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Bacterial communities in grassland turfs respond to sulphonate addition while fungal communities remain largely unchanged

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ABSTRACT

Sulphonates are often the major form of sulphur in soils where sulphate usually represents less than 5% of the total sulphur. The use of sulphonates as a S source is limited to a functional bacterial guild. However, fungi may assist bacteria in sulphonate desulphurization. In this study, grassland turfs were watered periodically with a modified Hoagland's solution that was i) sulphur free, ii) contained low molecular weight sulphonate iii) or high molecular weight sulphonate. DNA fingerprint analyses of fungal and bacterial communities revealed significant differences between the rhizosphere soil and the bulk soil. Sulphonate treatments had only significant effects on the bacterial and desulphonating bacterial communities and no significant effects on the fungal communities. However, sequencing of the fungal ITS region identified the presence of potentially endophytic fungi in sulphonate amended turfs. Analysis of the sulphur species in soil suggested that the added toluenesulphonate–sulphur was transformed despite the fact that the number of the desulphonating bacteria remained unchanged indicating no sulphonate limitation. The results showcase a robust fungal community in grassland turfs where only the bacterial community with its desulphonating bacterial guild is predominantly responding to the sulphonate amendment.

1. Introduction

Organically bound sulphur such as sulphate esters and sulphonates are the major sulphur (S) compounds in soil ecosystems where sulphate often represents less than 5% of the total S [1]. As plants almost entirely depend on sulphate as S source, they require microbial S-supply. Due to the vast reduction of air pollution, S has nowadays become a potential limiting factor of plant growth. It is therefore paramount to improve our understanding in organically bound S mobilization in order to implement a more sustainable land use approach, less dependent on inorganic fertilizers.

Organically bound S compounds arise in soils through deposition of S-containing biological material and are transformed through subsequent humification processes [2]. Animal-derived organic input in the form of sheep dung contains up to 80% of its S in carbon-bound form, largely as sulphonates [3]. Chemical and X-ray spectroscopic studies have shown that the dominant forms in a range of aerobic soils are sulphonates (30–70% of soil S) [4,5]. Plants are unable to utilize these organic compounds as S-sources [6]. However, 80–90% of all plant species associate with mycorrhizal fungal partners [7,8]. Their mycelial networks are several times longer than plant roots and often exceed 200 m per cm^3 [9]. These networks support plant hosts through the acquisition of limiting nutrients [10] acquired in partnership with other microorganisms including bacteria located in the mycorrhizosphere [11]. While some fungi are able to desulphurize sulphate-esters, the use of sulphonates as a S source seems to be limited to a defined functional bacterial guild [12]. Such soil bacteria can transform these compounds within weeks using a bacterial multi component monooxygenase system [12,13]. Work with *Pseudomonas putida* has shown that the desulphonation of aromatic sulphonates resulted in a plant growth promotion effect [6]. The gene *asfA* is the key marker in this desulphonation process and this marker has been used as a proxy in molecular approaches to study sulphonatase diversity [14–16]. Cultivation independent analysis of the *asfA* diversity allowed the identification of *Variovorax* and *Polaromonas* as the dominant sulphonate-metabolizing genera [14] in the rhizospheres of cereal crops and unmanaged *Agrostis* species [16,17].

So far, a direct involvement of mycorrhizal and saprophytic fungi in desulphonation has not been observed. Treatments of artificial

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sulphonates with several fungi and their enzymes for transformation showed no sign of desulphonation activity [18,19]. Brown and white rotters on minimal media plates with toluenesulphonate as the sole S source grew into larger colonies than on S free control media but growth of these saprophytic fungi on wood showed that sulphate esters and not sulphonates were used as the primary S source [20]. Soil isolates affiliated to the bacterial genera *Variovorax* and *Rhodococcus* are able to grow with 0.25 mM toluenesulphonate as sole S source with no apparent inhibition in growth [15,21]. While the former isolate was cleaving S from the aromatic ring, resulting in the accumulation of para-cresol, the latter isolate was capable of breaking the aromatic ring, using it as a carbon source, leaving no aromatic product behind [15,21].

The aim of this study was to find out whether addition of sulphonate S in form of the low molecular weight source toluenesulphonate (aromatic, TS) or the high molecular weight sulphonate lignosulphonate (aliphatic, LS) is impacting the structure and composition of the bacterial, the desulphonating bacterial guild, as well as the fungal community and the communities of the Ascomycetes, Basidiomycetes and Glomeromycota in grassland turfs. The hypotheses were that i) the rhizosphere soil community interact differently to the sulphonate treatment than the bulk soil community; ii) the sulphonate treatment is affecting the bacterial communities in particular the desulphonating bacterial guild and iii) bacterial desulphonation is potentially mediated by the fungal community due to depolymerization of large sulphonate molecules and S transport in the mycorrhizosphere of *Agrostis stolonifera*.

2. Materials and methods

2.1. Sampling, incubation of turfs, isolation of microorganisms and culture conditions

Turfs with *A. stolonifera* were sampled from the field site at Butt Close, Woburn Experimental Farm [22], Bedfordshire, United Kingdom (0°36′W, 52°2′N) in early October 2008 (self-sown, unmanaged grassland). Turf-subsamples (15 × 20 cm) from three locations at Butt Close (approximately 10 m apart) were transported to the laboratory, and placed into plastic boxes with drainage holes (nine boxes in total). These mesocosms were incubated in a plant growth chamber (Conviron, Winnipeg, Canada) at 20 °C with a photon flux of approximately 230 μmol m^{-2} s^{-1}, 50–60% humidity, and were watered with deionized water three times a week. The turf pieces were then additionally supplemented (150 ml twice a week) over a period of five weeks with either 0.25 times i) sulphate-free modified Hoagland's solution (SF) [23], or modified Hoagland's solution, amended with ii) 0.1 mM toluenesulphonate (TS) or iii) 0.1 mM lignosulphonate-S (average M_W 8000; LS). In order to ensure, no inorganic sulphate was present in the LS solution, the LS was purified over the course of 48 h in ultrapure water (replaced four times) using dialysis tubing (Float-A-Lyzer, Sigma–Aldrich, St. Louis, MO) at a cut off at 3.5 kDa. The nutrient concentrations in the modified Hoagland's solution were as described previously [16].

Soil, not directly attached to the roots was harvested in triplicates per mesocosm as bulk soil (3 g fresh weight (FW)). For rhizosphere soil, loosely attached soil was shaken off the roots and the remaining roots with closely attached soil were sampled (3 g of root FW). Soil or roots were added to sterile phosphate buffered saline (PBS, 20 ml) and shaken on a Genie roto-shaker (Scientific Industries, Bohemia, NY) for 30 min at 4 °C [21]. The obtained suspension was used directly for cultivation dependent experiments and ion chromatographic analysis (see below). Bacteria able to utilize sulphonate were quantified by cultivation at 25 °C (most probable number, MPN in microtiter plates [14] with 10 replicates) in modified minimal medium MM according to Beil and colleagues but without vitamin solutions [24] and TS or LS as sole S source (0.25 mM S). In parallel, MPN was also carried out in liquid R2A medium [25] to enumerate the abundance of cultivable heterotrophic bacteria.

2.2. DNA extraction and PCR conditions

DNA was extracted from the bacterial suspensions using the FastDNA extraction kit for soil (MPBio, Irvine, CA) as described previously [21]. Amplification of *asfAB* fragments (1.3 kbp *asfA* and 0.14 kbp *asfB*) from environmental DNA for T-RFLP analysis was carried out in a Tgradient thermocycler (Biometra, Göttingen, Germany) using a touch-down protocol of 10 cycles and a starting annealing temperature of 65 °C (1 min) and further 30 cycles with a denaturating temperature of 94 °C (1 min), annealing temperature of 55 °C (1 min), extension temperature of 72 °C (3 min) and a final extension time of 10 min. The initial denaturation took place at 95 °C (4 min). The amount of template DNA added for each PCR reaction was within the range of 1–10 ng, and final concentrations of 5% (v/v) DMSO, 0.5 U Kapa Robust HotStart Taq and 1× GC buffer (Kapa Biosystems, Woburn, MA), 2.5 mM $MgCl_2$, 0.5 μM primers and 200 μM dNTPs each were used for 25 μl reactions. All subsequent PCR applications used the Kapa Robust HotStart Taq system with the conditions as above but with Kapa Enhancer instead of DMSO and Kapa buffer A instead of GC as recommended by the manufacturer. PCR amplifications from environmental samples for 16S rRNA gene-based DGGE analysis were carried out in a PCR approach described previously [14] using the primers GC-341F and 518R [26]. The internal transcribed spacer (ITS) region was amplified using a nested PCR approach. In the first reaction ITS1F was selected as forward primer [27] and ITS4, ITS4A and ITS4B as reverse primers to amplify the higher fungi, the Ascomycetes and the Basidiomycetes, respectively [27,28]. In the second PCR, the forward primer contained a GC clamp (ITS1F-GC) and ITS2 was chosen as the reverse primer to facilitate the ITS-DGGE analysis as described by Anderson and colleagues [29]. For the Glomeromycota a nested PCR was conducted with the primer pairs AM1 [30] with NS31 [31] at the start and Glo1 with NS31-GC [32,33] as the second PCR. PCR cycle conditions for 16S and ITS amplification are described in the Supplementary Table S1.

2.3. Denaturing gradient gel electrophoresis (DGGE)

DGGE was carried out on 20 × 20 cm gels in a INGENYphorU electrophoresis chamber (Ingeny, Goes, The Netherlands). Gel electrophoresis of 16S and ITS fragments was conducted in 10% acrylamide gels with a gradient of 30–60% using urea and formamide as denaturing agents. Electrophoresis took place in 0.5 times TAE at 100 V for 18 h. Gels were stained for 30 min with SybrGold as recommended by the manufacturer (Invitrogen, Carlsbad, CA).

2.4. Cloning of ITS PCR products and genotyping

Amplified ITS fragments obtained with primers ITS1F and ITS4 from the bulk soil of the S free, TS and LS treatment were purified using the GeneJet PCR purification kit according to the manufacturer (Thermo Scientific, Waltham, MA), triplicates were pooled within each treatment and then ligated and transformed as described previously [21] using a pGEM-T ligation kit from Promega (Madison, Wisconsin) and competent cells of *Escherichia coli* DH5alpha. Recombinant plasmids containing the expected insert were amplified with primers ITS1F and ITS4 for RFLP analysis. RFLP was carried out with 139 clones in total, as described elsewhere [21] with restriction enzyme *Hinf*I (Thermo Scientific).