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The role of Variovorax and other Comamonadaceae in sulfur transformations by microbial wheat rhizosphere communities exposed to different sulfur fertilization regimes

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Abstract

Sulfonates are a key component of the sulfur present in agricultural soils. Their mobilization as part of the soil sulfur cycle is mediated by rhizobacteria, and involves the oxidoreductase AsfA. In this study, the effect of fertilization regime on rhizosphere bacterial asfA distribution was examined at the Broadbalk long-term wheat experiment, Rothamsted UK, which was established in 1843, and has included a sulfur-free treatment since 2001. Direct isolation of desulfonating rhizobacteria from the wheat rhizospheres led to the identification of several Variovorax and Polaromonas strains, all of which contained the asfA gene. Rhizosphere DNA was isolated from wheat rhizospheres in plots fertilized with inorganic fertilizer with and without sulfur, with farmyard manure or from unfertilized plots. Genetic profiling of 16S rRNA gene fragments (DGGE) from the wheat rhizospheres revealed that the level of inorganic sulfate in the inorganic fertilizer was correlated with changes in the general bacterial community structure and the beta-proteobacterial community structure in particular. Community analysis at the functional gene level (asfA) showed that 40% of clones in asfAB clone libraries were affiliated to the genus Variovorax. Analysis of asfAB-based T-RFLP fingerprints showed significant differences between sulfate-free treatments and those where sulfate was applied. The results suggest the occurrence of desulfonating bacterial communities that are specific to the fertilization regime chosen and that arylsulfonates play a major role in rhizobacteria sulfur nutrition. The genus Variovorax has been repeatedly identified both in this study and in a previous study in barley rhizosphere, where it dominated the desulfonating community in the wheat rhizosphere. This underlines its importance for terrestrial sulfonate mineralization, especially in cereal rhizospheres.
Introduction

In 1970 nearly 80% of the sulfur assimilated by plants in the United Kingdom originated from air pollutants, especially from atmospheric SO$_2$ (Zhao et al., 2003). Reductions in the use of coal, and the introduction of advanced filtering techniques in the 1990’s have led to a reduction in atmospheric pollution, but as a consequence, 11% of British agricultural land has a high risk of sulfur deficiency (McGrath and Zhao, 1995). Sulfur is essential not only for plants, but for all living organisms, mainly as the proteinogenic amino acids cysteine and methionine. Its redox characteristics also make it important in many enzymatic electron transfer processes and in protection against oxidative stress, and its metal-chelating properties are used by plants and microorganisms to deal with heavy metal stress (Meyer and Hell, 2005; Sharma and Dietz, 2006). Although plants require inorganic sulfate for assimilation into cysteine, most of the sulfur in soils is in an organically bound form - 60-90% of the sulfur in typical soil inputs such as decaying plant material consists of carbon-bound sulfur (Zhao et al., 1996), while sheep dung contains about 80% carbon-bound sulfur (Williams and Haynes, 1993). The oxidized carbon-bound sulfur fraction has been equated to the sulfonate pool in both chemical and spectroscopic investigations (Autry and Fitzgerald, 1990; Zhao et al., 2006), and its dynamics have been examined in short term soil incubation experiments (Ghani et al., 1992, 1993). The sulfonate fraction was found to be mineralised faster than other soil sulfur fractions, including the sulfate ester pool, suggesting that carbon-bound soil sulfur is quantitatively important for plant sulfur nutrition (Ghani et al., 1992). The importance of the sulfonate-sulfur has been confirmed in K-edge XANES spectroscopic studies on soils from the Broadbalk long-term experiment at Rothamsted, U.K. (Zhao et al., 2006). Microbial activity is thought to be critical in mineralization of sulfonate-sulfur as part
of the soil sulfur cycle (Kertesz and Mirleau, 2004; Kertesz et al., 2007), but there has
been little functional analysis of which specific microorganisms are most important in
this process and functional studies of soil sulfur metabolism have concentrated on
microbial sulfatase activities (Freeman and Nevison, 1999; Klose et al., 1999; Taylor
et al., 2002; Knauff et al., 2003; Vong et al., 2003).

A variety of bacteria can use aliphatic and aromatic sulfonates as a source of
carbon, nitrogen or energy (Cook et al., 1999; Cook and Denger, 2002; Denger et al.,
2004). Utilization of sulfonates as a sulfur source, by contrast, has been best studied in
the sewage sludge isolate *Pseudomonas putida* S-313, which can desulfurize a broad
range of aromatic and aliphatic sulfonates (Zürrer et al., 1987; Vermeij et al., 1999).
The desulfurization reaction is catalysed by a two-component FMNH$_2$-dependent
monooxygenase system consisting of the SsuD monooxygenase and SsuE FMN
reductase. Cleavage of sulfur from aromatic sulfonates requires the additional
reductase/ferredoxin couple AsfA and AsfB (Vermeij et al., 1999). Expression of the
corresponding genes is regulated *in vitro* by the sulfur supply to the cell, and is
repressed in the presence of inorganic sulfate (Vermeij et al., 1999). Recently, the
desulfurization of aromatic sulfonates has been studied in *Cupriavidus metallidurans*
and the rhizobacteria *Variovorax paradoxus* and *Acidovorax* sp. (Schmalenberger and
Kertesz, 2007) and a great diversity of *asfA* orthologues has been identified in barley
rhizospheres (Schmalenberger and Kertesz, 2007).

The rhizosphere is often regarded as a “hot spot” where the metabolic activity
of bacteria can increase up to one hundred-fold compared to bulk soil (Curl and
Truelove, 1986). The rates of sulfur cycling between organic and inorganic forms is
also greatly enhanced in the presence of crop plants (Castellano and Dick, 1991)
suggesting that the rhizosphere environment may stimulate specific groups of
rhizobacteria to carry out organosulfur mineralization processes, especially when inorganic sulfate levels are low. The Broadbalk long-term field experiment makes an ideal system with which to examine the microbial specialist species that are important for sulfur cycling in the rhizosphere of wheat. It was set up by Lawes and Gilbert in 1843 in order to study the impact of inorganic fertilizers (“mineral manures”), and winter wheat (*Triticum aestivum* L.) has been cultivated continuously at the site since then (Jenkinson, 1991). The plots receive different mineral fertilization regimes, and in 2001 a regime with sulfur free mineral fertilizer application was introduced. Several recent studies have looked at the sulfur content of Broadbalk soils (Knights et al., 2000; Knights et al., 2001; Zhao et al., 2003; Zhao et al., 2006), but nothing is yet known about the influence of the sulfur-free fertilizer application on the microbial community. The study of microorganisms in the Broadbalk long term experiment has so far focused mainly on the cycling of nitrogen (Hutsch et al., 1993; Vos et al., 1993; Harrison et al., 1995) and on the level of total microbial biomass (Abaye and Brookes, 2006).

In this study we explore the diversity of cultivable bacteria able to metabolize sulfonates in Broadbalk soils, and examine the changes in microbial diversity (total bacteria and beta-proteobacteria) with changes in sulfur fertilization, using cultivation-independent methods. In a third approach we characterize differences in the diversity of the desulfonation gene *asfA* in different Broadbalk rhizospheres. Together, the three approaches suggest that the *Variovorax* and *Polaromonas* genera, and related members of the Comamonadaceae family, are functional specialists for sulfonate cycling in wheat rhizosphere.
Results

Variations in rhizosphere sulfur and sulfate content with crop treatment.

Four continuous-wheat fertilizer treatments on the Broadbalk long term field experiment at Rothamsted, UK, were investigated in this study. Three of these have received uniform treatments for many years (NIL, FYM, NPKS; see below), while the fourth plot (NPK) has had a sulfur-free fertilization regime since 2001, but receives similar levels of N, P and K to the NPKS plot. The concentrations of total sulfur and of extractable inorganic sulfate were measured in the rhizosphere-attached soil of winter wheat exposed to these different fertilization regimes (Table 1). Sulfate concentrations in the rhizosphere soils were lowest in the unfertilized treatment (NIL), while the inorganic fertilizer treatment without sulfate (NPK) had almost two-fold more, and the plots treated with farmyard manure (FYM) or with sulfate-containing fertilizer (NPKS) contained significantly higher levels of sulfate. However, although extractable inorganic sulfate is the most directly plant-available, it does not represent the overall sulfur content of each soil. Total sulfur for each rhizosphere was therefore also measured, using X-ray Fluorescence (XRF). Total sulfur was again lowest in the NIL and NPK treatments. Concentrations of total sulfur in the NPKS plot were slightly higher, but the difference between NPKS and NPK plots was not as significant as for the sulfate content. Although the sulfate content of the FYM plot was comparable to that of the NPKS treatment, the total sulfur level was nearly three times higher than for the non organic fertilization regimes, consistent with the manure treatment introducing large amounts of organic sulfur into the soil. The contribution of inorganic sulfate to the total sulfur content therefore varied from 4% (NIL) to 9% (NPKS).
Quantification and identification of cultivable desulfonating bacteria in wheat rhizospheres.

Changes in sulfate availability in the soil may well affect both the overall rhizosphere bacterial population, and the proportion of this population that can desulfurize sulfonated organic molecules such as toluenesulfonate (TS). The populations of cultivable rhizosphere bacteria that were able to grow with toluenesulfonate as sole sulfur source were quantified using an MPN approach. The number of toluenesulfonate (TS)-utilizing bacteria varied around $10^6$ per gram root fresh weight, with highest values in the NPK and NPKS treatments ($0.8x10^6$ in NIL, $2x10^6$ in NPK and NPKS, $0.6x10^6$ in FYM). For comparison, the total bacterial community in the rhizosphere was estimated by MPN analysis after growth in R2A medium, and varied around $10^8$ cells per gram root fresh weight, again with higher values in the NPK and NPKS treatments ($0.3x10^8$ in NIL, $1X10^8$ in NPK, $2x10^8$ in NPKS, $0.5x10^8$ in FYM).

The percentage of potential TS-utilizing bacteria within the cultivable rhizosphere population was highest in the NIL treatment with 2.4%, followed by the NPK treatment with 2%. By contrast, rhizosphere soils from the NPKS and FYM treated plots contained significantly lower proportions of TS-utilizing bacteria (1.4% for NPKS and 1.2% for FYM). This contrasts with the number of cultivable enterobacteria in rhizospheres from the NIL, NPK, NPKS and FYM treatments ($0.7x10^6$, $6x10^6$, $7x10^6$ and $4x10^6$ cfu per gram root fresh weight) or of *Pseudomonas* spp. (about $10^5$ cfu per gram root fresh weight). The number of bacteria capable of utilizing TS as a carbon source was below detection level.

In order to characterize the dominant cultivable TS-utilizing bacteria in the rhizosphere, bacteria were recovered from the highest dilutions that still yielded...
significant growth with TS ($A_{600} > 0.2$) in the MPN analysis. Individual isolates were tested for the ability to grow with TS as sulfur source, and the presence of $asfA$ orthologues was investigated using PCR. Four isolates were found to be capable of reproducible growth *in vitro* with TS as sulfur source, and were characterized further by sequencing their 16S rRNA and $asfA$ genes. Isolate P6E3 was isolated from an NPK plot rhizosphere, and belonged to the genus *Polaromonas*, while isolates P1D3 (isolated from the NIL plot) and P7G10 (isolated from plot NPK), and P7D1 (isolated from plot NPK) were affiliated to the genus *Variovorax*. Interestingly, strains with identical 16S rRNA and $asfA$ gene sequences to isolate P7D1 were also identified from the NIL plot and twice from different parts of the NPKS plot. Analysis of the culture supernatant of isolates P7G10, P7D1 and P6E3 after growth with TS showed that desulfonation of TS by these strains produced $p$-cresol, as previously observed for *P. putida* S-313 (Zürrer et al., 1987). Isolate P1D3 grew poorly in shake flasks, and cresol could not be detected in the culture supernatant.

A range of different sulfur sources were supplied to the *Variovorax* (P7D1, P7G10) and the *Polaromonas* (P6E3) isolates during growth in microtiter plates, and the strains revealed distinct utilization patterns (Table 2). Isolate P7D1 grew well on all 23 sulfur sources tested, including aryl- and alkylsulfonates, sulfate-esters and amino acids, though it displayed reduced growth rates with DMSO and nitrocatecholsulfate. Isolate P7G10 grew similarly, but showed negligible growth with sodium dodecyl sulfate. By contrast, the *Polaromonas* isolate P6E3 grew at lower rates than the two *Variovorax* isolates, and was only able to desulfurize 13 of the 23 supplied sulfur sources, though these also included several different types of sulfur moieties (Table 2).
Bacterial rhizosphere community structures change with the levels of sulfur fertilization applied.

The overall bacterial community structure in the field plots was characterised by profiling of partial 16S rRNA genes to yield complex community fingerprints (Figure 1A). Visual analysis of the profiles shows clear separation between NIL/FYM, and the plots subjected to inorganic fertilizer treatment. This was confirmed by cluster analysis with Phoretix UPGMA software, which revealed a separation of all four treatments including the NPK and NPKS treatments, except that samples NPKS1 and 2 clustered distantly with the FYM profiles (Figure 2A). There is clear evidence for a pair wise structuring of the results, with replicate profiles from section 9 (replicates 1, 2) being highly similar, as were replicates from section 1 (replicates 3, 4). This suggests some heterogeneity within the Broadbalk field, since sections 1 and 9 are approximately 300 m apart; replicate sampling sites within each section were separated by about 10 m.

The desulfonating isolates described above all belonged to the beta-proteobacteria, as did those obtained in a previous study (Schmalenberger and Kertesz, 2007). DGGE community fingerprints were therefore generated using 16S primers specific for this clade (Figure 1B). The variability between the profiles within a treatment was higher than in the total bacterial community, and although cluster analysis showed the NPK plot to be significantly separate from the other three plot types (Figure 2B), there were considerable differences between the profiles of NPK1, 2 and NPK3, 4 (section 9 and 1 of the field, respectively). Samples NPK1 and 2 formed a separate branch, and a number of unique bands could be seen to be enhanced in the profile. These characteristic bands were sequenced from the NPK1 profile either directly or after cloning, and identified as *Variovorax*, *Polaromonas* and *Janthinobacterium*-related
(clones 22, 27 and direct sequenced bands: Figure 1B). Additionally, bands that seemed to occur in all treatments were sequenced after cloning from the NPK1 profile and directly from the NIL1 profile.

The overall bacterial community in sample NPK1 was further characterized by sequencing of 21 rhizosphere 16S gene sequences, revealing a range of phylogenetically distinct bacteria to be present (Figure 1A). By contrast, when a specific primer was used to amplify and sequence bands from the beta-proteobacterial community of plot NPK1 (Figure 1B) the sequences obtained were exclusively from this particular phylogenetic group, confirming that the primer used was highly selective (25 out of 25 sequences). A third of the beta-proteobacterial sequences did not match closely with sequences from known isolates (below 93% identity). The genus *Variovorax* occurred three times, *Nitrospira, Janthinobacterium, Polaromonas* and *Paucibacter* (only 96% similarity) were found twice each.

*Variation in asfAB gene diversity in wheat rhizospheres under different sulfur fertilization regimes.*

The *asfA* gene plays an important role in desulfonation processes in the rhizosphere (Schmalenberger and Kertesz, 2007), and so the response of *asfA* gene diversity to changes in sulfur fertilization was examined. Gene libraries of *asfAB* were constructed (containing 1.3kb of *asfA* and 0.14kb of *asfB*) with samples from each fertilization regime, and a total of 124 individual clones were screened, yielding 58 distinct *asfAB* genotypes (coverage:66%). Genotypes were sequenced that occurred more than once in the clone libraries (seventeen in total). Terminal restriction fragment length polymorphism (T-RFLP, (Liu et al., 1997)) was used to examine their relative frequency in differently treated rhizospheres.
Community \textit{asfAB} profiles were first obtained for all four samples from each fertilization regime, using T-RFLP with the forward primer labelled with the FAM fluorophore and the reverse primer labelled with HEX (Figure 3). Principal component analysis (PCA) revealed that the \textit{asfAB} signals in the 16 samples were quite dispersed, with the first three principal components accounting for 41 and 44\% (FAM-forward and HEX-reverse) of all signals when signals were treated as binary data. With the labelled forward primer it was found that NPKS, NPK1-2 and NPK3-4 signals were significantly different, and these differences were reproducible with binary and relative abundance data sets. Very similar results were obtained when the data were analysed by detrended correspondence analysis using DECORANA (Clampitt, 1985).

The occurrence of individual \textit{asfAB} genotypes across fertilization regimes was then compared using pooled rhizosphere samples for each of the four treatments. The labelled and digested PCR products from the four replicates from each treatment were mixed, and the profiles were compared with those of individual genotypes (i.e. \textit{in vitro} rather than \textit{in silico} analysis) (Figure 3). The genotypes for which co-migrating bands showed the greatest change with fertilization treatment were genotypes w1, w4 and w38, which were much more abundant in the NPK sample. Additionally, genotype w48 was only abundant in the NIL treatment, genotype w3 was enhanced in the FYM treatment, and genotype w25, which was almost absent in the FYM sample showed a progressive increase in intensity from NIL to NPK to NPKS. The co-migrating T-RF signals for genotypes: w16, w20, w51a, w51b, w54, and w55 occurred at positions where the \textit{asfAB} profiles showed no variability between the fertilization regimes. Several genotypes appeared to be correlated with two treatments, including genotype w51c (enhanced in the NIL and FYM treatments),
genotype w19 (NIL and NPK), and w11 and w42 (NIL and NPKS) (Figure 3). Quantitative PCR confirmed that the *Variovorax paradoxus*-like genotypes as a whole (w51, w54, w55, w25) did not significantly change in quantity in rhizospheres from the different fertilization treatments (data not shown). To check the relative importance of rhizosphere and bulk soil in the desulfonation process, we also compared the relative abundance of *Variovorax asfA* genes in each compartment (measured by quantitative PCR with *asfA*-specific primers and with eubacterial 16S rRNA gene primers). Bulk soils were sampled in both July 2005 and June 2006, but the former samples were lost, and data from the latter samples are reported here. The relative abundance of *Variovorax asfA* was 10-100 fold higher in the wheat rhizosphere than in the root-free bulk soil, with the NIL treatment showing the least difference between the two, and the NPKS treatment showing the greatest (Figure 4). This confirms that under the conditions tested here the rhizosphere plays a more important part in sulfonate cycling by *Variovorax* than does the bulk soil.

*AsfA sequences from pure cultures and environmental DNA*

The deduced AsfA peptide sequences obtained from four pure cultures and clones from 17 distinct genotypes were incorporated into a consensus AsfA tree obtained from a previous study (Schmalenberger and Kertesz, 2007), in order to detect affiliations to other isolates and to sequenced *asfA* genotypes from the rhizosphere of barley (Figure 5). Most of the obtained sequences fell into two main groups. The first included the AsfA of *Variovorax paradoxus* isolates P7D1 and P7G10, which clustered close to the AsfA of the *V. paradoxus* type strain, while *Variovorax* isolate P1D3 was slightly more removed (92% identity to the *V. paradoxus* AsfA). About 40% of all the AsfA sequences from clones obtained in this study (49 out of 124
clones, genotypes w51a-c, w55, w54, w25) also clustered within the \textit{V. paradoxus} group (Figure 5). Genotype w48 (4 clones) represented 3% of the clones and was more distantly \textit{Variovorax} affiliated (85% identity to the \textit{V. paradoxus} AsfA).

The second main group of AsfA sequences obtained was related to a large cluster of AsfA sequences retrieved from barley rhizospheres. This group included the \textit{Polaromonas} isolate (P6E3) AsfA sequence, but also 4% of the other genotypes obtained (5 clones out of 124, including genotypes w19 and w1, the latter was strongly represented in the NPK rhizosphere). Genotypes w11 and w42 (7 clones) represented about 6% of the AsfA clones and clustered in a deep branch of AsfA clones from the barley rhizosphere. All other sequenced genotypes were represented less frequently and were found across other smaller sub-branches of AsfA. For these genotypes, a cultivated representative has only been obtained for genotype w12, which had a high similarity (95%) to an \textit{Acidovorax} strain (WB62) isolated from winter barley (Schmalenberger and Kertesz, 2007).
discussion

the capability of bacteria to utilize sulfonates as a sulfur source is an important part of the biogeochemical sulfur cycle, since it allows the mineralization of carbon bound sulfur. the desulfonation of aromatics has only recently been linked to the presence of asfA genes in environmental isolates (Schmalenberger and Kertesz, 2007). in this report we have investigated the influence of distinct sulfur fertilization regimes in a long term field experiment with winter wheat (Broadbalk) on the total rhizobacterial community, the beta-proteobacterial community and the arylsulfonate desulfonating rhizobacterial (asfA) community. different fertilization regimes resulting in differences in inorganic sulfate levels had a strong influence on the rhizosphere bacterial communities present, though effects of field heterogeneity were also observed. Variovorax species and other members of the Comamonadaceae were identified as the most important players in cycling of sulfonate-sulfur, using both cultivation-dependent and cultivation-independent techniques.

Recent years have seen increased emphasis on the importance of sustainable agriculture, and the effect of fertilization regime on soil quality and soil microbial populations has been investigated in several long-term field studies (Marschner et al., 2003; Bohme et al., 2005; Widmer et al., 2006). The Broadbalk experiment is unique in including a treatment that has had no sulfur added since 2001, allowing the effect of sulfate depletion to be evaluated in this study. Four years after commencement of the treatment, total sulfur in the sulfate-depleted rhizosphere soil was found to be reduced to levels comparable to those found in the unfertilized plot, and the levels of inorganic sulfate were also reduced by 50% compared to the fertilized plot with sulfate (Table 1). It has been noted before that soil sulfate levels vary considerably during the growing season, and soil sulfate is therefore not necessarily a suitable
prognostic tool for plant sulfur nutritional status (Bloem et al., 2001). The data presented here do, however, confirm that the withdrawal of sulfate from the sulfur-free treatment has led to a reduction in soil sulfate and total sulfur content, despite the fact that no significant changes in grain yield have been seen between the sulfur-free and sulfur replete plots, up to and including harvest 2006 (Rothamsted Research, 2007).

In general, the rhizosphere contains lower concentrations of nutrients like nitrate, phosphate and sulfate than the bulk soil, since plants and microorganisms compete for these elements in this metabolically active habitat (Hinsinger et al., 2005). The region immediately surrounding the root is characterized by a phosphate-depletion zone that may be several millimetres wide (Jones et al., 2004; Hinsinger et al., 2005), and a similar effect has also been observed for sulfate, with sulfate-starvation-induced bacterial genes being strongly expressed on the rhizoplane of Arabidopsis plants (Fellows and Kertesz, unpublished). Under sulfate limited conditions, this depletion zone is expected to be enhanced, selecting for organisms in the rhizosphere microbial community that are specialized in the desulfurization of organic sulfur sources such as sulfonates. The rhizosphere bacterial communities observed in the different plots were distinct from each other (Figures 1, 2), with rhizosphere DGGE fingerprints from the sulfur-free plot clustering separately from the other plots (Fig 2). Changes in rhizobacterial community structure are well known to react to changes in land use, with significant effects seen for such factors as changes in soil type and plant species, including transgenic plants (Schmalenberger and Tebbe, 2002; Costa et al., 2006), herbicide application (Seghers et al., 2005), and fertilization (Clegg et al., 2003; Seghers et al., 2003; Seghers et al., 2005; Innerebner et al., 2006; Enwall et al., 2007; Stark et al., 2007). Importantly, the effects seen on
the total community level often reflect changes in the population of specific phylogenetic or functional groups. Actinobacteria and ammonia oxidising bacteria are important players responding to changes in grassland management regimes (Clegg et al., 2003), and other recent studies have shown an effect of organic and mineral fertilizers on actinobacteria (Stark et al., 2007), alpha-proteobacteria (Stark et al., 2007), ammonia oxidising bacteria (Innerebner et al., 2006; Enwall et al., 2007) and methanotrophs (Seghers et al., 2003; Seghers et al., 2005) in soil. With the Broadbalk soils we have also observed a general effect of fertilization and manure application on the bacterial community (Fig 1, 2), with bacterial communities from the different treatments clustering separately. It is important to note that because of the high number of species in soils, individual bands in “universal” DGGE fingerprints tend to consist of a high number of distinct sequences that co-migrate (Schmalenberger and Tebbe, 2003), and variability might be covered up. Cluster analysis therefore probably underestimates the differences between bacterial communities (here 80-90% similarity, Figure 2A). Considerable changes were seen in the beta-proteobacterial community in response to the sulfur fertilization regime (Figures 1, 2), with strong increases in bands corresponding to *Variovorax* species and members of related genera in the Comamonadaceae family. Interestingly, the beta-proteobacterial rhizosphere fingerprints obtained for the farmyard manure (FYM) and unfertilized (NIL) plots also appeared related (Figure 2B). However, this may well be due to factors concerning nitrogen limitation, since previous studies have shown that grain yields on the FYM plot are lower than those supplied with inorganic nitrogen, and the plants show expression of genes related to a nitrogen limitation response (Lu et al., 2005).
Cluster analysis of AsfA fragments from isolates and environmental DNA (molecular isolates) from this study revealed the significance of the genus *Variovorax* among the desulfonating rhizobacteria. Likewise, T-RFLP fingerprinting of *asfAB* showed a high abundance of environmental T-RFs that co-migrate with genotypes closely affiliated to *V. paradoxus* (Figure 3; w25, w51, w54, w55). These results confirm the importance of this genus for soil desulfonation that was reported in a previous study (Schmalenberger and Kertesz, 2007), in which three desulfonating isolates of *Variovorax paradoxus* (SB8, SB10, SB31) and several *Variovorax* like AsfA sequences were found in the rhizosphere of barley. Considerable diversity of AsfA sequences was found in the barley rhizosphere, with four main groups of *asfA* gene products found (Schmalenberger and Kertesz, 2007). The diversity of AsfA in wheat rhizospheres was similar, dominated by two of the same main groups observed previously (Figure 5). One of these groups is clearly associated with AsfA sequences derived from *Variovorax* strains, while the second major group is associated with a strain of *Polaromonas* obtained during the present study. *Polaromonas*-like AsfA did not dominate the clone library obtained from wheat rhizosphere, making up 4% of the clone library or 6% of the sequenced genotypes. However, T-RFLP analysis of the environmental DNA revealed that two of the *Polaromonas*-like *asfA* genotypes obtained were enhanced in the sulfur-depleted rhizospheres (Figure 3), suggesting that while the genus *Polaromonas* may play a less important role in wheat than in barley rhizosphere, it responded quite strongly to sulfur limitation conditions in the wheat rhizosphere. T-RFLP analysis also identified that genotypes w3 and w4 responded strongly to the fertilization treatment NPK (Figure 3B), though unfortunately these genotypes clustered in a branch of the AsfA tree that has not yet been affiliated to a cultivated representative. Identification of this clade in the future may give some vital
clues about the mechanisms that promote desulfonating rhizobacteria in soils of low sulfur levels.

Desulfonating bacteria in soil and rhizosphere can enhance plant growth in sulfate-limited soils by supplying available sulfur for plant growth, in an analogous manner to the way diazotrophic bacteria promote plant growth through fixing molecular nitrogen and hence making it available for plants. For nitrogen fixation the link between microbial specialists and ecosystem function is clear, but this is not true for many other functions, since they may carried out by a variety of microbes (Zak et al., 1994). This study suggests that the Comamonadaceae are the key players for mineralization of carbon bound sulfur in cereal rhizospheres, allowing cycling of soil sulfur between organic and inorganic forms, with the Variovorax genus being the key desulfonating genus in wheat rhizospheres. Polaromonas and Acidovorax strains also seem to be important as desulfurizing microorganisms in cereal rhizospheres. The presence of these bacteria may contribute to the ability of plants to grow in soils with low sulfur availability, and investigations into the plant-growth promoting properties of the isolates described here are currently being undertaken.

**Experimental procedures**

*Microorganisms, sampling, isolation and culture conditions.*

Bacterial isolates analysed in this study were cultivated aerobically at 25ºC in a minimal medium MM (Beil et al., 1995) with succinate, glucose and glycerol as carbon source (10 mM each) and on agarose plates (14 g/l, Eurobio, France) with different sulfur sources (250 μM). Wheat rhizosphere samples (Hereward cultivar) were collected from the Broadbalk long term experiment at Rothamsted, United Kingdom, (51°49’N 0°21’W (Jenkinson, 1991)). The Broadbalk experimental field is
a grid divided into 20 longitudinal strips that are subjected to different fertilization
regimes, and 10 transverse sections on which insecticide, weedkiller and crop rotation
treatments are applied. Two of the sections (1 and 9, at opposite ends of the test field)
are cropped with continuous wheat, and these were used to provide field replication of
the treatments. Wheat rhizosphere samples were collected in late July 2005 from each
of these two sections, choosing strips had been fertilized with farmyard manure (plot 2.2, FYM, receiving c 250 kg/ha N per year), inorganic fertilizer, (plot 9, NPKS, receiving 192 kg/ha N per year), sulphur-free inorganic fertilizer (plot 14, NPK, receiving 192 kg/ha N per year), and an unfertilized plot (plot 3, NIL). Four samples were collected for each treatment, two in section 9 (replicates 1 and 2, approx 10 m apart), and two in section 1 (replicates 3 and 4). Wheat plants (3 adjacent plants for each sample) were sampled three rows in from the edge of each strip, by loosening the roots with a fork and removing the plant together with root ball. The root ball was placed immediately on ice and taken to the laboratory for further analysis. Bulk soils were sampled adjacent to the plants in July 2005 and in June 2006, by pushing a sterile plastic tube into the soil to remove a sample, and immediately sealing the tube with the screw cap.

For rhizosphere analysis, the loosely attached soil was shaken off each sample, and bacteria were then extracted from the remaining attached soil (defined as rhizosphere soil by the “pull and shake” method) together with the root. Root and rhizosphere soil were suspended in sterile phosphate buffered saline (PBS: 20 ml per 3 g of root (FW)), shaken on a Genie roto-shaker (Scientific Industries, NY) for 30 min at 4°C. Soil bacteria able to desulfurize toluenesulfonate were identified by cultivating in most probable number (MPN) microtiter plates containing MM medium with toluenesulfonate (TS) as sole sulfur source (0.25 mM) on a rocker for seven days.
(200 μl, 10 replicates, 10⁻¹ to 10⁻⁸ dilutions). Total cultivable rhizosphere populations were quantified similarly in R2A liquid medium. *Pseudomonas* and enterobacteria in soil samples were quantified by plating on *Pseudomonas* agar (Fluka) and MacConkey agar (Oxoid), respectively, and growth for four days at 30°C. Individual toluenesulfonate-utilizing strains were identified for further study from the highest dilutions showing growth in MM-MPN microtiter plates. Strains were replated on MM agarose plates with TS as the sole sulfur source and single colonies were picked for subsequent analysis. Utilization of different sulfur sources by single bacterial isolates was tested during growth in microtitre plates with a range of 23 different sulfur sources including sulfonates and sulfate-esters, as described previously (Schmalenberger and Kertesz, 2007).

**Measurement of sulfate and total sulfur**

The total sulfur content of soils from each of the four fertilization regimes was measured by X-Ray Fluorescence (XRF) spectrometry (AXIOS wave-length-dispersive XRF instrument, Panalytical, Cambridge, UK). Four samples from each regime were dried (80°C), combined to obtain 14 g of dried rhizosphere soil, which was ground in a tungsten ball-mill and pressed to a pellet for analysis. Inorganic sulfate was measured using the barium chloride method (Barrow, 1961; Tabatabai, 1974), adapted to small scale work by using 1 ml of supernatant of washed soil and 0.1 ml of 0.5N HCl and BaCl₂-gelatin solution.

**DNA extraction and PCR conditions**

Suspensions of rhizosphere bacteria from plant roots were obtained by extraction into phosphate-buffered saline (PBS) (Schmalenberger and Kertesz, 2007). DNA was extracted from these bacterial suspensions using the FastDNA extraction kit for soil (QBiogene, Irvine, CA). The cell suspensions were collected by centrifugation (3000
g, 15 minutes, 4°C), resuspended in 2 ml of sterile PBS and aliquots (0.2 ml) were transferred to lysing matrix tubes E. DNA extraction was carried out using a bead beating protocol according to the manufacturer, and DNA was eluted with 0.1ml of sterile, deionised water. Genomic DNA from individual isolates was extracted for PCR analysis with a quick lysis protocol as published previously (Schmalenberger et al., 2001).

PCR was carried out in a T1 thermocycler (Biometra, Goettingen, Germany) in a final volume of 25 µl. Amplification of asfAB (1.3kb asfA and 0.14kb asfB) from pure cultures was carried out with Expand polymerase/buffer (Roche, Basel, Switzerland), 1.5 mM MgCl₂, 5% v/v DMSO, 0.5 µM primers, and each dNTP at 200 µM as described previously (Schmalenberger and Kertesz, 2007). For cloning asfAB and for T-RFLP analysis, PCR amplifications from environmental samples were carried out with HotMaster Taq polymerase, using the buffer and Enhancer provided by the manufacturer (Eppendorf, Germany), 5% v/v DMSO and a touch down protocol as described previously (Schmalenberger and Kertesz, 2007). PCR amplifications from environmental samples for 16S rRNA gene DGGE analysis were carried out with 1.0 U of HotMaster Taq polymerase (Eppendorf) in a total volume of 50 µl, using primers GC-341F and 518R (Muyzer et al., 1993) and a touch down PCR protocol (Cunliffe and Kertesz, 2006). Specific PCR for DGGE analysis of the beta-proteobacterial community was carried out using a nested-PCR approach. The initial PCR step used primers 27F (Lane, 1991) and 865R (GGTCAACTTCACGCGTTA), and 1 ng of the purified amplification product then served as template for a second PCR using universal DGGE primers GC-341F and 518R (Muyzer et al., 1993).
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 (Cunliffe and Kertesz, 2006) with a denaturant gradient of 35 to 65% and electrophoresis for 17 h at 64V and 60°C. Profiles from wheat rhizosphere were prepared with 500 ng of DNA, while samples with defined, mixed species contained 50 ng of DNA per species/band. Gels were stained for 30 min with SybrGold (Invitrogen, Carlsbad, CA).

Selected bands were excised from the gel, washed in dH2O for 24 h at 4°C, and then crushed in 0.5 ml of dH2O. 100-fold diluted template served for reamplification using identical primers but lacking the GC-clamp. Single bands were sequenced directly as described previously (Cunliffe et al., 2006). When multiple bands were retrieved, the original PCR products were cloned, and the position of bands from individual sequenced clones was compared with the community profiles by electrophoresis on a denaturing gel after reamplification with DGGE primers carrying a GC-clamp.

Cloning of asfAB PCR products and genotyping

Primers asfAF2 and asfBtoA (Schmalenberger and Kertesz, 2007) were used to amplify fragments of asfAB from wheat rhizosphere extracts obtained from each of the four treatments studied. PCR products were purified with the Qiagen PCR purification kit, ligated into the pGEM-T easy vector (Promega) and transformed into E. coli DH5α. Recombinant plasmids containing an insert of the correct size were then reamplified with primers asfAF2 and asfBtoA for RFLP analysis. RFLP was carried out with 124 clones in total (NIL:32, NPK:31, NPKS:31, FYM:30), as described previously (Schmalenberger and Kertesz, 2007). Clones with a similar restriction pattern were classified as a single genotype or OTU, using the Phoretix advanced 1D software (Nonlinear Dynamics, Newcastle upon Tyne, UK).
Terminal restriction fragment length polymorphism (T-RFLP) analysis

Primers asfAF2 and asfBtoA (Schmalenberger and Kertesz, 2007) were modified with fluorescent labels FAM and HEX respectively and used to amplify fragments of *asfAB* from wheat rhizosphere extracts and from individual cloned *asfAB* fragments. The amplification products were purified (Qiagen PCR purification kit, Germany) and 200 ng DNA was digested with 10 U of *AluI* and *RsaI* (Fermentas) at 37°C for 12 h. Samples were mixed with the LIZ GS500 size standard (Applied Biosystems, Warrington, UK) and run on an automated sequencer (ABI prism 3130xl) as described previously (Singh et al., 2006). Fluorescence signals for the forward or the reverse labelled primers were detected on separate channels, and data analysis was carried out with GeneMapper 3.7 software. Peaks with heights of less than 2% of the total peak height were discarded from the analysis. Signals were treated as identical when the calculated size identity was equal or below a mismatch of two base pairs. Statistical analysis of the wheat rhizosphere communities was carried out by principal component analysis (PCA) using Genstat9 (VSN International Ltd, Hampstead, UK) and correspondence analysis (detrended CA) using DECORANA.

DNA sequence analysis

OTUs (genotypes) of *asfAB* that were represented with more than one clone were sequenced in order to obtain the sequence information of *asfA* orthologues. Sequence fragments of *asfB* were truncated in order to obtain just the *asfA* fragment and were subsequently imported into an *asfA* database generated previously (Schmalenberger and Kertesz, 2007) using the ARB software package (www.arb-home.de). Phylogenetic trees for the encoded peptide sequences (AsfA) were calculated with the Maximum Likelihood (Dayhoff model), Maximum Parsimony, and Distance Matrix (Dayhoff model) methods, and a consensus tree was generated (Figure 5).
16S rRNA gene sequences from isolates obtained in this study were imported into the 2004 SSU database of ARB, together with closely affiliated sequences from Genbank identified using BLAST ((Altschul et al., 1990)) and FASTA3 ((Pearson and Lipman, 1988). The sequences were aligned, and the sequences with the highest similarity were identified. Affiliation of sequences from DGGE bands from this study were identified using BLAST and FASTA3.

Analytical methods

Analysis of culture supernatants was carried out by reversed-phase HPLC using a C18 column, with methanol gradient in 10 mM potassium phosphate buffer, as described previously (Vermeij et al., 1999).

Nucleotide sequence accession numbers

Isolates were identified by amplification and sequencing of the 16S rRNA genes with primers 27f and 1492r (Lane, 1991). The nucleotide sequences are available from Genbank, with the accession numbers AM492161-AM492164. Sequences of asfA (about 75% of the complete gene) obtained from isolates are available from Genbank with accession numbers AM494471-AM494474, while those obtained from molecular isolates have the accession numbers AM494454-AM494470.

Acknowledgements

We would like to thank Richard Preziosi for his assistance in the correspondence analysis. This work was supported by the Natural Environment Research Council (NERC). Rothamsted Research receives grant-aided support from the Biotechnology and Biological Science Research Council (BBSRC) of the UK. Brajesh Singh is funded by the Scottish Executive, Environment and Rural Affairs Department (SEERAD).
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### Table 1. Sulfur and total sulfur content in rhizosphere soil

<table>
<thead>
<tr>
<th>Fertilization regime</th>
<th>Sulfate (µg/g soil)</th>
<th>Total sulfur (µg/g soil)</th>
<th>Sulfate as proportion of total sulfur (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unfertilized (NIL)</td>
<td>16.1</td>
<td>406.2</td>
<td>4.0</td>
</tr>
<tr>
<td>Inorganic fertilizer, S free (NPK)</td>
<td>27.9</td>
<td>416.2</td>
<td>6.7</td>
</tr>
<tr>
<td>Inorganic fertilizer with sulfate</td>
<td>51.4</td>
<td>549.3</td>
<td>9.4</td>
</tr>
<tr>
<td>(NPKS)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Farmyard manure (FYM)</td>
<td>57.7</td>
<td>1388.9</td>
<td>4.2</td>
</tr>
</tbody>
</table>

a) Rhizosphere soils were sampled from 2-3 plants at four field locations for each treatment. Total sulfur was measured by XRF, inorganic sulfate was measured turbidimetrically.
Table 2. Utilization of different sulfur sources by *Variovorax* isolates P7D1 and
P7G10 and *Polaromonas* isolate P6E3

<table>
<thead>
<tr>
<th>Arylsulfonates (arenesulfonates):</th>
<th>P7D1</th>
<th>P7G10</th>
<th>P6E3</th>
</tr>
</thead>
<tbody>
<tr>
<td>o-Aminobenzenesulfonate</td>
<td>++</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>Nitrobenzenesulfonate</td>
<td>++</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>Naphthalene-2-sulfonate</td>
<td>++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Toluenesulfonate</td>
<td>++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Benzenesulfonate</td>
<td>++</td>
<td>++</td>
<td>+</td>
</tr>
</tbody>
</table>

| Alkylsulfonates (alkanesulfonates): |
|----------------------------------|------|-------|------|
| Methanesulfonate                 | ++   | +     | ++   |
| Pentanesulfonate                 | ++   | ++    | +    |
| Dodecanesulfonate                | ++   | +     | -    |
| 3 Morpholinopropanesulfonate     | ++   | ++    | +    |
| Taurine                          | ++   | +     | -    |
| Cysteate                         | ++   | ++    | +    |

<table>
<thead>
<tr>
<th>Sulfate ester:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methylsulfate</td>
</tr>
<tr>
<td>Nitrocatecholsulfate</td>
</tr>
<tr>
<td>Sodiumdodecylsulfate</td>
</tr>
<tr>
<td>Nitrophenolsulfate</td>
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<table>
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<tr>
<th>Amino acids:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutathione</td>
</tr>
<tr>
<td>Cysteine</td>
</tr>
<tr>
<td>Homocysteine</td>
</tr>
<tr>
<td>Methionine</td>
</tr>
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<table>
<thead>
<tr>
<th>Other sulfur sources:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium sulfate</td>
</tr>
<tr>
<td>Potassium thiocyanate</td>
</tr>
<tr>
<td>Dimethylsulfone</td>
</tr>
<tr>
<td>Dimethylsulfoxide</td>
</tr>
</tbody>
</table>

Cells were grown aerobically in minimal medium with 250 μM sulfur source. Growth was monitored as optical density at stationary phase: ++, growth to an OD$_{600}$ above 0.4; +, significant growth below an OD$_{600}$ of 0.4; -, no growth compared to the sulfur-free control.
Figure Legends

Figure 1. DGGE of 16S rRNA gene fragments from the general bacterial community (A) and the beta-proteobacterial community (B), amplified from the rhizosphere of winter wheat (Broadbalk long-term experiment, Rothamsted, UK) from the following fertilization regimes. NIL – no fertilizer application; NPK – inorganic fertilizer without sulfur; NPKS – inorganic fertilizer with sulfate; FYM – farmyard manure. Replicates 1 and 2 originated from section 9 and replicates 3 and 4 originated from section 1 of the Broadbalk experiment. Sequence information from selected signals was obtained through cloning and sequencing (clone number in brackets) or via direct sequencing (underlined text). Marker lanes (M) contained 16S rRNA gene sequences fragments retrieved from *Variovorax paradoxus*, *Rhodococcus erythropolis* and *Variovorax* like isolate P1D3 (two signals).

Figure 2. Dendrograms representing UPGMA analysis of the DGGE profiles shown in Figure 1 for the general bacterial community (A) and the beta-proteobacterial community (B) of Broadbalk rhizosphere soils. Profiles were evaluated by similarity analysis (Phoretix). Details of individual treatments are given in Figure 1.

Figure 3. T-RFLP profiles of *asfAB* amplified from the rhizosphere of winter wheat. A. *asfAB* profiles (position 1-700 *asfA*) visualised with fluorescent FAM-labelled forward primer. B. *asfAB* profiles (position 1-110 *asfB*, position >110 *asfA*) visualised with fluorescent HEX-labelled reverse primer. Fertilization regimes are detailed in the legend to Figure 1. Positions of *in vitro* co-migrating *asfAB* genotypes obtained by cloning are indicated with arrows.
Figure 4. Relative abundance of *Variovorax asfA* genes in wheat rhizospheres and in bulk soil. Hatched bars – rhizospheres, unhatched bars – bulk soil. Gene abundance was measured by quantitative PCR, using either *Variovorax asfA*-specific primers or eubacterial 16S rRNA gene primers. Details of individual field treatments are given in Figure 1.

Figure 5. 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), maximum-parsimony and maximum likelihood (Dayhoff model) methods. Polytomic nodes connect branches where a relative order could not be determined unambiguously. Closed circles indicate highly supported maximum likelihood 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 wheat rhizosphere (w1-w55, bold) and the isolates from the wheat rhizosphere (bold, underlined). *Nostoc* and *Anabaena* each contain two homologues of *asfA* (Table 1), designated A and B.