Structure and function of a *cysBJIH* gene cluster in the purple sulphur bacterium *Thiocapsa roseopersicina*

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A gene cluster containing homologues of the genes *cysB*, *cysJI* and *cysH* was found in the genome of the sulphur-oxidizing purple bacterium *Thiocapsa roseopersicina*. The nucleotide sequence indicated four open reading frames encoding homologues of 3'-phosphoadenylylsulphate (PAPS) reductase (*CysH*), sulphite reductase flavoprotein (*CysJ*) and haem protein (*CysI*) subunits, and a transcriptional regulator (*CysB*). Genes *cysJIH* are separated by a short *cis*-active intergenic region from *cysB* which is transcribed divergently. *cysB* encodes a polypeptide of 35.9 kDa consisting of 323 amino acid residues with 40% identity to the CysB regulator from enterobacteria. *cysH* encodes a protein with 239 amino acid residues and a calculated mass of 27.7 kDa; *cysJ* encodes a protein with 522 amino acid residues and a mass of 57.8 kDa; and *cysI* encodes a protein with 559 amino acid residues and a mass of 62.3 kDa. The *cysJIH* gene products have been expressed and used for complementation of *cys* mutants from *Escherichia coli* Biochemical analysis. The gene product CysH is a thioredoxin-dependent PAPS reductase (EC 1.8.99.4). It was repressed under photoautotrophic growth using hydrogen sulphide as electron donor and derepressed under conditions of sulphate deficiency. Products of the *cysJI* genes were identified as the two subunits of NADPH-sulphite reductase (EC 1.8.1.2). *cysJ* encoded the flavoprotein, with ≥39% identity to the protein from *E. coli*, and *cysI* encoded the haem protein, with ≥53% identity. A *cysI* clone was used to complement the corresponding mutant from *E. coli* and to express enzymically active methylviologen-sulphite reductase.

Keywords: sulphate-oxidizing bacterium, cysteine biosynthesis, phosphoadenylylsulphate (PAPS) reductase, sirohaem sulphite reductase, thioredoxin

INTRODUCTION

Assimilation of sulphate depends on a set of five enzymes which successively activate sulphate to an adenylylated form (3'-phosphoadenylylsulphate, PAPS) before it is reduced to sulphite and to sulphide. The main function of this pathway is to produce sulphide for the biosynthesis of amino acids. Sulphate dissimilation is described for sulphate-reducing bacteria which reduce sulphate to sulphide under anaerobic conditions using hydrogen or carbohydrates as electron donor. This respiratory reduction of sulphate is often coupled to

ATP synthesis (Kelly, 1990). Hydrogen sulphide and other reduced forms of sulphur are used by chemolithotrophic bacteria and photoautotrophic purple or green sulphur bacteria as electron donors for chemo- or photosynthetic reduction of carbon dioxide (Pfennig & Trüper, 1992). These organisms oxidize sulphur compounds such as sulphide or thiosulphate to sulphate. Sulphite is the pivotal intermediate in the oxidative reactions as it is in the reductive reactions, although many patterns of sulphur oxidation may be more complex than anticipated by a simple reversal of the reductive steps (Kelly *et al.*, 1997; Friedrich, 1998).

Assimilatory sulphite reductases can reduce sulphite to sulphide in a single step consuming six electrons, which can be provided from NADPH or reduced ferredoxin. This reduction is dependent on the occurrence of sirohaem in combination with iron-sulphur cluster(s) as

Abbreviations: APS, adenylyl sulphate; MVH, methylviologen, reduced form; PAPS, 3'-phosphoadenylyl sulphate.

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cofactors (Siegel, 1976; Crane & Getzoff, 1996). Both cofactors require two special pairs of cysteines that bind an orthorhombic $\text{Fe}_4\text{-S}_4$ cluster. Sirohaem is bound by ionic forces provided by the carboxyl groups and basic amino acid sidechains of the polypeptide backbone; one of the cysteines is also used to couple the haem with the $\text{Fe}_4\text{-S}_4$ cluster. Although virtually scattered over the entire apoprotein, residues shown to be involved in the binding of sirohaem are highly conserved among NADPH-sulphite reductases and ferredoxin-sulphite and nitrite reductases. Their role as haem ligands was elucidated most recently by the high-resolution three-dimensional structure of the haem protein of sulphite reductase from *Escherichia coli* (Crane & Getzoff, 1996; Crane *et al.*, 1997). Of the two pairs of cysteines required for the binding of the $\text{Fe}_4\text{-S}_4$ clusters, the first pair is found in a highly conserved CNPGC cysteine pentapeptide, and the second in a more variable CX₅C cysteine heptapeptide. C483 in the pentapeptide CNPGC of *E. coli* sulphite reductase is used as the bridging ligand between the iron-sulphur cluster and the central Fe of the haem, while the remaining three cysteine residues exclusively bind the $\text{Fe}_4\text{-S}_4$ cluster. The unique combination of cofactors is found in assimilatory sulphite reductases and nitrite reductases alike, suggesting that the apoprotein forms a highly conserved structure. Using the haem protein of NADPH-sulphite reductase as three-dimensional model, Crane *et al.* (1995) showed that functionally important subdomains of ferredoxin-sulphite and ferredoxin-nitrite reductase can be superimposed to give nearly identical structures.

At the beginning of this investigation, we assumed that this structure is encoded by related structural genes. Indeed, by using a DNA fragment of the *cysI* gene from *E. coli* as probe, and by nested PCR using degenerate primers deduced from conserved regions of the polypeptide, we isolated the ferredoxin-sulphite reductase gene from the cyanobacterium *Synechococcus* (Gisselmann *et al.*, 1993) and a higher plant (Brühl *et al.*, 1996). As a first step in dissecting the identity and function of sulphite reductases in the purple bacterium *Thiocapsa roseopersicina* the genome was probed with a 64-fold degenerate oligonucleotide deduced from the conserved cysteine pentapeptide CPNGC of assimilatory sulphite reductase (Haverkamp, 1992, 1996; Gisselmann *et al.*, 1993). In the present paper we report that the structural gene of this putative sulphite reductase is found in a complex gene cluster consisting of four open reading frames (ORFs) encoding a *cysB*-like regulator, a PAPS reductase encoded by a *cysH* homologue and a sirohaem sulphite reductase encoded by homologues of *cysJ* and *cysI*. The genes were sequenced and characterized by phenotypic complementation of corresponding *cys* mutants from *E. coli* and analysed for functionally active gene products.

METHODS

Bacterial strains and plasmids. The bacterial strains, plasmids and DNA fragments used in this study are listed in Table 1. *T. roseopersicina* was grown phototrophically at 500 lx

(21 mE m⁻² s⁻¹) in modified medium 1 (Pfennig & Trüper, 1992), replacing MgSO_4 by MgCl_2 and SL12 by SL4. Strain M1 was supplemented with 25 g NaCl l⁻¹. Sulphide/bicarbonate feeder solution was prepared as described by Siefert & Pfennig (1984). For phototrophic growth the basal mineral medium [containing, per litre: 0.5 g KH_2PO_4 , 0.62 g K_2HPO_4 , 0.5 g NH_4Cl , 0.33 g KCl, 0.05 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.33 g $\text{MgCl}_2 \cdot \text{H}_2\text{O}$ (Kämpf & Pfennig, 1980)] was supplemented with 1 ml SL4 l⁻¹ and 20 µg vitamin B12 l⁻¹, and adjusted to pH 7.3. A mixture of organic substrates was added to give a 2% (v/v) final concentration. The mixture consisted of sucrose, galactose, mannitol, glucose, fructose, 2-oxoglutarate, pyruvate, acetate, succinate and malate at 10 g l⁻¹ per compound in H_2O , adjusted to pH 7.0 using KOH and sterilized by filtration. For anaerobic growth 1 litre screw-capped bottles were filled completely but semiox cultures were grown on a shaker (120 r.p.m. at 30 °C) using notched 1 litre Erlenmeyer flasks half-filled and sealed with foam plastic plugs.

E. coli host strains were grown on LB supplemented with 100 µg ampicillin ml⁻¹ and/or 10 µg tetracycline ml⁻¹ if required for selection of transformed strains. In addition to the individual growth requirements, we grew the *cys*-complemented mutants on VBB medium (Vogel & Bonner, 1956) or supplemented with cysteine at 1 mM.

Cloning methods. Phage EMBL3 was used to construct a library harbouring genomic DNA from *T. roseopersicina* M1. DNA was partially restricted with *Sau*III and size-fractionated (15 kb average size) before ligation into the *Bam*HI site of EMBL3. We used *E. coli* LE392 as host. It was transfected using a λ coat packaging kit (Promega, Packagene *in vitro*). Plating, lysate preparation and isolation of DNA followed the methods outlined by Sambrook *et al.* (1989). For DNA sequencing the dideoxy chain-termination method (Sanger *et al.*, 1977) was used. Compressed sites were analysed with 7-deaza-dGTP mixes (Pharmacia) and universal primers (T3 and T7), and synthetic oligonucleotides were used for primer-hopping to sequence template and non-template strands.

The CPNGC-specific R3 oligonucleotide used as probe is described by Brühl *et al.* (1996). Five *cys*-positive clones were detected among 50 000 plaques, and subclones in pBluescript II SK+ were analysed as described below.

Nucleotide and amino acid sequences were analysed using the software provided by servers of EMBL or NCBI (Altschul *et al.*, 1997). Treeview 1.4 was written by R. Page, IBLS, University of Glasgow, UK.

Gene products. *cysJ*, *cysH* and *cysB* were expressed to allow their characterization by function and by immunological methods. Functionally active gene products were obtained by using pBTac1 (Boehringer) constructs; polypeptides required for the production of antibodies were produced from pQE vectors (Qiagen).

In order to express NADPH-sulphite reductase, *cysJ* from *T. roseopersicina* and *cysG* from *Salmonella typhimurium* were co-integrated into pBTac1. *cysG* was excised from pJYW609 as a 2.34 kb *Cl*AI-*Bam*HI fragment including its own promoter and blunt-end ligated into the *Pst*I site yielding pBTac1*cysG*. pTH11 was constructed by insertion of an *Nde*I-*Sal*I fragment (nt 582-4066) from pTH6 (5'-3' orientation) into pBTac1*cysG* using the *Sma*I site, while pTH12 was constructed by using the *Cl*AI-*Sal*I fragment (nt 602-4066). Both constructs harboured the *cys* genes in the order *cysJ*::*cysG* under control of the *tac* promoter. pTH13 containing DNA that encoded the β -subunit of sulphite reductase was obtained from pTH12 by deleting the *Pst*I fragment.

Table 1. Strains and plasmids

Strain or plasmid	Genotype	Source or reference
<i>T. roseopersicina</i>		
M1	Wild-type	H. van Gernerden, RU Groningen, Netherlands
6311	Wild-type	H. G. Trüper, Universität Bonn, Germany
<i>E. coli</i>		
JM96	<i>thr-1 leuB6 trp-1 hisG1 cysH56 argH1 thi-1 ara-13 lacY1 gal-6 malA1 xyl-7 mtl-2 strA9 tonA2</i> $\lambda^R \lambda^- supE44$	Genetic Stock Center, Yale, #5746
JM96 _{PAK}	As JM96 but <i>hsdR2zjj-202::Tn10(Tet^R)</i>	Krone <i>et al.</i> (1991)
JM246	<i>cysI53</i> λ^- IN(<i>rrnD-rrnE</i>)1	Genetic Stock Center, Yale, #5747
AT713	<i>supE44?</i> λ^- <i>cysJ43 argA21 lysA22 rpsL104 malA1</i> (λ^R) <i>xyl-7, mtl-2, thi-1?</i>	Genetic Stock Center, Yale, #4529
LE392	<i>e14</i> (McrA) <i>hsdR514 supE44 supF58 lacY1</i>	Stratagene
XL-1 Blue	<i>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac</i> [F' <i>proAB lacI^qZ</i> Δ M15Tn10(Tet ^R)]	Stratagene
TG1	<i>supE hsd</i> Δ 5 <i>thi</i> Δ (<i>lac-proAB</i>)F'(<i>traD36 proAB⁺ lacI^q lacZ</i> Δ M15)	Laboratory collection
Plasmids		
pBluescript II	2.96 kb phagemid	Stratagene
pBTac1	4.6 kb <i>tac</i> vector	Boehringer Mannheim
pQE30, 31, 32	His ₆ fusion expression vectors	Qiagen
pJYW609	pBR322::cysJ ^{Bc} + cysG St	Wu <i>et al.</i> (1991)
pUB5	pBTac1::cysH ^{Bc}	Berendt <i>et al.</i> (1995)
pQEB1	pQE30::cysB(<i>SacI</i> 131– <i>PstI</i> 545)	This work
pQEB11	pQE30::cysB(<i>KpnI</i> – <i>HindIII</i>)*	This work
pQEJ6	pQE31::cysJ(<i>PstI</i> 666– <i>PstI</i> 1794)	This work
pQE13	pQE32::cysI(<i>BglII</i> 3278– <i>KpnI</i> 4225)	This work
pQEH	pQE30::cysH(<i>BamHI</i> – <i>HindIII</i>)*	This work
pTH1	pBS::cysIH(<i>BamHI</i> 3295–5170)	This work
pTH2	pBS::cysHI(<i>BamHI</i> 5170–3295)	This work
pTH3	pBS::cysJI(<i>BamHI</i> 1129–3127)	This work
pTH4	pBS::cysIJ(<i>BamHI</i> 3127–1129)	This work
pTH5	pBS::cysIH(S1:3720– <i>BamHI</i> 5157)	This work
pTH6	pBS::cysBJIH(<i>Sall</i> 1–4066)	This work
pTH7	pBS::cysHIJB(<i>Sall</i> 4066–1)	This work
pTH8	pBS::cysBJ(<i>BamHI</i> – <i>BamHI</i> 1129)†	This work
pTH9	pBS::cysJB(<i>BamHI</i> 1129– <i>BamHI</i>)†	This work
pTH10	pBS::cysJ– <i>narK</i> (<i>BclI</i> 4 kb genomic)	This work
pBTac1cysG	pBTac1::cysG St (<i>ClaI</i> / <i>BamHI</i>) of pJW609 in <i>PstI</i> site of pBTac1	This work
pTH11	pBTac::cysG St + cysJI(<i>NdeI</i> 582– <i>Sall</i> 4066) in <i>SmaI</i> of pBTac1	This work
pTH12	pBTac::cysG St + cysJI(<i>ClaI</i> 602– <i>Sall</i> 4066)	This work
pTH13	pBTac::cysG St + cysJI(<i>PstI</i> 1794– <i>Sall</i> 4066)	This work
pKStrx1	pBS::trx– <i>rho</i> (<i>BamHI</i> 2 kb genomic)	This work

* Restriction site introduced by PCR primer.

† Includes *BamHI* site of the vector.

Active PAPS reductase that supported growth of *E. coli* JM96_{PAK} was expressed from a pBluescript II KS + derivative. The structural gene in pTH5 was contained in the bp 3720–5157 fragment obtained by S1 nuclease digestion of *cysI* up to

bp 3720 and restriction with *BamHI*. The His₆–CysH fusion protein in pQEH was expressed from a PCR-amplified gene using *cysH5* (3865: 5' GACTGGAT-CCATGAGTAAGCC-CCGATC 3') and *cysH3* (4561: 5' TCGCGGTACCGAAA-

CTAACGATGC 3') as primers. The *cysB* structural gene was isolated from a pBluescript II SK+ plasmid containing a 4 kb *Bcl*I genomic fragment identified by hybridization with a 0.58 kb *Nde*I–*Sal*I fragment of pTH6. Primers *cysB5* (960: 5' TCACGTACCAACGATATGGACGTACGCC 3') and *cysB3* (4: 5' CGCGAAGCTTAAGCA-CGCCCTCAGTC 3') were used to amplify the gene and the resultant *Kpn*I/*Hind*III (pQEB11) clone was expressed as a His₆ fusion protein in pQE30.

Enzyme assays, purification and immunological methods

Sulphite reductase. Frozen cells (23 g) from *E. coli* JM246 transformed with pTH3 and grown in VBB minimal medium (Vogel & Bonner, 1956) were ruptured by passage through a French press at 14.4 MPa using 50 mM phosphate buffer pH 7.7, 2 mM EDTA, 1 mM PMSF, 0.05 mg DNase ml⁻¹ and 0.05 mg lysozyme ml⁻¹. Cell debris was removed by centrifugation at 40000 g for 20 min. Active protein was recovered by fractionation with ammonium sulphate (from 40 to 90% saturation). It was collected by centrifugation and dialysed against 50 mM Tris/HCl pH 7.8, 0.1 mM EDTA. Anion-exchange chromatography on DEAE-Sephacrose CL 6B applying a gradient of 0–1 M NaCl in Tris/HCl buffer was used to enrich the protein. The concentration of enzyme protein was followed by immunodot-blotting of the fractions and testing for sulphite reductase protein using rabbit anti-sulphite reductase IgG. Activity was measured as methylviologen (MVH)-dependent sulphide formation from sulphite in stoppered cuvettes made anaerobic by repetitive evacuation and flushing with deoxygenated nitrogen. MVH was added to start the reaction. It was collected from a 10 mM stock solution in 50 mM KH₂PO₄/K₂HPO₄ buffer, pH 7.7, plus 0.1 mM EDTA, reduced electrochemically (Schwenn & Kemena, 1984). Sulphide was determined colorimetrically according to Siegel (1965).

PAPS reductase. The enzyme was measured as thioredoxin-dependent liberation of [³⁵S]sulphite from [³⁵S]PAPS using recombinant thioredoxin from *E. coli* (MBI Fermentas) and dithiothreitol as auxiliary reductant (Schwenn *et al.*, 1988; Berendt *et al.*, 1995). Rates were determined as nmol ³⁵SO₃ (mg protein)⁻¹ min⁻¹ (Schwenn & Schriek, 1986); [³⁵S]PAPS was prepared enzymically from ³⁵SO₄²⁻ (Schriek & Schwenn, 1984) and reaction products were analysed by HPLC (Schwenn & Jender, 1980).

His₆ fusion proteins and immunological methods. Rabbit polyclonal antisera were raised against His₆ fusion proteins. Expression of the genes was induced by 0.4 mM IPTG. The cells were extracted as described by the manufacturer (QIA-expression-ist, Qiagen) using the protocol for insoluble proteins. For immunization the regimen of Catty & Raykundalia (1988) was followed. The IgG fractions were recovered from whole antisera by anion-exchange chromatography and gel filtration as outlined. Protein–dye binding (Bradford, 1976), Western immunoblotting (Towbin *et al.*, 1979) and SDS-PAGE (Laemmli, 1970) were used to assess the quality and purity of gene products as described earlier (Krone *et al.*, 1991; Gisselmann *et al.*, 1993).

RESULTS

Structure of the *T. roseopersicina cysBJIH* DNA

A 5157 bp DNA fragment from *T. roseopersicina* containing four ORFs with homologies to *cys* gene products from entero- and cyanobacteria, yeast and

higher plants was detected by Southern hybridization with an oligonucleotide deduced from a conserved region of assimilatory sulphite reductases (Ostrowski *et al.*, 1989a, b; Gisselmann *et al.*, 1993; Brühl *et al.*, 1996). The relative size and orientation of the loci are depicted in Fig. 1. The ORFs represent homologues of the *cysJI* and *cysH* genes found in *E. coli* and *Synechococcus*. In *T. roseopersicina* the *cysJIH* homologues appear to form a multigene transcriptional unit which is separated by a non-translated intergenic region of 197 nt from a *cysB* homologue. *cysB* is located upstream of *cysJIH* and transcribed from the 3'–5' strand in the opposite direction. Shine–Dalgarno sequences are found at position 591–595 for the *cysJIH* cistron and at position 415–420 for *cysB*. A Shine–Dalgarno sequence is missing upstream of *cysI* and translation of *cysJ*–*cysI* may be coupled. *cysH* possesses its own Shine–Dalgarno site at position C3838GGAGG. The start codon A3850TG of *cysH* is located upstream of the termination codon T3855AA of *cysI* so that *cysI* and *cysH* overlap by eight nucleotides and correct translation of *cysH* would require reinitiation and restart in a different reading frame. The intergenic region between *cysB* and the *cysJIH* cluster contains two putative promoter sequences (*pcysB*, T592TGGCA–N₁₅–T571ATTCG; and *pcysJIH*, T444TCAAA–N₁₈–T468ACACT) that are similar to the consensus sequence of the σ^{70} promoters from *E. coli*. The intergenic region (Fig. 2) contains three palindromic sequences that could form stable hairpin structures (bp 507–535, bp 523–549 and bp 571–610). A small hairpin structure could form at position 4641–4665 of the sequence, with a ΔG of -9.7 kcal mol⁻¹ (-40.6 kJ mol⁻¹). With its G:C-rich stem followed by a short run of Us, the structure resembles a rho-independent termination site; however, its function remains to be investigated.

Structure and function of the gene products

CysB: a transcriptional regulator. The *cysB* gene product consisted of 323 residues with a predicted molecular mass of 35939 Da. Its amino acid sequence shows an overall identity of 39.9% to that of the enterobacterial CysB proteins. Alignment of CysB from *T. roseopersicina* with its homologues from enterobacteria, including also two species of closely related rRNA superfamilies, produced three regions with a high degree of conserved residues that corresponded to the position of the coinducer recognition/response domains observed in the LysR family of transcriptional regulators (Schell, 1993). In *T. roseopersicina* S38 is replaced by T in a relatively poorly conserved area, whilst T148 is unchanged and located in a stretch of highly conserved amino acids. Mutation of S38 destroyed its DNA-binding capacity whereas replacement of the T148 caused constitutive expression of positively regulated genes of the cysteine regulon from *S. typhimurium* (Colyer & Kredich, 1994).

CysJI: subunits of a flavo-haem sulphite reductase. *cysJ* encodes a polypeptide of 522 amino acids with a

Fig. 1. ORFs of the *cysB/JIH* multigene cluster and phenotypic complementation of *E. coli* *cys* mutants by *T. roseopersicina* *cys* homologues. The length, position and orientation of pTH subclones as obtained from the 5.1 kb genomic DNA fragment in relation to the ORFs of the *cysB/JIH* gene cluster are shown. Location of single restriction sites used for construction of the subclones is described in Table 1. *lacZ* and *tac* promoters were provided from pBTac1 and pBS cloning vectors; pTH1–7 are Bluescript derivatives; pTH11, 12 and 13 are pBTac1 derivatives, and pQE is a derivative of the pQE His₆ tag expression vector. ND, Not determined (in the complementation tests).

Fig. 2. Putative *cis*-active control region of the *cysB/JIH* gene cluster: promoters, ribosome-binding sites and secondary structures between the *cysB* gene and the *cysB/JIH* cluster. *cysJ1* and *cysJ2* indicate the position of the two possible start codons for *cysJ*. *cysB* is located upstream of the *cysB/JIH* cluster and transcribed divergently from the 3'–5' strand. A hypothesized promoter region of *cysB* overlaps with the transcriptional start region of *cysJ1* assuming a possible translational start at A₄₉₂TG. Putative genes are boxed, the –35 –10 region is emphasized (bold lettering), and possible secondary structures are enclosed by arrows showing the ΔG values of formation (in kcal; 1 kcal = 4.184 kJ).

molecular mass of 57791 Da. Comparison of the amino acid sequence with the sequences available [GenBank, PDB, SWISS-PROT and PIR (release 26.313)] aligned the *cysJ* gene product with the α subunit of NADPH-sulphite reductases from enterobacteria (score of 422 to 417). It shows 39.3% identity to the *E. coli* flavoprotein of the assimilatory NADPH-sulphite reductase that binds FAD and FMN as prosthetic groups. The putative flavoprotein from *T. roseopersicina* is shorter than the enterobacterial flavoproteins by 69 amino acid residues when the entire sequence is considered. These missing

residues cause three gaps in the second half of the sequence, where 59 residues are missing after T329, 21 residues after S343, and 5 residues after Q360. Additional amino acids are found in the first half of the polypeptide chain, where a small insertion of nine residues is found after N161 with no correspondence to the flavoproteins from *E. coli* and *S. typhimurium*. These differences in the primary structure apparently do not affect the flavin-binding domains. Of the typical sequence motifs that have been identified as prerequisites for binding the cofactors FMN, FAD and NADP, the

Table 2. Conserved residues and motifs in the structure of sulphite reductase flavoprotein from *T. roseopersicina*

Cofactor domain*	Position	Conserved residues†
FMN phosphate	59–78	RITILFGSQTGNAKAVAEQL
FMN ring (<i>Si</i>)	109–131	LIVVSTHGEGEPPENAYALHAFL
FMN ring (<i>Re</i>)	139–157	LEQLPFAVLGLGDSSYEHF
FAD ring (<i>Si</i>)	441–343	LHPTTVRGWSRAS
FAD ring (<i>Re</i>)	515–522	ECYATDDL
FAD adenine	344–352	GRTGAEDA
FAD pyrophosphate	353–360	ERLEREEQ
NADPH pyrimidine	451–488	KPYVQQRL....AHLYVCGATAMGQAV
NADPH pyrophosphate	381–400	LIMIGAGTGVAPYRAFLQQR

* Definition according to Wang *et al.* (1997).† Underlining indicates residues identical to *E. coli cysJ*.**Table 3.** Conserved residues and motifs in the structure of sulphite reductase haemprotein from *T. roseopersicina*

Function*	Amino acid type	Conserved residues
Binding of sirohaem	Basic	R73, R103, R107, Q111, H113, K215, K207, R204, Q385, R474
	Hydrophilic	T105, T428
	Aliphatic	V425
Substrate interaction on distal side of haem	Positively charged	R73, R107, R142, R204, K205, K207
Binding of Fe ₄ -S ₄ and bridging ligand	Cysteine groups	CVXXTC429PL, GCPNGC472

* Definition according to Crane *et al.* (1996, 1997).

isoalloxazin ring-binding motif of FMN, and the binding motifs of FAD and NADP as described by Wang *et al.* (1997), are fully conserved (Table 2).

An alternative N-terminus for the flavoprotein is predicted by the ORF-finder option from the *cysJ* DNA sequence. The protein is extended by 38 amino acids, resulting in a total length of 560 residues with a molecular mass of 62127 Da. If translation of the putative *cysJ* homologue starts at A492TG the CysJ polypeptide would be extended by a pre-sequence containing mainly positively charged amino acids like arginine that could interact with the negatively charged phospholipids of the cytoplasmic membrane.

cysI encodes the sulphite reductase haem protein consisting of 559 amino acid residues with a molecular mass of 62279 Da. It is 54% identical to the β subunit of *E. coli* NADPH-sulphite reductase. The polypeptide sequence NH₂-TGCPNGCAR474-COOH extracted from a multiple alignment of sulphite reductases from *E. coli*, *S. typhimurium*, *Synechococcus* and the nitrite reductase in order to design a DNA probe was fully conserved at

a corresponding position in the *Thiocapsa* haemprotein. In combination with the cysteines of the motif CVALPTC429 the cysteines in this sequence form covalent bonds with the Fe groups of the iron-sulphur cluster, while cysteine C472 serves as bridging ligand between the Fe₄-S₄ cluster and the Fe of the sirohaem. In addition to the cysteine clusters, the 15 amino acid residues that were proposed to bind the sirohaem are identical with the exception of LS259 of *E. coli*, which are replaced by TG247 in the *Thiocapsa* sequence. And secondly, the set of positively charged amino acids facing the distal anion-binding site of the haem is fully conserved (Table 3).

A catalytic function of the putative NADPH-sulphite reductase was demonstrated by phenotypic complementation of *cys* mutants deficient in flavo- and haem protein of sulphite reductase and by *in vitro* testing of the MVH-dependent formation of sulphide from sulphite. A complementation of the haemprotein-deficient *cysI* mutant of *E. coli* was observed with plasmids pTH6, 11 and 12, which contained the *cysI* fragment from *T. roseopersicina*. However, none of the constructs

containing also *cysJ* was able to complement the flavoprotein-deficient *E. coli* mutant (Fig. 1). pTH13 contained, in addition to *cysI* and the truncated *cysJ* genes, a complete *cysG* gene from *S. typhimurium* in order to increase the content of sirohaem (Wu *et al.*, 1991). Expression of the *cysI* gene from pTH13 in JM246 was verified by Western immunoblotting (data not shown). The size of 62 kDa corresponds to that predicted from the amino acid sequence. The *cysI* mutant complemented with pTH13 was tested for the occurrence of an active sulphite reductase haem protein using MVH as reductant. The activity of the recombinant *Thiocapsa* enzyme was hardly detectable [$0.9 \text{ mU (mg protein)}^{-1}$] and considerably lower than that of homologous recombinant sulphite reductase (65 mU mg^{-1}) expressed from pJYW609 with the authentic *cysJ* genes from *E. coli*. NADPH-sulphite reductase activity was not detectable *in vitro* from *E. coli* JM246 transformed with pTH13 but complementation of this mutant is seen as circumstantial evidence for its existence *in vivo*. The protein was enriched by ammonium sulphate precipitation and chromatography on a DEAE-cellulose column. A specific activity of 38.5 mU mg^{-1} was obtained from combined fractions of the DEAE-cellulose fractionation using MVH as reductant. The rate represented a 42-fold enrichment over the crude cell extract.

CysH: PAPS reductase. Translation of the *cysH* ORF yields a polypeptide of 239 amino acids with a molecular mass of 27 697 Da. Its amino acid sequence is 63.7% identical to the *cysH* gene product from *E. coli* representing the enzyme PAPS reductase. The *T. roseopersicina* PAPS reductase homologue is also highly related to the PAPS reductases from the cyanobacteria *Synechococcus* and *Synechocystis* and more distantly related to the PAPS reductases from fungi (*Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*). The putative PAPS reductase from the purple bacterium together with the aforementioned PAPS reductases belongs to a larger family of homodimeric PAPS reductases that do not contain (a) chromophore(s) but use reduced thioredoxin or glutaredoxin as reductant. A hallmark of this type of PAPS reductase is the cysteine motif ECGL/IH located near the C-terminus (Berendt *et al.*, 1995). In the active dimer two cysteine residues form a redox-active dithiol-disulphide. In addition to this cysteine motif, *T. roseopersicina* PAPS reductase also contained the GYxS/TI/VG motif with Y205 and a highly flexible loop rich in basic amino acids GxRR/KxQ with a flexible arginine residue. As deduced from the three-dimensional structure (Savage *et al.*, 1997) the arginine together with the DTG and SFG motifs form the active centre of PAPS reductase.

E. coli JM96 transformed with pTH5 regained its prototrophic growth, exhibiting a specific activity of 0.7 mU mg^{-1} measured as sulphite formed from PAPS with heterologous *E. coli* thioredoxin as reductant. The activity found in cell extracts was comparable to that of wild-type *E. coli* grown on sulphate. However, as JM96_{FAK} expressing *T. roseopersicina cysH* formed

Fig. 3. Immunological detection of native PAPS reductase from *T. roseopersicina* M1: Western immunoblot of cell extracts from bacteria (10 mg wet weight) grown as batch cultures, tested with monospecific antibodies against PAPS reductase from *T. roseopersicina*. (1) Photoautotrophic growth 2 d after feeding $\text{H}_2\text{S}/\text{CO}_2$; (2) as (1), but cells extracted 7 d after feeding $\text{H}_2\text{S}/\text{CO}_2$. Cells from (2) were used to inoculate cultures (4), (6) and (8) (20%, v/v) (termed plus SO_4^{2-}), while (3), (5) and (7) were washed in 2.5% NaCl before inoculation to remove inorganic sulphate of the pre-culture (termed minus SO_4^{2-}). (3) Phototrophic growth in the presence of organic carbon (see Methods), minus SO_4^{2-} ; (4) phototrophic growth as in (3), plus SO_4^{2-} ; (5) organoheterotrophic growth in the presence of organic carbon, dark, minus SO_4^{2-} ; (6) organoheterotrophic growth as in (5), plus SO_4^{2-} ; (7) organoheterotrophic growth as semioxic culture using Erlenmeyer shake flasks sealed with foam plastic plugs, supplemented with $\text{S}_2\text{O}_3^{2-}/\text{CO}_2$, minus SO_4^{2-} ; (8) organoheterotrophic growth as in (7), plus SO_4^{2-} , but $\text{S}_2\text{O}_3^{2-}$ omitted; (9) recombinant PAPS reductase, clone pQEH as expressed in *E. coli* JM96_{FAK}.

inclusion bodies, we compared the amount of readily extractable PAPS reductase with the amount of recombinant protein solubilized in the presence of 3 M urea by Western immunoblotting using polyclonal antibodies against PAPS reductases from *T. roseopersicina* and antibodies against PAPS reductase from *E. coli* (data not shown). The recombinant PAPS reductase was detectable at a position in the gel which corresponded to a molecular mass of 28 kDa. This is in good agreement with the size predicted from the amino acid composition of the *cysH* gene product from *T. roseopersicina*. The amount of soluble PAPS reductase detectable in the immunoblot decreased early at the beginning of the exponential growth phase when the cell density surpassed $\text{OD}_{595} 0.2\text{--}0.3$. Only traces of soluble protein were found in the late-exponential phase ($\text{OD}_{595} \leq 0.7$) falling below detectability in the stationary phase ($\text{OD}_{595} \geq 1.5$). A background caused by PAPS reductase from the host can be excluded because *E. coli* anti-PAPS reductase did not cross-react with PAPS reductase antigen from *T. roseopersicina*.

In Western immunoblots of cell extracts, native PAPS reductase in *T. roseopersicina* was only detected when the source of sulphur was manipulated (Fig. 3, lanes 3, 4, 5 and 8). *cysH* appeared to be derepressed when sulphate as supply of sulphur became limiting. In the presence of $\text{S}_2\text{O}_3^{2-}$ (lane 7) PAPS reductase became undetectable, implying that its biosynthesis was downregulated by this sulphur source in *T. roseopersicina*.

DISCUSSION

T. roseopersicina – a purple sulphur bacterium of the family *Chromatiaceae* – was found to contain a gene cluster consisting of four structural genes that were homologous to *cysB*, *cysJI* and *cysH* of enterobacteria and encoded a transcriptional regulator protein, both subunits of an NADPH-sulphite reductase, and PAPS reductase. The PAPS reductase and sulphite reductase genes form a small operon with a gene order of *cysJIH* which is assumed to be translationally coupled. The DNA structure upstream of *cysJIH* includes a homologue of the gene for the transcriptional regulator CysB, which is transcribed in the opposite direction. *cysB* and *cysJIH* are separated by a short intergenic region that could serve as target for the CysB protein overlapping the putative promoter of *cysJ*. *T. roseopersicina* CysB has 40% identical and 80% similar amino acids when compared with CysB from *S. typhimurium*. An identical function would imply that the CysB gene product autoregulates its synthesis and in addition, due to the very short distance between the *cysB* promoter and the promoter of the *cysJIH* gene, CysB as repressor would also control the transcription of the adjacent *cysJIH* genes. In enterobacteria, CysB controls the expression of *cysB* negatively as a true repressor whereas for expression of the *cysJIH* cluster it can also serve as transcriptional activator when it binds *N*-acetyl-L-serine (Kredich, 1993). By binding sulphide or thiosulphate it acts as repressor and sulphide or thiosulphate acts as co-repressor. Hryniewicz & Kredich (1995) observed that in *S. typhimurium* a single CysB protein tetramer covers the DNA from –34 to –72 of the *cysJ* promoter. Addition of *N*-acetyl-L-serine as inducer extended the DNA-binding region from –32 to +17, defining an additional accessory site that interacted with the protein. Projection of the footprints on an open cylinder showed that they form two adjacent sites, and binding of the two adjacent sites by CysB will probably cause the DNA to bend. The CysB DNA-binding site of *S. typhimurium* was 40 bp in length as defined by DNase I footprinting. In this respect, it seems noteworthy that the binding motifs found in the *cysK2* and *cysB* promoter regions of *E. coli* (Monroe *et al.*, 1990) and of *Klebsiella aerogenes* (*K. pneumoniae*) (Lynch *et al.*, 1994) are very similar with a sequence found in the promoter region of *Thiocapsa cysB* (Fig. 4).

Structural and mutational analysis of LysR-type regulator proteins indicated a structure consisting of a

helix–turn–helix DNA-binding motif (residues 6–66) located at the N-terminus. As shown for the *Klebsiella* CysB protein, residues 88–324 of the structure fold into two α/β domains that are connected by two short segments of polypeptide (Tyrrell *et al.*, 1997). The two domains form a substrate-binding cavity which is lined by P31, T100, T102, Q103, T149, E150, W166 and T202. T149, whose mutation caused constitutive expression (Colyer & Kredich, 1994), and all the other residues listed are conserved in the CysB homologue of *T. roseopersicina*.

cysJIH of *T. roseopersicina* encodes sulphite reductase and PAPS reductase. These gene products were analysed by phenotypic complementation and *in vitro* as recombinant enzymes. Of the two sulphite reductase mutants from *E. coli*, *cysI* was complemented by plasmids encoding either sulphite reductase holoenzyme or the sulphite reductase sirohaem subunit alone. The mutant with a defect in the flavoprotein subunit was not complemented by the homologous gene product from *T. roseopersicina*, but cumulative data from sequence alignments with NADPH:cytochrome P450 oxidoreductases and the known enterobacterial NADPH-sulphite reductases strongly implied that the enzyme is an NADPH-dependent sulphite reductase. This type of assimilatory sulphite reductase is composed of the two subunits that contain in the α subunit FAD and FMN as prosthetic groups and in the β subunit the $\text{Fe}_4\text{-S}_4$ cluster and the sirohaem.

The flavoprotein contained all the structural elements that have been defined most recently for the NAD(P)H:cytochrome P450 oxidoreductases. The close relation of *T. roseopersicina* flavoprotein moiety to the NADPH:cytochrome P450 oxidoreductases is indicated by its relative shorter distance in the unrooted phylogenetic tree, where it branches closer from the mammalian homologues than from the plant-type NADPH:cytochrome P450 oxidoreductases. With one exception, all residues suggested to form the FMN-binding domains and the FAD-NADP-binding pocket are conserved (Wang *et al.*, 1997). As in the *E. coli* sulphite reductase flavoprotein, only the inner ring-shielding tyrosine (Y154) is conserved while the exterior FMN-shielding tyrosine (Y115) is replaced by a histidine (see Table 2).

In an unrooted phylogenetic tree, sulphite reductase haem protein from *T. roseopersicina* is grouped together with the two haem proteins from enterobacteria. The group of bacterial haem proteins differ considerably from the fungal haem proteins of *Sacch. cerevisiae* and *Schiz. pombe*, which form a small group of eukaryotic NADPH-dependent sulphite reductases. The ferredoxin-dependent sulphite reductases are also related to the NADPH-dependent enzymes but, presumably due to the different electron donor, they form an individual third group of haem proteins. Ferredoxin nitrite reductases from higher plants and cyanobacteria were found as closest relatives whereas dissimilatory sulphite reductases appear as non-related using the search algorithms of Altschul *et al.* (1997).

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5' - AAATAAGAGATGGCTTATGCTGTCTCTTATTCATACTGATA-3' - Ec pcysB
5' - GAAACAGGGGTGGCTTATGCCGCCCTTATTCATCTTGCAT-3' - St pcysK2
5' - AGTGGTTATAGTTAGCACCTTTTATTATTAAATCGTATTA-3' - Ec pcysK2
3' - GGTCCGAGACGATTGAAATGACCATTATGCGTTATCACGG-5' - Tr pcysB
consensus      A      TTATt  at t

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Fig. 4. Sequence comparison of the *cysK2* and *cysB* promoter regions of *E. coli* (Ec), *S. typhimurium* (St) and *T. roseopersicina* (Tr).

A structural gene encoding PAPS reductase was not expected as Schmidt & Trüper (1977) observed sulphite formation in *Chromatium vinosum*, *T. roseopersicina* and *T. pfennigii* showing a 10-fold preference for adenylylsulphate (APS) versus PAPS, suggesting that an APS-sulphotransferase rather than thioredoxin-PAPS reductase occurred in these species of the *Chromatiaceae* and in *Rhodospirillum rubrum*. To date, only a structural gene related to *cysH* from enterobacteria rather than the plant-type GSH-dependent APS-reductase has been found in *T. roseopersicina*. PAPS reductase activity, however, was virtually undetectable from cells grown under photolithoautotrophic conditions, as the enzyme is expected to be down-regulated in the presence of sufficient supplies of sulphur. In this respect *T. roseopersicina* seems to control the enzymes of the assimilatory pathway like *E. coli* or *S. typhimurium*. Hence, sulphite formation from APS by APS sulphotransferase activity as observed by Schmidt & Trüper (1977) may have been produced by APS reductase, which is also present in *Chromatiaceae* (Dahl & Trüper, 1994).

The occurrence in *T. roseopersicina* of a sulphite reductase which is homologous to the assimilatory NADPH-dependent enzyme from enterobacteria does not exclude the possibility that the organism, like the closely related *Chromatium vinosum*, contains a dissimilatory sulphite reductase in addition to its assimilatory enzyme. Based on 16S rRNA cataloging and 5S rRNA sequence comparison (cited by de Ley, 1992), *T. roseopersicina* belongs to the family *Chromatiaceae*, which forms a phylogenetically coherent cluster with a binary matching coefficient (S_{AB}) of 0.66. In this context it is noteworthy that the oligonucleotide R3 that was used here to detect the *T. roseopersicina* *cysI* gene also gave similar hybridization signals with genomic DNA from *C. vinosum* digested with *Bam*HI (Haverkamp, 1992). *C. vinosum* was reported to contain the structural genes of a dissimilatory sulphite reductase (Hipp *et al.*, 1997) with a high homology to *dsr/dsv* genes from *Desulfovibrio vulgaris* and *Archaeoglobus fulgidus* respectively (Dahl *et al.*, 1993; Karkhoff-Schweizer *et al.*, 1995). As the polypeptides deduced from these *C. vinosum* genes give subunits of 46.8 and 39.9 kDa, which would be in good agreement with the subunit size of the reverse sulphite reductase described by Schedel *et al.* (1979), the authors suggested that dissimilatory sulphite reductase is the enzyme responsible for reverse sulphite reductase activity in *C. vinosum*. The finding that reverse sulphite reductase activity was only detected in *C. vinosum* grown autotrophically on hydrogen sulphide, but not in extracts from cells grown photoheterotrophically with malate and sulphate, does not conflict with the activity of an assimilatory sulphite reductase. As this type of enzyme appears to be under control of the CysB regulator it is expected to be detectable only when the supply of sulphur becomes limiting.

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