Characterization of a sulfur-regulated oxygenative alkylsulfatase from *Pseudomonas putida* S-313*

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**Running title:** *Oxygenative alkylsulfatase from P. putida*

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The *atsK* gene of *Pseudomonas putida* S-313 was required for growth with alkylsulfate esters as sulfur source. The AtsK protein was overexpressed in *E. coli* and purified to homogeneity. Sequence analysis revealed that AtsK was closely related to *E. coli* taurine dioxygenase (38% amino acid identity). The AtsK protein catalysed the α-ketoglutarate dependent cleavage of a range of alkylsulfate esters, with chain lengths ranging from C₄ to C₁₂, required oxygen and Fe²⁺ for activity, and released succinate, sulfate and the corresponding aldehydes as products. Enzyme activity was optimal at pH 7, and was strongly stimulated by ascorbate. Unlike most other characterised α-ketoglutarate-dependent dioxygenases, AtsK accepted a range of α-ketoacids as co-substrates, including α-ketoglutarate (*Kₘ* 140 µM), α-ketoadipate, α-ketovalerate and α-ketoctanoate. The measured *Kₘ* values for hexylsulfate and SDS were 40 and 34 µM, respectively. The apparent Mr of the purified enzyme of 121,000 was consistent with a homotetrameric structure, which is unusual for this enzyme superfamily, members of which are usually monomeric or dimeric. The properties and amino acid sequence of the AtsK enzyme thus define it as an unusual oxygenolytic alkylsulfatase, and a novel member of the α-ketoglutarate-dependent dioxygenase family.
Bacterial enzymes that cleave aliphatic sulfate esters to release the sulfate moiety have been the subject of considerable study, motivated originally by an awareness of the large-scale release of synthetic alkylsulfate esters into the environment. Due to their amphiphilic properties, long-chain aliphatic sulfate esters such as sodium dodecyl sulfate (SDS) are in common use as components of surfactant formulations and are consequently discharged into wastewater. A range of bacterial strains able to degrade aliphatic sulfate esters has been isolated from contaminated sources such as sewage sludge, on the basis of their ability to utilize aliphatic sulfate esters as carbon sources for growth (for a review, see (1)). In most cases, degradation of alkyl sulfate esters was found to be initiated by alkylsulfatase enzymes that catalyze the hydrolytic cleavage of the ester bond to liberate inorganic sulfate. The resulting parent alcohol is further degraded (1) or incorporated into cellular lipids (2). Cleavage of the sulfate moiety has been studied in some detail, and several alkylsulfatase enzymes have been purified from cell extracts (3-7).

The finding that many isolates from environmental sites that had not been contaminated by detergents also exhibit alkylsulfatase activity (8) suggests that such enzymes may play a role in natural environments as well. Naturally occurring alkyl sulfates include methyl, ethyl and propyl sulfate in avian eggs (9), and the long-chain alkylsulfates which have been found in membrane structures from unicellular algae (10) and seaweed (11). In aerobic soils, 40-50% of the total sulfur is present as sulfate esters bound to the soil organic matter (12,13), though the molecular structure of these compounds has not yet been determined in detail. It therefore seems likely that soil
bacteria may be able to mobilize organically bound sulfur for growth, and recent studies (14) have provided evidence that bacterial sulfatases indeed play a role in sulfur scavenging. From a genetic point of view, the best characterized sulfur-regulated sulfatases are the arylsulfatases, and much less is known about alkylsulfatases. A sulfur-regulated gene cluster encoding a general sulfate ester uptake system together with an arylsulfatase has been identified in *Pseudomonas aeruginosa* PAO1 (15), but the degradation pathway for aliphatic sulfate esters remains unknown in that species.

Here we report the identification and characterization of an α-ketoglutarate-dependent oxygenase which catalyses the liberation of sulfate from alkyl sulfate esters in the sewage isolate *Pseudomonas putida* S-313. The enzyme is one of a set of proteins that is expressed under sulfate-starvation conditions, and it enables its host to grow with aliphatic sulfate esters as sulfur source. To our knowledge, this is the first purified enzyme catalyzing an oxygenative alkyl sulfate ester cleavage reaction.

**EXPERIMENTAL PROCEDURES**

*Materials* – Restriction endonucleases, T4 DNA ligase, T4 DNA polymerase and polynucleotide kinase were obtained from MBI Fermentas. Amplification of DNA fragments was performed with the Expand High Fidelity PCR System (Boehringer Mannheim). Horse liver alcohol dehydrogenase was obtained from Fluka. NADH and DNAse I came from Boehringer Mannheim,
and RNAse I was purchased from Sigma. Hexylsulfate and methylsulfate were obtained from Aldrich, whereas the other linear alkylsulfate esters came from Lancaster. Sodium 2-ethyl-hexylsulfate was obtained from Fluka. DNA sequencing and oligonucleotide synthesis were done by Microsynth (Balgach, Switzerland).

Bacterial strains and growth conditions – All *P. putida* strains were grown aerobically at 30°C in succinate-salts minimal medium (16). Sulfur sources were added to a final concentration of 250 μM. *Escherichia coli* DH5α (*supE44 ΔlacU169 (φ80 lacZΔM15) hsdR recA1 endA1 gyrA96 thi-1 relA1*) and *E. coli* BL21(DE3) (*hsdS gal (λcIts857 ind1 Sam7 nin5 lacUV5-T7 gene 1)*) were grown aerobically in Luria-Bertani medium (17) at 30 or 37°C. Kanamycin was added at 25 μg/ml, tetracycline at 25 μg/ml and ampicillin at 100 μg/ml. Gentamicin was added at 15 μg/ml to *E. coli* growth media and at 25 μg/ml to *P. putida* growth media. When required in sulfate-free medium, kanamycin and gentamicin chloride were prepared from the corresponding sulfate salts as previously described (18). All solid media were prepared by addition of 1.5 % (w/v) molecular biology grade agarose.

Measurement of growth characteristics – Growth experiments were done in 100 ml Erlenmeyer flasks containing 10 ml succinate-salts minimal medium. The flasks were inoculated (1% v/v) with an overnight culture that had been grown in minimal medium with sulfate as sulfur source, and the cells then washed twice in sulfate-free medium. Growth was measured as absorbance at 600 nm after 24 hours.

DNA manipulations - Plasmid isolation, restriction enzyme digestion and transformation of *E. coli* DH5α were carried out using published procedures (19). *E. coli* BL21(DE3) and *P. putida* were transformed by electroporation in 0.1 cm cuvettes (12.5 kV/cm), using a GenePulser apparatus (Bio-
Construction of atsR and atsK expression plasmids for growth experiments – To construct the atsR expression plasmid pME4562, the atsR gene was placed under the control of the lac promoter in the broad-host range vector pBBR1MCS-3 (20). A 2.1 kb KpnI fragment from plasmid pME4429 was ligated with KpnI-digested pBBR1MCS-3 to give pME4562 (pME4429 contained a 7.6 kb genomic fragment of the ats cluster of P. putida S-313). pME4562 carried the atsR gene in parallel orientation to the lac promoter of the vector, and an additional 768 bp upstream of the atsR start codon, as well as 304 bp downstream of the stop codon. The atsK gene was cloned into the broad-host range cloning vector pUCP24 (21) by digesting pME4573, which contained an appropriate part of the ats gene cluster, with ClaI, blunting, and redigesting with NsiI. The resulting 1.7 kb fragment was ligated with Smal-PstI digested pUCP24 to give pME4596. Thus, the insert of pME4596 contained the atsK gene in parallel orientation to the lac promoter, together with 505 bp upstream and 314 bp downstream of atsK.

Construction of an atsK overexpression plasmid for enzyme purification – The atsK gene was placed under the control of the T7 RNA polymerase promoter of the vector pET24b(+) (Novagen). In a first step, it was amplified with the primers atsKfor (5′- CCCTGCATATGAGCAACGCTG-3′) and atsKrev (5′- GAATTGGCAAGCTTGCTCCC-3′) using pME4429 as a template (NdeI and HindIII sites underlined). The amplified 992bp DNA fragment was treated with T4 DNA polymerase and polynucleotide kinase (20 min, 25 °C) and ligated with Smal-digested pBluescript SK to give pME4576. The 976bp NdeI-HindIII fragment of pME4576 containing atsK was cloned into pET24b(+), to give pME4577.

Purification of AtsK – E. coli BL21(DE3)(pME4577) cells were grown at 30 °C in 5l
Erlenmeyer flasks containing 800 ml of LB medium. *atsK* expression was induced at an OD$_{600}$ of 0.5-0.8 by the addition of isopropyl-γ-thiogalactopyranoside to a final concentration of 168 µM. 2 ½ hours later the cells were harvested by centrifugation at 5200 x g for 10 min at 4 °C. Cells were 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). The suspension was incubated on ice for 30 min. Disruption of the cells was performed using a French Pressure cell. Cell-free crude extracts were obtained by centrifugation of the lysate at 100 000 x g for 1 h at 4 °C. Crude 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 BioCAD SPRINT apparatus (Perseptive Biosystems) at a flow rate of 5 ml/min. Proteins were eluted with an NaCl gradient: in a first step, NaCl concentration was increased from 0 to 200 mM in 6 column volumes, and in a second step, from 200 mM to 1 M in 5 column volumes. Protein samples were stored on ice after elution. Gel filtration was carried out at room temperature using a Superdex 200 column (Pharmacia Biotech). 20 mM Tris/HCl, pH 7.5/0.1 M NaCl was used as running buffer, at a flow rate of 1 ml/min. Protein fractions containing *AtsK* were collected and desalted into 20 mM Tris/HCl, pH 7.5 using PD-10 columns. When the gel filtration step was omitted, the fractions collected after ion-exchange chromatography were desalted in the same way. Glycerol was added (15% (v/v) final concentration) and the samples were snap-frozen and stored at −20 °C in 0.5 ml aliquots until further use.

*Enzyme activity assay* – Unless explicitly indicated in the text, the following standard assay conditions were used for all measurements of *AtsK* activity. The standard assay mixture (1 ml volume) contained 10 mM hexylsulfate, 1 mM α-ketoglutarate, 200 µM ascorbate, 100 µM of
freshly dissolved FeCl$_2$, and 100-200 µg/ml enzyme in 10 mM Tris/acetate buffer (pH 7.0). With the exception of NADH in the alcohol dehydrogenase coupled assay (see below), all reaction products were quantified by endpoint measurements. Assays were incubated at 30 °C for various times up to 30 min. and the time for the standard assay was then chosen at 5 min. Reactions were started by the addition of enzyme to the reaction mixture and were stopped by denaturing the protein in a boiling water bath for 2 minutes, and centrifugation in an Eppendorf centrifuge (13 000 rpm, 10 min) at room temperature.

For determination of $K_m$ values, the substrate concentrations used ranged from 25 µM to 1 mM for alkyl sulphate esters, and from 25 µM to 10 mM for α-ketoglutarate. The concentrations of all the other substrates were constant and corresponded to standard assay concentrations.

Analysis of enzyme reaction products – Sulfate, succinate and α-ketoglutarate were measured using a Dionex AS14 ion exchange column (4mm x 250mm) with an AG14 guard column on an Alliance HPLC (Waters) supplied with a conductivity detector and a self-regenerating suppressor (Dionex), using Millenium software (Waters). Isocratic runs were performed using 3mM NaHCO$_3$/1.2 mM Na$_2$CO$_3$ as running buffer. Sulfate present in aqueous solutions of the sulfate esters used was measured before use, and when sulfate was detected, the percentage hydrolysis was calculated and the enzyme activity values corrected accordingly. Qualitative detection of hexanal was done by gas chromatography using a Perkin Elmer GC 8700 supplied with a Poropack P Teflon/steel column (180x0.2 mm) and a flame ionization detector. In addition, production of hexanal was detected by coupling the AtsK reaction to horse liver alcohol dehydrogenase and following the oxidation of NADH by measuring the absorbance at 340 nm over 20 min. Coupled assay mixtures
contained 10 mM hexylsulfate, 1 mM α-ketoglutarate, 200 µM ascorbate, 100 µM of freshly dissolved FeCl₂, 175 µM NADH, 33 nmol/min protein of alcohol dehydrogenase and 5-20 nmol/min protein of AtsK in 30 mM sodium phosphate buffer (pH 6.9). Control assay mixtures containing either no alcohol dehydrogenase or no AtsK were included in the measurements, and used to calculate the amount of NADH consumed in the conversion of hexanal to hexanol.

Other methods – SDS-polyacrylamide gel electrophoresis (12% (w/v) polyacrylamide) was performed using a Mini-PROTEAN II system (Bio-Rad). Protein concentrations were measured using the Bradford method (22) with Bio-Rad reagent dye concentrate, following the manufacturer's instructions.

RESULTS

Identification of Genes Required for Alkylsulfate Ester Utilization – A miniTn5 mutagenesis experiment described in a previous study (23) led to the identification of various mutants of *P. putida* S-313 which were no longer able to grow with aliphatic or aromatic sulfate esters as sulfur sources. Application of transposon rescue techniques revealed that some of those mutants carried transposon insertions in a gene cluster displaying a high level of sequence identity to the the *ats* gene cluster of *P. aeruginosa*, which is required for the utilization of organic sulfate esters in that species (15). Like its *P. aeruginosa* homologue, the *P. putida ats* cluster\(^1\) contains the *atsRBC* genes, which presumably encode an ABC-type transport system (*P. putida* AtsB was 40-50% identical to known bacterial permeases, and AtsC was 45-55% identical to ATP binding proteins of ABC-transporters). In one of

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\(^1\) The nucleotide sequence for the complete sulfate ester utilisation gene cluster of *Pseudomonas putida* S-313 has been deposited in the GenBank database under GenBank Accession Number AF126201.
the *P. putida* mutants, strain PH3, the transposon was inserted 100 bp upstream of the translational stop codon of the *atsR* gene, which encoded a putative periplasmic sulfate ester binding protein (59% identical to the *P. aeruginosa* sulfate ester binding protein AtsR). PH3 was not able to desulfurize *p*-nitroocatecholsulfate (NCS; a representative of the aromatic sulfate esters), nor did it grow with the aliphatic sulfate esters hexylsulfate (HS) and sodium-dodecylsulfate (SDS) as the sulfur source. Growth with all other sulfur sources tested (including cysteine, methionine, and aliphatic or aromatic sulfonates) was not affected in strain PH3. The *atsR* gene was introduced into strain PH3 on the medium-copy plasmid pME4562, where it was expressed from a *lac* promoter. PH3(pME4562) was found to be able to grow with NCS, but not with HS or SDS as sulfur sources, suggesting that the loss of alkylsulfate utilization was not directly caused by the mutation in *atsR*, but might be due to a polar effect of the transposon insertion on downstream genes. Indeed, 39 bp downstream of *atsR* we located another open reading frame (903 bp), which we named *atsK*. It was preceded by a good consensus ribosome binding site and its predicted gene product shows similarity to members of the α-ketoglutarate dependent dioxygenase superfamily. The most similar characterized protein to AtsK (38% protein identity) is the α-ketoglutarate dependent taurine dioxygenase (TauD) which was first purified from *Escherichia coli* (24). Taurine dioxygenase catalyzes the desulfonation of 2-aminoethanesulfonate (taurine) to aminoacetaldehyde and sulfite, which is then channeled into the sulfate assimilation pathway, enabling *E. coli* to grow with taurine as a sulfur source. *P. putida* S-313 is also able to utilize taurine as a sulfur source, but this ability was not affected in strain PH3. When PH3(pME4562) was additionally provided with the *atsK* gene on pME4596, growth with both HS and SDS was restored, though this was not case for PH3(pME4596), which still lacks a functional *atsR* gene. We concluded that the AtsR protein was required for growth with all sulfate esters as sulfur
sources, and that the AtsK protein was specifically required for the utilization of aliphatic sulfate esters, but not aromatic sulfate esters. We proceeded to overexpress and characterize the AtsK enzyme further.

**Enzyme Purification and Measurement of Sulfate Release from Hexylsulfate**

The AtsK enzyme was overexpressed in *E. coli* BL21(DE3)(pME4577) as described in Experimental Procedures. SDS-PAGE of cell extracts after induction revealed an intense protein band with an apparent molecular mass of approximately 32 kDa (Fig. 1, Lane C), which corresponded well to the predicted mass for the AtsK monomer (33.2 kDa). Initial measurements of sulfate release from hexylsulfate in cell free crude extracts of *E. coli* BL21(DE3)(pME4577) were carried out under non-optimized conditions and incubated for 30 min at 30 ºC. Sulfate release was indeed detected, although a very low specific enzyme activity was observed (3.3 nmol/min*mg protein). No sulfate release was detected in the absence of hexylsulfate or α-ketoglutarate, or in assays prepared with either AtsK-containing crude extracts which had previously been heated to 100 ºC for 2 min, or crude extracts of *E. coli* BL21(DE3) devoid of the *atsK* expression plasmid pME4577.

AtsK was purified to homogeneity from *E. coli* BL21(DE3)(pME4577) crude extracts in a two-step purification procedure with a total recovery of 12% of enzyme activity. Using the standard assay conditions described in Experimental Procedures, the specific enzyme activity measured in crude extract was 22 nmol/min*mg protein. The protein eluted from the ResourceQ anion exchange
column at a NaCl concentration of 50mM as a single peak (Fig. 1, Lane D). The specific activity of
the partially purified enzyme after anion exchange chromatography was determined to be 39
nmol/min*mg protein, and the yield was 59%. In a next step, gel filtration chromatography was
carried out using a Superdex 200 column. This yielded pure enzyme (Fig. 1, Lane E), but the pure
enzyme exhibited a lower specific enzyme activity (32 nmol/min*mg) than the partially purified
enzyme. We concluded that this loss in specific activity was due to partial inactivation during the gel
filtration procedure, and we chose to use the partially purified enzyme for further assays, since it was
estimated to be >95% pure by SDS PAGE (Fig. 1). Using gel filtration chromatography, the M₀ of
native AtsK was estimated to be 121,000 kDa. The calculated molecular mass of the atsK gene
product was 33.5 kDa, and we conclude that AtsK is present as a tetramer. The high molecular mass
of the native AtsK protein was somewhat surprising, since most α-ketoglutarate dependent
dioxygenases investigated so far are monomers or homodimers (25).

*Optimisation of Assay Conditions* – In analogy to other reactions catalyzed by α-
ketoglutarate dependent dioxygenases, we propose the reaction scheme shown in Fig. 2 for the
oxygenative hexylsulfate ester cleavage catalyzed by AtsK. The carbon atom forming the
hexylsulfate ester bond is hydroxylated by one atom of oxygen derived from molecular oxygen, to
give 1-hydroxy-hexylsulfate. Simultaneously, the cosubstrate α-ketoglutarate is oxidatively
decarboxylated to succinate and carbon dioxide, with incorporation of the second atom of molecular
oxygen into CO₂. 1-Hydroxy-hexylsulfate spontaneously decomposes to hexanal and sulfate. The
oxygenation reaction is dependent on ferrous iron.

The effect of pH on enzyme activity was examined using various buffer systems over a pH
range of 4.6-10.0. Enzyme activity displayed an optimum between pH 6.5-7.5, depending on the
buffer system tested. Highest activity was obtained with a Tris/acetate buffer at pH 7 (not shown).

The dependence of iron concentration on enzyme activity was determined over a range of FeCl₂ concentrations between 0 and 150 µM. Iron was required for the reaction, and maximal specific enzyme activity was observed at a concentration of 100 µM Fe²⁺. No enzyme activity was measured when FeCl₂ was replaced by chloride salts of other divalent metals at a final concentration of 100 µM. Metals tested included Ni²⁺, Co²⁺, Mn²⁺, Cu²⁺, Zn²⁺, Mg²⁺ and Ca²⁺. The addition of 100 µM EDTA to the standard assay mixture abolished enzyme activity. When ascorbate (200 µM) was added to the reaction mixture, enzyme activity increased threefold. Ascorbate has previously been shown to enhance α-ketoglutarate dependent dioxygenase reactions, although its requirement is not strict since the reactions do not require an external reducing agent for turnover. It has been proposed that it may play a part in reducing inactive Fe(III) and additionally protecting the enzymes from oxidative self-inactivation (26).

Products and Stoichiometry of the Oxygenative Sulfatase Reaction — Sulfate and succinate were quantified by ion chromatography, as was α-ketoglutarate disappearance. The disappearance of hexylsulfate in the assay was monitored qualitatively, since the conductimetric response of hexylsulfate was too small to allow precise quantification. The amount of sulfate released was plotted against the amount of consumed α-ketoglutarate obtained in a series of assays using the substrates hexylsulfate, heptylsulfate, octylsulfate, nonylsulfate, decylsulfate and SDS at various concentrations between 10 µM and 10 mM. The data obtained with heptylsulfate are shown in Fig. 3A. Linear regression analysis of the data in Fig. 3A revealed that the ratio of sulfate produced to α-ketoglutarate consumed was 0.92. When the same ratio was calculated for the assays in which other
substrates were used (data not shown), an average value of 0.98 was obtained for the different substrates. We conclude that one molecule of sulfate is produced per molecule of α-ketoglutarate consumed in the oxygenative alkylsulfatase reaction. It has been reported earlier that the α-ketoglutarate dependent dioxygenases prolyl 4-hydroxylase and lysyl hydroxylase are able to catalyse the uncoupled oxygenolytic decarboxylation of 2-oxoglutarate in the absence of the peptide substrates, by consumption of ascorbate (27). If the oxygenative alkylsulfatase AtsK were able to catalyze such uncoupled reactions, a significant decrease of α-ketoglutarate in the absence of the alkylsulfate ester substrates should have been observed, which was not the case (data not shown). When the amount of succinate produced in the reaction was plotted against consumed α-ketoglutarate (Fig. 3B), linear regression yielded a ratio of 1.2. We conclude that one molecule of succinate is produced per molecule of α-ketoglutarate. Hexanal, which is formed by spontaneous decomposition of the hydroxylated product of the reaction, 1-hydroxy-hexylsulfate, was detected qualitatively by gas chromatography in the standard assay. It was not present when no AtsK protein was added to the standard assay mixture. In addition, hexanal was detected indirectly by coupling the oxygenative alkylsulfatase reaction to alcohol dehydrogenase, catalyzing reduction of hexanal to hexanol, which was followed by the consumption of NADH (Fig. 4). A continuous decrease in NADH concentration was observed in the complete coupled assay mixture over a time period of 20 min, showing that hexanal was formed in the reaction catalysed by AtsK. Since it was possible that the low specific activity of AtsK observed under standard assay conditions was due to oxidative damage to the enzyme caused by the aldehyde, we tested whether the activity could be increased by continuous removal of hexanal in the coupled assay. However, the specific sulfate ester cleavage activity observed in the coupled assay was no higher than in the absence of alcohol dehydrogenase
(the concentration of alcohol dehydrogenase had been experimentally optimised to rule out the possibility that it formed a kinetic bottleneck in the coupled assay). The coupled assay was then used to examine whether sulfate inhibited the oxygenative sulfatase reaction, by adding sodium sulfate to the reaction mixture at various concentrations between 50 µM and 10 mM. No reduction in enzyme activity was observed for sulfate concentrations up to 10 mM.

**Substrate Range and $K_m$ values** - In order to investigate the substrate specificity of the AtsK enzyme, we measured sulfate release using different aliphatic sulfate esters as substrates in the standard assay. The substrates tested included linear primary alkylsulfate esters with carbon chain lengths of C$_1$, C$_4$ to C$_{10}$, and C$_{12}$. In addition, we tested 2-ethyl-hexylsulfate as a representative of branched alkylsulfate esters. Of all the substrates tested, only methylsulfate yielded a specific enzyme activity significantly lower than the one obtained with hexylsulfate (the specific enzyme activity for methyl sulfate was 1.1 nmol/min*mg protein). Kinetic studies were therefore done with all sulfate esters except methylsulfate, using α-ketoglutarate as cosubstrate. The enzyme activity showed a Michaelis-Menten-type saturation curve in response to increasing substrate concentrations when substrates were added at concentrations below 1 mM. At substrate concentrations between 1mM and 10mM, nonylsulfate and SDS inhibited the enzyme, though this effect was not observed with any of the other substrates tested. In order to collect kinetic data that would allow a relative comparison of substrate affinities at lower, more physiologically relevant alkyl sulfatase ester concentrations, we determined $K_m$ values according to Michaelis-Menten using alkyl sulfate ester concentrations between 50 µM and 1 mM. The $K_m$ values measured for hexyl-, heptyl-, octyl-, nonyl- and decylsulfate and for SDS and 2-ethyl-hexylsulfate are shown in Table 1. No $K_m$ values could be
obtained for butyl- and pentylsulfate, because the commercially available substrates contained high sulfate levels (butylsulfate was 5% hydrolysed and pentylsulfate 7% hydrolysed) which prevented sufficiently accurate measurements at low substrate concentrations. However, when added to the standard assay at a concentration of 10 mM, the specific enzyme activity obtained with butylsulfate was 20 nmol/min*mg protein, and 19 nmol/min*mg with pentylsulfate, indicating that these compounds are desulfated by the AtsK enzyme.

The $K_m$ for $\alpha$-ketoglutarate was 140 $\mu$M. The abilities of alternative 2-oxo-acids to act as cosubstrates in the oxygenative alkylsulfatase reaction were tested by measuring sulfate release from hexylsulfate. $\alpha$-Ketoacids were added at a concentration of 2 mM; all other assay conditions corresponded to the standard assay. 2-Ketoacids tested supported desulfation at the following rates, relative to the rate obtained with $\alpha$-ketoglutarate: 2-oxo-valerate 87%, 2-oxo-adipate 81%, 2-oxo-octanoate 31%, 3-methyl-2-oxo-butyrate 25%, oxaloacetate 15%, and no desulfation was obtained with pyruvate.

Since the AtsK enzyme was 38% identical to the taurine dioxygenase TauD, we tested its ability to catalyse the taurine dioxygenase reaction by using previously published methods (24). No sulfite release was measured from taurine. When taurine was added as a substrate to the AtsK-alcohol dehydrogenase coupled assay no NADH consumption was observed, indicating that no aldehyde was produced. Thus the AtsK enzyme is not involved in the utilisation of taurine as a sulfur source in $P.\ putida$ S-313, confirming the taurine-positive growth phenotype of mutant PH3.

DISCUSSION
Investigations of microbial biodegradation of alkylsulfate esters have concentrated on the cleavage of the aliphatic sulfate ester bond in a hydrolysis reaction (3-7,28,29). A new aspect was added to the understanding of alkylsulfate ester cleavage with the unexpected discovery that desulfation of methyl sulfate in the methylotrophic strains *Agrobacterium* sp. M3C and *Hyphomicrobium* MS223 is dependent on oxygen availability (30,31). Methyl sulfate can also serve as a substrate for a multicomponent NADH-dependent methanesulfonic acid monooxygenase isolated from another methylotrophic strain (32). In this paper we report the identification, purification and characterization of an α-ketoglutarate dependent dioxygenase which catalyzes desulfation of a broad range of aliphatic sulfate esters in *P. putida* S-313. In addition to the sulfated ester, the desulfation reaction requires molecular oxygen and α-ketoglutarate, and the products formed in the reaction are sulfate, CO₂, succinate and an aliphatic aldehyde. To our knowledge, the present study describes the first oxygenolytic enzyme cleaving aliphatic sulfate esters other than methyl sulfate, and the first enzyme of this type whose synthesis is regulated by the sulfur supply to the cell.

The biochemical properties of the oxygenative alkylsulfatase AtsK and sequence analysis of the *atsK* gene demonstrate that it belongs to the α-ketoglutarate dependent dioxygenase superfamily of enzymes (25,26,33). These enzymes catalyze a variety of significant metabolic reactions including hydroxylations, desaturations and epoxidations, and require an α-ketoacid cosubstrate. One oxygen atom from molecular oxygen is incorporated into the α-ketoacid, which subsequently decomposes to give succinate and CO₂. Activation of O₂ hence occurs via a mechanism that is distinct from the one catalyzed by oxygenases using a porphyrin ring or a second metal ion, since the driving force necessary for dioxygen cleavage is probably provided by the energy released in the decarboxylation
of the 2-oxoacid (34).

The degree of protein sequence similarity between α-ketoglutarate dependent dioxygenases is low, indicating that the members of this superfamily of enzymes arise from different evolutionary origins (33). The only common sequence motif is a 2-His-1-carboxylate facial triad, which has been shown to anchor the Fe(II) ion in the binding site of several crystallographically characterized α-ketoglutarate dioxygenases, including deacetoxycephalosporin C synthase (35), 4-hydroxyphenylpyruvate dioxygenase (36), and 2,4-dichlorophenoxyacetate dioxygenase (TfdA) from *Ralstonia eutropha* (37). Of these enzymes, only TfdA is significantly related to the oxygenative alkylsulfatase AtsK (29% protein sequence identity). Fig. 5 shows a partial amino acid sequence alignment of the *P. putida* S-313 oxygenative alkylsulfatase AtsK with its *P. aeruginosa* homologue, TfdA and taurine dioxygenase from *E. coli*. The iron binding motif His-X-Asp-X51-57-His is provided by histidine 108, aspartate 110 and histidine 162 in AtsK, and these residues might therefore also constitute the iron binding site in the oxygenative alkylsulfatase.

Previous studies on hydrolytic alkylsulfatases revealed that substrate binding affinities depend on the length of the aliphatic chain of the sulfate esters (3-5), and led to the conclusion that hydrophobic interactions play a major part in substrate binding in these enzymes. No regular dependence of $K_m$ values on substrate carbon chain length is observed in the case of AtsK (Table 1), suggesting that substrate binding is based instead on recognition of the aliphatic sulfate ester group. However, the presence of the aliphatic chain also plays a role, since of all the substrates tested, methyl sulfate reacted most slowly.
An intriguing aspect of alkylsulfatase investigations to date has been the question of what enzymic properties allow alkylsulfatases to tolerate high concentrations of their detergent substrates. Activity of AtsK was not inhibited when the aliphatic sulfate esters were added at concentrations up to 10 mM, with the exception of nonyl sulfate and SDS, where an inhibitory effect was observed above 1 mM. Although it cannot be ruled out that a denaturing effect of the detergents is the cause of this inhibition, it is also possible that micelle formation under the specific buffer conditions used may have led to reduced substrate availability. Micelle formation has been observed earlier in a study on an SDS-degrading enzyme (5) and resulted in a similar type of Michaelis-Menten plot as we obtained for AtsK activity when nonyl sulfate and SDS were added at concentrations up to 10 mM.

AtsK exhibits greatest efficiency with α-ketoglutarate as a cosubstrate, but significant activities were obtained with other mono- and dicarboxylic 2-oxoacids, when added at concentrations exceeding the $K_m$ for α-ketoglutarate by tenfold. 2-Ketoadipate has previously been reported to act as a cosubstrate for other α-ketoglutarate-dependent dioxygenases such as 2,4-dichlorophenoxyacetate dioxygenase ($k_{cat}/K_m$ was 7% of the value observed with α-ketoglutarate) (38), and taurine dioxygenase (4-10% of the desulfonation rate observed with α-ketoglutarate) (24). These findings led to the conclusion that the presence of a second carboxyl group significantly increases the binding affinity. In the case of AtsK, most alternative 2-oxoacids tested supported the reaction at unexpectedly high levels, and it was especially surprising to find that 2-ketovalerate was even a better substrate than 2-ketoadipate. The other monocarboxylic acids tested, 2-ketoctanoate and 3-methyl-2-ketobutyrate, also supported significant reaction rates, which together suggests that cosubstrate recognition by AtsK is less restricted to dicarboxylic acids than in other characterized α-
Oxygenative alkylsulfatase from P. putida

ketoglutarate-dependent dioxygenases.

Hydrolytic alkylsulfatases acting on long-chain aliphatic substrates are located in the periplasm (7, 39, 40). The discovery of a cytoplasmic short-chain (C₃-C₇) alkylsulfatase in a coryneform led to the proposal that the different locations of the sulfatases might be related to the relative potential toxicity of their substrates (40). Thus, the long chain aliphatic sulfate esters, which are more efficient surfactants, would be degraded outside the cell to ensure protection of the cell from membrane and protein damage, while the synthesis of an exocytoplasmic enzyme would be wasteful for cleaving the relatively harmless short-chain sulfate esters. The lack of a typical signal sequence in the atsK gene strongly suggests that AtsK is a cytoplasmic protein. However, AtsK was found to act on sulfate esters with carbon chain lengths ranging from C₄ to C₁₂. Together with the finding that strain PH3(pME4562), in which the atsK gene is not expressed, is not able to utilize hexyl sulfate or SDS as a sulfur source for growth, this suggests that under the sulfate-limited conditions used, no second, periplasmic long-chain sulfatase is expressed in P. putida S-313. In P. aeruginosa PAO1, the situation is somewhat different, since the sulfur-regulated ABC-type transporter AtsRBC was required for growth with hexyl and octyl sulfate, but not for growth with SDS (15). In the latter species it appears that medium- and short-chain-specific alkylsulfatases are present in the cytoplasm, whereas an SDS sulfatase is localized in the periplasm. Since P. aeruginosa expresses an AtsK homologue under sulfate-starvation conditions (protein PA4, (41)), we speculate that in this species the oxygenative alkylsulfatase is required for desulfation of medium-and short-chain sulfate esters, but that an additional periplasmic SDSase is present which does not exist in P. putida S-313. A good candidate for this SDSase is the uncharacterized product of the sdsA gene (42). sdsA encodes
a periplasmic SDSase found in Pseudomonas sp. ATCC19151, whose expression is regulated in that strain by a LysR-type transcriptional regulator, SdsB, which is encoded adjacent to sdsA. Homologues of sdsA can be found in the genome sequences of P. aeruginosa PAO1 (www.pseudomonas.com) and P. putida KT2440 (www.tigr.org), though it is not yet known how their expression is regulated in these species, and whether the SDSase is synthesized in response to sulfate limitation, or as part of the carbon cycle. It will therefore be interesting to compare the substrate specificities of the AtsK proteins from each of these pseudomonads with that of the products of the sdsA genes. Further investigations in this direction are continuing in our laboratory.

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**Table 1**

*K_m* values of the oxygenative alkylsulfatase AtsK for different aliphatic sulfate ester substrates

*K_m* values were determined using an assay mixture containing 100 µM FeCl₂, 200 µM ascorbate, 1 mM α-ketoglutarate, 0.17-0.25 mg/ml enzyme and 50 –1000 µM of the corresponding substrate in 10 mM Tris/acetae buffer (pH 7.0).

<table>
<thead>
<tr>
<th>Alkylsulfate ester substrate</th>
<th><em>K_m</em> [µM]</th>
</tr>
</thead>
<tbody>
<tr>
<td>hexylsulfate</td>
<td>40</td>
</tr>
<tr>
<td>heptylsulfate</td>
<td>271</td>
</tr>
<tr>
<td>octylsulfate</td>
<td>437</td>
</tr>
<tr>
<td>nonylsulfate</td>
<td>60</td>
</tr>
</tbody>
</table>
decylsulfate 97
SDS 34
2-ethyl-hexylsulfate 66

**Fig. 1.** SDS polyacrylamide gel of protein samples obtained during purification of the AtsK enzyme. A: Marker (kDa); B: cell-extract of *E. coli* BL21(DE3)(pME4577) before induction of expression of the *atsK* gene; C: cell-extract of *E. coli* BL21(DE3)(pME4577) harvested after 2 ½ hours of induction of AtsK expression; D: pooled fractions containing AtsK after ResourceQ chromatography; E: purified AtsK enzyme after Superdex200 gel filtration chromatography.

**Fig. 2.** Oxygenative alkylsulfate ester cleavage reaction catalysed by the α-ketoglutarate dependent dioxygenase AtsK from *P. putida* S-313.

**Fig. 3.** Appearance of sulfate (A) and of succinate (B) plotted against disappearance of α-ketoglutarate in the oxygenative alkylsulfate ester cleavage reaction. The sulfate/α-ketoglutarate data (A) were obtained using different amounts of the substrate heptylsulfate (10 µM - 10 mM) under otherwise standard assay conditions, as described in Experimental Procedures. The succinate/α-ketoglutarate data (B) were obtained with different hexylsulfate concentrations (50 - 500 µM). All concentrations were measured by ion chromatography.
**Fig. 4.** Detection of hexanal produced in the oxygenative alkylsulfatase reaction by enzymatic reduction to hexanol. The reaction catalysed by AtsK was coupled to horse liver alcohol dehydrogenase. NADH oxidation was followed continuously by measuring the absorbance at 340 nm. Measurements include control assays without the AtsK enzyme, hexylsulfate or α-ketoglutarate.

**Fig. 5.** Partial protein sequence alignment of the AtsK protein with other α-ketoglutarate dependent oxygenases. The proteins shown are AtsK from *Pseudomonas putida* S-313 (301 amino acids), AtsK from *Pseudomonas aeruginosa* PAO1 (300 amino acids), taurine dioxygenase (TauD) from *Escherichia coli* (283 amino acids) and 2,4- dichlorophenoxyacetate dioxygenase (TfdA) from *Ralstonia eutropha* (287 amino acids). The conserved iron binding motif His-X-Asp-X_{51}-His is marked with asterisks.
\[
\text{AtsK} \rightarrow \text{Fe}^{2+} \\
\text{[Oxidation]} \\
\text{[Reaction]} \\
\text{[Product]}
\]
Characterization of a sulfur-regulated oxygenative alkylsulfatase from Pseudomonas putida S-313
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