Evidence for redundancy in cysteine biosynthesis in *Rhizobium leguminosarum* RL3841: analysis of a *cysE* gene encoding serine acetyltransferase

G. Parker, D. Walshaw, K. O'Rourke, S. Broad, A. Tingey,
P. S. Poole and R. L. Robson

Author for correspondence: R. L. Robson. Tel: +44 118 9316639. Fax: +44 118 9316562.
e-mail: r.l.robson@reading.ac.uk.

Microbiology Division,
School of Animal and
Microbial Sciences, The
University of Reading,
Reading RG6 6AJ, UK

A *cysE* gene encoding a serine acetyltransferase (SAT) potentially involved in the biosynthesis of cysteine was identified ~ 4 kb upstream of the previously described *aapJQMP* gene cluster that encodes an amino acid permease in *Rhizobium leguminosarum* strain 3841. The gene exhibits >40% identity to the family of SATs containing N-terminal extensions that have been described for other bacteria and plants. The ORF has three possible translation initiation sites which potentially encode polypeptides of 311, 277 and/or 259 amino acid residues, respectively. All three ORFs complemented the *cysE* mutation in an *Escherichia coli* cysteine auxotroph, strain JM39. Insertion of *Tn5-lacZ* into *cysE* in the genome of *R. leguminosarum* (strain RU632) lowered SAT activity in crude extracts by >95%. However, RU632 was not a cysteine auxotroph, which suggests that *R. leguminosarum* possesses some redundancy in cysteine biosynthesis. Additional copies of *cysE* could not be detected in the genome when the *R. leguminosarum* *cysE* gene was used as a hybridization probe. Therefore it is possible that *R. leguminosarum* possesses an alternative pathway for cysteine biosynthesis which avoids O-acetylserine. Strain RU632 was unaffected in its ability to nodulate *Pisum sativum*, and the nodules were effective for N₂ fixation (measured by C₂H₂ reduction). Transcriptional activity of *cysE* was determined by measuring the β -galactosidase arising from *cysE::Tn5-lacZ* fusions. Maximal levels of expression were observed during early exponential growth and were not influenced by the level of sulphur (supplied as sulphate). However, transcription was repressed by approximately twofold in ammonium-grown, as opposed to glutamate-grown, cultures. Repression by ammonium was not seen in a strain defective for *ntrC*.

Keywords: sulphur metabolism, symbiotic nitrogen fixation, SAT, nodulation

INTRODUCTION

Members of the soil-dwelling genus *Rhizobium* form specific N₂-fixing symbioses with leguminous plants: the bacterium receives fixed carbon from the plant and supplies fixed nitrogen in return. This exchange of nutrients is highly co-ordinated and involves the in-

duction of specific transport systems in the bacteroid. In the course of an investigation into the mechanisms by which fixed nitrogen compounds may be passed from *Rhizobium leguminosarum* to the plant, a general L-amino acid transporter was discovered which catalyses high rates of exchange between intracellular and extracellular amino acid pools (Walshaw & Poole, 1996). The genes encoding this transporter were originally identified on the basis that they increased [¹⁴C]glutamate uptake when supplied in additional copies in *R. leguminosarum*. One such clone which conferred this phenotype is pRU3024, a cosmid that contains a relatively large

Abbreviations: DTNB, 5,5'-dithobis-(2-nitrobenzoic acid); SAT, serine acetyltransferase.

The GenBank accession number for the sequence reported in this paper is AJ271648.

Table 1. Bacterial strains and plasmids used in this workStr^R, streptomycin-resistant; Amp^R, ampicillin-resistant; Tc^R, tetracycline-resistant; Kan^R, kanamycin-resistant.

Strain or plasmid	Phenotype/genotype	Reference/source
<i>Rhizobium leguminosarum</i>		
RL3841	Str ^R derivative of strain 300	Glenn <i>et al.</i> (1980)
RU502	RL3841(pRU3031)	This work
RU504	RL3841(pRU3033)	This work
RU543	<i>aapJ</i> ::Tn5- <i>lacZ</i> derivative of RL3841	Walshaw & Poole (1996)
RU632	<i>cysE</i> ::Tn5- <i>lacZ</i> derivative of RL3841	This work
RU929	<i>ntrC</i> :: Ω derivative of RL3841	Walshaw <i>et al.</i> (1997)
RU981	<i>ntrC</i> :: Ω (pRU3031) derivative of RL3841	This work
RU982	<i>ntrC</i> :: Ω (pRU3033) derivative of RL3841	This work
RU1017	<i>aapJ</i> ::Tn5- <i>lacZ</i> <i>ntrC</i> :: Ω derivative of RU929	Walshaw <i>et al.</i> (1997)
RU1027	<i>cysE</i> ::Tn5- <i>lacZ</i> <i>ntrC</i> :: Ω derivative of RU632	This work
<i>Escherichia coli</i>		
JM39	(F' <i>cysE51 tfr8</i>)	Jones-Mortimer (1968)
DH5 α	ϕ 80 <i>dlacZ</i> Δ M15 <i>recA1 endA1 gyrA96 hsdR17</i> (<i>r_K⁻ m_K⁻</i>) <i>supE44 relA1 deoR</i> Δ (<i>lacZYA-argF</i>)U169	Hanahan (1983)
BL21(DE3)	F ⁻ <i>ompT hsdS_B</i> (<i>r_B⁻ m_B⁻</i>) <i>dcm gal</i> (λ <i>clts857 ind1 Sam7 nin5 lacUV5-T7</i> gene 1)	Studier & Moffat (1986)
<i>Sinorhizobium meliloti</i>		
1021	SU47 <i>str-21</i>	Mead <i>et al.</i> (1982)
Plasmid/cosmid		
pBluescript SK +	Amp ^R cloning vector	Mead <i>et al.</i> (1986)
pTZ18	Amp ^R cloning vector	
pT7-7	Amp ^R T7 promoter expression vector	
pRU3024	Tc ^R , 15 kb genomic segment from RU3841 in pLAFR, <i>cysE aapJQMP</i>	Walshaw & Poole (1996)
pRU3031	pRU3024, Tc ^R Km ^R <i>cysE</i> ::Tn5- <i>lacZ</i>	This work
pRU3033	pRU3024, Tc ^R Km ^R <i>cysE</i> ::Tn5- <i>lacZ</i>	This work
pRU383	Amp ^R , 0.8 kb <i>Bam</i> HI fragment with most of <i>cysE</i> from pRU3024 in pBluescript SK +	This work
pATRLCE13	Amp ^R , <i>cysE</i> , 3.4 kb <i>Eco</i> RI fragment from pRU3024 in pTZ18	This work
pSS1	Amp ^R , ORF2 ¹ in pT7-7	This work
pSS2	Amp ^R , ORF2 ² in pT7-7	This work
pSS3	Amp ^R , ORF2 ³ in pT7-7	This work

fragment (~ 30 kb) of the *R. leguminosarum* genome. Random Tn5 mutagenesis of pRU3024 led to the localization of an approximately 7 kb region that was responsible for the enhanced glutamate-uptake phenotype. Insertion mutations in this region abolish enhanced glutamate uptake. When this region was subcloned and sequenced it was found to contain a cluster of four genes (*aapJ*, *aapQ*, *aapM* and *aapP*) that encode polypeptides with a high degree of amino acid sequence identity to components of ATP-binding cassette (ABC) superfamily of transporters (Walshaw & Poole, 1996). Further analysis established that the *aap* genes in *R. leguminosarum* not only enhance the accumulation of a broad range of amino acids, including glutamate, but also affect the efflux of those amino acids directly or independently via a second efflux channel or transporter. The *aapJQMP* operon in *R. leguminosarum* was shown to be responsive to nitrogen regulation. In *R. legumino-*

sarum, nitrogen status is probably sensed by a mechanism similar to that known for *Escherichia coli*, in which a uridylyltransferase/uridylyl-removing enzyme senses the ratio of glutamine to α -ketoglutarate and changes the uridylylation state of the P_{II} protein. This, in turn, alters the ability of NtrB to act either as a kinase or as a phosphatase for the phosphorylation or dephosphorylation of NtrC, which is a transcriptional activator of nitrogen-regulated operons (Patriarca *et al.*, 1993). Mutants in *ntrC* caused derepression of the *aap* operon, which induced high rates of amino acid uptake even when the organisms were grown with ammonium as a nitrogen source. Therefore NtrC appears to negatively regulate the *aap* genes (Walshaw *et al.*, 1997).

This work describes a second locus present on the cosmid pRU3024, in which Tn5 mutations lower the enhanced accumulation of glutamate but, unlike the

mutations in the *aap* genes, do not abolish it completely. We show here that the mutations are located in a *cysE* gene encoding a serine acetyltransferase (SAT), an enzyme which catalyses the first unique step in cysteine biosynthesis.

METHODS

Strains and media. The strains and plasmids used in this work are listed in Table 1. *E. coli* strains were routinely grown at 37 °C on nutrient agar or broth. To test for cysteine auxotrophy, M9 minimal salts agar (Sambrook *et al.*, 1989) was used and cysteine was added at 60 µg ml⁻¹ for growth of cysteine auxotrophs. *R. leguminosarum* strains were grown at 28 °C on either tryptone yeast extract (TY) (Berlinger, 1974) or acid minimal salts (AMS) medium (Poole *et al.*, 1994a). Antibiotics were added, where required, at the following concentrations (µg ml⁻¹): for *E. coli* strains, ampicillin, 100; kanamycin, 20; and for *R. leguminosarum* strains, streptomycin, 500; kanamycin, 40, tetracycline, 2 in acid minimal salts medium and 5 in tryptone yeast extract medium.

Molecular biology methods. Methods used for preparation of genomic and plasmid/cosmid DNA, restriction analysis, agarose gel electrophoresis, cloning, production of digoxigenin-labelled probes, and automated DNA sequencing using a Pharmacia ALF Express and Cy5-labelled primers were as described by Walshaw & Poole (1996) or in Parker *et al.* (1999). Hybridizations were carried out at various stringencies as stated in the text. The PCR reactions were routinely performed with the following cycle: a hot start at 94 °C for 2 min, then 30 cycles of 1 min at 94 °C for, 1 min at 60 °C and 1.5 min at 65 °C, after which samples were chilled at 4 °C. The cloning primers for the PCR were as follows: RLCEM1, 5'-CGAAAACATATGCTAAAAAACCATGGC-3'; RLCEM2, 5'-AACGAGGAGAGCGCATATGGTCCG-3'; RLCEM3, 5'-CCGCTGAAGCATATGGATCCC-3'; RLCEND1, 5'-GGCCCTCCCCGCTCGGATCCCGCCG-3'; RLCEND1B, 5'-GGCCCTCCCCGCTCAGATCTCGC-CG3' (restriction sites underlined). The DNA sequencing primers were as follows: RLCEM, 5'-CAATGGGCGAGAC-GATGTGT-3'; RLCEM2, 5'-TGGTGCCGACGCCGATCA-GC-3'; RLCEM3, 5'-CCAAGATCCTCGGCAATATC-3'; Tn5 primer, 5'-GGATCCATAATTTTTTCTCC-3'.

Bacterial genetic methods. The transformation of *E. coli* with plasmids, the conjugation in *R. leguminosarum*, the procedures for Tn5-*lacZ* mutagenesis, and the homogenization of mutations into the genome of *R. leguminosarum* were as described by Walshaw & Poole (1996).

Bioinformatics. Protein-similarity searches were performed using BLAST or ψ -BLAST (Altschul *et al.*, 1997), using the server for The National Centre for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/blast/blast.cgi>). Multiple sequence alignments were carried out using MULTALIN (Corpet, 1988) at the Server of the Institut National de la Recherche Agronomique (<http://www.jouy.inra.fr/>) or CLUSTAL W (Thompson *et al.*, 1994) at the server of the European Bioinformatics Institute (<http://www2.ebi.ac.uk/clustalw/>). Protein relationships were determined using the neighbour-joining method of Saitou & Nei (1987), and the relational cluster diagram constructed from these analyses was drawn using TREEVIEW (<http://taxonomy.zoology.gla.ac.uk/rod/treeview.html>). Searches for protein targeting signals were conducted using SIGNALP (Nielsen *et al.*, 1997: <http://www/cbs.dtu.dk/services/SignalP>).

Measurement of enzyme activities and protein contents. SAT activity was measured spectrophotometrically in a real-time assay at 30 °C by following the serine-dependent cleavage of acetyl-CoA in the presence of DTNB, essentially as described by Kredich & Tomkins (1966). Units are defined as nmol (mg protein)⁻¹ min⁻¹. β -Galactosidase activity was measured essentially as described by Miller (1972) but with the modifications (for *R. leguminosarum*) of Poole *et al.* (1994b). Protein contents were measured using Folin-Ciocalteu reagent with BSA as the standard.

Determination of nodulation and nitrogen fixation. Nodulation and N₂ fixation in nodules was determined in triplicate as described by Trinick *et al.* (1976). N₂-fixation activity was expressed as µmol C₂H₂ reduced h⁻¹ per plant.

RESULTS AND DISCUSSION

Mutations in pRU3024 affecting enhancement of glutamate uptake in *R. leguminosarum*

Cosmid pRU3024 (Fig. 1) was described previously and contains the *aap* gene cluster within a segment of approx. 30 kb of the genome of *R. leguminosarum* strain 3841 cloned into pLAFR1. When introduced into *R. leguminosarum*, pRU3024 enhanced the accumulation of [¹⁴C]glutamate by more than sixfold in organisms grown in a nitrogen-sufficient medium. Only insertions of Tn5::*lacZ* into the *aap* cluster completely abolish the enhanced glutamate-uptake phenotype, but the insertions in plasmids pRU3031 and pRU3033 located 3.4 and 4 kb upstream of the *aap* gene cluster lower the enhancement of glutamate uptake by approximately 2.5-fold (Walshaw & Poole, 1996).

Analysis of DNA sequences immediately flanking the Tn5-*lacZ* insertions in plasmids pRU3031 and pRU3033 indicated that both insertions disrupt a gene with high sequence identity to *cysE* genes from bacteria such as *E. coli* and *Azotobacter* sp. and from plants such as *Arabidopsis*. A 3.4 kb *Eco*RI fragment from pRU3024 covering the sites of insertions in pRU3031 and pRU3033 was subcloned into pTZ18 to give plasmid pATRLCE13. The DNA sequence for 2712 bp of this fragment was determined. Within this region, two ORFs were found which have codon usages consistent with other genes from *R. leguminosarum*. ORF1 is preceded by a good potential ribosome-binding site and encodes a polypeptide of 261 amino acid residues showing significant identity to a family of bifunctional enzymes with both esterase and halogenoperoxidase activities. A second ORF (ORF2A) of 936 bp was located 36 bp downstream of ORF1 (Fig. 2). ORF2A encodes a polypeptide of 311 amino acid residues with > 40% identity to SATs from a wide variety of bacteria and plants (Fig. 3), the closest relatives being the CysE2 and CysE4 from *Arabidopsis thaliana* (Fig. 4). Alternative potential translation initiation sites are located 129 and 183 bp 3' to ORF1, giving ORF2B and ORF2C, which encode shorter polypeptides of 277 and 259 residues, respectively. Of the three potential translation initiation sites, only that of ORF2B (5'-AACGAGGAGAGCGCGGATG-3') is significantly purine rich and conforms well

Fig. 1. Location and cloning of the *cysE* gene from *R. leguminosarum* 3841. The figure shows the location and orientation of ORF1 and ORF2B (*cysE*) relative to the *aapJQMP* cluster previously described for cosmid pRU3024. The 3.4 kb *Eco*RI fragment of pRU3024 bearing these genes was cloned into pTZ18 to give plasmid pATRLCE13 (shown in the lower part of the figure). The extent of the DNA sequence obtained for this fragment is shown by the continuous line. The sites of Tn5-*lacZ* mutations in *cysE* in the plasmids pRU3031 and pRU3033 are indicated. The mutation in pRU3031 was selected for most of the phenotypic analysis and for the construction of the chromosomal homegene, since it is located closest to the N-terminal coding region of the gene.

Fig. 2. The nucleotide sequence for the region containing parts of ORF1 and ORF2. The sequence of a region extending from base 983 to base 1221 from within the 2712 bp of the sequenced portion of the insert in pATRLCE is shown. The deduced C-terminal amino acid sequence of ORF1 and potential alternative N-terminal sequences for ORF2 are shown using single-letter notation. The potential alternative translation starts for ORF2 are indicated by the arrows starting at positions 1041 (ORF2A), 1147 (ORF2B) and 1201 (ORF2C). Codon usage does not allow discrimination of the most likely translation product *in vivo*, but only ORF2B is preceded by a convincing potential ribosome-binding site starting at position 1135 (underlined).

to the consensus sequence for potential ribosome-binding sites observed in other *R. leguminosarum* genes. Sequence analysis revealed a potential transcription terminator located 31 bp downstream of ORF2. No other significant matches at the DNA or protein levels were found in the sequences flanking ORFs 1 and 2. ORF1 and ORF2 are organized in such a way that they could be co-transcribed, but this remains to be established.

SAT catalyses the acetyl-CoA-dependent acetylation of serine, giving O-acetylserine, which serves as the acceptor for sulphide in the biosynthesis of cysteine catalysed by O-acetylserine-thiol lyase. SAT-encoding genes are found in eubacteria, microfungi, algae, higher

plants and some protozoa but not in the *Archaea* or higher animals. In the enteric bacteria *E. coli* and *Salmonella typhimurium*, SAT is rate limiting for cysteine biosynthesis and is subject to feedback inhibition by cysteine. Also, O-acetylserine acts as a co-activator in the genetic control of the expression of proteins and enzymes involved in the assimilation of sulphur via the assimilatory sulphate-reduction pathway (Kredich, 1987). Irrespective of which of the three ORFs is correct, the SAT encoded by the *R. leguminosarum* gene clearly belongs to one of the three known SAT families. All members have a relatively high degree of identity extending over approximately 170 residues (which probably constitutes the catalytic core of the enzyme). Group 1 SATs contain little more than this

	1				50
RleCysESS2					
AthSAT2	MVDLSSLYNI	HIPPLDSSLF	AFSVLSLFFVQ	SKRVCDSSLS	SPWRDMNGDE
EcoCysE					
AchnifP					
HpyCysE					
	51				100
RleCysESS2	M	VAKTDIRAFE	TGHPLKVMDF	IWDSLREEAR	LAAERDPVLA
AthSAT2	LPFESGFVY	AKGTHKSEFD	SNLLDPRSDP	IWDAREEAK	LEAEKEPILS
EcoCysE					
AchnifP					
HpyCysE					
	101				150
RleCysESS2	AFLYSTVINY	HSLEECVIHR	ICERLDHPDM	QANLLRQTFE	EMLLDWPWDS
AthSAT2	SFLYAGILAH	DCLEQALGFV	LANRLQNPTL	LATQLLDIFY	GVMHDKGIQ
EcoCysE	SFYHATLLKH	ENLGSALSYM	LANKLSSPIM	PAIAIREVVE	EAYAADPEMI
AchnifP					MSLL
HpyCysE					M
	151				200
RleCysESS2	SILRVDIAI	YDRDPAQLRF	MEAVLYFKGF	HALQTHRLAH	WLLNRGRRDF
AthSAT2	SSIRHDLQAF	KDRDPAQLSY	SSAILHLKGY	HALQAYRVAH	KLWNEGRKLL
EcoCysE	ASAACDIQAV	RTDPAVDKY	STPLLYLKGF	HALQAYRIGH	WLNQGRRL
AchnifP	AQWREDIRCV	FERDPAARTT	FEVLLTYPGV	HAIMLYRLAH	RLWRPNALPR
HpyCysE	LDLSYSLERV	LQEDPAARNK	WEVLLLYPGI	HALLCYRLAH	ALHRRFPYFI
	201				250
RleCysESS2	ALYLQSRSSS	VFQTDINPAA	RIGKGIFLDH	ATGLVVGETA	VIGDNVSILH
AthSAT2	ALALQSRISF	VFGIDIHPAA	RIGEGILLDH	GTGVVIGETA	VIGNGVSIH
EcoCysE	AIFLQNVQSV	TFQVDIHPAA	KIGRGIMLDH	ATGIVVIGETA	VIENDVSILQ
AchnifP	PAAVVR.ARL	VSNVDIHPGA	VIGARFFIDH	GACVVIGETA	EIGRDVTLXH
HpyCysE	ARALSQARLF	ITGIEIHPGA	KIGRGLFIDH	GMCVVIGETT	EIGDDVTIYH
	251				300
RleCysESS2	GVTLLGGTGKE	GADRHPKIGS	GVMIGAGAKI	LGNIEIGYCS	RVAAGSVVLK
AthSAT2	GVTLLGGTGKE	TGDRHPKIGE	GALLGACVTI	LGNISIGAGA	MVAAGSLVLK
EcoCysE	SVTLGGTGKKS	GGDRHPKIRE	GVMIGAGAKI	LGNIEVGRGA	KIGAGSVVLQ
AchnifP	GVTLLGGTGGA	KGRRHPPLGD	VVLVGAGAKI	LGPITIGANA	RVGANSVVVQ
HpyCysE	GVTLLGGTGKF	KGRRHPPLGN	RVVVGAGAKV	LGAICVGGDV	RIGANAVVLS
	301				350
RleCysESS2	AVPPKKTIVAG	VPAKVVEAG	CSEPS...RN	MDQVIGADI	
AthSAT2	DVPSHSHVAG	NPAKLIRVME	EQDPS...LA	MKHDKATKEFF	RHVADGYKG
EcoCysE	FVFPHTTAAG	VPARIVGKPD	SDKPS...MD	MDQHENG...I	NHTFEYGDG
AchnifP	DVPEGCTVVG	IPGKVVKLRE	AGQLNPYGD	LDHHLIPDPV	GKAIACLLER
HpyCysE	DLPTGSTAVG	AKAKTITKDR			
	351				400
RleCysESS2					
AthSAT2	AQSNQPSLSA	GDTEKGHTNS	TS.....		
EcoCysE	IDI.....				
AchnifP	IDSLEKRVEA	GGLVAAAASS	TFYEGCNPDN	SICETNLRRS	APWSSGRPRR
HpyCysE					
	401				416
RleCysESS2					
AthSAT2					
EcoCysE					
AchnifP	PAHAGDRVSG	RAKGSD			
HpyCysE					

Fig. 3. Multiple alignments of amino acid sequences of SATs of bacterial and plant origin. The sequences are as follows: RleCysESS2, ORF2B from *R. leguminosarum*; HpyCysE, putative SAT from *Helicobacter pylori* (type 1 SAT); AchnifP, SAT from the N₂-fixation gene cluster of *Azotobacter chroococcum* (type 3 SAT with C-terminal extension), EcoCysE, SAT from *E. coli* (type 2 SAT with N-terminal extension), AthSAT2, SAT 2 from *A. thaliana* (type 2 SAT with N-terminal extension including N-terminal targeting sequence). Amino acid residue identities are shown in bold.

conserved core, whereas group 2 SATs, including the example identified here, contain N-terminal extensions of between 40 and 200 amino acid residues. Group 3 SATs lack the long N-terminal extensions but have significant C-terminal extensions. The N-terminal domains of the group 2 SATs from plants are likely to be chloroplast target signals. This possibility was worth considering in the case of *R. leguminosarum* because of the plant–bacterium symbiosis, but when the three ORFs were subjected to analysis using the SIGNALP

Fig. 4. Inter-relationships of serine acetyltransferases from different sources. The methods for multiple alignment and reconstruction of the unrooted phylogenetic tree are described in the text. The sources of the SAT amino acid sequences are as follows: Ace, *Allium cepa* (onion); Ach1, *Azotobacter chroococcum niff*; Ath1, Ath2, Ath4 and AthSAT52, *A. thaliana* isoforms; Avi1 and Avi2, *Azotobacter vinelandii*; Bap, *Buchnera aphidicola*; Bsu, *Bacillus subtilis*; Cme, *Cyanidioschyzon merolae* (red alga, chloroplast isoform); Cla, *Citrullus lanatus* (water melon); Cje, *Campylobacter jejuni*; Cst, *Clostridium sticklandii*; Eco, *Escherichia coli*; Edi1 and Edi2, *Entamoeba dispar* isoforms; Ehi, *Entamoeba histolytica*; Hin, *Haemophilus influenzae*; Hpy, *Helicobacter pylori*; Mle, *Mycobacterium leprae*; Rle, *Rhizobium leguminosarum* ORF2B; S6803, *Synechocystis* sp. (strain 6803); S7942, *Synechococcus* sp. (strain 7942); S8801, *Synechococcus* sp. (strain 8801); Spo, *Schizosaccharomyces pombe*; Sty, *Salmonella typhimurium*; Sxy, *Staphylococcus xylosus*; Tma, *Thermotoga maritima*.

analytical tool, none appeared to encode N-terminal secretion signals.

Demonstration of SAT functionality

To test if pATRLCE13 contains a functional *cysE* gene, it was transformed into *E. coli* JM39, a *cysE* cysteine auxotroph. JM39(pATRLCE13) was able to grow on M9 minimal agar without cystine addition, whereas JM39 containing the parent vector, pTZ18, remained a cysteine auxotroph. To localize the *cysE* gene more precisely, the PCR was used to amplify the three alternative ORF2s (with pATRLCE13 as the template). Three different N-terminus-encoding primers were used (RLCEM1, RLCEM2 and RLCEM3) together with a common C-terminus-encoding primer (RLCEND1B) to amplify ORFs 2A, 2B and 2C, respectively. The amplified DNA segments were cloned between the *Nde*I and *Bam*HI restriction sites in plasmid pT7-7 in order to exploit the strong translation initiation signals present in this expression vector for potential overexpression and further characterization of the *R. leguminosarum* SAT. The resultant plasmids, pSS1, pSS2 and pSS3, containing ORF2A, ORF2B and ORF2C, respectively,

were transformed into *E. coli* JM39. All three plasmids complemented the mutation in JM39 after growth at 37 °C for 48 h on M9 minimal medium. Attempts to overexpress the three ORFs from pSS1, pSS2 and pSS3 in *E. coli* BL21(DE3) were unsuccessful. The SAT activities of crude extracts of the strains prepared after induction were not significantly above background in any case. This was surprising, since overexpression of CysE from *E. coli* and from *Azotobacter* sp. had been obtained in a similar vector/host system. We have observed previously that the minimum levels of SAT activity needed to complement the *cysE* mutation in JM39 are below the limits of detection of the biochemical assay in crude extracts. The native SAT in *R. leguminosarum* probably corresponds to the polypeptide of 277 amino acid residues encoded by ORF 2B, since it is preceded by the best potential ribosome-binding site. The question of the correct translation initiation start can only be resolved by purification and determination of the N-terminal sequence of SAT from *R. leguminosarum*.

Mutagenesis of *cysE* in *R. leguminosarum*

The Tn5-*lacZ* mutants in the *cysE* gene in cosmid pRU3024 were obtained by random mutagenesis. Plasmids pRU3031 and pRU3033 carry mutations in *cysE* (see Fig. 1). The mutation in pRU3031 was chosen for further work. Transfer of the insertion into the genome of RU3841 by homogenization gave strain RU632. We were surprised to find that RU632 exhibited no requirement for cysteine or cystine for growth in the defined medium AMS. This suggests a potential redundancy in cysteine biosynthesis in *R. leguminosarum* RL3841. We measured enzyme activity in crude extracts prepared from the wild-type strain RU3841 and the mutant RU632. The specific activity for SAT observed in RU3841 was 1.38 nmol (mg protein)⁻¹ min⁻¹, which appears low, at only 17% of that observed in *E. coli* (Denk & Böck, 1987). However, such levels of activity are likely to be more than sufficient to satisfy cysteine needs because the *R. leguminosarum* *cysE* complemented the *cysE* mutation in *E. coli* JM39 even though the levels of SAT activity in extracts of the complemented strain were also below the detection limits of our assay. However, in the mutant, RU632, activity was not detectable above background. We estimated that the minimum detectable activity was not greater than 0.08 nmol (mg protein)⁻¹ min⁻¹. These data suggest that the *cysE* gene described here accounts for at least 95% of the SAT activity in *R. leguminosarum*.

Although we could detect no residual SAT activity in the mutant, a second *cysE* gene may nevertheless exist, especially since several organisms contain multiple copies of *cysE* genes, including *A. thaliana*, which has four *cysE* genes (Murillo *et al.*, 1995; Howarth *et al.*, 1997; Bogdanova & Hell, 1997), and *Azotobacter* species (which also fix N₂) in which two *cysE* genes are known (Evans *et al.*, 1991; Zheng *et al.*, 1998). Therefore, DNA encoding the *cysE* gene cloned here was used to probe genomic DNA from *R. leguminosarum* under a

variety of stringencies. In the *Eco*RI digest, a single 3.4 kb fragment was identified which probably corresponds to the *Eco*RI fragment cloned in pATRLCE13. No other hybridizing fragment was present in this digest. In the *Eco*RI/*Bam*HI double digest, two hybridizing bands were seen: a more intense band at 0.8 kb, and a much less intense band at 1.2 kb. Since the *cysE* probe contains a *Bam*HI site located 65 bp from the N-terminal coding region, it was anticipated that two fragments of different intensities would be detected. Also, the DNA sequence predicts that the probe should detect two *Eco*RI/*Bam*HI fragments of 1205 and 804 bp, which agrees well with the observed results. Prolonged exposure of the blot and careful scrutiny provided no evidence for the existence of other hybridizing bands in *R. leguminosarum*.

Either a second copy of *cysE* exists in *R. leguminosarum* RL3841 which is quite divergent from the gene cloned here and is expressed but unstable during extraction and/or assay, or cysteine can be made by a pathway avoiding O-acetylserine. One possibility is that cysteine can be synthesized from methionine; this appears to be the case in *Pseudomonas aeruginosa* (Fogliano *et al.*, 1995) and, more pertinently, *Rhizobium etli* (Taté *et al.*, 1999). These organisms contain a methionine-biosynthesis pathway, first established for *Saccharomyces cerevisiae*, in which sulphide is incorporated not into O-acetylserine but into O-succinylhomoserine to form homocysteine, which is converted to methionine. Cysteine is formed from methionine via a *trans*-sulphurylation reaction involving cystathionine as an intermediate, obviating the need for O-acetylserine (Cherest & Surdin-Kerjan, 1992).

Evidence for regulation of the expression of CysE

The transcriptional activity of the *R. leguminosarum* *cysE* promoter was measured under a number of different growth regimes by following the β -galactosidase activity arising from the Tn5-*lacZ* inserted into *cysE*. The experiments were performed with cosmid- or chromosomal-borne *cysE*::Tn5-*lacZ* fusions (Table 2). There was no significant difference between growth in sulphate-sufficient and sulphate-limited media. However, the nitrogen status influenced the expression of *cysE*, which was similar in degree in both chromosomal and cosmid-borne fusions. Ammonium, as opposed to glutamate, as a nitrogen source repressed transcription of *cysE* by ~50% in the chromosomal fusion strain RU632, and by ~35% in the cosmid-borne fusion strains RU502 and RU504. However, in an *ntrC* background, the repression by ammonium was not observed either in the chromosome (strain RU1027) or in the cosmid (strains RU981 and RU982). These results are similar to those obtained previously (Walshaw *et al.*, 1997) for the *aap* operon, as illustrated here by new data for the *aapJ*::Tn5-*lacZ* fusion in the *ntrC*⁺ (strain RU543) and *ntrC*⁻ (strain RU1017) backgrounds. Also, as reported earlier for the *aap* cluster, the repression of *cysE* appears, unusually, to require NtrC, since no

Table 2. Influence of nitrogen status and *ntrC* on transcription of the *cysE* and *aapJ* genes in *R. leguminosarum* RL3841, as measured in transcriptional Tn5::lacZ fusions

The data indicate specific activities for β -galactosidase (expressed as modified Miller units) in cultures of the various strains grown in the presence of the nitrogen sources indicated.

Strain	Relevant genotype	Glucose/ NH ₄ Cl	Glucose/ glutamate
3841	Wild-type	15 ± 2	22 ± 3
RU929	<i>ntrC</i> :: Ω	27 ± 1	23 ± 1
RU543	<i>aapJ</i> ::Tn5-lacZ	155 ± 23	690 ± 39
RU1017	<i>aapJ</i> ::Tn5-lacZ <i>ntrC</i> :: Ω	603 ± 106	678 ± 49
RU632	<i>cysE</i> ::Tn5-lacZ	139 ± 10	269 ± 23
RU1027	<i>cysE</i> ::Tn5-lacZ <i>ntrC</i> :: Ω	289 ± 28	309 ± 44
RU1013	<i>ntrC</i> :: Ω / <i>cysE</i> ⁺	34 ± 1	34 ± 2
RU502 (3841/pRU3031)	Wild-type host <i>cysE</i> ::Tn5-lacZ	878 ± 189	1313 ± 156
RU504 (3841/pRU3033)	Wild-type host <i>cysE</i> ::Tn5-lacZ	1325 ± 103	2096 ± 103
RU981 (RU929/pRU3031)	<i>ntrC</i> :: Ω <i>cysE</i> ::Tn5-lacZ	1213 ± 150	1285 ± 134
RU982 (RU929/pRU3033)	<i>ntrC</i> :: Ω <i>cysE</i> ::Tn5-lacZ	2118 ± 168	2162 ± 267

repression by ammonium was observed in an *ntrC*⁻ background. Since the *cysE* and *aap* genes are divergently transcribed, the repressive effects of NtrC appear to be mediated at two independent promoters.

Influence of the *cysE* mutation on symbiotic nitrogen fixation

The *cysE* mutant, RU632, was indistinguishable from the parent strain in its ability to infect and nodulate peas. The mutation was stable in the host, since all 25 isolates from nodules infected with RU632 retained the kanamycin-resistance phenotype associated with the Tn5-lacZ insertion. Rates of C₂H₂ reduction for the parent and mutant strains were shown to be 0.45 ± 0.02 and 0.83 ± 0.01 μ mol h⁻¹ plant⁻¹, respectively.

The finding of redundancy in cysteine biosynthesis and of a *cysE* gene in proximity to (and subject to) a similar pattern of regulation as the *aap* genes required for maximal symbiotic N₂ fixation in *R. leguminosarum* RL3841 echoes the presence of *cysE* genes closely linked to *nif* genes in other organisms. Of the two *cysE* genes known for *Azotobacter* sp., one is intercalated into the major (*nif*) gene cluster (Evans *et al.*, 1991; Zheng *et al.*, 1998). A *cysE* gene is located adjacent to *nifB* in a large cluster of genes for N₂ fixation in *Synechococcus* sp. strain RF-1 (Huang *et al.*, 1999), but as yet there is no evidence for redundancy in cysteine biosynthesis in this cyanobacterium. It has been suggested that the link between cysteine metabolism and N₂ fixation may be explained by a high requirement for inorganic sulphur obtained from cysteine for the synthesis of metal-sulphur clusters. Alternative or parallel pathways for cysteine biosynthesis may boost the provision of sulphur for the biosynthesis of nitrogenase often present in high levels (Evans *et al.*, 1991). However, deletion of the *nif*-related *cysE* gene in *Azotobacter* has no marked effects

on the development of nitrogenase activity. In *R. leguminosarum* RL3841, it is unclear how the bacteroid obtains sulphur for housekeeping purposes and for the synthesis of nitrogenase, but, like *Azotobacter*, RU632 (the strain defective in the *cysE* gene) was unaffected in terms of N₂ fixation, though there may be conditions (e.g. nutrient stress) under which a phenotype is expressed.

ACKNOWLEDGEMENTS

This work was supported, in part, by BBSRC grant CO4183 to R.L.R., and by The Nuffield Foundation (via a Vacation Studentship awarded to K.O.) We also like to thank Liz Pontin and Mike Taylor (both of AMSEQ) for the automated DNA sequencing.

REFERENCES

- Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W. & Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25**, 3389–3402.
- Beringer, J. E. (1974). R factor transfer in *Rhizobium leguminosarum*. *J Gen Microbiol* **84**, 188–198.
- Bogdanova, N. & Hell, R. (1997). Cysteine synthesis in plants: protein-protein interactions of serine acetyltransferase from *Arabidopsis thaliana*. *Plant J* **11**, 251–262.
- Cherest, H. & Surdin-Kerjan, Y. (1992). Genetic analysis of a new mutation conferring cysteine auxotrophy in *Saccharomyces cerevisiae*: updating of the sulfur metabolism pathway. *Genetics* **130**, 51–58.
- Corpet, F. (1988). Multiple sequence alignment with hierarchical clustering. *Nucleic Acids Res* **16**, 10881–10890.
- Denk, D. & Böck, A. (1987). L-Cysteine biosynthesis in *Escherichia coli*: nucleotide sequence and expression of the serine acetyltransferase (*cysE*) gene from the wild-type and a cysteine excreting mutant. *J Gen Microbiol* **133**, 515–525.

- Evans, D. J., Jones, R., Woodley, P. R., Wilborn, J. R. & Robson, R. L. (1991). Nucleotide sequence and genetic analysis of the *Azotobacter chroococcum* *nifUSVWZM* cluster, including a new gene (*nifP*) which encodes a serine acetyl transferase. *J Bacteriol* **173**, 5457–5469.
- Fogolino, M., Borne, F., Bally, M., Ball, G. & Patte, J. C. (1995). A direct thiolation pathway is used for methionine biosynthesis in *Pseudomonas aeruginosa*. *Microbiology* **141**, 431–439.
- Glenn, A. R., Poole, P. S. & Hudman, J. F. (1980). Succinate uptake by free-living and bacteroid forms of *Rhizobium leguminosarum*. *J Gen Microbiol* **119**, 267–271.
- Hanahan, D. (1983). Studies on transformation of *Escherichia coli* with plasmids. *J Mol Biol* **166**, 557–580.
- Howarth, J. R., Roberts, M. A. & Wray, J. L. (1997). Cysteine biosynthesis in higher plants: a new member of the *Arabidopsis thaliana* serine acetyltransferase small gene-family obtained by functional complementation of an *Escherichia coli* cysteine auxotroph. *Biochim Biophys Acta* **1350**, 123–127.
- Huang, T. C., Lin, R. F., Chu, M. K. & Chen, H. M. (1999). Organization and expression of nitrogen-fixation genes in the aerobic nitrogen-fixing unicellular cyanobacterium *Synechococcus* sp. strain RF-1. *Microbiology* **145**, 743–753.
- Jones-Mortimer, M. C. (1968). Positive control of sulphate reduction in *Escherichia coli*. The nature of the pleiotropic cysteineless mutants of *E. coli* K12. *Biochem J* **110**, 597–602.
- Kredich, N. M. (1987). Biosynthesis of cysteine. In *Escherichia coli and Salmonella typhimurium: Cellular and Molecular Biology*, pp. 419–428. Edited by F. C. Neidhardt and others. Washington, DC: American Society for Microbiology.
- Kredich, N. M. & Tomkins, G. M. (1966). The enzymic synthesis of L-cysteine in *Escherichia coli* and *Salmonella typhimurium*. *J Biol Chem* **241**, 4955–4965.
- Mead, D. A., Long, S., Ruvkin, G., Brown, S. & Ausubel, F. (1982). Physical and genetic characterization of symbiotic and auxotrophic mutants of *Rhizobium meliloti*. *J Bacteriol* **149**, 114–122.
- Mead, D. A., Szczesna-Skorupa, E. & Kemper, B. (1986). Single-stranded DNA 'blue' promoter plasmids: a versatile tandem promoter system for cloning and protein engineering. *Protein Eng* **1**, 67–74.
- Miller, J. H. (1972). *Experiments in Molecular Genetics*, pp. 352–355. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Murillo, M., Foglia, R., Diller, A., Lee, S. & Leustek, T. (1995). Serine acetyltransferase from *Arabidopsis thaliana* can functionally complement the cysteine requirement of a *cysE* mutant strain of *Escherichia coli*. *Cell Mol Biol Res* **41**, 425–433.
- Nielsen, H., Engelbrecht, J., Brunak, S. & von Heijne, G. (1997). Identification of prokaryotic and eukaryotic signal peptides and prediction of the cleavage sites. *Protein Eng* **10**, 1–6.
- Parker, G. F., Higgins, T. P., Hawkes, T. & Robson, R. L. (1999). *Rhizobium* (*Sinorhizobium*) *meliloti* *phn* genes: characterisation and identification of their protein products. *J Bacteriol* **181**, 389–395.
- Patriarca, E. J., Riccio, A., Taté, R., Colonna-Romano, S., Iaccarino, M. & Defez, R. (1993). The *ntrBC* genes of *Rhizobium leguminosarum* are part of a complex operon subject to negative regulation. *Mol Microbiol* **9**, 569–577.
- Poole, P. S., Blyth, A., Reid, C. & Walters, K. (1994a). *myo*-Inositol catabolism and catabolite regulation in *Rhizobium leguminosarum* bv. *viciae*. *Microbiology* **140**, 2787–2795.
- Poole, P. S., Schofield, N. A., Reid, C. J., Drew, E. M. & Walshaw, D. L. (1994b). Identification of chromosomal genes located downstream of *dctD* that affect the requirement for calcium and the lipopolysaccharide layer of *Rhizobium leguminosarum*. *Microbiology* **140**, 2797–2809.
- Saitou, N. & Nei, M. (1987). The neighbour-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Studier, F. W. & Moffatt, B. W. (1986). Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. *J Mol Biol* **189**, 113–130.
- Tabor, S. & Richardson, C. C. (1985). A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes. *Proc Natl Acad Sci USA* **82**, 1074–1078.
- Taté, R., Riccio, A., Caputo, E., Iaccarino, M. & Patriarca, J. (1999). The *Rhizobium etli* *metZ* gene is essential for methionine biosynthesis and nodulation of *Phaseolus vulgaris*. *Mol Plant-Microbe Interact* **12**, 24–34.
- Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673–4680.
- Trinick, M. J., Dilworth, M. J. & Grounds, M. (1976). Factors affecting the reduction of acetylene by root nodules of *Lupinus* species. *New Phytol* **77**, 359–370.
- Walshaw, D. L. & Poole, P. S. (1996). The general L-amino acid permease of *Rhizobium leguminosarum* is an ABC uptake system that influences efflux of solutes. *Mol Microbiol* **21**, 1239–1252.
- Walshaw, D. L., Reid, C. J. & Poole, P. S. (1997). The general amino acid permease of *Rhizobium leguminosarum* is negatively regulated by the Ntr system. *FEMS Microbiol Lett* **152**, 57–64.
- Zheng, L., Cash, V. L., Flint, D. H. & Dean, D. R. (1998). Assembly of iron-sulfur clusters. Identification of an *iscSUA-hscBA-fdx* gene cluster from *Azotobacter vinelandii*. *J Biol Chem* **273**, 13264–13272.

Received 15 February 2001; revised 1 June 2001; accepted 12 June 2001.