Sulfate treatment affects desulfonating bacterial community structures in Agrostis rhizospheres as revealed by functional gene analysis based on asfA.

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Abstract

Sulfonates are major soil sulfur components that can be desulfurized by certain soil-born bacteria as a sulfur source, but application of sulfate through fertilization could affect this natural sulfur mobilizing process. This study investigates the effects of sulfate treatments on bacterial communities in semi-natural grasslands. *Agrostis stolonifera*-dominated turfs with their autochthonous bacteria from Woburn Experimental Farm, UK, were incubated with and without additional sulfate over a period of eight weeks and compared to soil and rhizosphere samples taken from the field directly. Cultivable rhizobacteria able to desulfurize toluenesulfonate were dominated by strains affiliated to the *Variovorax, Polaromonas* and *Rhodococcus* genera. The betaproteobacteria communities (16S rRNA gene-based denaturing gradient gel electrophoresis) and the desulfonating bacterial communities (*asfA* based terminal restriction fragment length polymorphism) revealed clear differences between field rhizospheres and bulk soil and the two types of incubated rhizospheres. Clone libraries of *asfA* from the sulfate-limited treatment were dominated by a new AsfA type, affiliated to *Polaromonas*. The results from this study suggest that the community of desulfonating bacteria in the *Agrostis* rhizosphere adapts quickly to changing levels of inorganic sulfate.
1. Introduction

Although biological mineralization and mobilization of organically bound sulfur (organo-S) is well known in soils, little is known about the microorganisms responsible for these processes. Organo-S such as sulfate-esters and sulfonates are the major sulfur compounds in many soils, where sulfate often represents less than 5% of the total sulfur [2, 21, 22]. These organo-S compounds occur in soils due to the deposition of biological material and its subsequent humification [16, 24]. Plants are unable to utilize the sulfur part from these organic compounds directly [22] and rely on prior mineralization of the sulfur by soil microbes to make it available which can be quite rapid in agricultural soils [12].

Two to three decades ago, up to 78% of sulfur in plant tissue originated from pollution in Britain but nowadays plants are likely to be deficient in sulfur unless sulfate-containing fertilizer is applied [27, 39, 44]. The uptake of nutrients by the plant root creates a rhizosphere zone depleted of inorganic N, P and K [19] and according to recent in vitro studies also depleted of inorganic sulfur [24]. Microbes capable of desulfurizing sulfonates could have a significant influence on the cycling of nutrients, the growth of sulfur-limited plants and on the microbial biomass. However, in agricultural systems plant nutrient depletion is avoided by fertilization and this could have a negative effect on the microbial diversity and its function to cycle nutrients such as sulfur, especially when the application of fertilizers is carried out over a long-term period. This is not the case in semi-natural grassland soils, where the microbial biomass is critical for nutrient cycling and organic matter decomposition [14]. Grassland plants often form symbioses with mycorrhizal fungi that improve plant nutrient uptake [20]. Agrostis plants grow with arbuscular mycorrhizal fungi such as Glomus [13] as a symbiont but little is known about the capability of
mycorrhizal fungi to utilize organo-sulfur as a sulfur source. Filamentous and saprophytic fungi have been reported to have sulfatase (desulfurization of sulfate-ester) activity [26]. The white rot fungus *Phanerochaete* is capable of transforming linear alkylbenzensulfonates but only without desulfonation [42], and fungal laccases and peroxidases used for decolorization of sulfonated dyes do not desulfurize sulfonates [41]. To date, reports of utilization of sulfonates as a sulfur source are exclusively limited to specific functional bacterial guilds that are often associated with plants [24].

No bacteria-based cell-free desulfonation assay has been established to date since a multi-enzyme reaction with several unstable components is required, but one gene of the sulphonatase complex called *asfA* has been successfully employed as a molecular marker [40, 34]. *AsfA* is essential for arylsulfonate desulfonation [40] since mutation of *asfA* in *Pseudomonas putida* and *Variovorax paradoxus* led to the loss of the ability to desulfurize arylsulfonates [40, 34]. Recent studies with wheat rhizospheres, using *asfA* as a marker, revealed long-term changes in the desulfonating bacterial community related to the long-term application of different types of fertilizers [37]. Since these studies were carried out in rhizospheres of a fertilized monoculture, established over 150 years ago, the bacterial diversity and function in soil sulfur cycling might have been impaired.

This study explored the diversity of sulfonate-desulfurizing bacteria in the rhizosphere of *Agrostis stolonifera* from grassland at Woburn Experimental Farm (UK), using both isolation techniques and cultivation-independent methods targeting *asfA*. Treatments with and without inorganic sulfate (eight weeks) had a significant effect on the β-proteobacteria and desulfonating bacteria rhizosphere communities, with desulfonating *Polaromonas* sp. predominantly found under sulfate-limited conditions.
2. Materials and methods

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

Bulk soil and turfs (20x20 cm) with *Agrostis stolonifera* were sampled from the field site at Butt Close, Woburn Experimental Farm [6], Bedfordshire, United Kingdom (0°36’W, 52°2’N) in early October 2006 (self-sown, unmanaged two year old grassland). *Agrostis* rhizosphere (method, see below) and soil samples were collected from the turfs on the same day to serve as references for the samples in the incubation experiment (see below). Turf-subsamples from three locations at Butt Close (approximately 10 m apart) were transported to the laboratory, split in half (6 pieces in total, approx. 10x8cm each) and placed into plant propagators with drainage holes, incubated at 18-22 ºC under greenhouse conditions with natural light, and were watered with deionised water (3 times a week, approx. 200ml at a time) for a period of four weeks. The turf pieces were then additionally supplemented (75ml twice a week) over a period of four weeks with either 0.25X sulfate-free modified Hoagland’s solution [18] (three turf pieces, triplicate), or modified Hoagland’s solution, amended with 0.1mM sodium sulfate (three turf pieces, triplicate). The nutrient concentrations in the modified Hoagland’s were as follows (mM): K 3.0, Ca 2.5, Mg 1.0, Fe 0.05, Mn 0.0045, Zn 0.00038, Co 0.00016, Na 0.0001/0.1, NO₃ 7.5, PO₄ 0.5, Cl 2.0, BO₃ 0.023, MoO₄ 0.00006.

Rhizospheres were harvested from the turf pieces (contained approx. 10 shoots) after removing the turf edges. Loosely attached soil was first shaken off each sample and bacteria were then extracted from the root surface and the remaining attached soil (3 g of root fresh weight (FW)) or bulk soil (3 g FW) into sterile phosphate buffered saline (PBS, 20 ml) by shaking on a Genie roto-shaker (Scientific Industries, Bohemia, NY).
for 30 min at 4°C [34]. Bacteria able to utilize toluenesulfonate were quantified by
cultivation at 25 °C (most probable number, MPN in microtiter plates [37] with 10
replicates) in MM medium [4] and toluenesulfonate (TS) as sole sulfur source (0.25
mM). Total cultivable bacteria populations were quantified using MPN assays in
liquid R2A medium [30]. Individual TS-utilizing strains were isolated for further
study from the highest MPN dilutions showing growth with MM-TS medium. Strains
were sub-cultivated on MM-TS plates solidified with agarose that was low in sulfur
content (12 g l⁻¹, Eurobio, Ulis, France; about 10X lower sulfate concentration
compared to Noble agar) for subsequent analysis.

2.2 DNA extraction and PCR conditions

DNA was extracted from the bacterial suspensions using the FastDNA extraction kit
for soil (QBiogene, Irvine, CA) as previously described [34]. Genomic DNA from
individual isolates was extracted for PCR analysis with a quick lysis protocol [35].
Amplification of asfAB fragments (1.3kb asfA and 0.14kb asfB) from pure cultures
and environmental DNA for cloning and T-RFLP was carried out in a T-gradient
thermocycler (Biometra, Goettingen, Germany) using a touch-down protocol (with a
decrease of 1 °C per cycle) starting with an annealing temperature of 65 °C (1 minute)
for a period of 10 cycles and further 25 cycles with an annealing temperature of 55 °C
as described previously [34, 37, 36], but the extension time was increased to 3
minutes. The amount of template DNA added for each PCR reaction was within the
range of 1 to 10 ng, and final concentrations of 5 % v/v DMSO, 0.625 U HotMaster
Taq (5 PRIME, Hamburg, Germany), 2.5 mM MgCl₂, 0.5 M primers and 200 M
dNTPs each were used [34, 37, 36] for 25 l reactions. PCR amplifications from
environmental samples for β-proteobacterial 16S rRNA gene-based DGGE analysis
were carried out in a nested PCR approach described previously [37] using primers
27F [25] and 865R [37] in the first PCR and primers GC-341F and 518R [28] in the second PCR.

2.3 Denaturing gradient gel electrophoresis (DGGE)

DGGE was carried out on 20 x 16 cm gels in a D-code electrophoresis chamber (Biorad, Hercules, CA) as described previously [10] with a denaturant gradient of 35 to 65%. Gels were stained for 30 min with SybrGold (Invitrogen, Carlsbad, CA).

2.4 Cloning of asfAB PCR products and genotyping

Amplified fragments of asfAB from incubated turfs (triplicates) were purified, pooled within each treatment and then ligated and transformed as described previously [34]. Recombinant plasmids containing an insert of about 1.5kb were then reamplified with primers asfAF2 and asfBtoA for RFLP analysis. RFLP was carried out with 96 clones in total (48 from the rhizospheres incubated with and without sulfate, respectively), as described elsewhere [34] with restriction enzymes AluI and RsaI. The richness of the clone libraries was estimated using the Chao1 estimator [7], calculated with EstimateS 8.0 (http://purl.oclc.org/estimates).

2.5 Terminal restriction fragment length polymorphism (T-RFLP) analysis

Primers asfAF2 and asfBtoA [34] for the desulfonating bacteria were modified with fluorescent labels FAM and HEX (5’FAMasfAF2, 5’HEXasfBtoA) and used to amplify fragments of asfAB from bulk soil and rhizosphere extracts. PCR reactions were set up with only one fluorescent primer each and products were subsequently pooled. The amplification products were purified (Qiagen PCR purification kit, Germany) and approximately 200 ng DNA was digested with 10 U of AluI and RsaI (Fermentas) at 37°C for 12 h. Samples (1 µl) were mixed with formamide (9 µl) and a modified ROX500 size standard (Applied Biosystems, Warrington, UK) containing additional 585, 685 and 785 bp signals (obtained with the amplification of fragments
of an M13mp18 vector with primers: 585 bp Standard ATT GTA AAC GTT AAT
ATT TTG TTA, 685 bp Standard GAT GAA CGG TAA TCG TAA AA, 785 bp
Standard TTC TAG CTG ATA AAT TAA TG, M13 Reverse ROX-TCA CAC AGG
AAA CAG CTA TGA C), and analyzed on an automated sequencer (ABI Prism
3730). Signals from the forward or the reverse labeled primers were detected on
separate channels, and data analysis was carried out with the GeneMapper 3.7
software. Peaks with heights of < 2% of the total peak height were discarded from the
analysis. Signals were treated as identical when the calculated size was equal or below
a difference of two bases (statistical analysis described below).

2.6 DNA sequence analysis
Genotypes of asfAB that were represented by two or more clones were sequenced, and
the asfA sequence fragments were imported into an asfA database generated
previously [34]. Phylogenetic trees for the encoded peptide sequences (AsfA, 5’
truncated and approximately 400 amino acids in length) were calculated with the
Maximum Likelihood (Dayhoff model) and Distance Matrix (Dayhoff model)
methods, and a consensus tree was generated.
16S rRNA gene sequences from isolates obtained in this study were imported into
ARB alongside closely affiliated sequences from Genbank identified using BLAST
[1]. The sequences were aligned, and phylogenetic affiliations of sequences from this
study were identified.

2.7 Analytical methods
Measurement of sulfate, nitrate and phosphate was carried out by ion chromatography
(IC). Rhizosphere soil from Agrostis stolonifera roots (3 g) from the incubated turfs
was detached in 10 ml of ultra purified water (18 mΩ, Elga labwater, purelab ultra,
High Wycombe, UK) using a vortex mixer for 1 minute. Root material was removed
and the soil suspension was centrifuged at 3500 rpm for 15 minutes. The supernatant was subjected to IC analysis using a Dionex DX-120 Ion Chromatograph with an AS14 column for anion separation (eluent 8 mM Na₂CO₃ / 1 mM NaHCO₃) coupled with an electro-conductivity detector (CDM-3) at a flow rate of 1.2 ml min⁻¹ (4 technical replicates) and the soil dry weight was determined (soil pellets dried at 80 °C, 72 h).

Analysis of incubated culture media after removal of the bacterial biomass via centrifugation was carried out by reversed-phase HPLC using a C18 column, with a methanol gradient in 10 mM potassium phosphate buffer, as described previously [40].

2.8 Statistical analysis

Cultivable bacteria were quantified via the MPN method [9] as described previously [37] employing the U.S. Food and Drug Administration (FDA) Biological Analytical Manual MPN table (http://www.cfsan.fda.gov). MPN values were considered as significantly different when the difference of the log(MPN) was higher than 0.516 (95% confidence interval). Standard deviations were calculated for measured sulfate, nitrate and phosphate concentrations and two sided T-tests were carried out (see P-values in the results section).

Binary data sets of the DGGE image were obtained employing the Phoretix advanced 1D software (Nonlinear Dynamics, Newcastle upon Tyne, UK). Binary data sets of T-RFLP fingerprints comprising signals from the forward and the reverse labeled primer were combined. DGGE and T-RFLP data sets were subjected to detrended correspondence analysis (DCA) in order to remove arch effects in the correspondence analysis, using DECORANA [8, 17] as described earlier [37, 36] and available at www.ceh.ac.uk/products/software/index.html. Results were summarized in ordination
diagrams where replicates were displayed as means with standard deviations and
visualized as error bars. To test the effect of experimental variables (application of
Hoagland’s solution with or without sulfate and incubation conditions) on the β-
proteobacterial and the desulfonating community structures, permutation tests were
performed available in CANOCO using 9999 replicates as described elsewhere [33,
29].

2.9 Nucleotide sequence accession numbers
Isolates were identified by amplification and sequencing of the 16S rRNA genes with
primers 27f and 1492r [25] and sequences were deposited in the EMBL Nucleotide
Sequence Database (FM986388 to FM986394). Sequences of asfA from bacteria and
environmental DNA isolated from soil and rhizosphere were deposited in the same
database (FM986395 to FM986401 for isolated bacteria and FM986799 to FM986816
for cloned environmental DNA).
3. **Results**

3.1 *Effect of sulfate treatment on populations of cultivable desulfonating bacteria in* Agrostis stolonifera *rhizospheres*

A mesocosm study was set up with turfs taken from an *A. stolonifera*-dominated grassland (Woburn experimental farm, UK [6]) to examine how changes in sulfate concentrations over a short-term period of eight weeks affected the microbial community in the rhizosphere of a semi-natural environment. Macro nutrient concentrations were monitored in the soil rhizosphere at the end of the incubation period to confirm that only the level of sulfate differed between the two microcosm treatments. Application of sulfate-containing Hoaglands solution resulted in significantly (P < 0.001) elevated levels of sulfate-S in the rhizosphere soil (12.7 ± 0.8 mg kg\(^{-1}\) dry weight). Rhizosphere soil watered with sulfate-free Hoaglands had half the concentration of sulfate-S (6.3 ± 0.6 mg kg\(^{-1}\) dry weight). Concentrations of nitrate and phosphate were not significantly different in both treatments (29.5 ± 7.1 and 35.4 ± 8.8 mg kg\(^{-1}\) dry weight nitrate (P = 0.08); 123.3 ± 27.3 and 160.1 ± 21.5 mg kg\(^{-1}\) phosphate (P = 0.34)).

The numbers of cultivable bacteria capable of growing with toluenesulfonate as sole sulfur source (sulfonate utilizers) were compared with the total population of cultivable heterotrophs. Values (MPN g\(^{-1}\) root or soil (FW)) for the heterotrophs were between 1.1 x 10\(^7\) and 8.7 x 10\(^7\) and for the sulfonate utilizers between 0.5 x 10\(^6\) and 3.0 x 10\(^6\) (Table 1). The incubation with nutrient solution containing sulfate led to a statistically significant (P ≤ 0.05) increase in the total cultivable bacterial population and an increase (although non-significant) in cultivable sulfonate utilizers in the rhizospheres. Whereas this was in part due to the addition of N and P, the main driver for this stimulation was sulfate supply, since a decrease (although non-significant) of
the total population was seen in the absence of added sulfate.

A total of 43 isolates were obtained from the highest MPN dilutions that showed growth that were capable of utilizing toluenesulfonate as sole sulfur source. For six different types of isolates (classified by 16S rRNA gene sequencing), reproducible toluenesulfonate disappearance was observed from the culture supernatant after incubation for 1-2 weeks (strains A7, A22, A30, A35, A37, A38). Release of p-cresol was also observed for three isolate types (strains A7, A37, A38) corresponding to mono-oxygenolytic desulfurization of toluenesulfonate. Taxonomic affiliations of these strains were determined by 16S rRNA gene sequencing, and revealed that the isolates were affiliated to *Variovorax paradoxus* (strain A7; 99% similarity to the type strain), *Polaromonas gingsengioli* (strains A37 and A38; 99%) and *Rhodococcus erythropolis* (strains A22, A30, A35; 99%). All isolated strains under investigation contained the *asfAB* gene fragment, demonstrated by amplification with primers specific for proteobacteria and rhodococcales *asfAB* [34, 37, 36]. *Variovorax* isolates were retrieved from rhizospheres sampled directly from the field (reference), and from rhizospheres harvested from the incubated turfs (sulfate-limited and sulfate-amended), but *Polaromonas* isolates A37 and A38 were found exclusively in the rhizospheres of the turfs incubated under sulfate limitation. *Rhodococcus* isolates were only isolated from the incubated turf rhizospheres (both treatments).

### 3.2 Effect of sulfate treatments on β-proteobacterial rhizosphere community structures.

Rhizobacterial community structures in the field and in the incubated turfs were analyzed by profiling β-proteobacterial 16S rRNA gene fragments using DGGE, and fingerprints were subjected to statistical analysis. Community profiles from the incubated rhizospheres (turfs with and without addition of sulfate) showed significant
differences between the two treatments (permutation test: F-ratio = 2.067, P-value = 0.0257). Reference samples from rhizospheres harvested directly from the field showed significant differences in the community structure when compared to the incubated rhizospheres and the directly harvested reference soil (F-ratio ≥ 2.251, P-value ≤ 0.0151). The significant differences between the samples were visualized by detrended correspondence analysis (DCA) (Fig. 1a).

3.3 Effect of sulfate treatment on desulfonating bacteria (asfAB gene diversity) in Agrostis stolonifera rhizospheres

The diversity of desulfonating bacteria was characterized by fingerprinting the proteobacterial asfAB gene fragment from the field (both rhizosphere and bulk soil) and from the incubated turfs by T-RFLP. The number of T-RF peaks included in the subsequent analysis was in the range of 29 to 37 in the rhizosphere incubated under sulfate limitation and 29-30 in the rhizosphere incubated under sulfate amendment. In the reference samples 23-28 and 16-36 T-RF peaks were found in the rhizosphere and bulk soil harvested directly from the field, respectively. Statistical analysis (DCA and permutation tests) showed a significant separation between the asfAB communities of the sulfate limited and the sulfate-amended rhizospheres (F-ratio = 1.612; P-value = 0.0163; Fig. 1b). The DCA analysis (Fig. 1b) clearly separated the reference soil samples and the reference rhizosphere samples (both directly from the field) from the incubated rhizospheres. Permutation tests confirmed a significant separation between the reference soil and the incubated rhizospheres (F-ratio ≥ 1.338; P-value ≤ 0.0125; Fig. 1b), between the rhizosphere reference samples and the rhizospheres incubated under sulfate limitation (F-ratio = 1.592; P-value = 0.0450; Fig. 1b), but not between the reference rhizospheres and the rhizospheres under sulfate amendment (F-ratio = 1.273; P-value = 0.0906).
3.4 AsfA sequences from pure cultures and environmental DNA

Amplified asfAB sequences from the two turf treatments (incubated under sulfate amendment and sulfate limitation, respectively) were cloned (96 in total) and 48 unique operational taxonomic units (OTU) were identified, which of the 18 most abundant OTUs were sequenced. The estimated richness [7] of asfAB OTUs in the sulfate-limited and sulfate-amended rhizosphere clone library was 79 and 68, respectively, while the combined estimated richness was 86. Indeed, 47 out of the 96 clones belonged to OTUs that contained clones from both clone libraries. Sequences obtained from the clones and from the isolates were translated into protein sequences and added to a phylogenetic tree of AsfA (Fig. 2). Variovorax A7 AsfA was closely affiliated to AsfA from the Variovorax paradoxus type strain and several Variovorax isolates from spring barley and wheat [34, 37]. Additionally, OTU CA4 was found in the Variovorax cluster with 4/4 clones originating from the sulfate limited turf incubation. Although Variovorax isolates were the most abundant cultivable desulfonating organisms in this and previous studies [34, 37, 36], Variovorax strains did not dominate the desulfonating community. Isolates Polaromonas A37 and A38 formed a clade (Polaromonas 2) with AsfA from environmental DNA from the rhizosphere of spring barley and wheat [34, 37]. Previous studies have associated the genus Polaromonas with a different clade with high similarities to the Variovorax cluster [37]. Four of the dominating OTUs were found in the Polaromonas A37/A38 cluster (CA1, CA7, CA14, CA42, 10/14 clones from sulfate limited treatment) and three OTUs (CA19, CA28, CA33) were found to be distantly related to Cupriavidus metallidurans, (9/11 clones from sulfate-amended treatment). Rhodococcus isolates A22, A30 and A35 clustered with sequences from Rhodococcus isolates retrieved from wheat rhizospheres and Rhodococcus sp. IGTS8 [36].
4. Discussion

In this study, the diversity of sulfonate-desulfurizing (desulfonating) bacteria and β-proteobacteria in Agrostis stolonifera-dominated grassland was investigated after incubation with and without sulfate amendment. Significant shifts were detected in both communities and the genus Polaromonas was dominant in the sulfate-limited treatment.

Agrostis-dominated grasslands are usually characterized by relatively low levels of biomass production but with high levels of soil organic matter and fungal biomass [3, 14, 15]. Nutrient limitation is one plausible explanation for this low productivity. Sulfur is regarded as an absolute requirement for growth of all organisms [24] and is an essential macro nutrient just like nitrogen and phosphorus. The application of inorganic fertilizer to grasslands increases the plant productivity significantly and shifts the microbial community from fungal dominance to bacterial dominance [3, 14]. Up till now, bacterial abundance has not been linked to sulfur limitation, but a lack of an essential nutrient such as sulfur should cause clear growth limitations. In this study, incubation of turfs (and their rhizospheres) with the addition of inorganic sulfate caused an eight-fold increase in the cultivable heterotrophic bacterial population and a considerable rise in the toluenesulfonate utilizing bacterial population (50% increase, not significant), suggesting that sulfur is indeed a limiting factor for bacteria in the A. stolonifera-dominated grassland under study. So far, sulfur deficiency has been linked to reduced seed yield, lower seed quality and growth limitations in crops such as wheat in plant pot experiments ([31], Schmalenberger et. al., unpublished) and in the field [43, 5].

In the last decade, sites at Woburn Experimental Farm have been used for several
studies including sulfur deficiency of wheat (Butt Close) [31] and grassland lysimeter
studies (Great Hill) [32]. Previous analysis of bulk soil at Butt Close reported slightly
lower soil concentrations of sulfate-S (4 mg kg$^{-1}$ air dry soil) [31] than found for
rhizosphere soil in the present study. The values obtained here, however, were quite
similar to results obtained in a previous study with wheat rhizospheres obtained from
sulfate-limited plots of Broadbalk at Rothamsted (5.4 mg kg$^{-1}$ sulfate-S fresh weight
[37]). Indeed, sulfate contents of soils vary considerably with time for any given soil
and measurements of free sulfate are not necessarily a very useful indicator for plant-
available sulfur over a growing season [38].
A previous study in wheat rhizospheres showed that long-term differences in the
sulfate supply can alter bacterial communities and the ones from the β-proteobacteria
in particular [37]. Here, communities of β-proteobacteria were significantly different
in the short-term incubation treatment with and without sulfate and also showed
significant differences to the reference samples harvested from field site (Fig.1a). The
latter results suggested that the application of Hoagland’s solution and the sulfate had
an effect on dominant members of the β-proteobacterial community. Changed
temperature and water supply during incubation have been identified as significant
factors for determining the soil bacterial community composition before [11, 29], but
a significant short-term influence of sulfate on the β-proteobacterial community is a
novel observation.
The analysis of the functional guild of desulfonating bacteria revealed clear (in all but
one case also significantly different) separations (Fig. 1b) between the incubated
rhizospheres and the reference samples from the field (bulk soil and rhizospheres).
These and the cultivation dependent results suggest that desulfonating bacteria were
not able to take advantage of the addition of sulfate at the same level as the β-
proteobacteria and cultivable heterotrophic bacteria did. Cloned sequences related to
AsfA from *Polaromonas* and *Variovorax* were much less abundant in the sulfate
amended treatment suggesting that sulfate application has a negative effect on some
desulfonating bacteria. However, an increase of *Cupriavidus*-like AsfA sequences in
the sulfate amended treatment, suggest that at least some desulfonating bacteria can
also take advantage of the elevated sulfate levels. The estimated richness of *asfA* in
the sulfate amended treatment and the total number of detected T-RF peaks was only
marginally lower compared to the sulfate limited treatment suggesting that the short-
term application of sulfur had only a limited effect on the overall *asfA* diversity.

While desulfonating communities in wheat rhizospheres were dominated by
*Variovorax paradoxus* [37], the present study found much smaller numbers of clones
with AsfA associated to *Variovorax* (4 out of 96 clones). Nevertheless, *V. paradoxus*
was isolated in this study four times, confirming that it is also a common
desulfonating strain in the rhizospheres of *A. stolonifera*.

The cultivation of bacteria from the *A. stolonifera* rhizospheres also identified
desulfonating *Rhodococcus* isolates (Fig. 2). Primers targeting the corresponding *asfA*
gene have been used in this study to investigate the diversity of *Rhodococcus*-like
*asfA* as described earlier [36] but found no sulfate related incubation effect (data not
shown).

Desulfonating *Polaromonas* strains were isolated from both wheat and *Agrostis*, but
the *asfA* gene from the wheat isolate (*Polaromonas* sp. P6E3) was not closely
affiliated to the ones from *Polaromonas* sp. A37 and A38 isolated from *A. stolonifera*,
which define the *Polaromonas* 2 clade (Fig. 2). The results suggest that horizontal
gene transfer might be involved in the way different *Polaromonas* strains have
obtained different types of *asfA*. Analysis of the genome sequence of the related
Polaromonas naphthalenivorans reveals no asfA homologue, suggesting that not all Polaromonas species contain the asf cluster. Sequences of asfA affiliated to Polaromonas have been retrieved from agricultural wheat and naturally occurring Agrostis rupestris rhizospheres before where levels of sulfate were low [33, 37]. These and the findings in this study suggest that Polaromonas plays an important role in sulfate limited environments on both agricultural and non agricultural sites. In contrast, sequences affiliated to Cupriavidus dominated sulfate amended A. stolonifera rhizospheres in the present study. The conclusion from this study is that different types of desulfonating rhizobacteria of A. stolonifera fulfill different roles depending on the sulfate levels in the rhizosphere. Transport of sulfonates into the cell and desulfurization are energy demanding processes, and the proteins involved (such as AsfA) must be synthesized de novo, since they are part of the sulfate starvation-induced (SSI) stimuion, and are not expressed in the presence of sulfate [23, 21]. However, the degree of sulfate-mediated asfA repression in vitro was lower in Variovorax paradoxus than in Pseudomonas putida S-313 [37], suggesting that the regulation of these proteins varies between bacterial genera. Desulfonating bacteria such as Polaromonas, adapted to environments with low levels of sulfate could have a different regulation of their SSI proteins, with higher levels of SSI expression or more efficient SSI proteins but this needs to be explored further in the future.

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Table 1: Cultivable bacteria

<table>
<thead>
<tr>
<th></th>
<th>Total heterotrophic bacteria/g root or soil * 10^7</th>
<th>TS utilizing bacteria/g root or soil * 10^6</th>
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<tbody>
<tr>
<td>Bulk soil</td>
<td>1.2 (0.52-2.70) ^1^A</td>
<td>0.5 (0.22-1.16) ^1^C</td>
</tr>
<tr>
<td>Rhizosphere</td>
<td>2.3 (0.99-5.30) ^1^A</td>
<td>1.1 (0.47-2.55) ^1^CD</td>
</tr>
<tr>
<td>Sulfate limited incubation</td>
<td>1.1 (0.47-2.55) ^1^A</td>
<td>3.0 (1.29-6.95) ^1^D</td>
</tr>
<tr>
<td>Sulfate amended incubation</td>
<td>8.7 (3.75-20.16) ^1^B</td>
<td>2.1 (0.59-6.66) ^1^CD</td>
</tr>
</tbody>
</table>

^1^ 95% confidence interval

^A^B^C^D^ indicate significant differences
Figure legends

Figure 1. Ordination plot (detrended correspondence analysis, DCA) of DGGE profiles of β-proteobacteria with 16S rRNA gene fragments (A) and ordination plot (DCA) of T-RFLP profiles of desulfonating bacteria with proteobacterial asfAB (B) amplified from different soil and rhizosphere samples from the Butt Close field site. Reference field bulk soil samples S1-S4 (circle); reference field Agrostis stolonifera rhizospheres R1-R4 (square); Rhizospheres of A. stolonifera A, B, C after incubation with sulfate free Hoagland’s solution (diamond); Rhizospheres of A. stolonifera D, E, F after incubation with sulfate-amended Hoagland’s solution (triangle). Error bars indicate the standard deviation of the mean of each sample group. Italic letters indicate significant differences (P ≤ 0.05, permutation test) between the sample groups. Eigenvalues (α) of the axis are noted next to the axis labels.

Figure 2. Consensus tree of partial N-terminally truncated peptide sequences of the oxidoreductase AsfA and its orthologues deduced from the sequenced genes. Trees were constructed for the consensus using distance matrix (Dayhoff model) and maximum likelihood (Dayhoff model) methods. Polytomic nodes connect branches where a relative order could not be determined unambiguously. Closed circles indicate highly supported bootstrap analysis (over 80%), while open circles indicate 60-80% support. Sequences retrieved from this study are the molecular isolates from the clone library of Agrostis stolonifera rhizosphere (CA2-CA45, underlined) and the isolates from the A. stolonifera rhizosphere (A7-A38, underlined and bold). Sequences retrieved from previous studies include isolates and culture collection strains of Variovorax [34, 37], Cupriavidus [34], Pseudomonas [40], Rhodococcus [36] and Acidovorax [34]. Sequences retrieved from clones from earlier studies are labeled with sb (spring barley, [34]) and W (wheat [37]).