and abundant hyphospheric bacterial community involved in sulfonate desulfurization

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ABSTRACT

Hyphae of symbiotic arbuscular mycorrhizal (AM) fungi extend into the soil, affecting the hyphosphere and interact with beneficial soil bacteria. This study aimed to elucidate differences in hyphosphere, hyphoplane and bulk soil bacterial communities and their role in mobilization of sulfonate-sulfur. Abundances of cultivable hyphosphere and hyphoplane bacteria were significantly increased over bulk soil. Cultivation independent fingerprinting revealed significantly different community structures of both hyphosphere and hyphoplane bacteria, fungi and AM fungi over bulk soil. However, cultivation dependent and independent analysis did not identify a difference between bacterial hyphoplane and hyphosphere (hyphospheric) communities. Isolated bacteria capable of aromatic sulfonate desulfurization were almost exclusively of hyphospheric origin. Members of the hyphospheric Gammaproteobacteria and Actinobacteria were found to possess marker gene *asfA* for aromatic sulfonate desulfurization and *hrcR* for attachment to fungal hyphae with a type III secretion system, that were not detected in bulk soil. These findings suggest that AM hyphae host a distinct population of sulfonate desulfurizing bacteria putatively capable of hyphal attachment with potential to increase plant sulfur supply.

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1. Introduction

Arbuscular mycorrhizal (AM) fungi form symbiosis with 80% of land plant species, where they penetrate root cortical cells forming arbuscules for plant-fungus metabolite exchange (Smith and Read, 2008). AM fungi obtain essential nutrients via their extra-radicular hyphae (2–10 μm in diameter) that can reach microsites within the soil structure, thus, increasing the volume of soil and quantity of nutrients accessible to the host plant (Smith and Read, 2008). In addition, AM bestow several other benefits on the host plant including increased drought tolerance, protection from plant pathogens, improved soil aggregation, alleviation of toxic metal stress, and provision of an increased surface area for interaction with beneficial soil microbes (Frey-Klett and Garbaye, 2005; Kaldorf et al., 1999; Ruiz-Lozano et al., 1995). Given the carbon (C) limited nature of soil, plant roots and their associated mycorrhizae create nutritional hot spots for bacteria to proliferate due to exudation of C rich compounds (Toljander et al., 2007).

Recent studies of bacteria-mycorrhiza interactions have identified the presence of a type III secretion system (T3SS) which allows bacteria to attach and co-migrate with extra-radicular hyphae (Warmink et al., 2011). Bacterial attachment to the hyphoplane may be mutualistic in nature with bacteria improving the availability of soil nutrients in return for C rich exudates from their fungal partner. Indeed, the mycorrhizosphere has been shown to influence bacterial mobilization of soil nutrients most extensively studied for phosphorus (P) (Johansson et al., 2004). This is of critical importance as AM fungi and associated bacteria may support plant growth considerably in grassland ecosystems where soil disturbances are kept to a minimum (van der Heijden et al., 2008). However, to date a knowledge gap exists regarding mobilization of organically bound sulfur in the mycorrhizosphere.

Sulfur (S) is required for the growth of all living organisms. In recent years, inorganic S deposition to soils through air pollution has been greatly reduced, while at the same time fertilizer formulations were refined to contain less sulfate. This has led to a reduction in plant available sulfate in soil (McGrath et al., 2003). In soil, S is up to 95% organically bound, primarily as sulfate esters and sulfonate-S, and not directly plant available (Autry and Fitzgerald, 1990; Kertesz and Mirleau, 2004). Plants rely on microbes to facilitate organo-S mobilization (Kertesz et al., 2007).

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While hydrolysis of the sulfate ester bond is facilitated by bacteria and fungi alike, aromatic sulfonate-S mobilization is achieved via a bacterial multi-enzyme complex of which the *asfA* gene is a marker (Schmalenberger and Kertesz, 2007).

The growth of the AM fungus *Glomus intraradices* (*Rhizophagus intraradices*) on *Trifolium* plants in sand culture was strongly stimulated by addition of 2-(*N*-morpholino)-ethanesulfonic acid, but this stimulation was related to metabolites released by sulfonate desulfurizing bacteria (Vilariño et al., 1997). Sulfonate desulfurizing bacteria may have been enriched on the AM hyphal surface (hyphoplane) and the soil directly affected by mycorrhizal fungi (hyphosphere) as opposed to the bulk soil, in analogy to the rhizosphere effect. Limited information exists on the role of hyphoplane and hyphosphere bacteria in nutrient mobilization. This is attributable to the inherent delicate nature of the hyphosphere making it more difficult to analyze experimentally than the rhizosphere (Scheublin et al., 2010; Singh et al., 2004).

The aim of this study was to investigate the bacterial and fungal diversity associated with hand-picked fungal hyphae selectively enriched in AM in comparison to bulk soil. Additionally, the ability of these bacteria to desulfurize sulfonate-S and putatively attach to fungal hyphae was investigated. The hypotheses were that (i) fungal hyphae select for a distinct bacterial community, and (ii) sulfonate utilizing bacteria would be enriched in the hyphoplane over both the hyphosphere and bulk soil due to their putative role in plant S supply and ability to attach to fungal hyphae via T3SS.

2. Materials and methods

2.1. Site description

Intact sods of grass (10 × 10 cm) were obtained in triplicate from Teagasc, Johnstown Castle (52°16′N, 6°30′W, 30 m above sea level). The soil type is a poorly drained gley soil with a pH of 6, organic matter (11%), and loamy topsoil (18% clay), classified as Mollic Histic Stagnosol (WRB 2006). The soil used has not received P or S fertilizer since 1968 and has not been ploughed since 1970 (P0-0, site 5A). Swards are mixtures of *Lolium perenne, Dactylis glomerata,* and various meadow grass species (Tunney et al., 2010). Entire sods and adjacent bulk soil were taken to the laboratory, where soil attached to roots and soil free of visible roots (defined as bulk soil) was subsampled to extract DNA and bacteria from the hyphoplane, hyphosphere and bulk soil.

2.2. Extraction and quantification of bacteria from AM hyphae

A number of techniques for separating bacteria directly colonizing the AM hyphoplane and those in the hyphosphere were compared including; sucrose density gradient centrifugation (Tommerup, 1992), Gentodenz extraction (Scheublin et al., 2010), and Winogradsky's rapid fractionated centrifugation (Faegri et al., 1977). The modification to these methods lay in the starting material. For this study, in-depth morphological assessment was used to pick 0.5 g of hyphae (H) from the roots of each of the sods using a compound microscope (×100 magnification) and fine forceps (Hodge and Fitter, 2010) to enrich the sample in AM hyphae. Excess soil was removed from the hyphal-root surface using a soft bristled brush. The hyphae were picked carefully as to minimize the bias toward picking only robust hyphal morphologies. Detailed microscopic observations of the picked hyphae confirmed typical anatomical features of AM fungi and the absence of defined septa (Humphreys et al., 2010). Bulk soil (BS, soil free of any visible roots) was sampled at each site and used for downstream experimentation in parallel to the picked hyphae. Both H and BS were separated into supernatant and pellet (hyphosphere (hs) and hyphoplane (hp), respectively) via centrifugation (4500 rpm, 15 min) and each fraction underwent further size fractionation using sterile sieving to yield 212 and 64 μm treatment sizes (Supplementary information – Extraction Methods). Serial dilutions of these separation fractions were plated on R2A (Reasoner and Geldreich, 1985) to calculate colony forming units (CFU g^{-1}, wet weight) of H and BS. The separation suspensions were aseptically filtered and stored in a freezer for subsequent fingerprinting analysis (see Section 2.4).

2.3. Functionality of dominant isolates

Based on CFU results and preliminary fingerprinting analysis, sucrose density gradient centrifugation was selected as the best available bacterial separation method. Using the CFU plates of this separation method, 200 random colonies were sub-streaked (50 isolates each for all 4 hs fractions from H and BS – hs212, hs64, hsbs212, hsbs64) on both R2A and agarose plates with toluenesulfonate as sole S source (MM2TS (Fox et al., 2014) solidified with 6 g l^{-1} agarose, Eurobio, Courtaboeuf, France). Bacterial pure cultures that exhibited advantageous growth on solid MM2TS were identified and subsequently transferred into liquid MM2TS and MM2SF (medium with toluenesulfonate and S free control; (Fox et al., 2014)). The optical density at 590 nm was used to compare relative growth in MM2TS and MM2SF and was recorded using an ELX808IU spectrophotometer (Bio Tek Instruments Inc., Winooski, VT).

Genomic DNA from isolates growing significantly better in MM2TS than MM2SF ($P \leq 0.05$) was extracted for polymerase chain reaction (PCR) analysis using a modified quick lysis protocol (Schmalenberger et al., 2001). The extracted DNA was subjected to 16S rRNA gene amplification and the final concentration per 25 μl reaction was 1 × buffer (2 mM $MgCl_2$), 0.2 mM dNTP mix, 0.4 μmol of each primer 27 F and 1492 R (Lane, 1991), and 0.5 U of DreamTaq polymerase (Fisher Scientific, Waltham, MA). Cycling conditions were as follows: 32 cycles of 94 °C denaturation (45 s), 55 °C annealing (45 s) and 72 °C extension (90 s). In order to ascertain taxonomic diversity of cultivated bacterial isolates, restriction fragment length polymorphism (RFLP) was carried out on PCR amplicons using the restriction enzymes *Rsa*I and *Taq*I (5 U per reaction; Thermo Scientific) for 4.5 h at 37 °C. Following digestion, DNA fragments were separated on a 10% acrylamide gel (110 V for 100 min). Operational taxonomic units (OTUs) were established and re-amplified as above in volumes of 50 μl and purified (GenElute, Sigma–Aldrich, St. Louis, MO). OTUs that were identified more than once or showed a growth advantage of over 0.5 (OD_{590}) were subjected to sequence identification (GATC Biotech, Konstanz, Germany) and compared with similar sequences using BLAST (Altschul et al., 1990). Taxonomically distinct dominant OTUs were subjected to PCR of *asfA* and *hrcR* (Section 2.5).

2.4. Community fingerprinting

The frozen filter paper (Section 2.2) was used for community DNA extraction (hp, hs in H and BS) using the UltraClean Soil DNA extraction kit from MoBio (Carlsbad, CA) according to the manufacturer. The DNA was quantified using a Nano Drop ND-1000 (Thermo Scientific) and diluted to 10 ng μl^{-1}.

Bacterial 16S rRNA gene amplification was carried out with this DNA using the primer pair GC-341F/518R targeting the V3 region for denaturing gradient gel electrophoresis (DGGE) (Muyzer et al., 1993). The final concentration per 25 μl reaction was 1 × buffer (2 mM $MgCl_2$), 0.2 mM dNTP mix, 0.4 μmol of each primer, and 0.5 U of DreamTaq polymerase (Fisher Scientific). A touchdown PCR protocol was used with the following cycling conditions: 20 cycles of 94 °C denaturation (45 s), 65–55 °C touchdown (45 s),

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