Regulation of the sulfate starvation response in *Pseudomonas aeruginosa*: role of cysteine biosynthetic intermediates

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***Pseudomonas aeruginosa* PAO1 grew in defined synthetic medium with any of a broad variety of single sulfur sources, including sulfate, cysteine, thiocyanate, alkanesulfonates, organosulfate esters and methionine, but not with aromatic sulfonates, thiophenols or organothiocyanates or isothiocyantes. During growth with any of these compounds except sulfate, cysteine or thiocyanate, a set of 10 sulfate starvation-induced (SSI) proteins was strongly up-regulated, as observed by two-dimensional protein electrophoresis of total cell extracts. A comparable level of up-regulation was found for the hydrolytic enzyme arylsulfatase, which has previously been used as a marker enzyme for the sulfate starvation response. One of the SSI proteins was identified by N-terminal sequencing as a high-affinity periplasmic sulfate-binding protein, and another was related to thiol-specific antioxidants, but the N-terminal sequences of the other SSI proteins revealed no similarity to N-termini of proteins of known function, and they probably represent uncharacterized enzymes involved in sulfur scavenging when preferred sulfur sources are absent. To study the role that cysteine biosynthetic intermediates play in the synthesis of these proteins *in vivo*, we isolated mini-Tn5 transposon mutants of *P. aeruginosa* with insertions in the *cysN* and *cysI* genes, which encode subunits of ATP-sulfurylase and sulfite reductase, respectively. These two genes were cloned and sequenced. *cysI* showed high similarity to the cognate gene in *Escherichia coli*, whereas *cysN* encoded a 69.3 kDa protein with two domains corresponding to the *E. coli* CysN and CysC proteins. Sulfate no longer repressed synthesis of the SSI proteins in *cysN* mutants, but repression was restored by sulfite; in the *cysI* mutant, sulfate, sulfite and sulfide all led to repression of SSI protein synthesis. This suggests that there are at least two independent corepressors of the sulfate starvation response in this species.**

Keywords: *Pseudomonas aeruginosa*, sulfate starvation, cysteine biosynthesis

INTRODUCTION

Cysteine biosynthesis by the sulfate assimilation pathway has been well-characterized in both plants and micro-organisms (Kredich, 1996; Leustek, 1996). It

Abbreviations: SSI, sulfate starvation-induced; SSIS, sulfate starvation-induced stimulon.

The GenBank accession numbers for the sequences reported in this paper are AF026066 (*cysI*) and AF035608 (*cysDN*).

proceeds by activation of inorganic sulfate, followed by reduction of the intermediate phosphoadenosyl phosphosulfate to sulfite and hence to sulfide, and transfer of this molecule onto an organic backbone. In limnic and enteric environments, sulfate is present in excess of microbial requirements, but in aerobic soils sulfur is available almost exclusively (>95%) in organically bound form, and inorganic sulfate is rare (Autry & Fitzgerald, 1990). The sulfur pool in the soil is, in addition, a dynamic one. Isotopic studies with ³⁵S have shown not only that inorganic sulfate is rapidly in-

corporated into soil organic matter (Strickland *et al.*, 1986), but that soil organic sulfur (which may be present as sulfate ester-sulfur, amino acid/protein-sulfur or sulfonate-sulfur) is also cycled between forms (Watwood *et al.*, 1986). This cycling is assumed to be due to microbial activity in the soil, or to microbially released soil enzymes, but the mechanisms involved in sulfur soil cycling and in the response of soil bacteria to sulfate limitation are not yet well understood.

For *Escherichia coli*, in contrast, the response to sulfur limitation has been studied in some detail. When this species is grown with sulfonate-sulfur as sulfur source, in the absence of inorganic sulfate, a set of proteins is synthesized which is completely absent during growth in the presence of inorganic sulfate (Quadroni *et al.*, 1996; van der Ploeg *et al.*, 1996). These proteins include a taurine sulfonate (Eichhorn *et al.*, 1997), high-affinity uptake systems for sulfate and cystine, and several unidentified proteins. The proteins induced by sulfate starvation in *E. coli* therefore appear to be 'sulfur-scavenging enzymes' involved in utilization of non-favoured sulfur sources.

In soil environments, such bacterial 'sulfur-scavenging enzymes' may be critically important in the *in situ* degradation of sulfur-containing xenobiotics. Desulfonation of a broad range of aromatic sulfonates, which are released into the environment in large quantities each year as, for example, detergents and dyestuffs, has been demonstrated in several soil isolates (Dudley & Frost, 1994; Kertesz *et al.*, 1994; Zürrer *et al.*, 1987). Synthesis of the enzyme responsible is strongly repressed in the presence of sulfate or cysteine (Beil *et al.*, 1996). Similar results have been obtained for the desulfurization of dibenzothiophene by *Rhodococcus* species, as part of studies aimed at biodesulfurization of coal and oil (Kayser *et al.*, 1993). Another well-characterized sulfur-scavenging enzyme is the hydrolytic enzyme aryl-sulfatase, which releases sulfate from aromatic sulfate esters, and is one of the commonest enzymes found in soil. Synthesis of this enzyme is repressed by sulfate in pseudomonads (Beil *et al.*, 1995; Kertesz *et al.*, 1993) and in other bacterial and fungal species (Dodgson *et al.*, 1982).

In the present study, we have investigated the regulation of the sulfate starvation response in a ubiquitous soil bacterium, *Pseudomonas aeruginosa*. We report here that *P. aeruginosa* responded to sulfate limitation with the synthesis of at least 10 novel proteins, and that expression of the corresponding genes was mediated by at least two separate corepressors.

METHODS

Chemicals. Sulfur-containing chemicals used as sulfur sources for bacterial growth were of the highest purity commercially available, and were obtained from Fluka, except for 4-phenylbutane sulfonate and *n*-hexyl sulfate (Aldrich). The sulfate content of all sulfur sources was checked by ion chromatography before use (Kertesz *et al.*, 1994) and was found to be less than 0.5 mol%. DNase I and RNase A were from Boehringer Mannheim. Ampholyte mixtures (pH 3–10)

for two-dimensional PAGE were obtained from Bio-Rad. Oligonucleotides were supplied by Microsynth.

Bacterial strains, media and growth conditions. Bacterial strains used in this study are listed in Table 1. *P. aeruginosa* strains were routinely cultivated in the sulfur-free succinate salts medium described previously (Kertesz *et al.*, 1993), with the addition of all the naturally occurring protein amino acids (40 µg ml⁻¹), except methionine and cysteine. Sulfur-limited solid media were prepared by addition of 0.6% molecular biology grade agarose (FMC BioProducts). Sulfur sources (500 µM) were added as described in the text. All glassware used in sulfate-limited experiments was washed with 3 M HCl and rinsed thoroughly with glass-distilled water before use. Cultures were grown either aerobically on a rotary shaker (180 r.p.m.) (30 ml cultures in 300 ml flasks) at 37 °C, or in a vigorously stirred, flat-bottomed 500 ml flask held in a thermostat-controlled water bath at 37 °C, with a continuous air supply (Harvey *et al.*, 1968). Growth was monitored spectrophotometrically at 650 nm, and the cells were harvested in the mid-exponential phase. Growth rates were determined by measuring total protein in samples removed from the cultures during growth (Beil *et al.*, 1996). Antibiotics were added at the following concentrations (ml⁻¹): ampicillin, 100 µg for *E. coli*; tetracycline, 25 µg for *E. coli* and 125 µg for *P. aeruginosa*; streptomycin and carbenicillin, 500 µg for *P. aeruginosa*.

Two-dimensional protein gel electrophoresis, protein extraction and sequencing. *P. aeruginosa* cells were prepared for two-dimensional gel electrophoresis by lysis with SDS followed by incubation with urea/CHAPS/DTT by the method of Hochstrasser *et al.* (1988). For analytical gels (1.0 mm thickness), 5–20 µg protein was loaded, and the separated proteins were visualized by silver staining (Ausubel *et al.*, 1987). Preparative gels were thicker (1.5 mm), and up to 300 µg protein was loaded. Isoelectric focusing was carried out in tube gels with carrier ampholytes for 16 h at 500 V. The tube gels were then equilibrated in transfer buffer (70 mM Tris/HCl, 3.0% SDS, 0.003% bromophenol blue, pH 6.8) for approximately 2 min before transfer onto a 12% polyacrylamide second-dimension gel. Isoelectric focusing and SDS-PAGE were both done with the Protean 2-D PAGE apparatus (Bio-Rad). For analysis purposes, the gels were scanned with a Personal Densitometer (Molecular Dynamics), and image analysis and spot matching were carried out with the Investigator software package (Millipore). Isoelectric points and molecular masses were determined by co-electrophoresis with a set of 2-D protein standards (Bio-Rad). For preparative purposes, individual proteins from 10–20 gels were excised, concentrated as described previously by Hatt *et al.* (1997), and blotted onto PVDF membrane. The N-terminal sequence was determined by automated Edman degradation (Applied Biosystems 120A PTH Sequenator).

Enzyme assays and preparation of cell extracts. Cell extracts for enzyme assays were prepared from cells harvested in the mid-exponential growth phase. The cells were harvested (7000 g, 15 min, 4 °C), washed and treated with lysozyme (1 mg ml⁻¹, 60 min), and then lysed by three passages through a chilled French pressure cell at 135 MPa. Cell debris and membranes were removed by ultracentrifugation (250 000 g, 30 min, 4 °C). The cell extract was then desalted by passage over a small Sephadex G-25 column (PD-10; Pharmacia). When necessary, the extract was stored at -20 °C before assaying enzyme activities; however, cystathionine β-lyase was always assayed immediately, as it proved to be unstable to freezing.

Arylsulfatase was assayed in whole cells or cell extracts as

Table 1. Bacterial strains and plasmids used in this study

Strain/plasmid	Genotype/relevant features	Reference/source
<i>P. aeruginosa</i>		
PAO1	Prototroph	Holloway (1955)
PAO1S	Spontaneous streptomycin-resistant mutant of PAO1	Beil <i>et al.</i> (1995)
PAO716	<i>cys54 rif96</i>	Ratnaningsih <i>et al.</i> (1990)
PAO1219	<i>trp54 cys50::Tn5 rif5 fon1</i>	B. Holloway, Monash University, Australia
AC309	<i>cysN::mini-Tn5-Tc</i>	This study
AX18	<i>cysI::mini-Tn5-Tc</i>	This study
<i>E. coli</i>		
DH5 α	<i>supE44 ΔlacU169 (ϕ80 <i>lacZ</i>ΔM15) <i>hsdR recA1 endA1 gyrA96 thi-1 relA1</i></i>	Hanahan (1983)
S17-1 <i>λpir</i>	<i>hsdR thi pro recA</i> ; RP4 integrated into the chromosome (<i>kan::Tn7 ter::Mu</i>) <i>λpir</i>	De Lorenzo & Timmis (1994)
Plasmid		
pBluescript II KS	Cloning vector, Amp ^R	Stratagene
pLAFR3	IncP-1, <i>cos</i> , Tra, Tc ^R	Staskawicz <i>et al.</i> (1987)
pUT-mini-Tn5-Tc	Delivery vector for mini-Tn5-Tc	De Lorenzo & Timmis (1994)
pME4007	pLAFR3-derived cosmid clone from <i>P. aeruginosa</i> including the <i>cysDN</i> genes	This study
pME4008	pLAFR3-derived cosmid clone from <i>P. aeruginosa</i> including the <i>cysI</i> gene	This study
pME4062	3.8 kb <i>Pst</i> I- <i>Xho</i> I fragment (Tc ^R) ex strain AX18, in pBluescript KS, <i>cysI</i>	This study
pME4311	3.3 kb <i>Pst</i> I fragment (Tc ^R) from strain AC309 in pBluescript SK, <i>cysDN'</i>	This study
pME4318	1.8 kb <i>Not</i> I- <i>Stu</i> I fragment (Tc ^R) from strain AC309 in pBluescript SK, <i>cysN</i>	This study

release of 4-nitrocatechol from 4-nitrocatechol sulfate as described previously (Beil *et al.*, 1995; Kertesz *et al.*, 1993). Rhodanese was assayed as thiocyanate production (Westley, 1981). Cystathionine β -lyase was measured by the method of Uren (1987), and cystathionine γ -lyase was assayed as 2-oxobutanoic acid formation from L-homoserine (Nagasawa *et al.*, 1987).

DNA manipulations. Molecular genetic methods were carried out using published methods (Ausubel *et al.*, 1987). Competent *E. coli* cells for transformation were prepared by the method of Inoue *et al.* (1990), and *P. aeruginosa* cells were transformed by electroporation, using 0.1 cm cuvettes in a Gene Pulser apparatus (Bio-Rad). Conjugative transfers were carried out by patch mating on nutrient plates at 37 °C.

Isolation of cysteine auxotrophic mini-Tn5 mutants of *P. aeruginosa*. Transposon mutagenesis of *P. aeruginosa* was carried out using the mini-Tn5 system (De Lorenzo & Timmis, 1994). The transposon was introduced into *P. aeruginosa* by plate conjugation at 37 °C for 8–24 h, using late-exponential-phase cultures of *P. aeruginosa* PAO1S. *E. coli* S17-1 *λ pir* (pUT-mini-Tn5-Tc) was used as donor strain. To screen for cysteine auxotrophy, tetracycline-resistant colonies which grew on proline-free minimal plates with cysteine as sulfur source were picked to minimal plates with either inorganic sulfate or cysteine as sulfur source (250 μ M). Colonies showing no growth with sulfate after 24 h were tested further in liquid media.

Cloning of the *cysI* and *cysDN* genes. The interrupted genes in cysteine auxotrophic strains AX18 and AC309 were cloned by transposon rescue techniques. Suitably sized transposon-containing DNA fragments from strains AX18 and AC309 were identified by Southern analysis using the *tet* gene as

probe, and were then cloned into linearized pBluescript to give the plasmids pME4062, pME4311 and pME4318, respectively. After subcloning, the DNA sequences of the regions immediately flanking the transposon were determined using a primer specific for the mini-Tn5 (mkomega: 5'-cttgctcaatca-atcacc-3'), and the sequences obtained were used to design oligonucleotides for PCR-based screening of a cosmid library of *P. aeruginosa* (Visca *et al.*, 1994). Cosmids containing the *cysI* and *cysDN* genes (pME4008 and pME4007, respectively) were isolated, subcloned into pBluescript and the genes of interest were sequenced, using sequence-specific primers and a primer-walking strategy.

DNA sequence analysis. DNA sequences were determined on both strands using the dideoxy chain-termination method (Sanger *et al.*, 1977). Analysis of DNA and protein sequences was done with the Genetics Computer Group Package of the University of Wisconsin, version 8.

Other procedures. Total protein in cell suspensions was determined by a modified Lowry method (Kennedy & Fewson, 1968), and soluble protein was measured with the Bio-Rad protein reagent, following the manufacturer's instructions. Bovine serum albumin was used as standard in both cases.

RESULTS

Growth of *P. aeruginosa* PAO1S with different sulfur sources

Cells of *P. aeruginosa* PAO1S grew aerobically in a synthetic sulfur-free medium with sulfur for growth supplied either as inorganic sulfate, or as one of a range of different, non-sulfate sulfur sources (Table 2).

Table 2. Growth rates and selected enzyme activities observed during growth with sulfur sources used in this study

Relative enzyme activities were determined in extracts from cells harvested in the mid-exponential growth phase (OD_{650} 0.8). They were assayed as described in Methods, and represent three independent experiments. Activities are normalized to the activity observed after growth with sulfate. Typical values seen with sulfate were: arylsulfatase, $0.84 \text{ nmol min}^{-1} \text{ mg}^{-1}$; rhodanese, $130 \mu\text{mol min}^{-1} \text{ mg}^{-1}$; cystathionine γ -lyase, $1.83 \text{ nmol min}^{-1} \text{ mg}^{-1}$; cystathionine β -lyase, $0.16 \text{ nmol min}^{-1} \text{ mg}^{-1}$.

Sulfur source for growth (500 μM)	Doubling time (min)*	SSI response†	Arylsulfatase	Rhodanese	Cystathionine γ -lyase	Cystathionine β -lyase
Sulfate	42	–	1.0	1.00	1.00	1.00
Thiocyanate	50	–	3.5	1.22	1.10	1.13
Cysteine	79	–	12.0	0.84	2.43	2.19
Methionine	88	+	57.0	1.16	3.49	2.56
Cystine	78	+	18.0	1.20	2.64	2.31
<i>n</i> -Hexyl sulfate	61	+	35.0	0.93	1.77	3.81
4-Nitrocatechol sulfate	45	+	45.0	1.04	3.59	4.00
Ethanesulfonate	55	+	36.0	0.95	2.26	3.88
Pentanesulfonate	57	+	41.0	0.92	1.96	1.69
Cyclohexylsulfamate	74	+	67.0	0.89	2.23	3.81
SDS	56	+	43.0	0.95	1.62	1.81

* Growth rates were measured during cultivation in vigorously stirred flasks with continuous air supply (Harvey *et al.*, 1968) for all substrates except methionine. Growth rate with methionine was measured in shaken flasks, due to rapid oxidation of the methionine under the former conditions.

† The presence or absence of an SSI response was determined by inspection of two-dimensional gels prepared with cells grown with the respective sulfur source.

Fig. 1. Two-dimensional electropherograms of total cell protein from *P. aeruginosa* PAO15 grown aerobically in succinate salts medium with different sulfur sources: (a) sulfate, cysteine or thiocyanate; (b) other sulfur sources from Table 2. The numbers indicate proteins that are up-regulated under sulfate starvation conditions (SSI proteins; Table 3).

Table 3. SSI proteins in *P. aeruginosa* PAO

SSI proteins were identified by computer-assisted comparison of silver-stained two-dimensional gels. For preparative purposes proteins were extracted from cells grown with sulfate or with ethanesulfonate as sulfur source, and were purified and analysed as described in Methods. Protein numbering corresponds to that in Fig. 1. The results are representative of three independent experiments.

Protein	Mol. mass (kDa)	pI	Up-regulation (-fold)*	N-terminal amino acid sequence†	Comments
PA1	38.8	6.70	4.8	ATQLLNVSYPDPTXE	Sulfate-binding protein
PA2	38.8	6.30	2.6	XEQSANTLRIXYQRYXXLV	No similarity found
PA4	37.5	6.20	SSI	XNAAQALAVPRLDIYP	No similarity found
PA6	23.8	6.30	6.2	—	Not Coomassie-blue-detectable
PA7	28.0	6.25	2.2	AEKLIVAATPIPHAEILELIKPTLA	No similarity found
PA8	28.0	6.00	2.6	—	N-terminally blocked
PA9	36.4	5.80	SSI	ADITVGYQTGIDPXX	No similarity found
PA11	28.0	5.65	5.0	SLRLGDIAPDFEQD	Similarity to liver spot 40 (Hochstrasser <i>et al.</i> , 1993)
PA12	38.8	6.05	SSI	XQGALMXQP	No similarity found
PA13	44.5	6.00	SSI	XLEIFAFLPTHGD	Similarity to OrfM (Quirk <i>et al.</i> , 1994)

* SSI indicates that proteins were not visible by silver staining after growth with sulfate, cysteine or thiocyanate.

† X indicates amino acid residues which could not be assigned with certainty.

Growth yields in this medium were found to be limited by sulfur when the sulfur concentration was below 120 μ M (not shown), and since we wished to examine the bacterial response to sulfate limitation but not total sulfur deprivation, we always supplied excess sulfur (500 μ M). *P. aeruginosa* proved quite flexible in the range of sulfur-containing compounds which it could utilize for growth. The cells were able to grow well on all the sulfur sources listed in Table 2, and these were therefore used in subsequent experiments for analysis of the SSI response. *P. aeruginosa* was also able to utilize all alkanesulfonates tested (including, amongst others, 4-phenylbutanesulfonate, taurine, ethanesulfonate, MOPS, HEPES, bromoethanesulfonate), as well as aromatic and aliphatic sulfate esters, thiosulfate, glutathione, thioglycollate, isethionate and dimethylsulfoxide. No utilization of aromatic sulfonates such as toluenesulfonate, aromatic sulfides such as dibenzothiophene, aromatic thiols such as thiocresol, or organic thiocyanates or isothiocyanates was observed, though control experiments showed that these compounds were not toxic at the concentrations used (500 μ M). The doubling times observed with a variety of sulfur sources (Table 2) lay between 40 and 80 min, but no correlation was found between synthesis of the SSI proteins and the relative growth rates observed for each sulfur source.

Sulfate starvation-induced (SSI) proteins of *P. aeruginosa* PAO

Total cellular protein was extracted from cells harvested during the exponential growth phase (OD_{650} 0.5), and separated by two-dimensional electrophoresis (Fig. 1)

followed by silver staining. Visualization with 35 S was not used, since there is evidence that proteins that are specifically induced under sulfate-limiting conditions may contain lower than average levels of cysteine and methionine (Hellinga & Evans, 1985; Mazel & Marlière, 1989), and would therefore only be poorly labelled by 35 S compared to other cellular proteins. Computer-assisted analysis of the gels revealed a set of 10 SSI proteins which was partly or completely repressed whenever sulfate, thiocyanate or cysteine was present in the growth medium, but whose synthesis was up-regulated during growth with all the other sulfur sources tested (Fig. 1, Table 3). The degree of up-regulation of each of the SSI proteins was measured by densitometric analysis (Table 3), and varied from 2.2-fold (protein PA7) to several proteins which could not be detected at all by silver staining after growth with sulfate.

To characterize the SSI proteins further, they were excised from the gels and their N-terminal amino acid sequences were determined (Table 3). Using this method, protein PA1 could be identified with certainty as the sulfate-binding protein, since its N-terminal sequence was 85% identical to that of the periplasmic sulfate-binding protein of *E. coli*, and its size and pI were also similar to those of the *E. coli* protein (Hellinga & Evans, 1985). The sulfate-binding protein has not yet been reported in *P. aeruginosa*, but has been the subject of intensive study in *E. coli* (Jacobson *et al.*, 1991) and in *Salmonella typhimurium* (Pflugrath & Quioco, 1985). In *E. coli* it has also been identified as an SSI protein using two-dimensional PAGE (Quadroni *et al.*, 1996). Protein PA6 could not be analysed by the method used,

since it could not be visualized by Coomassie staining, and protein PA8 was apparently N-terminally blocked. For protein PA11, 67% identity was found to the N-terminus of protein 40 from human liver (Hochstrasser *et al.*, 1993). This protein is related to the large family of thiol-specific antioxidant proteins (Chae *et al.*, 1994), which includes alkylhydroperoxide reductase, a protein whose synthesis was also shown to be partially controlled by sulfate in *E. coli* (Quadroni *et al.*, 1996). The N-terminal sequence of protein PA13 revealed 61% identity to the N-terminus of the *orfM* gene product from *Bacillus subtilis* (Quirk *et al.*, 1994), and 70% identity to that of the Ssi6 protein in *E. coli* (Quadroni *et al.*, 1996), both of unknown function. All of the remaining SSI proteins yielded sequences with no significant similarity to sequences in the databases.

To test whether the SSI proteins are general stress proteins, arylsulfatase activities and synthesis of the SSI proteins were measured under conditions of phosphate limitation (0.2 mM phosphate), heat shock (43 °C) and during anaerobic growth. Sulfate-mediated repression of both arylsulfatase and SSI proteins was not overcome by any of the environmental stress signals tested.

Synthesis of enzymes of sulfur metabolism by *P. aeruginosa* PAO during growth with different sulfur sources

In *E. coli*, the SSI proteins have all proved to be involved in sulfur metabolism, and several play a role in the metabolism of organosulfur compounds (Quadroni *et al.*, 1996; van der Ploeg *et al.*, 1996; Eichhorn *et al.*, 1997). In *Pseudomonas* species, by contrast, little is known about the sulfur assimilation pathway, and very few of the corresponding genes have been characterized. Since it seemed likely that the *Pseudomonas* SSI proteins might also be directly involved in sulfur metabolism, we measured the levels of several enzymes that play a role in cysteine or methionine biosynthesis during growth with a variety of sulfur sources (Table 2). Thiosulfate: cyanide sulfurtransferase (rhodanese) was chosen to represent a broad range of sulfurtransferases (Sörbo, 1975), cystathionine β -lyase plays a part in the methionine biosynthetic pathway, and cystathionine γ -lyase is important in cysteine biosynthesis in *P. aeruginosa* (Fogolino *et al.*, 1995). Arylsulfatase levels were also measured, since this enzyme has been used before as a marker enzyme for the sulfate starvation response (Beil *et al.*, 1995; Kertesz *et al.*, 1993). The results are shown in Table 2. In *P. aeruginosa* a high degree of repression was seen for arylsulfatase, but the other enzymes assayed displayed much less stringent control by sulfur supply. Significant levels of thiosulfate: cyanide sulfurtransferase activity could be measured, but the enzyme did not appear to be subject to repression by sulfate. The other enzymes examined showed up-regulation by a factor of up to threefold (cystathionine γ -lyase) and two- to fourfold (cystathionine β -lyase) in the absence of sulfate, significantly less than the up to 67-fold induction seen for the arylsulfatase, or the *de novo* appearance of several of the SSI proteins seen on two-dimensional

PAGE (Fig. 1). Levels of cystathionine β -lyase observed were found to be significantly lower than those reported in *E. coli* (Uren, 1987), which may reflect the fact that this enzyme plays a less important role in *P. aeruginosa* than in the former organism, due to the presence of an alternative transsulfuration pathway leading directly to homocysteine (Fogolino *et al.*, 1995). Interestingly, the two lyases did not appear to be fully repressed by cysteine, though the presence of cysteine led to repression of the SSI stimulon (SSIS) and of arylsulfatase expression. This may suggest that cysteine and sulfate regulate different systems in this organism, or that cysteine does not play the key regulatory role in *P. aeruginosa* that it does in *E. coli*.

Isolation of cysteine auxotrophic mutants of *P. aeruginosa* and characterization of the *cysI* and *cysDN* genes

Although physiological studies have shown that the synthesis of arylsulfatase is repressed during growth with sulfate or cysteine (Beil *et al.*, 1995), it was not yet known whether sulfate itself was responsible for this repression *in vivo*. To investigate this question, cysteine auxotrophic mutants of *P. aeruginosa* PAO1S were generated using the mini-Tn5-Tc system. After screening of 19200 mutants, 112 were found to be unable to grow on solid media with sulfate as sole sulfur source. Growth of these mutants was further characterized in liquid medium with sulfur supplied as sulfate, sulfite, pentane-sulfonate, sulfide or cysteine. However, this physiological characterization was hampered by the fact that when cultivated with sulfate as sole sulfur source, many of the mutant strains reverted to a cysteine prototrophic phenotype after an 8–24 h lag phase. Southern analysis of selected strains revealed that the position of the transposon insertion in the revertants had remained stable (not shown), and we therefore concluded that the 'pseudoreversion' effect was due to selection for strains with suppressor mutations (see Discussion). We also observed suppression of the cysteine auxotrophic phenotype during cultivation with sulfate for two cysteine auxotrophic mutants which had been obtained previously, PAO716 and PAO1219 (O'Hoy, 1987; Ratnaningsih *et al.*, 1990). The mutations in these latter strains have been mapped on the PAO chromosome to 56 min and 45 min, respectively (Holloway *et al.*, 1994), but it is not known in what genes the mutations are situated, nor have the mutants been further characterized biochemically.

For further work, we chose to use two of the isolated mutants, strains AC309 and AX18. On solid media, strain AX18 was unable to grow with sulfate, pentane-sulfonate or sulfite, but grew well with sulfide or cysteine. This suggested that the strain was defective in sulfite reductase. Strain AC309 could grow with pentane-sulfonate or sulfite, but not with sulfate, suggesting a defect in the sulfate activation pathway. This strain was also unable to grow with methionine, and there may therefore be a link between the sulfate activation pathway and the mechanism of methionine trans-

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1  AAGGCGCGTTGCGAAGCGGTATGCTTGCCTGCGGACTAGTCTTTTGGTTCTTACCATATACCTAAACATCACCTTTACGCATAAAT
91  ATGAACGTGTATCGTGCGCCACCTCCGCGTAGAGCGCGTAGCGGCTGGTTAACGCAGTAAGCCGCATAGCGCTGACAGCAAGGA
181  TATTCATGTACGTATACGACGAGTACGACCAACGGATCGTCGAGGACCGCGTCAAGCAGTCCGCGACACGCCCGCTTACCTGGCGG
cysI M Y V Y D E Y D Q R I V E D R V K Q F R D Q T R R Y L A G
271  GTGAATTGACGGTGAGGAGTTCGCCCGCTGCGCTGCGAAGCGTCTCTATATCCAGCGCTATGCCCGATGCTGCGCATCGCGCTTC
E L T G E E F R P L R L O N G L Y I Q R Y A P M L R I A V P
361  CCTATGCGCTGCTCTCTCGCGCAACTGCGCAAGCTGGCGCAGATCGCCCGGACTACGACAAGGGCTACGCCACATCAGCACCCGGC
Y G L L S S R Q L R K L A Q I A R D Y D K G Y A H I S T R Q
451  AGAACGTGACGTTCAACTGCGCGAGCTGGAAGACGTGCGGAAATCCTCGCGAGCTGGCCACCGTGCAGATGCACGCGATTGACACCA
N V Q F N W P E L E D V P E I L A E L A T V Q M H A I Q T S
541  GCGGCAATGCATCCGCAACACCAACCCGACCGAGTTCGCGCGGCTGCGCCGCGACGAGCTGGTGGACCCGCGCCCTGGTGAGAGATCA
G N C I R N T T T D Q F A G V A R D E L V D P R P W C E I I
631  TCGGCAATGGTGCACCTTCCACCCGAGTTCGCCCGGCTGCGCGCAAGTTCAAGATCGCGGTGAACGCGCGGTGAGCGACCGTGGCG
R Q W S T F H P E F A H L P R K F K I A V N G A V S D R A A
anion-binding site
721  CCATCGAAGTCGACGACATCGGCTGGAGCGGTGAAGAACGAGCGCGGAGCTCGGCTTCGCGTCTCGGTGCGCGCGCGCTGGGCG
I E V H D I G L E A V K N E A G E L G F R V S V G G G L G R
811  GAACCCCATCGTGGCAGCTTCATCACTGCGCTGCGCAGCAGCTGCTCGGCTACCTGGATGCCATCTGCGCGCTTACAAAC
T P I V G S F I N E F L P W Q H L L G Y L D A I L R V Y N R
901  GCTATGGCGCGCGACAAAGTACAAAGCGCGGATCAAGATCTGGTCAAGCGCTGACCCCGGAAGTCTTCGCGGAGCGCTGCTCGAG
Y G R R D N K I L V K A L T P E V F A E R R R G
mini-Tn5 insertion site in strain AX18
991  GCGCAATGGCGCAACCTCAAGGACGCGGACACCTCACCGAGCGGAAGTCCAGCGCGTCCGCGCGCATTTCTGTCGATCCGGCTACA
R M G Q P Q G R T E H L T E A E V Q R V A A H F V D P A Y K
1081  AGGCCCTCGACGACCTCGACGAGTCCCTCGCGCGCTCGACGCGGAGCAGCCCGGCTTCGCGCGCTGGCGCGCAGCAACACCTTCGCGC
A L D D L D E S L A R L D A E H P G F A R W R A R N T F A H
1171  ACAAGAAGCCGCGTACGCTGCGCTGCGCTGAGCGCCACCGGACCGCGCGGAGACGTCACCGCAACACAGCTGGAGGCCA
K K P G Y V A V T L S L K P T G T A P G D V T D K Q L E A I
1261  TCGCGCATCTCGCGCAGCGCTACAGTTCGCGGAGGTGCGCAACAGCATAACAGAACATCATCTTCGCGGAGCTGAGCAGGGCGAAC
A D L A D R Y S F G E V R N S H N Q N I I L A D V E Q G E L
1351  TCTTCGCGCTTCGGGCGAAGTTCGCGGAGTTCGCGGAGTTCGCGGAGTTCGCGGAGTTCGCGGAGTTCGCGGAGTTCGCGGAGTTCGCGGAG
F A L W G E L R E L G F A T P N V G L L T D I I C C P G G D
1441  ACTTCTGCTCCCTCGCGCAGCGCTGCGGAGTTCGCGGAGTTCGCGGAGTTCGCGGAGTTCGCGGAGTTCGCGGAGTTCGCGGAGTTCGCGGAG
F C S L A N A K S I P I A E A I O P G F D N L D Y L F D I G
1531  CGGACATCGACCTGAACATCTCCGTTGATGAACGCTGCGGCGATCACCATGTCGCGCACATCGGCGATCTCGGGGTGACAGAAG
D I D L N I S G C M N A C G R H H V G H I G I L G V D K K G
siroheme binding site
1621  GCGCGGAGTTCTACAGGTTCTCCCTCGCGCGGAGCTCGCGGAGCTCGCGGAGCTCGCGGAGCTCGCGGAGCTCGCGGAGCTCGCGGAGG
A E F Y Q V S L G S S A R D A S L G Q I L G P S F A Q E Q
1711  AGATGGCCGACGTGATCGACAAGATCATCGAGTCTACGTGGAACGCGGACGAGGAAGAACCTTCATCGACACCTTCGCGCGTATCG
M A D V I D K I I Q V Y V E R R T E E P F I D T F R R I G
1801  GTATCGACCCGTTCAAGGAGCGGTATATGACGCAATCATTAAGGACGCGAGGTGGTGCAGCAGCGCTGCGACTTGTGCCCCAAGGAC
I D P F K E R V Y A A N H *
1891  GCGACCTGGAAGCTGCCAAGCAGCAGCAGTGCATCTCCGCTGCGCCTGTGGCTCGAGCAGCGCC

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Fig. 2. DNA sequence of the *cysI* gene of *P. aeruginosa*, and the deduced amino acid sequence. The putative ribosome-binding site is underlined and the stop codon is shown with an asterisk. The proposed siroheme binding site and anion-binding site are boxed, and a putative rho-independent transcription terminator in the upstream region is shown with arrows. The site of mini-Tn5 insertion in strain AX18 is indicated.

sulfuration in this species (Günther *et al.*, 1979). Interestingly, both strains grew normally with thiocyanate, suggesting that thiocyanate-sulfur is not oxidized to sulfate before assimilation, but can enter the cysteine biosynthetic pathway directly, probably at the level of sulfide. Both strains were shown by Southern analysis to contain only a single transposon insertion.

To identify and characterize the interrupted loci in each of the two mutants, the insertion sites were cloned by transposon rescue techniques and the flanking regions were used to identify the corresponding clones in a *P. aeruginosa* library in the cosmid pLAFR3 (Visca *et al.*, 1994). Sequencing of the transposon flanking regions in strain AX18 revealed an open reading frame encoding a 62 kDa protein of pI 6.3, with 27% identity to the haemoprotein subunit of *E. coli* sulfite reductase (CysI). The protein included the typical Fe_4S_4 -siroheme binding motif of sulfite and nitrite reductases (see Fig. 2), and also the conserved sequence LPRKFK, which has been associated with substrate anion binding in the *E. coli* sulfite reductase (Crane *et al.*, 1995). Although the *E. coli* *cysI* gene is transcribed as part of the *cysJIH* operon, in *P. aeruginosa* it appeared to be monocistronic, since it was preceded by an inverted repeat and an AT-rich putative promoter region, and no further reading frames could be detected downstream. Interestingly, the *P.*

aeruginosa *cysH* gene, which was recently described (Delic-Attree *et al.*, 1997), also appears to be encoded as a single-gene transcript. Similar analysis of strain AC309 revealed that in this strain the transposon had inserted into an open reading frame encoding a 69.3 kDa protein whose N-terminal domain showed 60% identity to the GTP-binding subunit of the *E. coli* ATP-sulfurylase protein (CysN). The C-terminal domain of this gene product showed high similarity to the product of the *E. coli* *cysC* gene, adenosine 5'-phosphosulfate kinase (Fig. 3), and the *P. aeruginosa* CysN protein therefore corresponds to an apparent functional fusion of the *E. coli* CysN and CysC proteins. Sequencing upstream and downstream of the *cysN* gene revealed that it was preceded by *cysD* (encoding a 35.5 kDa protein with 78% identity to *E. coli* CysD), which encodes the small subunit of ATP-sulfurylase. The *cysD* and *cysN* genes of *P. aeruginosa* therefore appear to encode a single enzyme complex responsible for sulfate activation in this species. Divergent to *cysD* lay the start of a further open reading frame, *orfX*, with similarity to the *E. coli* *mltB* gene. The mol% G+C content of the *cysI* and *cysDN* coding regions (65% and 64%, respectively) was slightly lower than that normally seen for *P. aeruginosa* (West & Iglewski, 1988) but agreed well with the 64 mol% G+C content found for other cysteine biosynthetic genes in this species (Delic-Attree *et al.*, 1997).

Fig. 3. Sequence alignment of the product of the *P. aeruginosa* *cysN* gene with the *E. coli* CysN and CysC proteins, showing the two-domain structure of the *P. aeruginosa* CysN protein. The alignment was done with the program PILEUP. Residues which are identical (*) and similar (:) in both sequences are indicated.

Chromosomal location of the *cysI* and *cysDN* genes

The *cysI* and *cysDN* genes were localized on the chromosome of *P. aeruginosa* PAO by hybridization of digoxigenin-labelled probes to genomic DNA digested with *SpeI* or *DpnI* as described previously by Römmling *et al.* (1992). The *cysI* gene hybridized to *SpeI* fragment L and *DpnI* fragment C, corresponding to the 55–57.5 min region of the chromosome, where the *cys-54* and *cysG1* loci have previously been located (Holloway *et al.*, 1994). The *cysDN* genes hybridized to *SpeI* fragment E and *DpnI* fragment I, corresponding to the 71–75 min region.

Regulation of the sulfate starvation response in cysteine auxotrophic mutants of *P. aeruginosa*

The regulation of the sulfate starvation response was investigated in the mutant strains AX18 and AX309, initially by using arylsulfatase activity as a marker for the two-dimensional PAGE response. Cells were grown with a derepressing sulfur source (methionine for strains PAO1S and AX18 and pentanesulfonate for strain

AC309, since the latter strain did not grow with methionine), and arylsulfatase activities were measured in the exponential phase. Low-molecular-mass sulfur compounds (sulfate, sulfite, sulfide, thiocyanate) were added to the growth medium to test their effect as repressors of *atsA* expression (Fig. 4), and control experiments were included to check that the pseudo-reversion effect described above did not occur in the time frame of the experiment. In the wild-type strain, arylsulfatase expression was completely repressed by sulfate, sulfite, sulfide or thiocyanate, as expected. In AC309, which is unable to activate sulfate and reduce it to sulfite, sulfate no longer repressed *atsA* expression, showing clearly that sulfate is not the active corepressor *in vivo*. Sulfite, sulfide and thiocyanate repressed *atsA* expression in all three strains. Since strain AX18 cannot convert sulfite to sulfide, this implies that separate effects are responsible for the repressive nature of these two compounds. Two-dimensional PAGE gels were also prepared with extracts of the PAO1S, AX18 and AC309 cultures used for the arylsulfatase assays. Under the growth conditions tested, synthesis of the SSI proteins

Fig. 4. Arylsulfatase activities in *cysI* and *cysN* strains of *P. aeruginosa*. Cells were cultivated with methionine (strains AX18, PAO15) or pentanesulfonate (strain AC309) as sulfur source (100 μ M), and sulfate, sulfite or sulfide (500 μ M) was added. Arylsulfatase activity was assayed in whole cells and is given as a percentage of the value observed after growth with methionine or pentanesulfonate [Met/Pn; 14–20 nmol min⁻¹ (mg protein)⁻¹]. ■, Strain PAO15; ▨, strain AX18 (*cysI*); □, strain AC309 (*cysN*).

described above (Table 3) was controlled in exactly the same way as that of arylsulfatase, even though the latter was present at too low a level to be visible on the two-dimensional gels (Beil *et al.*, 1995). Intermediate levels of arylsulfatase activity (e.g. with strain AX18 after growth with sulfate/methionine; Fig. 4) gave rise to SSI protein spots on the gels of reduced intensity (not shown). We conclude that at least two separate corepressors exist to mediate repression of the sulfate starvation response, one located before and one after sulfite in the cysteine biosynthetic pathway.

DISCUSSION

Synthesis of at least 10 proteins is regulated in *P. aeruginosa* by the sulfur supply during steady state growth (SSI proteins; Fig. 1, Table 3). This number represents a minimum value, since enzymes involved in sulfur metabolism are only required at low levels (sulfur constitutes 0.5% of dry weight in *Pseudomonas* cells; Kertesz *et al.*, 1993), and many more may be expressed at concentrations below the detection limit of the conditions used in this study. This is the case for arylsulfatase, where the enzyme constitutes only 0.04% of cell protein even when fully induced, and is not visible in two-dimensional PAGE of cell extracts (Beil *et al.*, 1995). Although sulfur-limiting conditions could in principle lead to induction of the stringent response (Cashel *et al.*, 1996), the doubling times for *P. aeruginosa* were comparable for all the sulfur sources we examined (Table 2) and we concluded that the increased synthesis of the SSI proteins was not part of a general starvation response, but due to repression exerted by sulfite, cysteine or thiocyanate, or a related metabolite. The identification of Sbp as one of the SSI proteins in *P.*

aeruginosa (Table 3) confirms that the SSI proteins include sulfur-scavenging proteins in this species, and hence the unidentified SSI proteins might include enzyme systems for assimilation of alternative sulfur sources such as alkanesulfonates (Kertesz, 1996; Kertesz *et al.*, 1994) or arylsulfates (Beil *et al.*, 1995). For further studies of the SSI proteins we are using the sequences obtained (Table 3) to isolate the corresponding genes, and hope thereby to arrive at a fuller understanding of the functions of these proteins.

The broad range of sulfur sources which *P. aeruginosa* can assimilate was divided into two groups on the basis of repression or derepression of the SSI proteins (Table 2). This division is reminiscent of previous classifications of sulfur sources into 'preferred' and 'non-preferred' compounds in other species [e.g. *E. coli* (Roberts *et al.*, 1955; Uria-Nickelsen *et al.*, 1994), *Comamonas* (Seitz *et al.*, 1993), *Pseudomonas putida* (Beil *et al.*, 1996)]. For *P. putida*, detailed growth measurements have shown that the sulfur sources that repress the SSIS are also utilized preferentially by the cell (Beil *et al.*, 1996). This represents an exact parallel to other nutrient starvation systems – in the case of phosphorus, for example, it is the absence of the preferred phosphorus source (inorganic phosphate) which leads to synthesis of the proteins that constitute the *pho* regulon. Whether the analogy between these two systems (*pho* and SSIS) can be extended further is not yet clear, since the *pho* system is essentially regulated only by phosphate levels (Wanner, 1993), whereas the SSIS in *P. aeruginosa* is repressed by sulfate, cysteine and thiocyanate. Up until now thiocyanate has primarily been explored as a source of carbon (Boucabeille *et al.*, 1994), energy (Katayama *et al.*, 1995) or nitrogen (Betts *et al.*, 1979; Stratford *et al.*, 1994) for bacteria, and very few data are available on its utilization as a sulfur source (Laudenbach & Grossman, 1991). Although the mechanism by which thiocyanate-sulfur is assimilated has not yet been elucidated, our results suggest that the sulfur atom enters the cysteine biosynthetic pathway at the level of sulfide, possibly by direct sulfo transfer to an acceptor such as acetylserine or succinylhomoserine.

The chemical and structural diversity of the SSIS-repressing substrates poses the interesting mechanistic question of whether all of these substrates are active as corepressors *in vivo*, whether they are interconverted, or whether regulation is effected by conversion to a single, as yet unidentified effector. The best characterized system that is regulated by sulfur is the *cys* regulon (Kredich, 1996), whose components are also repressed by cysteine and sulfate. Nothing is known of the effect of thiocyanate, but most studies of the *cys* regulon have been done in *E. coli*, which does not use thiocyanate as a sulfur source (Kertesz *et al.*, 1993). For the *cys* regulon, dual regulation by cysteine and sulfate may be traced to two effects – feedback regulation by cysteine to prevent synthesis of the positive effector *N*-acetylserine, and conversion of sulfate to sulfide, which exerts an anti-induction effect on several genes of the regulon (Kredich, 1996). A different type of mechanism has been reported

for arylsulfatase synthesis by *Klebsiella pneumoniae*. In this organism, studies with cysteine biosynthesis mutants have shown that sulfate can act to repress arylsulfatase synthesis without prior activation and reduction, and that cysteine acts as a corepressor independently from sulfate (Adachi *et al.*, 1975). Our results with mutants of *P. aeruginosa* (Fig. 4) suggest another mode of regulation. As in *K. pneumoniae*, at least two sulfur-containing species can act as corepressors, but intracellular sulfate itself is not active as a corepressor molecule. This, and the results of enzyme measurements reported above (Table 2) suggest that for sulfur assimilation several regulatory circuits may overlap, and the regulatory situation may be more complex than previously thought.

One of the proteins identified as part of the SSIS in *P. aeruginosa*, the periplasmic sulfate-binding protein (Sbp), is also a member of the *cys* regulon in enteric bacteria (Kredich, 1996), and is especially important in scavenging sulfate at low concentrations. Since *sbpA* mutants of *Synechococcus* can still utilize sulfate (Laudenbach & Grossman, 1991), the Sbp protein is clearly not always required for sulfate transport, and it may constitute a second, high-affinity sulfate-uptake system in this species, analogous to the Pst system for phosphate uptake (Wanner, 1993). A similar duplication of function is also seen with the Sbp protein and the thiosulfate-binding protein in *E. coli* (Sirko *et al.*, 1995). We and others (Calhoun & Feary, 1969) have had difficulty isolating *P. aeruginosa* mutants in some loci of the *cys* pathway (e.g. sulfate uptake), and this and the rapid appearance of pseudorevertants of our *cys* mutants suggest that several of the *cys* genes may be duplicated on the chromosome. Duplication of *cys* gene activities has been reported for *Salmonella*, for CysI, CysB and CysM activities. In this species, specific expression of the pairs of homologues is controlled by the oxygen status of the cells (Kredich, 1996). In *P. aeruginosa*, genes involved in cysteine and methionine biosynthesis are quite scattered, being located at 20 and 40 min (methionine biosynthesis), and at 45, 56, 69 and 71–75 min (cysteine biosynthesis) on the PAO chromosome. Three genes have been characterized at a DNA sequence level until now – *metZ* (O-succinylhomoserine sulfhydrylase; Foglino *et al.*, 1995), *cysH* (phosphoadenosine-phosphosulfate sulfotransferase; Delic-Attree *et al.*, 1997) and the regulatory gene *cysB*, which also appears to play a role in alginate biosynthesis in this species (Delic-Attree *et al.*, 1997). More work is clearly required on cysteine and methionine biosynthesis in *P. aeruginosa*, since several differences to the pathways that have been characterized in *E. coli* are already apparent, both at a biochemical level (Foglino *et al.*, 1995; Günther *et al.*, 1979) and at the level of gene organization (Delic-Attree *et al.*, 1997; this paper).

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