

# Purification and characterization of the arylsulfatase synthesized by *Pseudomonas aeruginosa* PAO during growth in sulfate-free medium and cloning of the arylsulfatase gene (*atsA*)

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An arylsulfatase (EC 3.1.6.1) was extracted from *Pseudomonas aeruginosa* PAO1 and purified 2700-fold to homogeneity. Synthesis of this enzyme was repressed when sulfate, cysteine or thiocyanate was supplied as the sole sulfur source for growth, but derepressed with all other sulfur sources tested. The apparent molecular mass was determined by SDS/PAGE to be 57 kDa, and the enzyme was presumed to be a monomer after gel filtration chromatography. The arylsulfatase showed maximal activity at 57°C and pH 8.9, and a  $K_m$  of 105  $\mu$ M for 4-nitrocatecholsulfate. Despite previous reports that both inducible and derepressible forms of arylsulfatase exist in *P. aeruginosa*, we found only one enzyme under a variety of growth conditions: a sulfate-repressed enzyme with a native isoelectric point of 4.76. The gene encoding this enzyme (*atsA*) was isolated by complementation of a Tn5-751 mutant of *P. aeruginosa* PAO1. Sequencing revealed a 1602-bp reading frame encoding a 534-amino-acid protein with sequence similarity to known bacterial and eukaryotic arylsulfatases (30–40% and 25–30% identity, respectively), but lacking the signal peptide which is present in all known sequences. The lack of this signal peptide suggests that the *P. aeruginosa* arylsulfatase is neither periplasmic nor membrane-associated, unlike other known arylsulfatases. The *atsA* gene was located at 15–17' on the *P. aeruginosa* genome by Southern hybridization. Only a single copy was observed under moderate stringency conditions.

**Keywords.** Arylsulfatase; *Pseudomonas aeruginosa*; sulfur metabolism; sulfate limitation.

Arylsulfatase hydrolyzes arylsulfate esters to the corresponding phenols and inorganic sulfate. Its synthesis is subject to complex regulation in bacteria and other microorganisms. Though some arylsulfatases are known which are specifically induced by their substrates [1, 2], most are subject to repression by inorganic sulfate and intermediates of cysteine biosynthesis; their synthesis is derepressed when other substrates (organosulfates, organosulfonates, methionine) are used to provide sulfur for cell growth [3, 4]. We have recently devoted more attention to the sulfate-controlled regulation of arylsulfatase since discovering that synthesis of a number of other proteins outside the cysteine biosynthetic pathway is also subject to strict sulfate repression, in several bacterial species [4]. These proteins constitute the sulfate-starvation-induced stimulon, a species-specific set of proteins whose synthesis is repressed by sulfate and is coregulated with that of arylsulfatase during growth with a variety of sole sulfur sources. The sulfate-repressed proteins were observed in two-dimensional polyacrylamide gels of extracts from *Pseudo-*

*monas putida*, *Escherichia coli*, and *Staphylococcus aureus* [4], and in *Pseudomonas aeruginosa* (unpublished results). Though they have not been identified, it seems probable that they, like arylsulfatase, are involved in sulfur metabolism.

The best investigated bacterial arylsulfatase is that from *Klebsiella aerogenes*, which is subject to a complex set of controls. In this species, repression of arylsulfatase expression by sulfate or cysteine can be reversed by the addition of tyramine to the growth medium [3, 5]. This effect has been traced to coregulation of the arylsulfatase (*ats*) and monoamine oxidase (*mao*) operons as part of the monoamine regulon [6]. A further regulatory link appears to exist with C<sub>1</sub> metabolism, since in *K. aerogenes* a mutation in the negative regulator gene *atsR* was found to be complemented by the *E. coli folA* gene, which encodes dihydrofolate reductase [7]. A putative positive regulator gene (*atsB*) has also been discovered in the arylsulfatase operon of *K. aerogenes*, but has not yet been studied in detail [8]. The reasons for this regulatory complexity are still unclear.

In *P. aeruginosa*, several different arylsulfatase activities have been described in cells cultivated under different growth conditions. One of these enzymes was induced by the presence of arylsulfates as sulfur source, whereas a second enzyme was not substrate-induced, but was repressed by certain sulfur-containing metabolites (sulfate, sulfite, thiosulfate, cysteine), and was only synthesized in their absence [9]. This latter sulfate-repressed activity was later reported to be catalysed by two iso-

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Abbreviation. X-sulfate, 5-bromo-4-chloro-3-indolyl sulfate.

Enzyme. Arylsulfatase (EC 3.1.6.1).

Note. The novel nucleotide sequence data published here have been deposited with the EMBL sequence data bank and are available under the accession number Z48540. The novel amino acid sequence data have also been deposited with the PROSITE database.

**Table 1. Bacterial strains, plasmids and phages used in this study.**

Strain or plasmid	Relevant characteristics	Source or references
<i>P. aeruginosa</i>		
PAO1	prototroph	[46]
PAO1S	spontaneous Sm <sup>R</sup> mutant of PAO1	this study
AS1	<i>atsA</i> ::Tn5-751	this study
<i>E. coli</i>		
ED8654	<i>supE supF hsdR metB lacY gal trpR</i>	[47]
S17-1	<i>hsdR thi pro recA</i> ; RP4-2 integrated into the chromosome ( <i>kan</i> ::Tn7 <i>ter</i> ::Mu)	[48]
DH5 $\alpha$	<i>supE44 <math>\Delta</math>lacU169 (<math>\phi</math>80 <i>lacZ</i><math>\Delta</math>M15) <i>hsdR17 recA1 endA1 gyrA96 thi-1 relA1</i></i>	[49]
JM109	<i>recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi <math>\Delta</math>(<i>lac-proAB</i>)</i>	[50]
Plasmids		
pME9	IncP-1 <i>oriT trfA</i> [= Rep(ts)] Tc <sup>R</sup> Cb <sup>R</sup> Tn5-751 (Tp <sup>R</sup> Km <sup>R</sup> )	[19]
pKT240	IncQ, <i>mob</i> <sup>+</sup> Amp <sup>R</sup> Km <sup>R</sup>	[51]
pLAFR3	IncP-1, <i>cos</i> , Tra, Tc <sup>R</sup>	[20]
pME4012	24.5-kb chromosomal DNA fragment from <i>P. aeruginosa</i> PAO1 in pLAFR3; <i>atsA</i> <sup>+</sup>	this study
pME4019	4.5-kb <i>EcoRI</i> – <i>Bam</i> HI fragment from pME4012 in pLAFR3; <i>atsA</i> <sup>+</sup>	this study
pME4026	3.2-kb <i>EcoRI</i> – <i>Bam</i> HI fragment from pME4019 in pKT240	this study
Phages		
M13 mp18/mp19	single-stranded bacteriophage used for sequencing	[50]
4026-mp18	1.8-kb <i>EcoRI</i> – <i>Sal</i> I fragment from pME4026 in M13mp18	this study
4026-mp19	1.8-kb <i>EcoRI</i> – <i>Sal</i> I fragment from pME4026 in M13mp19	this study
4019Eco-mp19	1.4-kb <i>EcoRI</i> – <i>Eco</i> RI fragment from pME4019 in M13mp19	this study

enzymes which differed slightly in charge and in their catalytic properties [10]. As part of further studies of the sulfate-starvation-induced stimulon in this species, we here report the identification, purification and characterization of a single sulfate-repressed arylsulfatase from *P. aeruginosa* PAO1 grown with ethanesulfonate as sole source of sulfur, and the cloning and sequencing of the gene which encodes it.

## MATERIALS AND METHODS

**Chemicals.** Sulfur-containing chemicals used as sulfur sources for bacterial growth were of the highest purity commercially available, and with the exception of methyl sulfate (Aldrich) and 5-bromo-4-chloro-3-indolyl sulfate (X-sulfate) (Sigma), they were all obtained from Fluka. The sulfate content of all sulfur sources was checked by ion-exchange chromatography before use [11] and found to be less than 0.05 mol/100 mol. DNase I and RNase A were from Boehringer Mannheim. Restriction enzymes were obtained from Boehringer or from New England Biolabs and were used according to the manufacturer's instructions.

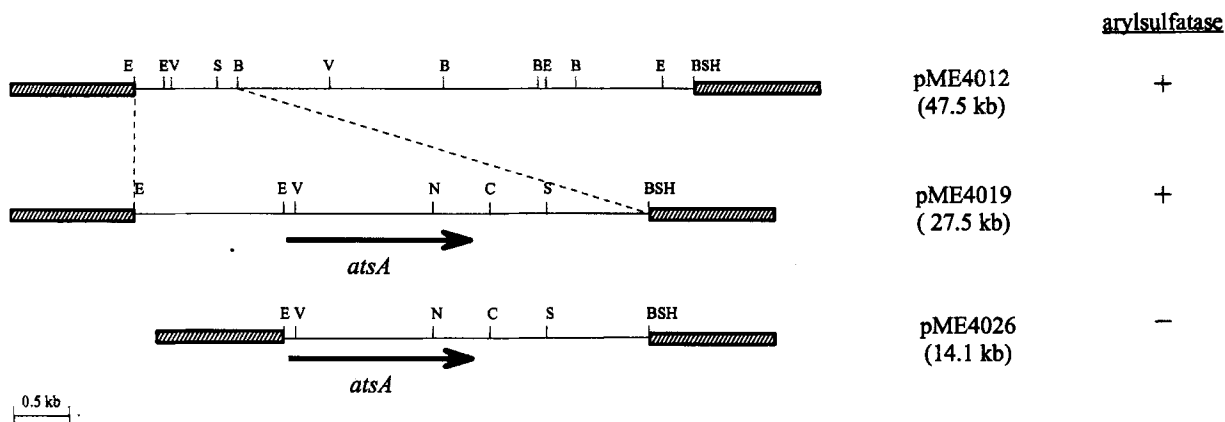
**Bacterial strains, plasmids, and growth media.** Bacterial strains used in the present study are listed in Table 1. *E. coli* strains were all grown routinely at 37°C in Luria-Bertani medium [12]. Strains of *P. aeruginosa* were grown either in nutrient yeast broth [13] or in a synthetic acetate/salts medium containing 50 mM sodium acetate, 20 mM ammonium chloride, 20 mM potassium chloride, 20 mM sodium chloride, 2 mM potassium phosphate, 0.5 mM magnesium chloride, 50 mM Tris/HCl, a sulfate-free trace element solution (10 ml/l) [14] and all the naturally occurring protein amino acids except cysteine and methionine (40 µg/ml each); the pH of the medium was adjusted to 7.3. Sulfur sources were added as described in the text. Solid media were prepared by addition of 1.5% (mass/vol.) agar to complex media or 0.6% (mass/vol.) Seaplaque agarose (FMC Bioproducts, Rockland ME) to minimal media. Antibiotic concentrations used were, for *E. coli* kanamycin 25 µg/ml, ampicillin 100 µg/ml, tetracycline 20 µg/ml; for *P. aeruginosa* strepto-

mycin 500 µg/ml, kanamycin 300 µg/ml, trimethoprim 300 µg/ml, carbenicillin 500 µg/ml and tetracycline 125 µg/ml. Relevant plasmids constructed in the course of the present work are shown in Table 1 and Fig. 1. All glassware used in sulfate-limited experiments was washed with 3 M HCl and rinsed thoroughly with glass-distilled water before use.

**Enzyme assays.** Arylsulfatase was routinely assayed as 4-nitrocatechol release from 4-nitrocatechol sulfate at 37°C. The assay mixture (250 µl) contained 70 mM Tris/acetate pH 8.8 and 10 mM 4-nitrocatechol sulfate, and the reaction was started by addition of enzyme. After suitable times, the reaction was stopped by diluting portions tenfold into 0.66 M NaOH, and the 4-nitrocatechol produced was quantified spectrophotometrically ( $\lambda = 515$  nm,  $\epsilon = 13$  mM<sup>-1</sup> cm<sup>-1</sup>). Arylsulfatase was routinely assayed over 10–30 min but in the presence of sufficient substrate the reaction was linear over several hours.

For studies of the substrate range of arylsulfatase, the purified enzyme (0.1 µg protein) was incubated for 30 min at 37°C with 10 mM substrate in 100 mM Tris/acetate pH 8.8; the reaction was stopped by heating to 100°C for 15 min. All substrates tested were stable under these conditions in the absence of arylsulfatase. Sulfate released during the enzyme reaction was quantified by ion-exchange chromatography with chemical suppression, as previously described [11].

**Purification of arylsulfatase.** *P. aeruginosa* PAO1S was grown in acetate/salts medium containing 500 µM ethanesulfonate as sole sulfur source (1-l cultures in 5-l conical flasks), and incubated aerobically at 37°C on a rotary shaker (180 rpm). Growth was monitored as absorbance at 650 nm. Cells were harvested in the mid-exponential phase ( $A_{650} = 0.8$ ) by centrifugation (7000×g, 25 min, 4°C) and washed with 50 mM Tris/HCl pH 7.3 containing 1 mM EDTA (8000×g, 20 min, 4°C). They were then resuspended in the same buffer (0.3 g wet mass/ml) and ruptured by four passages through a chilled French pressure cell at 135 MPa. DNase I (50 µg/ml), RNase A (10 µg/ml) and MgCl<sub>2</sub> (2 mM) were added, and cell debris was removed by centrifugation (20000×g, 30 min, 4°C). After an ultracentrifugation step (250000×g, 60 min, 4°C), the supernatant fluid was stored at –20°C. This extract was thawed and refrozen several



**Fig. 1. Restriction maps of recombinant plasmids carrying the *atsA* gene of *P. aeruginosa* PAO.** The size of the plasmid and the ability of the plasmid to complement the arylsulfatase-negative phenotype of mutant strain AS1 are shown on the right-hand side. (—) *P. aeruginosa* DNA; (▨) vector fragments. Restriction sites: B, *Bam*HI; E, *Eco*RI; C, *Cla*I; H, *Hind*III; N, *Not*I; S, *Sal*I; V, *Eco*RV.

times, leading to selective precipitation of contaminating proteins, and an increase in the specific activity of about 10%.

Purification of arylsulfatase to homogeneity was achieved in five chromatographic steps. An initial, high-capacity separation over a DEAE-Sepharose CL-6B column [15] was performed using a standard, automated protein liquid chromatography system (Pharmacia) at 4°C. The remaining steps were done with commercially packed columns on an apparatus which has been described elsewhere [15]. These steps were performed at room temperature, with samples collected at 4°C. Where required between chromatographic steps, protein solutions were concentrated by ultrafiltration using Centriprep or Centricon concentrators (Amicon). These were pretreated overnight with Tween-20 (5%, by vol.) and washed thoroughly with distilled water before use. The concentrators then afforded reproducible enzyme recoveries of 95% and greater; without pretreatment, recovery was 40–50%.

**Step 1.** The DEAE-Sepharose column (30 mm×135 mm) was equilibrated with 50 mM Tris/HCl pH 7.5 (2.5 ml/min). Crude extract (about 400 mg protein) was applied to the column, which was then washed with the start buffer for 48 min. A linear gradient of Tris/HCl pH 7.5 was then applied (50–600 mM over 110 min), fractions were collected (8 ml) and tested for arylsulfatase activity. Arylsulfatase eluted at about 220 mM Tris/HCl. Active fractions were pooled and concentrated to a volume of 1.5 ml.

**Step 2.** The Fractogel TSK Butyl 10/150 hydrophobic interaction column was equilibrated with 50 mM Tris/SO<sub>4</sub> pH 7.5, containing 1.7 M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (2.0 ml/min). The concentrated material from step 1 was diluted to 10 ml with distilled water, and solid ammonium sulfate added to a concentration of 1 M, with stirring at 4°C. The solution was centrifuged to remove particulates (25000×g, 45 min, 4°C), applied to the column (about 100 mg protein) and the column was washed with the start buffer for 23 min. Proteins were eluted by decreasing the ammonium sulfate concentration linearly from 1.7 M to zero over 60 min, and collected in 1.5-ml fractions. Arylsulfatase eluted at 0.12 M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>. The active fractions were pooled, diluted with 20 mM Tris/acetate pH 7.5, to reduce the sulfate concentration to 10 mM, and concentrated by ultrafiltration.

**Step 3.** The Mono Q HR 5/5 anion-exchange column was equilibrated with start buffer (20 mM Tris/SO<sub>4</sub> pH 7.5), at a flow rate of 0.5 ml/min. The concentrated sample from step 2 was loaded (about 10 mg protein), the column was washed with start buffer for 34 min, and proteins were eluted with an increasing gradient of Na<sub>2</sub>SO<sub>4</sub> (0–85 mM over 34 min). Fractions were col-

lected (0.5 ml), and the arylsulfatase activity was recovered at about 50 mM sulfate. Active fractions were pooled and concentrated to about 150 µl.

**Step 4.** The Superose 12 HR 10/30 column was equilibrated with 50 mM Tris/SO<sub>4</sub>, 250 mM Na<sub>2</sub>SO<sub>4</sub>, pH 7.5 (0.25 ml/min). The concentrated material from step 3 (about 1 mg protein) was loaded onto the column, and then eluted isocratically with start buffer while 125-µl fractions were collected. Arylsulfatase eluted in a single symmetrical peak, although the fractions containing arylsulfatase activity revealed two proteins on SDS/PAGE. This preparation was stored at –20°C, and used for enzyme studies. Homogeneous, but no longer stably catalytically active, enzyme was obtained by a further purification step.

**Step 5.** The ProRPC 5/10 C<sub>4</sub> reverse-phase column was equilibrated with 0.1% (by vol.) aqueous trifluoroacetic acid (0.5 ml/min). Material from step 4 was diluted 3.5-fold with distilled water and applied to the column (0.4 mg protein). Proteins were eluted with an increasing gradient of methanol/0.1% trifluoroacetic acid (0–70% methanol, 0–30 min; 70–100% methanol, 30–60 min), while fractions were collected (0.25 ml). Arylsulfatase eluted at about 70% methanol but enzyme activity rapidly diminished due to denaturation. Fractions containing pure arylsulfatase were stored at –20°C and used subsequently for amino acid sequence determination.

**Electrophoretic methods.** Protein purification was routinely monitored by SDS/PAGE [16], using a 12% separating gel and a 4% stacking gel in a MiniProtean slab gel apparatus (Bio-Rad). Proteins were visualized either with Coomassie blue R-250 [15] or by silver staining [17]. Non-denaturing gel electrophoresis was performed similarly, but SDS was not added to gels, loading buffer or running buffer. Arylsulfatase was visualized in non-denaturing gels by incubation for 1–18 h at 37°C in 1 mM X-sulfate, 100 mM Tris/acetate pH 8.8.

Non-denaturing isoelectric focussing was performed at 10°C under a layer of DC200 silicone oil (Fluka), using preformed pH gradients (pH 3–10 and 4–7) in Immobiline Drystrip gels (Pharmacia) on a Multiphor flatbed apparatus (Pharmacia). The gels were reconstituted as described by the manufacturer and protein (up to 85 µg) was loaded onto the gel and allowed to enter the gel at low voltage (300 V, 2 h; 500 V, 3 h). The voltage was then increased to 3500 V over 2 h and electrophoresis continued at this voltage for 17 h (total 67000 V h). Denaturing isoelectric focussing was performed with Immobiline Drystrip gels in the presence of 8 M urea at 20°C. Standard proteins for isoelectric focussing were obtained from Pharmacia and standards for SDS/PAGE were supplied by Bio-Rad.

**Protein-sequencing methods.** N-terminal amino acid sequences were determined by automated Edman degradation using an Applied Biosystems 120A PTH sequenator. Internal amino acid sequences were obtained as follows. Purified arylsulfatase (200 pmol) was digested sequentially with cyanogen bromide (200 mM CNBr, 70% HCOOH, 18 h, room temperature, dark) and trypsin (0.5  $\mu$ g, 0.5 M urea/50 mM ammonium hydrogen carbonate pH 8.5, 12 h). The resulting peptides were separated by reverse-phase HPLC as previously described [18] and analysed in a TSQ710 triple quadrupole mass spectrometer (Finnigan MAT) with an electrospray ionization interface (Analytica, Branford CT). Scans were accumulated from 400–2000 Da in 4 s and the amino acid sequences of individual peptides were determined using the PEPMAP program (Finnigan MAT).

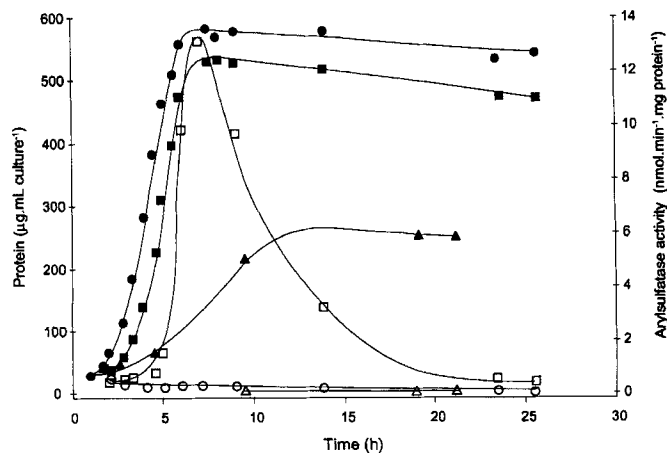
**DNA techniques and sequencing.** DNA was isolated, purified and manipulated by standard techniques [12, 17]. DNA sequencing of the *atsA* gene was performed by subcloning the 1.4-kb *EcoRI*–*EcoRI* fragment of pME4019 and the 2.6-kb *EcoRI*–*SalI* fragment of pME4026 (Fig. 1) into M13mp18 and M13mp19 phages (Table 1), and sequencing by the dideoxy chain-termination method, using either universal primers or specific synthetic primers. Both DNA strands were sequenced.

**Generation and complementation of arylsulfatase-negative mutants.** Transposon mutagenesis of *P. aeruginosa* PAO1 with the Tn5 derivative Tn5-751 was performed as previously described [19]. Mutants with reduced or zero expression of the arylsulfatase gene were identified by plating cells on the synthetic acetate/salts medium containing 200  $\mu$ M ethanesulfonate and the chromogenic arylsulfatase substrate X-sulfate (40  $\mu$ g/ml). A genomic library of *P. aeruginosa* PAO1 in *E. coli* S17-1 was prepared from chromosomal DNA by partial digestion with *Sau3A* and ligation of the 18–30-kb fraction into the *Bam*HI restriction site of the cosmid vector pLAFR3 [20] (Serino, L., unpublished). Complementation of the Tn5-751 mutation in strain AS1 was done by plate conjugation with sub-pools of the library. The conjugation was performed by patching concentrated suspensions of donor and recipient cells together on nutrient agar for 4 h at 37°C. The recipient cells (strain AS1) were then screened for recovery of the ability to hydrolyze 4-nitrocathechol sulfate during growth in selective medium with methionine (70  $\mu$ M) as sulfur source.

**Other procedures.** Total protein in cell suspensions was determined by a modified Lowry method [21]. Soluble protein was measured by the method of Bradford [22], using bovine serum albumin as standard. Molecular mass values for native proteins were determined by gel filtration through a Superose 12 column at a flow rate of 0.25 ml/min, using the following proteins as standards: ribonuclease A (13.7 kDa), chymotrypsinogen (25 kDa), ovalbumin (43 kDa), bovine serum albumin (67 kDa), aldolase (158 kDa).

## RESULTS

**Derepression and purification of arylsulfatase.** *Pseudomonas aeruginosa* PAO1 did not synthesise significant levels of arylsulfatase in acetate/salts medium containing sulfate as the sole added source of sulfur for growth. However, when ethanesulfonate was used as sole source of sulfur, arylsulfatase was produced in significant amounts (Fig. 2). Repression of arylsulfatase synthesis was also observed with cysteine or thiocyanate as sulfur sources, whereas with all other sulfur sources tested (alkyl and aryl sulfates, sulfamates, methionine) arylsulfatase synthesis was derepressed (not shown). Growth with tyramine in the presence of sulfate did not reverse the repressive effect of sulfate on arylsulfatase synthesis, either in the presence of ammonium ions



**Fig. 2.** Arylsulfatase activity in *P. aeruginosa* PAO during growth. *P. aeruginosa* was grown at 37°C in synthetic mineral salts/acetate medium either with ammonium as N source (20 mM) and sulfate (●, ○) or ethanesulfonate (■, □) as S source (500  $\mu$ M), or with tyramine as N source (5 mM) and sulfate (500  $\mu$ M) as S source (▲, △). Samples were removed at intervals for determination of protein content (●, ■, ▲) and arylsulfatase activity (○, □, △) as described in Materials and Methods.

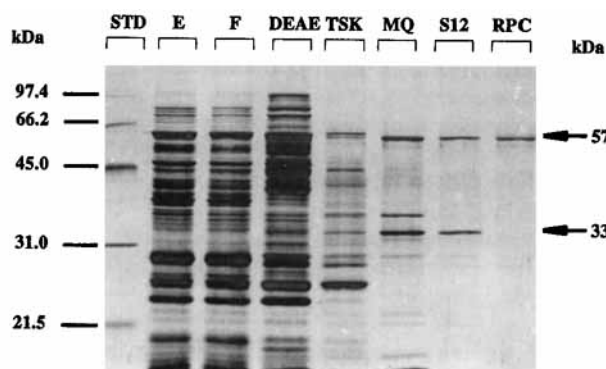
(not shown) or when tyramine was the sole nitrogen source present (Fig. 2). This is in contrast to the situation found in enteric bacteria, where tyramine overcomes sulfate-mediated repression [3].

As part of our studies of the sulfate-starvation-induced stimulon [4], we were interested in characterization of the sulfate-repressed enzyme previously reported [10]. During growth with ethanesulfonate, we observed a clear maximum in arylsulfatase activity at the end of the exponential growth phase (Fig. 2). The cells were therefore harvested in the mid-exponential phase of growth, disrupted in a French pressure cell, and the membrane fraction removed by ultracentrifugation. Repeated freeze/thawing (–20°C) and centrifugation of this crude extract led to an initial purification, since contaminating proteins could be denatured and removed without any loss of arylsulfatase activity. The enzyme was then purified to apparent homogeneity using a series of chromatographic steps (Table 2, Fig. 3). An initial crude fractionation over a DEAE-Sepharose column was followed by a hydrophobic interaction-exchange chromatography step. Arylsulfatase eluted very late from this column, at 0.12 M  $(\text{NH}_4)_2\text{SO}_4$ , indicating that the protein was quite hydrophobic. The hydrophobic nature of the enzyme was also apparent from the substantial losses which were experienced during concentration by ultrafiltration with commercial Centricon and Centriprep ultrafiltration units (Amicon). These losses presumably arose from binding of the enzyme to the ultrafiltration units used, since full recovery could be obtained after passivization of these units before use by overnight treatment in 5% (by vol.) Tween 20 [23].

Further purification was obtained by anion exchange (Mono Q column) and gel filtration (Superose 12 column). At this stage, the fractions exhibiting arylsulfatase activity still contained two proteins, 57 kDa and 33 kDa in size, which had copurified throughout the procedure described (Fig. 3). These two proteins could be separated by chromatography over a  $\text{C}_4$  reverse-phase column in methanol/0.1% trifluoroacetic acid. The arylsulfatase activity was unstable in the solvent used for reverse-phase chromatography (70% methanol/0.1% trifluoroacetic acid) but could still be measured immediately after elution, and was found only in fractions containing the 57-kDa protein. The 33-kDa species was hence not required for enzyme activity and did not consti-

**Table 2. Purification of arylsulfatase from *P. aeruginosa* PAO1S.** Arylsulfatase activity was measured as formation of nitrocatechol from nitrocatechol sulfate as detailed in Materials and Methods. The corrected activity values after reverse-phase chromatography (last line in Table) take into account the inactivation of arylsulfatase enzyme caused by the 70% methanol/0.1% trifluoroacetic acid used as FPLC eluent.

Step	Total activity	Total protein	Specific activity	Recovery	Purification
	$\mu\text{mol} \cdot \text{min}^{-1}$	mg	$\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg} \cdot \text{protein}^{-1}$	%	-fold
Crude extract	27.0	443	0.061	100	1.0
Freeze-thawing	29.6	296	0.100	110	1.6
DEAE-Sepharose	76.8	96.6	0.795	284	13
TSK-Butyl	54.7	11.4	4.80	202	79
Mono Q	21.4	1.40	15.2	79.1	250
Superose 12	21.9	0.40	54.4	81.2	892
Reverse-phase chromatography	2.5	0.04	55.4	9.2	908
Reverse-phase chromatography (corr.)	6.6	0.04	165.0	24	2700



**Fig. 3. SDS/PAGE of the purification of arylsulfatase from *P. aeruginosa* PAO1.** Proteins were visualized by silver staining [17]. The arrows show the arylsulfatase (57 kDa) and the co-purifying 33-kDa protein. Lanes: E, crude extract (3  $\mu\text{g}$  protein); F, freeze-thawing step (2.5  $\mu\text{g}$  protein); DEAE, proteins eluting from the DEAE-Sepharose column (12  $\mu\text{g}$  protein); TSK, proteins eluting from the TSK-Butyl column (2.0  $\mu\text{g}$  protein); MQ, proteins eluting from the Mono Q column (1.0  $\mu\text{g}$  protein); S12, proteins eluting from the Superose 12 column (0.5  $\mu\text{g}$  protein); RPC, proteins eluting from the ProRPC column (0.2  $\mu\text{g}$  protein). STD, standard protein markers (each  $\approx 0.2$   $\mu\text{g}$  protein).

tute a functional second subunit of the enzyme. The kinetics and properties of the enzyme were studied further with the eluate from the Superose column.

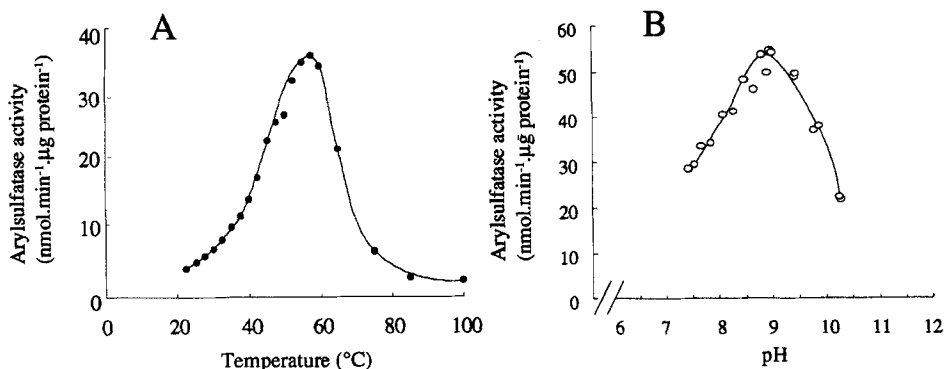
**Characterization of arylsulfatase.** *P. aeruginosa* arylsulfatase was stable both in crude cell extracts and in the highly purified preparation obtained after Superose 12 chromatography, and could be stored at  $-20^{\circ}\text{C}$  for several months without loss of enzyme activity. It was also stable to incubation for 20 min at  $50^{\circ}\text{C}$ , though extended incubation at temperatures above this value led to inactivation. The enzyme activity displayed a sharp temperature optimum at  $57^{\circ}\text{C}$  (Fig. 4A), in contrast to previous studies [10] where a flat temperature profile with an optimum of about  $37^{\circ}\text{C}$  was reported. The pH optimum observed for the enzyme was 8.9 (Fig. 4B), which was very similar to that previously reported both for the *P. aeruginosa* enzyme [10] and for arylsulfatases from various other species [3]. The  $K_m$  measured for 4-nitrocatecholsulfate was 105  $\mu\text{M}$ . The denatured molecular mass of 57 kDa determined by SDS/PAGE was close to the value of 60 kDa previously reported [10]. Presumably because of the enzyme's highly hydrophobic nature, an accurate measure of native molecular mass could not be obtained by gel filtration. The enzyme bound strongly to the Superose column, even when

increased levels of salt were included in the buffer, and was eluted at an apparent molecular mass of 39 kDa, much lower than the value of 57 kDa measured by SDS/PAGE. Interactions of this sort between hydrophobic proteins and Superose columns are well known [24] and can lead to inaccurate measurements of native molecular mass as seen here. From the apparent molecular mass observed, the enzyme is probably present as a monomer, as found previously for other bacterial arylsulfatases [3].

In order to determine whether one or two isozymes were present, native electrophoresis was performed with samples taken from different stages of the purification process, and arylsulfatase activity in the gel was visualized by staining with X-sulfate. In each case, a single, sharp band with arylsulfatase activity was found. Cell extracts prepared from cells cultivated with ethanesulfonate, nitrocatechol sulfate or methionine as their sole source of sulfur were also compared by native isoelectric focussing, to check for the presence of various forms of arylsulfatase under different growth conditions. The isoelectric point observed for the native enzyme was 4.76. Under denaturing conditions a value of 7.6 was obtained and, in each case, a single band of arylsulfatase activity was observed, which corresponded to the purified enzyme from ethanesulfonate-grown cells. This, and the fact that in all the protein purification steps the arylsulfatase activity was found to elute in a single peak, suggested that, in contrast to the *P. aeruginosa* strains studied previously [9, 10, 25], strain PAO1 contains a single sulfate-repressed arylsulfatase.

**Substrates and inhibitors of arylsulfatase.** The range of substrates hydrolysed by the *P. aeruginosa* arylsulfatase was measured as sulfate release from a range of sulfate esters and quantified by ion-exchange chromatography [11]. The enzyme was able to hydrolyse various aromatic sulfate esters. Highest activity was seen with nitrophenyl sulfate as substrate, and lower values were found for nitrocatechol sulfate (57% of the activity found for nitrophenyl sulfate), X-sulfate (41%), and indoxyl sulfate (7%). The sulfonate analogue of phenyl sulfate, phenylmethanesulfonate, was not hydrolysed at all by the enzyme, nor was the sterically similar alkyl sulfate dimethylcyclohexyl sulfate.

The response of *P. aeruginosa* arylsulfatase to a number of compounds known to inhibit different classes of arylsulfatase was tested with nitrocatechol sulfate as substrate, and is shown in Table 3. *P. aeruginosa* arylsulfatase appears to be a typical class I arylsulfatase [26], since cyanide ( $> 10$  mM) inhibited its activity, but no effect was seen with sulfate, phosphate or chloride, nor with thiol reagents such as 2-mercaptoethanol or gluta-



**Fig. 4.** Effect of temperature and pH on arylsulfatase activity from *P. aeruginosa* PAO1. (A) Effect of temperature. The assay was started by the addition of purified arylsulfatase (0.22 µg protein) to the warmed assay mixture and was terminated after 10 min. The results shown are the average of three experiments. (B) Effect of pH. The reaction was performed with crude extract (260 µg protein) for 15 min at 37°C in 50 mM Tris/50 mM glycine adjusted to the appropriate pH. The pH was checked after the enzyme reaction using a microelectrode.

**Table 3.** Inhibitors of arylsulfatase from *P. aeruginosa* PAO1. Residual activity was determined in a standard assay (30 min, 37°C).

Inhibitor	Concentration	Residual activity
	mM	%
Na <sub>2</sub> SO <sub>4</sub>	40	100
Na <sub>2</sub> HPO <sub>4</sub>	40	91
KCN	1	95
	10	64
NaCl	240	103
NaF	1	94
	70	79
Glutathione	7	95
2-Mercaptoethanol	10	94
Sodium dodecyl sulfate	0.1	23
	1	3
Sodium dodecyl sulfonate	0.1	89
	1	19
Sodium hexyl sulfate	1	96
	10	89
Sodium pentane sulfonate	1	95
	10	97
Sodium ethane sulfonate	1	95
	10	96
Phenylmethanesulfonyl fluoride	1	57
	10	9
Sodium phenylmethane sulfonate	1	68
	10	17
<i>p</i> -Toluenesulfonate	1	93
	10	100
Tyramine	1	91
	10	66
<i>p</i> -Cresol	1	79
	10	23

thione. By contrast, the presence of sodium dodecyl sulfate led to complete inhibition of the enzyme even at low concentrations. This was presumably due to binding of the detergent alkyl chain to the hydrophobic protein, since sodium dodecyl sulfonate also inhibited the enzyme strongly, whereas the short-chain ana-

logues pentanesulfonate and hexylsulfate displayed much less inhibition. Phenylmethanesulfonyl fluoride also inhibited arylsulfatase, but appeared to act as a substrate analogue rather than by covalent binding to the enzyme, since the inhibitory effect was not time-dependent and could be reversed after gel filtration to remove the inhibitor. Incubation with phenylmethanesulfonate gave levels of inhibition similar to those observed for phenylmethanesulfonyl fluoride. Toluene sulfonate, by contrast, did not inhibit the enzyme, whereas cresol and tyramine were both moderate inhibitors.

**N-terminal sequence and mass spectroscopic peptide fingerprint studies.** The N-terminal amino acid sequence determined for arylsulfatase was as follows: Asp-Lys-Arg-Pro-Asn-Phe-Leu-Val-Ile-Val-Ala-Asp-Asp-Leu-Gly-Phe-Ser-Asp-Ile-Gly-Ala-Phe-Gly-Gly-Glu-Ile. For further comparison with other known arylsulfatases and with the gene sequence (Fig. 5), arylsulfatase from *P. aeruginosa* was subjected to cleavage with cyanogen bromide and subsequent tryptic digestion; the resulting peptides were separated by HPLC and analysed by mass spectrometry. The peptide sequences determined for the main peptide peaks observed are shown in Fig. 5; they corresponded exactly with the amino acid sequence derived from the gene.

**Generation of an arylsulfatase-negative mutant of *P. aeruginosa*.** In order to isolate and characterize the gene encoding arylsulfatase, *P. aeruginosa* PAO was mutagenized with the transposon Tn5-751 [19]. This transposon is a derivative of Tn5 encoding both kanamycin and trimethoprim resistance, carried on the temperature-sensitive plasmid pME9. Mutants in arylsulfatase expression were identified by plating on the synthetic acetate/salts medium containing X-sulfate and ethanesulfonate. One mutant was obtained which could not grow with arylsulfate esters as sole sulfur source, and which showed no arylsulfatase activity after cultivation with methionine, alkanesulfonates, or alkylsulfate esters as sulfur source, although growth with these compounds was identical to that of the wild-type strain. This mutant was named AS1. Several other mutant strains were identified which displayed reduced but non-zero arylsulfatase activity under derepressing conditions, but these organisms have not yet been further investigated.

**Cloning and sequencing of the arylsulfatase gene.** A genomic cosmid library of *P. aeruginosa* PAO in the cosmid vector pLAFR3 [20] was used for complementation of the mutation in strain AS1. Plate conjugation of individual clones of the library with strain AS1 and screening for arylsulfatase activity led to

1	CAGTGCAGGAAGAGTTGCTGCGATCCGCCGGCGCACCGTATCACCACCTGTCTGGTACACCCACGACGTGGAGGAGCGCTCTACCTGGCC	90
91	GACCGGGTGGTGGTGGTGGAGCCCGTCCGGGGCGGATCAGCGGGTGGTTCGAGGTGGCCCTGGCGCATCCGCGCGAGCGGGTGGCTTC	180
181	GA <sup>EcoRI</sup> CTTCCTGGCGCCAGCGGAAGCGTGTGCACGA <sup>EcoRI</sup> ACTGACCGCCGACGGCGAGTACGTCCGCCCGCCGCGAGCGGGTGGAAAACT	270
271	GCCCTTCGAATTCATCGCTTGTGAAAAGCCCTTGCCTTGGCCCTTCCCTCCCTGCGGGAAGGGCGGGGGCGAGGGCCCCACCTTCTCCT	360
361	CCCATCCAGAC <b>CGAGAC</b> CCCGCGCATGAGCAAACGCCCAACTTCCTGGTGGTATCGTCCGCCGACGACCTGGGCTTCTCCGATATCGGGCGCTT	450
	<b>at#A M S K R P N F L V I V A D D L G F S D I G A F</b>	
451	CGGCGCGAGATCGCCACCGCCGAACCTCGACGCCCTGGCCATCGCCGGGCTGCGCCTGACCGACTTCCACACCGCCTCGACCTGCCTCGCC	540
	<b>G G E I A T P N L D A L A I A G L R L T D F H T A S T C S P</b>	
541	GACCCGCTCGATGCTGCTCACCGGCACCGACCACCATCGCCGGGATCGGCACCATGGCCGAGGCGCTGACCCCGGAACCTGGAAGGCAA	630
	<b>T R S M L L T G T D H H I A G I G T M A E A L T P E L E G K</b>	
631	GCCGGTTACGAAGGCATCTCAACGAGCGTGGTGGCGCTGCGGAGCTGCTCCGCGAGGCGGCTACCGAGCCCTCATGGCCGGCAA	720
	<b>P G Y E G H L N E R V V A L P E L L R E A G Y Q T L M A G K</b>	
721	GTGGCACCTCGGTCTGAAGCCGAACAGACGCCCATGACCGGTTTCGAGCGTTCCTTCTCGCTGCTGCCGGGCGCCCAACCACTA	810
	<b>W H L G L K P E Q T P H A R G F E R S F S L L P G A A N H Y</b>	
811	TGGTTTCGAGCGCCCTACGACGAAAGCACTCCCGCATCTCAAGGGTACGCCAGCGCTCTACGTGGAAGACGAGCGCTACCTCGACAC	900
	<b>G F E P P Y D E S T P R I L K G T P A L Y V E D E R Y L D T</b>	
901	GCTGCGGAGGGCTTCTATTCCTCCGACGCCCTTCGGCGACAAGCTGCTCAATACCTCAAGGAGCGCGACCGAGCGCGCTTCTTCGC	990
	<b>L P E G F Y S S D A F G D K L L Q Y L K E R D Q S R P F F A</b>	
991	CTACCTGCGGTTCTCCGCGCACTGGCCGCTGCAAGCGCCGGGAGATCGTCGAGAAGTACCGCGTTCGCTACGACCGCCGTCGCA	1080
	<b>Y L P F L P L A P H W P L Q A P R E I V E K Y R G R Y D A G P E</b>	
1081	AGCGTGCGCCAGGAACCGCTGGCCGGCTCAAGGAGCTGGCCCTGGTGGAAAGCGGACGTGAAGCCATCCGGTGCCTCGCCCTGACCCG	1170
	<b>A L R Q E R L A R L K E L G L V E A D V E A H P V L A L T R</b>	
1171	CGAGTGGGAGGCCCTGGAGGACGAGGAACGGGCTAAGTCGGCGCGGGCGATGGAGTCTACGCGCGATGGTTCGAGCGCATGGACTGGAA	1260
	<b>E W E A L E D E E R A K S A R A M E V Y A A M V E R M D W N</b>	
1261	CATCGCCAGGGTCTGGACTACCTGCGCCGGCAGGGCGAGCTGGACAACACCTTCGCTCTGTTCATGTCCGACACCGCCGAAGGGCC	1350
	<b>I G R V V D Y L R R Q G E L D N T F V L F M S D N A P K A P</b>	
1351	CTGCTGGAGGCGTCCCGAATCGGCGGACCTGCTGGCTTCTCGACCGCACTACGACAAAGCCCTGGAAAACATCGGCGCGCCCAATTC	1440
	<b>C W R R S R I G R T C W L L D R H Y D N S L E M I G R A N S</b>	
1441	CTACGTCTGGTATGGCCCGCTGGGCCACGGCGCCACCGACCATCGCGCTGTACAGGCGTTCACCACCCAGGGCGGGATTCCGCT	1530
	<b>Y V W Y G P R W A Q A A T A P S R L Y K A F T T Q G G I R V</b>	
1531	GCCAGCGCTGGTGCCTACCCCGGCTAAGCCGGCAGGCTGCGATCAGCCATGCCTTCGCCACGGTGTGACGTCACCCCGACCCCTCCT	1620
	<b>P A L V R Y P R L S R Q G A I S H A F A T V M D V T P T L L</b>	
1621	CGACCTCGCCGTTGTCGCCACCCAGGCAAGCGCTGGCCGCGCGGAGATCGCCGAGCCGCGCGGAGGTCTGGCTGGGTTGGCTTTC	1710
	<b>D L A G V R H P G K R W R G R E I A E P R G R S W L G W L S</b>	
1711	CGGCGAGACCGAGGCGCCACGACGAGAACCCTGACCGGCTGGGAGCTGTTTCGGCATGCGTGCATCCGCCAGGGCGACTGGAAGGC	1800
	<b>G E T E A A H D E N T V T G W E L F G M R A I R Q G D W K A</b>	
1801	GGTGTACCTGCGCGCCCGTGGCCGCCACCTGGCAGCTCTACGACCTGGCCCGCAGCCGGGCGAGATCCACGACCTCGCTGACAGCCA	1890
	<b>V Y L P A R W P A T W Q L Y D L A R D P G E I H D L A D S Q</b>	
1891	GCCGGCAAGCTGGCGGAGCTGATCGAGCATTGAAGCGATACGTCAGCGAGACCGGTTCGCTAGAGGGGGCTTCGCTTCTCTGGTGG	1980
	<b>P G K L A E L I E H W K R Y V S E T G V V E G A S P F L V R</b>	
1981	ATAAGCGGAACCTCTCCCTCGCCCTCTCCCTCAGGAGGGAACGCTTGGCCAGAAGGTGGGGAGAGGGGCTACTTCGCGGGTGGAAAC	2070
	<b>*</b>	
2071	GAGCGACCCGGGTCAAGCCGAGAAACGCCATCGATGGTCAAGGCTGGCCCGGTTGATGTAGGCCCTTCCGCGCCGGCCAGTAGGCGA	2160
	<b>Clai</b>	
2161	CGAAGCTGGCGATTCTCTCGCTGGCGCGTAGCGCGCAGCCATCAGGCCCTTGAGGGCTCGGCGAAGTCGCGCTCGTCCGGTTC	2250
2251	TGTCGGTATCCACCGGACCCGCTGCACGTTGTTACCGTATCCCGCGCGGCGAGGTCGCGGGCCAGGCCCTTGGTTCAGGCCGACCA	2340
2341	GCGCCGACTGCTCATCGCTAGGTGGCCCGCGCGAAGCGCATGCGTTCGGCATTGGTCTGCCGATGCTGATACCCCGCCCGCGCTG	2430
2431	CCCATGTGCTTACCGCCGCTGGGTGGCGACGAAGCGCTGCGCAGTTGATCGCCAGGTCGCGCTCGAAGTCGCGCCAGCGCGAGCTC	2520
	<b>Sali</b>	
2521	TTCACCGAACCAGGGCGAGGACGCGCGCATGTTGACCGAGATGTCGATCGTCCGAGCGAGCCGCTTCGTCGAC	2601

Fig. 5. Nucleotide sequence of the 2600-bp sequence containing the *atsA* gene and the deduced amino acid sequence. Regions identified by N-terminal automated Edman sequencing or HPLC/MS sequencing of the arylsulfatase protein are shown in bold face. The putative ribosome binding site for *atsA* is boxed. The stop codon is shown with an asterisk.

identification of a single cosmid (pME4012) which contained the arylsulfatase gene (*atsA*). The gene was then localized to a 4.5-kb fragment in plasmid pME4019 by deletion analysis (Fig. 1), and primers designed from the N-terminal sequence of the protein were used to localize the exact position of the open reading frame. Both strands of the arylsulfatase gene and adjacent sequences were then sequenced; this sequence has been submitted to the EMBL data bank. The open reading frame thus identified encodes a protein consisting of 534 amino acids with

a molecular mass of 60.14 kDa, which is in accord with the value of 57 kDa obtained for the purified arylsulfatase protein by SDS/PAGE. All the amino acid sequences determined from the purified protein could be confirmed. The G+C content of the *atsA* open reading frame is 68.8%, which is normal for *P. aeruginosa* [27], and the codon usage observed also corresponds to that previously reported for *P. aeruginosa* [27]. A putative ribosome binding site immediately preceding the *atsA* open reading frame was also identified (Fig. 5). The amino acid se-

quence encoded by *atsA* showed considerable similarity to known sulfatase proteins from several bacterial and eukaryotic species (30–40% and 25–30% identity respectively), but unlike other sulfatasases, the protein from *P. aeruginosa* did not contain a signal sequence to facilitate transport across the cell membrane (Fig. 6), and processing appeared to be limited to removal of the terminal *N*-formylmethionine.

**Localization of the *atsA* gene on the *P. aeruginosa* chromosome.** The *atsA* gene was localized on the *P. aeruginosa* PAO chromosome by hybridization to chromosomal DNA digested with *SpeI* or *DpnI* as previously described [28]. Under medium stringency hybridization conditions (40 mM sodium phosphate pH 7.2, 5% SDS) the gene hybridized to a single band on *SpeI* fragment O and *DpnI* fragment E [28], corresponding to 15–17 min on the new *P. aeruginosa* PAO genetic map [29].

## DISCUSSION

We have purified the arylsulfatase from *P. aeruginosa* 2700-fold to homogeneity, with an overall recovery of 24%. After growth with ethanesulfonate as sulfur source the enzyme therefore constitutes some 0.04% of the total cell protein. From the specific activity of the purified protein given in Table 2, and assuming a molar growth yield of about 4 kg protein/mol sulfur [30], the amount of enzyme present would therefore be sufficient to support normal rates of growth with aromatic sulfates as sole sulfur source. However, the arylsulfatase is clearly not substrate-induced, as it is also synthesized in the absence of aromatic sulfates as part of a response to sulfate limitation (presumably as a 'sulfur-scavenging' enzyme), even when excess sulfur is present in the form of a different, non-sulfate, sulfur source. This phenomenon is comparable to the synthesis of alkaline phosphatase, which is induced as part of the Pho regulon even in the presence of excess phosphonate or organophosphate if concentrations of inorganic phosphate are low. The direct corepressor in the case of the Pho regulon appears to be inorganic phosphate itself, in conjunction with the Pst/PhoU system [31]. For the sulfate-starvation-induced stimulon the situation is more complex, since sulfate, cysteine, and in some cases thiocyanate all repress synthesis of the sulfate starvation-induced proteins [4]. In *K. aerogenes*, studies with mutants in the cysteine biosynthetic pathway have led to the conclusion that sulfate and cysteine act independently as corepressors of arylsulfatase synthesis [32]. Thus, in a sulfate-uptake mutant arylsulfatase formation was effectively repressed by cysteine, whereas in a strain with a mutation late in cysteine biosynthesis, sulfate was able to repress arylsulfatase synthesis, even though it could not be converted to cysteine (sulfur for growth was provided by methionine). In *P. aeruginosa* the precise nature of the corepressor(s) which are responsible for arylsulfatase repression at a molecular level is unknown. Sulfate, cysteine, thiocyanate, sulfite and sulfide all lead to repression in this organism [9] (and our unpublished results).

Interestingly, the arylsulfatase makes up such a small proportion of cell protein that it can not be seen on two-dimensional PAGE of cell extracts, even when the proteins are visualized by sensitive methods such as silver staining. The sulfate starvation-induced proteins which have previously been observed by two-dimensional PAGE of cell extracts of *E. coli*, *P. putida*, *S. aureus* [4] and *P. aeruginosa* (our unpublished results) are hence present at much higher levels than is arylsulfatase. The number of sulfate-starvation-induced proteins reported [4] is therefore an underestimate of the total number of proteins in the cell which are regulated by sulfate levels.

Several different arylsulfatase isoenzymes have previously been reported in *P. aeruginosa* but, by isoelectric focussing and by FPLC analysis, we have only found evidence for one form of the enzyme. The *atsA* open reading frame (Fig. 5) contained all the partial amino acid sequences (N-terminal sequence and internal sequences) which had been determined for the purified arylsulfatase, confirming unambiguously that it indeed encodes the sulfate-repressed enzyme which we have purified and characterized. The protein encoded by *atsA* also showed considerable similarity to other sulfatase sequences, with the most conserved regions located in the N-terminal region (Fig. 6). This region also contained the two sulfatase consensus patterns listed in the PROSITE database, which were almost exactly conserved, with only one small alteration at the second residue of the sulfatase-1 consensus sequence (an atypical threonine). The encoded protein (534 amino acids) contains three cysteine and ten methionine residues, suggesting that although it is synthesized only under sulfate-limited conditions, the protein itself is not low in sulfur amino acids. This contrasts, for instance, with the phyco-bilosomal proteins produced by cyanobacteria under sulfate-limited conditions, which contain much less cysteine and methionine than the equivalent proteins produced when excess sulfate is present [33].

The most significant new characteristic of the *P. aeruginosa* arylsulfatase, however, is the absence of the N-terminal signal peptide which is found in arylsulfatase precursors in other species (Fig. 6). To eliminate the possibility that translation begins further upstream than indicated, we examined the region preceding the *atsA* gene. An in-frame stop codon was found 90 bp upstream of *atsA* and no alternative translation start codons were present. The beginning of the *atsA* open reading frame in *P. aeruginosa* corresponds well with the N-termini of the mature forms of other arylsulfatase proteins. The *E. coli* arylsulfatase-like protein is an exception here, since it carries no leader peptide and, although its synthesis is apparently controlled by sulfate levels like other arylsulfatasases, the protein has no arylsulfatase activity and has only been detected by cross-reaction with antibodies raised against the *K. aerogenes* arylsulfatase [34]. The putative signal peptide deduced from the DNA sequence of the *K. aerogenes* arylsulfatase is clearly active in protein translocation, since the enzyme has been shown to be localized in the periplasm [35]. Several other bacterial or fungal arylsulfatasases are also periplasmic or cell-wall-associated, or associated with lysosomal particles [3], cellular locations which are in good agreement with the postulated role of microbial arylsulfatasases as 'sulfur-scavenging enzymes' for the cell.

In *P. aeruginosa*, transport of proteins into the periplasm is catalysed by a complex very similar to the Sec system of *E. coli* [36], which has an absolute requirement for a defined N-terminal signal peptide [37]. The *P. aeruginosa* arylsulfatase does not carry an N-terminal signal peptide and is therefore an atypical arylsulfatase in that it is unlikely to be either periplasmic or cell-wall-associated. Whereas a small group of proteins lacking an N-terminal leader sequence (e.g. alkaline protease [36],  $\alpha$ -hemolysin [38]) is known to be transported across the cytoplasmic membrane, these proteins are exclusively extracellular, whereas *P. aeruginosa* arylsulfatase could not be detected in the external growth medium. Since we have failed to detect release of arylsulfatase from *P. aeruginosa* cells in spheroplasting experiments (unpublished) using several methods [35, 39, 40], the enzyme is probably indeed intracellular. A system for uptake of aromatic sulfates into the cell must therefore also be present, though this has not yet been studied. Further confirmation of arylsulfatase localization awaits immunocytological studies in whole cells.

The presence of several isozymes of arylsulfatase is not unusual in bacteria, and has been reported for *P. aeruginosa* [10],



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