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The LysR-type regulator SftR is involved in soil survival and sulphate ester metabolism in *Pseudomonas putida*

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Summary

Sulphate esters make up a large proportion of the available sulphur in agricultural soils, and many pseudomonads can desulphurize a range of aryl- and alkylsulphate esters to provide sulphur for growth. After miniTn*5* transposon mutagenesis of *Pseudomonas putida* S-313, we isolated 19 mutants that were defective in cleavage of the chromogenic sulphate ester 5-bromo-4-chloro-3-indoxylsulphate (X-sulphate). Analysis of these strains revealed that they carried independent insertions in a gene cluster that comprised genes for a sulphate ester/sulphonate transporter (*atsRBC*) a LysR-type regulator (*sftR*), an oxygenolytic alkylsulphatase (*atsK*), an arylsulphotransferase (*astA*) and a putative TonB-dependent receptor (*sftP*). The SftP protein was localized in the outer membrane, and the arylsulfphotransferase was identified as an intracellular enzyme. Expression of *sftR* was repressed in the presence of inorganic sulphate, and the *sftR* gene was required for the expression of *atsBC*, *atsRK* and *sftP–astA*. An *sftR* mutant was unable to grow with aryl- or alkylsulphate esters in laboratory media and showed significantly reduced survival compared with the parent strain during incubation in Danish agricultural and grassland soils. This effect suggests that sulphate esters are an important sulphur source for microbes in aerobic soils and highlights the importance of the microbial population in the soil sulphur cycle.

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Introduction

Owing to their surfactant properties, aliphatic sulphate esters such as sodium dodecyl sulphate (SDS) form part of a variety of synthetic formulations and are discharged into wastewater in considerable quantities. The metabolic pathways by which aliphatic sulphate esters are degraded have consequently been the subject of considerable study. The first step in their biodegradation is catalysed by alkylsulphatases, which liberate sulphate in a hydrolytic reaction to yield the corresponding alcohol (for a review, see Dodgson *et al*., 1982). A variety of bacterial isolates from sewage sludge and contaminated soils have been found to contain alkylsulphatase enzymes, which suggests that the widespread ability to degrade aliphatic sulphate esters might primarily represent an evolutionary answer to the challenge of large-scale anthropogenic release of such compounds.

However, alkylsulphatases have also been found to occur in a range of isolates from uncontaminated sites (White *et al*., 1985), and these enzymes probably catalyse desulphurization of naturally occurring sulphate esters. Compounds of this type may derive from plant and animal debris (e.g. polysaccharide sulphates, glycosaminoglycans) or from microbially catalysed incorporation of inorganic sulphate into humic material (Scherer, 2001). Studies on sulphur speciation in aerobic forest and agricultural soils show that a major fraction of the sulphur is present as organically bound sulphur, including a high proportion of sulphate esters (Fitzgerald, 1976; Autry and Fitzgerald, 1990; Scherer, 2001). This soil sulphate ester pool is a labile one (McLaren *et al*., 1985). Although sulphate esters cannot be used directly as sulphur sources by plants (Castellano and Dick, 1991), they are hydrolysed by microbial sulphatases to provide a source of plant-available sulphur that is almost as accessible for plants as adsorbed inorganic sulphate (Shan *et al*., 1997). The sulphatases concerned have been found to be partly associated with microbial biomass and partly exist free in the soil; the latter population probably represents extracellular microbial sulphatases (Klose and Tabatabai, 1999). Expression of sulphatases in agricultural soil is dependent on plant growth and on the cropping cycle (Klose *et al*., 1999) and, hence, seems to be linked to interactions between the microbe and the plant. *In vitro*, expression of most sulphatases is repressed in the presence of inorganic sulphate, and these enzymes therefore

form part of a broader sulphate starvation-induced stimulon (Kertesz, 1999).

In this study, we report the identification of a gene cluster encoding both sulphate ester desulphurization enzymes and sulphonate/sulphate ester uptake systems in *Pseudomonas putida* S-313. This strain is a representative of the broad group of soil pseudomonads that play a crucial role as plant growth-promoting bacteria in the rhizosphere. Expression of the genes involved in sulphate ester utilization is regulated by the LysR-type transcriptional activator SftR, which is synthesized when the cells grow with sulphate esters, sulphonates or methionine as the sulphur source. We demonstrate that survival of an SftR mutant is impaired in different soils and in the rhizosphere, implying that the utilization of sulphate esters plays a part in determining bacterial soil competence.

Results

Mutants of P. putida *that are unable to use arylsulphate esters as a sulphur source*

Bacterial desulphation of aromatic sulphate esters is usually catalysed by arylsulphatases. *P. putida* S-313 was able to grow with aromatic sulphate esters as sulphur sources (Beil *et al*., 1996), but Southern analysis of the *P. putida* S-313 chromosome with the arylsulphatase gene (*atsA*) of the closely related species *Pseudomonas aeruginosa* did not give a hybridization signal (Kahnert and Kertesz, 2000). In order to investigate the assimilatory pathway for aromatic sulphate esters in *P. putida*, we mutagenized *P. putida* S-313 with the miniTn*5*Km transposon and screened 5000 kanamycin-resistant exconjugant colonies for loss of the ability to desulphurize the chromogenic aromatic sulphate ester 5-bromo-4-chloro-3-indoxylsulphate (X-sulphate). Twenty-three of these colonies showed reduced or zero desulphurization of X-sulphate on plates. These were classified into different types according to the range of sulphur sources they were able to use during growth in liquid culture with a variety of organosulphur compounds as the sole sulphur source (Table 1). Selected strains from each group were then chosen for further genetic studies. All classes of mutants displayed similar growth to the wild type when cultivated with sulphate or cysteine, but were unable to grow in liquid culture with nitrocatechol sulphate as sulphur source. Classes I and III were deficient in growth with all sulphonates and sulphate esters tested and contain transposon insertions in the *ssu* gene cluster, which encodes a two-component sulphonatase and an uptake system for sulphonates (Kahnert *et al*., 2000). Classes II, IV and V grew normally with aliphatic sulphonates as sulphur source. Class V mutants were deficient in growth with 4-nitrocatecholsulphate (NCS), class IV mutants were unable to use *n*-hexyl sulphate or NCS, and class II mutants could not grow with either type of sulphate ester or with benzenesulphonate. The DNA regions flanking each transposon insertion were cloned by transposon rescue techniques, and sequence analysis revealed that miniTn*5*Km insertions in type II, IV and V mutants had taken place in the *ats–sft* gene cluster (Fig. 1; GenBank accession no. AF126201). This cluster contained seven open reading frames (ORFs), each preceded by a putative ribosome binding site, and the overall GC content was 65%. Sequence analysis indicated that the insertions had occurred in putative ORFs encoding a sulphotransferase (*astA*), a putative TonB-dependent receptor (*sftP*), an ABC-type transporter (*atsRBC*) and a regulator protein (*sftR*). The *sftR* mutant, strain PH18, was unable to grow with aliphatic and aromatic esters, but transformation of

Table 1. Growth phenotypes of *P. putida* S-313 miniTn*5* mutants affected in desulphation of 5-bromo-4-chloro-3-indoxylsulphate (X-sulphate).

	Sulphur source[a]					
	NCS	HS	BS	PS	Met	Mutant strains
Class I	–	–	–	–	–	SN34
Class II	–	–	–	+	+	PW1, SN57
Class III	–	–	–	–	+	PW2, PW7, PW10
Class IV	–	–	+	+	+	PH3, PH4, PH18
Class V	–	+	+	+	+	PH5, PH7, PH11

a. –, no growth in liquid culture after 24 h. +, growth similar to the parent strain *P. putida* S-313.
NCS, *p*-nitrocatecholsulphate; HS, sodium hexylsulphate; BS, benzenesulphonate; PS, pentanesulphonate; Met, methionine.

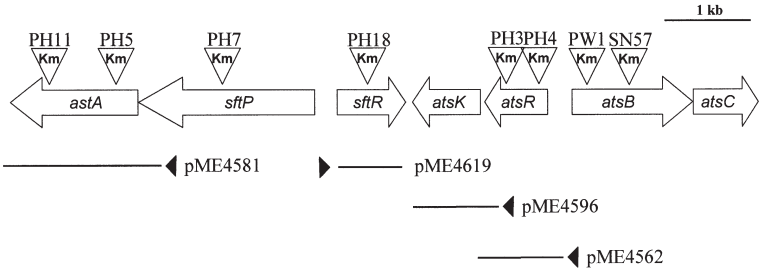

Fig. 1. Map of the *P. putida* S-313 *ats–sft* gene cluster, with insertion sites of miniTn*5*Km in strains PW1, PH3, PH4, PH5, PH7, PH11 and PH18, obtained by screening for the inability to release sulphate from ester 5-bromo-4-chloro-3-indoxylsulphate (X-sulphate), and strain SN57, which was obtained in a previous study (Vermeij *et al*., 1999). Several plasmids described in the text are shown, and the location of the *lac* promoter in the vector is indicated by a solid triangle.

this strain with the *sftR* gene on pME4619 led to restoration of the wild-type phenotype. The protein encoded by *sftR* was very similar to LysR-type regulators that are known to be involved in the desulphurization of aromatic sulphonates (44% identity to AsfR; Vermeij *et al*., 1999) or sulphated surfactants (39% identity to SdsB from *Pseudomonas* sp. ATCC 19151; Davison *et al*., 1992). These findings suggested that the *ats–sft* cluster played an important role in sulphate ester utilization, and that SftR might control a specific sulphate ester utilization regulon. We therefore carried out initial studies to investigate possible ecological functions for such a regulon.

SftR is important for survival in soil environments

Pseudomonas putida is an important inhabitant of aerated soils and rhizosphere, where sulphate esters make up a large proportion of the sulphur content (Fitzgerald, 1976; Autry and Fitzgerald, 1990; Scherer, 2001). We tested whether the *sftR* mutant strain PH18 displayed reduced rhizosphere or soil competence compared with the parent strain. The mutant and wild-type strains were each inoculated into microcosms constructed with forest, grassland or agricultural soils from central Denmark that had been washed to remove inorganic sulphate. Survival of the inoculated strains was monitored over 30 days. The forest and agricultural soils were also used to test survival of the cells in the rhizosphere of *Arabidopsis thaliana* (the washed grassland soil was not used, as it gave unsatisfactory plant growth). Figure 2 shows the changes in the bacterial population in the three soils and two *A. thaliana* rhizospheres that were examined. In all conditions tested, the bacterial density increased from 10^6 cfu g^{-1} up to 10^8–10^9 cfu g^{-1} dry soil 5 days after inoculation. The initial density of strain PH18 was higher (2×10^6 cfu g^{-1} dry soil) than for the wild type (8×10^5 cfu g^{-1} dry soil), and this difference was maintained until the second sampling date. Both wild-type and mutant populations then decreased in all conditions until the end of the experiment. Regression analysis showed that there was no significant difference ($P = 0.05$) in the population decrease of the wild-type strain in the three soils tested, whereas the decrease in the mutant population was significantly more pronounced than that of the wild type in both agricultural and grassland soils. Survival of both wild-type and mutant strains in agricultural soil was significantly better ($P = 0.05$) in the rhizosphere of *A thaliana* than in the absence of the plant, but the mutant strain displayed significantly lower survival than the wild type under these rhizosphere conditions. The mutation affecting the *sftR* gene in strain PH18 therefore appears to have an effect in lowering its soil competence compared with the wild-type strain.

Expression of sftR and identification of SftR-controlled genes

In order to test whether expression of *sftR* was regulated in response to growth with organosulphur sources, an *sftR*::*xylE* chromosomal transcriptional reporter gene fusion was constructed in *P. putida* S-313, and catechol-2,3-dioxygenase (C23O) activity was measured during growth with sulphate, hexylsulphate, NCS, pentanesulphonate, benzenesulphonate and methionine as sulphur sources. Almost no activity was present when the cells were grown with sulphate, but strong upregulation of C23O activity was observed with sulphate esters, methionine and sulphonates (Fig. 3). Additionally, chromosomal *atsK*::*xylE*, *astA*::*xylE* and *atsB*::*xylE* transcriptional fusions were constructed in both the wild-type strain and the SftR mutant PH18. These were used to test the expression of the other *ats–sft* cluster genes during growth with different sulphur sources and to measure the effect of the mutation of the *sftR* gene. The results are shown in Fig. 4. Expression of the alkylsulphatase gene *atsK*, the sulphotransferase gene *astA* and the membrane component of the transporter (*atsB*) was upregulated during growth with hexylsulphate, NCS, benzenesulphonate and pentanesulphonate, whereas methionine enhanced transcription to a lesser extent than in the case of *sftR*. Upregulation of *atsK*, *astA* and *atsB* expression was dependent on the presence of SftR during growth with pentanesulphonate, whereas with aromatic sulphonates, another regulatory mechanism seems to contribute to the expression of the *ats–sft* genes. These results suggest that SftR is a positive transcriptional regulator required specifically for the utilization of sulphate esters as sulphur sources in *P. putida* S-313.

The closely related strain *P. aeruginosa* also contains an *sftR* homologue, which is located 5 kb downstream of *atsR* and is closely associated with an *atsK* homologue, as observed in *P. putida*. The encoded protein (protein PA0191, accession no. D83621) is 65% identical to SftR in *P. putida* S-313. In *P. aeruginosa*, aromatic sulphates are cleaved by the arylsulphatase protein AtsA (Beil *et al*., 1995). To test whether SftR is also involved in sulphate ester metabolism in *P. aeruginosa*, we constructed a mutant (strain PA191) that was deficient in the expression of gene PA0191, as described in *Experimental procedures*. Strain PA191 was still able to use NCS, but was unable to grow with hexylsulphate as a sulphur source and displayed reduced growth with SDS. Interestingly, strain PA191 could also no longer use the aliphatic *N*-sulphated ester *N*-cyclohexylsulphamic acid (cyclamate), suggesting that desulphurization of sulphamates and aliphatic sulphate esters is co-regulated in this species.

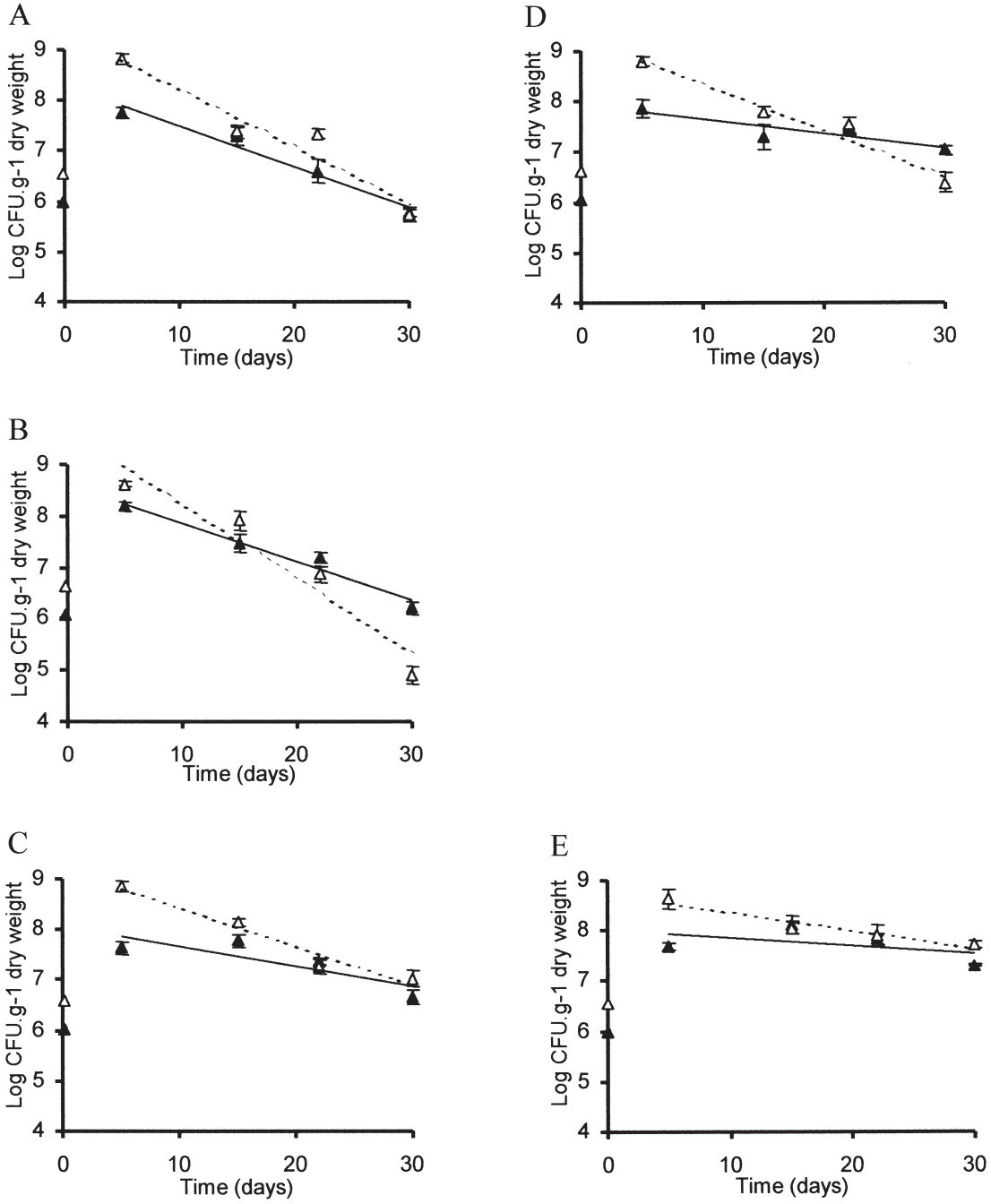

Fig. 2. Soil competence of *P. putida* strains in soils and rhizosphere. Survival of *P. putida* S-313r (closed triangles) and the *sftR* mutant strain PH18 (open triangles) was measured in (A) agricultural soil, (B) grassland soil, (C) forest soil, (D) rhizosphere of *A. thaliana* grown in agricultural soil and (E) rhizosphere of *A. thaliana* grown in forest soil. All soils were obtained from a small region in central Denmark and were washed and sieved before use. Each data point represents data from five independent microcosms, as described in *Experimental procedures*.

The atsRBC *genes are required for the uptake of sulphate esters*

In order to characterize the enzyme systems controlled by SftR, we analysed the *ats–sft* genes further. Class II mutants were found to carry transposon insertions in a gene encoding a putative permease component of an ABC-type transport system (*atsB*), which displayed up to 36% identity to known permeases. These mutants were also unable to grow with aromatic sulphonates, suggesting that AtsB constitutes the permease component of a general sulphonate/sulphate ester transporter. The *atsC*

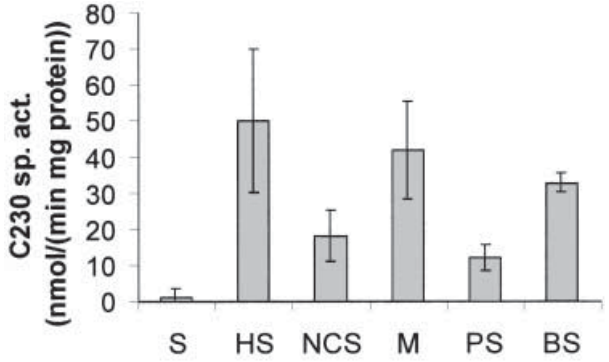

Fig. 3. Expression of *sftR*::*xylE* fusion in *P. putida* strain WTX-R, grown in minimal medium with different sulphur sources (S, sulphate; HS, hexylsulphate; NCS, *p*-nitrocatecholsulphate; PS, pentanesulphonate; BS, benzenesulphonate; M, methionine). C23O, catechol-2,3-dioxygenase.

gene downstream of *atsB* presumably encodes the ATP-binding component of this transport system, as AtsC displayed up to 47% sequence identity to known ATP-binding components. Hydrophobicity analysis of the AtsB protein suggested the presence of 11 transmembrane domains, and AtsC was found to contain the ATP/GTP binding site motif expected for ATP-binding cassettes of ABC-type transporters (Higgins, 1992). The AtsR protein encoded adjacent to AtsB is up to 27% identical to known periplasmic solute-binding proteins. Class IV mutants PH3 and PH4 carried transposon insertions in *atsR* and were deficient for growth with NCS and hexylsulphate, but not with benzenesulphonate. This indicated that, although AtsR–AtsB–AtsC acts as a sulphate ester transporter, a different periplasmic-binding protein specific for aromatic sulphonates interacts with AtsBC for sulphonate uptake. When the *P. putida atsR* mutants PH3 and PH4 were transformed with the *atsR* gene on plasmid pME4562, growth on NCS was restored. However, growth with aliphatic sulphate esters was recovered only by double complementation with both the *atsR* gene (pME4562) and the downstream *atsK* gene on plasmid pME4596. *atsK* encodes an α-ketoglutarate-dependent dioxygenase catalysing sulphate release from aliphatic sulphate esters such as hexylsulphate and SDS (Kahnert and Kertesz, 2000). Thus, AtsR is a periplasmic-binding protein for the uptake of aromatic and aliphatic sulphate esters.

Arylsulphate metabolism in P. putida *S-313 requires the arylsulphotransferase AstA*

Class V mutants were defective in growth with aromatic sulphate esters but grew normally with other sulphur sources tested. These strains carried transposon insertions in *sftP* (PH7) and *astA* (PH5 and PH11). Complementation of PH5, PH7 and PH11 with the *astA* gene on plasmid pME4581 restored growth with NCS in all cases. *astA* encodes a protein displaying 43–44% identity to sulphotransferases from enteric bacteria (Baek *et al*., 1996; Kwon *et al*., 1999), which catalyse the transfer of the sulphate moiety from phenolic sulphate esters to an unknown acceptor. As reported previously, cell extracts of *P. putida* S-313 grown with sulphate esters catalyse desulphation of NCS when phenol is added as an acceptor (Kahnert *et al*., 2000). No sulphotransferase activity was measured in PH7, PH5 and PH11, when the cells were grown with hexylsulphate as a sulphur source. The pH optimum for the sulphotransferase assay was determined using phenol as an acceptor, and most activity was observed at pH 9. The AstA protein was partially purified from *P. putida* S-313(pME4581) grown with NCS as a sulphur source, using anion exchange chromatography

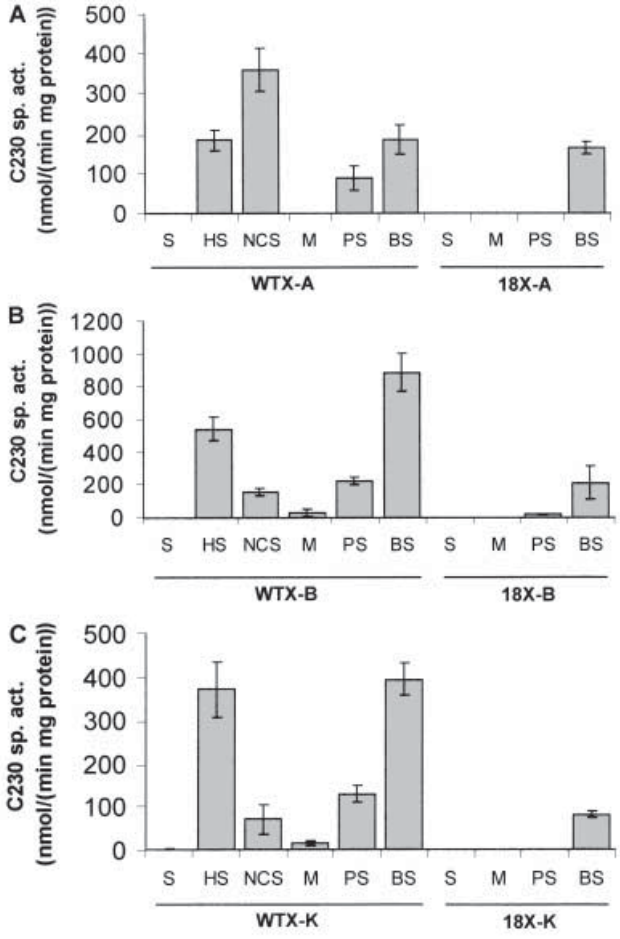

Fig. 4. Expression of (A) *astA*::*xylE*, (B) *atsB*::*xylE* and (C) *atsK*::*xylE* fusions in the *P. putida* wild-type-derived strains WTX-A, WTX-B and WTX-K, as well as in the corresponding PH18-derived strains 18X-A, 18X-B and 18X-K. All strains were grown in minimal medium with different sulphur sources (S, sulphate; HS, hexylsulphate; NCS, *p*-nitrocatecholsulphate; PS, pentanesulphonate; BS, benzenesulphonate; M, methionine). C23O, catechol-2,3-dioxygenase.

and gel filtration chromatography, as described in *Experimental procedures*. The sulphotransferase enzyme was estimated to be 80% pure after gel filtration chromatography. The 10 N-terminal amino acids were determined as N-A-K-T-E-T-P-A-L-P, which corresponded to the predicted protein encoded by the *astA* gene after cleavage of the formylmethionine residue. This finding confirmed the conclusion from sequence analysis that AstA lacks a signal peptide. Unlike other bacterial sulphotransferases (Baek *et al*., 1996; Kwon *et al*., 1999), the *P. putida* S-313 AstA enzyme is therefore very unlikely to be a periplasmic enzyme.

SftP – a putative TonB-dependent receptor

The *sftP* gene encodes a protein displaying sequence identity to various outer membrane receptors that are involved in the translocation of siderophores across the outer membrane [25% identity to IrgA from *Vibrio cholerae* (Goldberg *et al*., 1992) and 22% identity to FhuA from *Escherichia coli* (Coulton *et al*., 1983)]. These receptors contain a TonB box motif that is also present in SftP (PROSITE accession no. PS01156), suggesting that SftP is involved in the translocation of large compounds across the outer membrane in a TonB-dependent manner. Strain PH7 was unable to grow with NCS as a sulphur source, but this phenotype could be complemented by transformation with the *astA* gene, demonstrating that *sftP* and *astA* are co-transcribed and that SftP is not strictly required for growth with aromatic sulphate esters. SftP was predicted to carry a signal sequence for export from the cell, and the mature protein has a predicted molecular mass of 81.0 kDa. In order to investigate whether SftP is a sulphur-regulated outer membrane protein, outer membrane proteins were isolated by sucrose density gradient centrifugation from *P. putida* S-313 grown with either hexylsulphate or sulphate and separated by SDS–PAGE. Three proteins exhibiting molecular masses of 85–90 kDa were found in the hexylsulphate-grown sample but were not present in sulphate-grown cells (Fig. 5). The proteins were excised from the SDS gels and subjected to LC-QTOF mass spectrometry. The protein marked with an arrow in Fig. 5 was conclusively identified as the *sftP* gene product by analysis of peptide sequence data from three separate tryptic peptides obtained from this protein. This confirmed that SftP is an outer membrane protein, and that its synthesis is upregulated during growth with hexylsulphate.

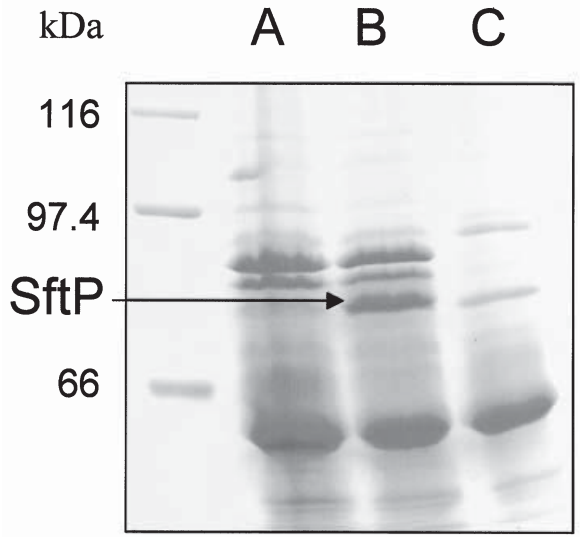

Fig. 5. SDS–PAGE of outer membrane proteins of (A) *P. putida* strain PH7 (*sftP*::*miniTn5Km*) grown with hexylsulphate as the sulphur source, (B) *P. putida* S-313 grown with hexylsulphate, and (C) *P.* putida S-313 grown with sulphate. The gel contained 8% polyacrylamide, and LPS were removed as described in *Experimental procedures* before loading the samples. The arrow indicates the SftP protein, which was identified by mass spectroscopic analysis (see text).

Discussion

In this study, we report the identification of the *ats–sft* gene cluster encoding sulphate ester utilization enzymes in *P. putida* S-313. Expression of the *ats–sft* genes was controlled by the SftR regulator protein, which was synthesized during growth with sulphate esters, sulphonates and methionine as sulphur sources, but not with inorganic sulphate. In addition to SftR, enzymes encoded in the *ats–sft* cluster comprise an unusual α-ketoglutarate-dependent dioxygenase that catalyses the release of sulphate from aliphatic sulphate esters (AtsK) (Kahnert and Kertesz, 2000), a putative ABC-type transport system (AtsRBC), an outer membrane protein (SftP) and a sulphotransferase that transfers the sulphate moiety from aromatic sulphate esters onto an unknown acceptor (AstA). A mutant in SftR was deficient in the expression of all the other genes of the *ats–sft* cluster and showed significantly reduced survival in soil environments.

Desulphurization of sulphonates and sulphate esters occurs by quite different mechanisms in *P. putida* S-313. Both aromatic and aliphatic sulphonates are cleaved by the $FMNH_2$-dependent monooxygenase SsuD, whereas sulphate esters are desulphurized either by an α-ketoglutarate-dependent dioxygenase (AtsK) or via an arylsulphotransferase (AstA). Despite this biochemical diversity, sulphonate and sulphate ester utilization as sulphur sources for growth are closely linked. Growth with both arylsulphonates and sulphate esters requires the *ats* transporter described above, and also the *ssuF* gene product, a small protein with sequence similarity to clostridial molybdopterin-binding proteins (Kahnert *et al*., 2000). This latter protein is encoded in the *ssu* operon,

together with the ABC-type transporter responsible for alkanesulphonate transport and the desulphonating monooxygenase. Arylsulphonate and sulphate ester utilization are regulated by two closely related members of the LysR-type transcriptional activator family. AsfR, which plays a role in arylsulphonate degradation (Vermeij *et al*., 1999), shares 48% amino acid identity with SftR, the sulphate ester utilization regulator identified in this study. Both are also quite similar to SdsB, the regulator of the long-chain sulphatase SdsA in *Pseudomonas* sp. ATCC 19051 (Davison *et al*., 1992) (31% and 39% identity respectively). These enzymes clearly form a small subfamily within the LysR group.

However, there are significant differences in the ways in which AsfR and SftR expression is regulated, and how they control their target genes. SftR is not expressed during growth with sulphate as a sulphur source, and the gene is switched on only under sulphate starvation conditions in the presence of a variety of organosulphur sources (Fig. 3). This is not the case for AsfR, which is also synthesized during growth with sulphate (Vermeij *et al*., 1999). In the presence of toluenesulphonate, AsfR mediates the expression of the *asfABC* genes, and it seems likely that, under these conditions, AsfR is also probably responsible for the residual expression of the *ats–sft* genes that is observed in the SftR mutant during growth with benzenesulphonate (Fig. 4). However, the possibility of cross-talk between AsfR and the *ats–sft* promoters has not yet been examined in detail. The periplasmic sulphonate-binding protein AsfC is only synthesized in the presence of aromatic sulphonates, but has been shown to facilitate the uptake of both aromatic sulphonates and sulphate esters in *P. aeruginosa* (Vermeij *et al*., 1999). Its presence allows the *atsR* mutants PH3 and PH4 to grow with arylsulphonates, although no growth is seen with aromatic sulphates because *asfC* is only expressed in the presence of the sulphonate substrates.

The observation of upregulation of SftR during growth with methionine represents a further link to sulphonate metabolism in *P. putida* S-313, as in this strain the sulphonatase-encoding gene *ssuD* is required for the utilization of methionine as a sulphur source. A mechanism has been proposed in which methionine sulphur is released as methanethiol, oxidized to methanesulphonate and subsequently cleaved by SsuD to release sulphite, which can be directly reincorporated into cysteine (Vermeij and Kertesz, 1999). SftR may be involved in regulating the expression of the *ssu* operon during growth with methionine or aliphatic sulphonates, but it cannot be strictly required, as strain PH18 is still able to grow with these sulphur sources. An additional regulatory mechanism must be present that prevents the SftR-mediated upregulation of the other *ats–sft* genes when the cells grow with methionine, but this mechanism remains to be elucidated.

The finding of a sulphotransferase instead of an arylsulphatase in *P. putida* S-313 was surprising, as in the closely related *P. aeruginosa*, cleavage of sulphate from aromatic sulphate esters is catalysed by a member of the widespread arylsulphatase family of enzymes. Bacterial arylsulphotransferases have so far exclusively been characterized from mammalian intestinal bacteria (Kim *et al*., 1986; 1992; Lee *et al*., 1995; Kang *et al*., 2001), where they catalyse the transfer of sulphate from phenolic sulphate ester donors onto as yet unidentified acceptors. Their physiological function remains unclear. With the exception of one enzyme exhibiting relatively low (26%) identity to AstA (Goldberg *et al*., 2000), all characterized prokaryotic arylsulphotransferases are periplasmic enzymes, and it has been speculated that they play a role in the detoxification of xenobiotic phenolic compounds in the mammalian gut (Baek *et al*., 1996). No evidence has yet been given on the regulation of their expression as part of the bacterial sulphur cycle. AstA from *P. putida* S-313 is 43–44% identical to these enzymes and, therefore, clearly belongs to the same family, but its role in sulphur assimilation together with its cytoplasmic localization add new aspects to the understanding of bacterial arylsulphotransferases. One can speculate that their occurrence in soil bacteria has been widely underestimated, and that routine measurements of arylsulphatase activity in soils largely represent sulphotransferase activity, because of the presence of acceptor substrates in soil organic matter.

The role of the putative TonB-dependent receptor protein in the context of sulphate ester utilization also remains to be clarified. Despite the lack of an observable growth phenotype in SftP mutants, its clustering and co-expression with the *ats–sft* genes suggest an involvement in either the uptake or the biodegradation of sulphate esters. TonB-dependent receptors have been shown to be involved in the translocation of iron siderophores and vitamin B12 across the outer membrane (for a review, see Folschweiller *et al*., 2000). The dioxygenase AtsK, which catalyses the release of sulphate from alkylsulphate esters, requires iron as a cofactor (Kahnert and Kertesz, 2000), and it is therefore possible that SftP contributes to iron acquisition under conditions in which AtsK is expressed. This assumption is underlined by the fact that, on the *P. aeruginosa* genome, SftP (protein PA0192; 63% identical to SftP from *P. putida* S-313) is encoded adjacent to AtsK (protein PA0193) (Stover *et al*., 2000). However, LC-QTOF MS of outer membrane proteins from sulphate- and hexylsulphate-grown cells revealed that not only SftP, but also at least one other putative TonB-dependent receptor was synthesized in the absence of sulphate, and it will be interesting to investigate further

a possible link between sulphate starvation and iron metabolism. It cannot be excluded that SftP has a different function from the one observed for TonB-dependent receptors so far, e.g. the translocation of sulphated substrates across the outer membrane. Indeed, it has been observed previously that SDS could diffuse through the related *Escherichia coli* FhuA receptor when the latter was transformed into a channel upon phage binding (Bonhivers *et al*., 1996).

It has been suggested that microbial hydrolysis of sulphate esters is an important source of inorganic sulphate for plants (Castellano and Dick, 1991; Scherer, 2001). We propose that the connected sulphonate- and sulphate ester utilization response evolved as an adaptation to sulphate starvation conditions in aerobic soils, where the majority of the sulphur is present in organically bound forms. This is reflected by the reduced survival of the *sftR* mutant in grassland and agricultural soils, which gives an indication of how important the ability to utilize sulphate ester is to microbial fitness in the environment. In forest soils with higher organic matter content, the effect was reduced, possibly because of the lower pH of the forest soil and a resultant higher concentration of adsorbed inorganic sulphate (Martini and Mutters, 1984; Kparmwang *et al*., 1997). We assume that the microbial switch between sulphate starvation and the assimilation of inorganic sulphate might play a role in regulating fluxes in the soil sulphur reservoir for plant nutrition. Previous studies have shown that a considerable fraction of plant-available sulphur is derived from ester sulphate in soil organic matter (Freney *et al*., 1975; Nguyen and Goh, 1994), but the experiments did not allow any conclusions concerning the relative importance of enzymatic mobilization, as opposed to chemical sulphate ester hydrolysis. To date, more efforts are clearly required to elucidate the importance and role of microbial organosulphur assimilation, and investigations are currently being undertaken in our laboratory to quantify expression of the *ats–sft* and other sulphur-regulated genes in different soils.

Experimental procedures

Bacterial strains and growth conditions

Bacterial strains, plasmids and phages used in this study are listed in Table 2. *P. putida* S-313r was a spontaneous rifampicin-resistant mutant of strain S-313 that was indistinguishable from the parent strain in growth on a range of different sulphur sources. All *P. putida* strains were grown aerobically at 30°C in succinate minimal medium (Kertesz *et al*., 1993). Sulphur sources were added to a final concentration of 250 μM. *Escherichia coli* strains were grown aerobically in Luria–Bertani medium at 30 or 37°C. Antibiotics used were: kanamycin (25 μg ml^{-1}), ampicillin (100 μg ml^{-1}), tetracycline (25 μg ml^{-1}), chloramphenicol (20 μg ml^{-1}) and cycloheximide (100 μg ml^{-1}). Gentamicin was added at 15 μg ml^{-1} to *E. coli* growth media and at 25 μg ml^{-1} to *P. putida* growth media. When required in sulphate-free medium, kanamycin and gentamicin chloride were prepared from the corresponding sulphate salts as described previously (van der Ploeg *et al*., 1996). All solid media were prepared by the addition of 1.5% (w/v) molecular biology grade agarose.

Enzyme assays

Catechol-2,3,-dioxygenase activity was measured in extracts prepared from cells in the mid-exponential growth phase (Karkhoff-Schweizer and Schweizer, 1994). Arylsulphotransferase assays (500 μl) contained 10 mM *p*-nitrocatecholsulphate, 10 mM phenol (pH 9), 50 μl of cell extract and 100 mM Tris acetate, pH 9. The assay mixture was incubated at 30°C, and 4-nitrocatechol was quantified spectrophotometrically at 515 nm (Kahnert *et al*., 2000).

DNA manipulations

For plasmid isolation, restriction enzyme digestion and transformation, published procedures were used (Ausubel *et al*., 1987). Polymerase chain reaction (PCR) was carried out in a Biometra DNA thermal cycler. Reaction mixtures consisted of 100 pmol of primers, 50 nmol of dNTP, 2 U of *Taq* DNA polymerase and 1 ng of template in a final volume of 100 μl. *P. putida* was transformed by electroporation in 0.1 cm cuvettes (12.5 kV cm^{-1}), using a Gene Pulser apparatus (Bio-Rad). Southern analysis was carried out by standard methods (Ausubel *et al*., 1987) using digoxigenin-labelled probes.

Transposon mutagenesis and sequencing

Transposon mutagenesis of *P. putida* S-313 was done by patch mating equal cell numbers of overnight cultures of *E. coli* S17-1λpir (pUT miniTn*5*-Km) and *P. putida* S-313 on LB agar at 30°C for 8 h. The cells were resuspended in 0.9% NaCl and plated out on succinate salts medium agar plates containing chloramphenicol and kanamycin chloride, with 20 μM cysteine and 250 μM X-sulphate as sulphur sources. White and pale blue colonies were selected for further study. Insertion sites of transposons were identified by cloning the transposon-containing fragment into pBluescript using transposon rescue techniques described earlier (Vermeij *et al*., 1999) and sequencing the transposon-flanking regions with a transposon-specific primer, π (5′-GCATTAACAATCTAGC GAGG-3′). Transposon insertion sites in strains PH3 and PH4 were verified by PCR.

Cloning of the ats–sft *genes and construction of xylE fusions*

pME4581 was constructed by inserting a 900 bp *Pst*I–*Sac*II fragment from pME4568 and a 1.5 kb *Pvu*II–*Pst*I fragment from pME4566 into *Sac*II–*Sma*I-cut pBBR1MCS-3. A 1.4 kb *Xho*I fragment was cloned into pBBR1MCS-3 to give pME4619.

Chromosomal *xylE* reporter gene fusions were constructed as follows. A fragment with the translational start site of the

Table 2. Strains and plasmids used in this study.

Strain	Relevant features	Reference or source
Escherichia coli		
DH5α	*supE44* Δ *lacU*169 (ϕ80 *lacZΔM15*) *hsdR17 recA1 endA1 gyrA*96 *thi-1 relA1*	Laboratory collection
S17-1λpir	*hsdR thi pro recA;* RP4 integrated into the chromosome (*kan*::Tn*7 ter*::Mu) λ*pir*	De Lorenzo and Timmis (1994)
Pseudomonas putida		
S-313	Prototroph	Zürrer *et al.* (1987)
S-313r	Rifampicin-resistant mutant of strain S-313	This study
SN57	*atsB*::miniTn*5*Km	Vermeij *et al.* (1999)
PH3	*atsR*::miniTn*5*Km	Kahnert and Kertesz (2000)
PH4	*atsR*::miniTn*5*Km	This study
PH5	*astA*::miniTn*5*Km	This study
PH7	*sftP*::miniTn*5*Km	This study
PH11	*astA*::miniTn*5*Km	This study
PH18	*sftR*::miniTn*5*Km	This study
PW1	*atsB*::miniTn*5*Km	This study
PW2	*ssuB*::miniTn*5*Km	Kahnert *et al.* (2000)
PW7	*ssuB*::miniTn*5*Km	Kahnert *et al.* (2000)
PW10	*ssuC*::miniTn*5*Km	Kahnert *et al.* (2000)
WTX-B	S-313 *atsB*::*xylE Gm Tc*	This study
18X-B	PH18 *atsB*::*xylE Gm Tc*	This study
WTX-K	S-313 *atsK*::*xylE Gm Tc*	This study
18X-K	PH18 *atsK*::*xylE Gm Tc*	This study
WTX-R	S-313 *sftR*::*xylE Gm Tc*	This study
WTX-A	S-313 *astA*::*xylE Gm Tc*	This study
18X-A	PH18 *astA*::*xylE Gm Tc*	This study
Pseudomonas aeruginosa		
PAO1	Prototroph	Holloway (1955)
PA191	Co-integrate of pME4626 in PAO1 (*sftR* homologue PAO191 disrupted)	This study
Plasmids		
pBluescript KS/SK	Cloning vectors, Ap^r	Stratagene
pBBR1MCS-3	Broad-host-range cloning vector, Tc^r	Kovach *et al.* (1995)
pUCP24	Broad-host-range cloning vector, Gm^r	West *et al.* (1994)
pUT miniTn*5*-Km	Delivery plasmid for miniTn*5*-Km	De Lorenzo and Timmis (1994)
pX1918GT	Amp^r, *xylE*, Gm^r	Schweizer and Hoang (1995)
pKNOCK-Tc	Mobilizable suicide vector, Amp^r, Tc^r	Alexeyev (1999)
pTrc99a	Expression vector, Amp^r,	Amersham Pharmacia
pUC18Not	Cloning vector, Amp^r	Herrero *et al.* (1990)
pME4429	6.5 kb *Not*I–*Mun*I chromosomal DNA fragment (Km^r) from SN57 in pBluescript KS	Kahnert and Kertesz (2000)
pME4562	*atsR*$^+$*;* 2.1 kb *Kpn*I fragment from pME4429 in pBBR1MCS-3	Kahnert and Kertesz (2000)
pME4564	≈ 13kb *Cla*I chromosomal DNA fragment (Km^r) from PH5 in pBluescript KS	This study
pME4565	≈ 5 kb *Cla*I–*Sac*II chromosomal DNA fragment (Km^r) from PH7 in pBluescript SK	This study
pME4566	≈ 10 kb *Sac*II chromosomal DNA fragment (Km^r) from PH11 in pBluescript SK	This study
pME4568	≈ 3.5 kb *Not*I deletion of pME4564	This study
pME4573	≈ 4 kb *Cla*I chromosomal DNA fragment (Km^r) from PH18 pBluescript KS	Kahnert and Kertesz (2000)
pME4581	*astA*$^+$*;* 900 bp *Pst*I–*Sac*II fragment from pME4568 and 1.5 kb *Pvu*II–*Pst*I fragment from pME4566 in *Sma*I–*Sac*II-digested pBBR1MCS-3	This study
pME4593	mobilizable suicide vector with *xylE* reporter gene –2.4 kb *EcoR*I fragment from pX1918GT in *Kpn*I-cut pKNOCK-Tc	This study
pME4596	*atsK*$^+$, 1725 bp *Cla*I–*Nsi*I fragment from pME4573 in *Sma*I–*Pst*I-cut pUCP24	Kahnert and Kertesz (2000)
pME4599	mobilizable suicide vector with *xylE* reporter gene and downstream *trc* promoter –1.5 kb *Pae*I–*Kpn*I fragment from pTrc99a in *Not*I site of pME4593	This study
pME4619	*sftR*$^+$*;* 1.4 kb *Xho*I fragment from pME4429 in pBBR1MCS-3	This study
pME4620	*atsB*::*xylE* in pME4599	This study
pME4621	*atsR*::*xylE* in pME4599	This study
pME4622	*sftR*::*xylE* in pME4599	This study
pME4623	*astA*::*xylE* in pME4599	This study
pME4624	472 bp *Hin*dIII–*Pag*I fragment from pTrc99a in *Sma*I site of pUC18Not	This study
pME4625	mobilizable suicide vector with *xylE* reporter gene and downstream transcriptional terminator – 600 bp *Not*I fragment from pME4624 in pME4593	This study
pME4626	600 bp *Sma*I fragment of *P. aeruginosa* PAO191 gene in pME4625	This study

gene of interest was cloned upstream of the *xylE* gene in the suicide plasmid pME4599 and recombined onto the chromosomes of *P. putida* S-313 and PH18 respectively. The delivery vector used, pME4599, was based on pKNOCK-Tc and was constructed by cloning the *lacI*^{q} gene and the *trc* promoter (1.5 kb *Pae*I–*Kpn*I fragment from pTrc99a) in the *Not*I site, and a *xylE*::*Gm*^{r} cassette (2.4 kb blunted *Eco*RI fragment from pX1918GT) into the blunted *Kpn*I site. The *trc* promoter was included to prevent polar effects of the insertion in the merodiploid strain. For construction of the *atsB*::*xylE* fusion, a 1.1 kb fragment containing the *ats* promoter region was amplified by PCR and cloned into *Sma*I-digested pME4599 to give pME4620. pME4620 was integrated into the chromosome of *P. putida* S-313 and PH18 to give WTX-B and 18X-B, respectively, using *E. coli* S17-1 λpir(pME4620) as the plasmid delivery strain for conjugational transfer. All other fusion strains were constructed in a similar procedure, using the constructs listed in Table 2.

Construction of strain PA191

PA191 is a co-integrate of pME4626 in *P. aeruginosa* PAO1. pME4626 was constructed by cloning a 600 bp internal fragment of the gene encoding protein PA0191 [resulting from a *Sma*I digest of a fragment amplified with primers 191for (5′-TTACTTCATCGCCCTCGCC-3′) and 191rev (5′-TCCAGT TCCACCAGCGTC-3′), using chromosomal DNA of *P. aeruginosa* as a template] into the *Sma*I site of pME4625. pME4625 was derived from pKnock-Tc and contained a transcriptional terminator originating from pTrc99a upstream of the MCS and a *xylE*::*Gm*^{r} cassette downstream of it. Integration of pME4626 into the *P. aeruginosa* chromosome therefore generates a merodiploid strain containing an *sftR*::*xylE* fusion and a second copy of *sftR* that is silenced by the polar effect of the transcriptional terminator in the vector. *P. aeruginosa* was transformed with pME4626 by conjugational transfer from *E. coli* S17-1λpir (pME4626).

Purification of arylsulphotransferase

Pseudomonas putida S-313(pME4581) was grown at 30°C in 5 l Erlenmeyer flasks containing 800 ml of succinate salts minimal medium, with NCS as the sole sulphur source. The cells were harvested at mid-exponential growth phase by centrifugation at 5200 *g* for 10 min at 4°C, washed once with 50 mM Tris-HCl, pH 7.5, and resuspended in 8 ml of the same buffer containing lysozyme, DNase I and RNase I (each 10 μg ml^{-1}). The suspension was incubated on ice for 30 min. Cell-free extracts were obtained by two passes through a French pressure cell at 4°C and centrifugation of the lysate at 100 000 *g* for 1 h at 4°C. Cell extracts were desalted into 20 mM Tris-HCl, pH 7.5, using PD-10 columns (Amersham Pharmacia). The desalted lysate was chromatographed at room temperature on a 1 ml Resource-Q anion-exchange column (Amersham Pharmacia) with a fast protein liquid chromatography (FPLC) system (Amersham Pharmacia) at a flow rate of 5 ml min^{-1}. Proteins were eluted with an NaCl gradient, and the arylsulphotransferase was eluted at a concentration of 140 mM NaCl. Active fractions were purified further by gel filtration chromatography using a Superdex 200 column (Pharmacia Biotech). A mixture of 50 mM Tris-HCl (pH 7.5) and 0.15 M NaCl was used as running buffer at a flow rate of 1 ml min^{-1}.

Isolation of outer membrane proteins

Outer membrane proteins were isolated by sucrose density gradient centrifugation (Nikaido, 1994) using a discontinuous gradient. Cytoplasmic membranes appeared in a 35% (w/w) sucrose top layer, and outer membranes were recovered as a distinct white band in the lower layer of 50% sucrose. LPS were removed before SDS–PAGE by phenol extraction (Hancock and Nikaido, 1978).

Mass spectroscopic fingerprinting methods

Tryptic in-gel digestion of proteins that were subjected to mass spectroscopic fingerprinting was carried out by published methods (Jeno *et al*., 1995; Shevchenko *et al*., 1996; Wilm *et al*., 1996) modified for use with a robotic digestion system (Wait *et al*., 2001) (Investigator ProGest; Genomic Solutions). Cysteine residues were reduced with dithiothreitol (DTT) and derivatized with iodoacetamide before digestion with trypsin (Promega; 10 μl at 6.5 ng μl^{-1} in 25 mM ammonium hydrogen carbonate, 8 h at 37°C). Products were recovered by sequential extractions with 25 mM ammonium hydrogen carbonate, 5% formic acid and acetonitrile and then lyophilized.

Tandem electrospray mass spectra were recorded using a Q-Tof hybrid quadrupole/orthogonal acceleration time of flight spectrometer (Micromass) interfaced to a Micromass CapLC capillary chromatograph. Samples were desalted on a 300 μm × 15 mm Pepmap C18 column (LC Packings) and eluted into the mass spectrometer with a 0.1% formic acid–acetonitrile gradient (5–70% acetonitrile over 20 min: 1 μl min^{-1}). Proteins were identified by correlation of uninterpreted tandem mass spectra to entries in SWISSPROT/TREMBL using PROTEINLYNX global server (Version 1, Micromass) or by searching deduced amino acid sequences against the NCBI's non-redundant database using BLAST (Altschul *et al*., 1997).

Soil and rhizosphere methods

The experiments were performed in agricultural (AS), forest (FS) and grassland (GS) soils collected at Karup (Denmark), differing in their sulphur content (AS: 176 mg kg^{-1}; FS: 177 mg kg^{-1}; GS: 82 mg kg^{-1}) and carbon content (AS: 1.48% C; FS: 3.00% C; GS: 2.60% C). The soils were washed with distilled water in order to elute inorganic sulphate, air dried and sieved (<4 mm). Population dynamics of *P. putida* S-313r and of the mutant strain PH18 were compared in bulk soil and in the rhizosphere of *A. thaliana* grown in either soil.

The microcosms used for this experiment were 10 ml syringes filled with 15 g of soil and suspended in test tubes. A gauze wick was used to absorb water provided in the bottom of the glass tube. *A. thaliana* (Landsberg erecta) seeds were sterilized in a 1.25% (v/v) solution of NaOCl for 20 min, washed three times with sterile distilled water, placed

on agar plates and germinated for 5 days in a growth chamber on a cycle of 16 h light (25°C) and 8 h dark (22°C). Ten seedlings were transferred to each microcosm used for rhizosphere analysis. For the preparation of bacterial inocula, the respective strains were grown for 24 h on LB–cycloheximide plates containing rifampicin (strain S-313r) or kanamycin (strain PH18). Bacterial colonies were resuspended, washed twice (6000 *g*; 5 min) and percolated into the surface of the microcosm to obtain a bacterial density of 10^6 colony-forming units (cfu) g^{-1} dry soil. The cultivated and uncultivated microcosms were then placed in the growth chamber.

At various time intervals (0, 5, 15, 22 and 30 days), bulk soil was collected from five independent uncultivated microcosms, and rhizosphere soil was collected, after a gentle brushing of root systems, from five independent cultivated microcosms. Each soil sample was suspended in 5 ml of sterile dilution buffer (0.1% NaPP, 1% peptone) (Trevors and Cook, 1992) and mixed for 60 s with a Vortex shaker. The soil suspensions were diluted in the dilution buffer and plated on LB containing the appropriate antibiotics. After enumeration of colonies, bacterial densities were expressed as cfu g^{-1} dry soil. As populations of bacteria approximate a log normal distribution (Loper *et al*., 1984), values were log transformed before analysis. The kinetics of survival was approximated by linear regression of the log-transformed values against time. Linear regression analysis was performed according to the method of Gardner and Altman (1989), using a 95% confidence interval.

Sequence analysis and nucleotide sequence accession number

DNA sequencing was carried out by Microsynth. The *P. putida* S-313 *sft–ats* cluster sequence has been deposited in the GenBank database with accession no. AF126201.

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