Analysis of a *Rhizobium leguminosarum* gene encoding a protein homologous to glutathione S-transferases

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A novel *Rhizobium leguminosarum* gene, *gstA*, the sequence of which indicated that it was a member of the gene family of glutathione S-transferases (GSTs), was identified. The homology was greatest to the GST enzymes of higher plants. The *Rhizobium gstA* gene was normally expressed at a very low level. The product of *gstA* was over-expressed and purified from *Escherichia coli*. It was shown to bind to the affinity matrix glutathione-Sepharose, but no enzymic GST activity with 1-chloro-2,4-dinitrobenzene as substrate was detected. *gstA* encoded a polypeptide of 203 amino acid residues with a calculated molecular mass of 21990 Da. Transcribed divergently from *gstA* is another gene, *gstR*, which was similar in sequence to the LysR family of bacterial transcriptional regulators. A mutation in *gstR* had no effect on the transcription of itself or *gstA* under the growth conditions used here. Mutations in *gstA* and *gstR* caused no obvious phenotypic defect and the biological functions of these genes remain to be determined.

Keywords: glutathione, glutathione S-transferase, *Rhizobium*, transcriptional regulator

INTRODUCTION

Bacteria of the genera *Rhizobium*, *Azorhizobium* and *Bradyrhizobium* infect legume roots, inducing nodules in which the bacteria reduce atmospheric N₂, making the resultant ammonia available to the host. In exchange, the bacteria obtain an exclusive source of fixed carbon from the plant. Over the past few years, there have been major advances in the understanding of the functions of many of the bacterial genes that are required for the early steps of the infection process and in the process of N₂ fixation *per se* (Fischer, 1994; Spaink, 1995). There is, however, less information on the 'housekeeping' genes of the bacteria, some of which may also be required for symbiotic N₂ fixation.

We had previously identified and sequenced several genes in a region of the chromosome of *Rhizobium leguminosarum* bv. *viciae*, the symbiont of peas and

vetches. This region contained the *cyc* genes that are involved in the biosynthesis of cytochrome *c* (Delgado *et al.*, 1995) and the regulatory genes *feuP* and *feuQ*, which are required for the efficient uptake of iron (Yeoman *et al.*, 1997).

During the course of studying this region, we identified another gene that specifies a protein with a striking similarity in sequence to proteins of the glutathione S-transferase (GST) family. These enzymes are multifunctional proteins that catalyse the conjugation of GSH via the thiol group to a large variety of hydrophobic electrophilic compounds (Jakoby & Habig, 1980), resulting in their detoxification. They can also catalyse the reduction of peroxide molecules (Pickett & Lu, 1989). In mammals, the cytosolic GST isozymes are of at least four distinct classes, alpha, mu, pi and theta, according to their substrate specificity, immunochemical reactivity and their primary amino acid sequences (Ogura *et al.*, 1991; Mannervik *et al.*, 1992; Meyer, D. J. *et al.*, 1991). These enzymes have been widely studied in mammals and, to a lesser extent, in plants. In plants, GSTs can detoxify herbicides such as atrazine (Timmerman, 1989); they are also induced by pathogenic infection and are likely to be involved in defence responses (Dudler *et al.*, 1991).

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Abbreviations: CDNB, 1-chloro-2,4-dinitrobenzene; GST, glutathione S-transferase.

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There have been relatively few studies on GSTs in prokaryotes, although a recent survey showed that a wide range of Gram-negative soil bacteria have GST activity, albeit at a low level in most cases (Zablotowicz *et al.*, 1995). Allocati *et al.* (1994) reported that in *Proteus mirabilis*, a GST-like protein was located in the bacterial periplasmic space. Morohoshi (1991) and Masai *et al.* (1991, 1993) cloned and sequenced a gene, *ligF*, from *Pseudomonas paucimobilis* which could direct the breakdown of aryl β -ether, resulting in the degradation of various dimeric lignin-based compounds. Sequence analysis revealed the deduced product of *ligF* to be similar to GSTs. Bacterial GST-like proteins have also been implicated in the bacterial breakdown of xenobiotics. Thus, Hofer *et al.* (1994) noted that the product of a gene involved in the catabolism of polychlorinated biphenyls by a strain of *Pseudomonas* had similarity to the GSTs of higher organisms. Furthermore, in two methylotrophs, *Methylobacterium* and *Methylophilus*, a gene, *dcmA*, specifying a dichloromethane dehalogenase enzyme showed some similarity in sequence to members of the GST gene family (LaRoche & Leisinger, 1990; Bader & Leisinger, 1994). Interestingly, *dcmA* of *Methylobacterium* is regulated by a divergently transcribed gene, *dcmR* (LaRoche & Leisinger, 1991) for which the substrate dichloromethane is an inducer.

Iizuka *et al.* (1989) described some of the properties of the GST of *Escherichia coli*. It is a homodimer with a substrate specificity towards 1-chloro-2,4-dinitrobenzene (CDNB), a commonly used substrate for this family of enzymes. More recently, the *gst* gene of *E. coli* has been characterized at a molecular level; some low-level homology between the product of the *E. coli* gene and those of eukaryotes was detectable (Nishida *et al.*, 1994) but the function of this gene is unknown.

Here, we describe the structure of a *gst*-like gene of *R. leguminosarum*, together with studies on its regulation and the properties of its purified gene product.

METHODS

Materials. GSH, equine liver GST, CDNB and Paraquat were from Sigma and atrazine was a gift from Ciba-Geigy. Restriction enzymes, T4 DNA ligase and *Taq* DNA polymerase were used according to the manufacturer's instructions (Boehringer Mannheim). Plasmid pET-21a was from Novagen. A pre-packed GSH-Sepharose 4B column was from Pharmacia. Specific 24-mer oligonucleotides were synthesized by Genosys or by Richard James (University of East Anglia) on a Bioresearch Cyclone Oligonucleotide Synthesizer.

Bacterial strains and plasmids. Bacterial strains and plasmids used in this study are listed in Table 1. General growth conditions and antibiotic concentrations for *E. coli* and *R. leguminosarum* were as described by Beringer (1974), unless otherwise stated.

DNA manipulations. DNA manipulations including cloning, Southern hybridization and transformations were done according to standard procedures (Sambrook *et al.*, 1989). DNA sequencing was carried out with the aid of an ALF Pharmacia

semi-automated sequencer according to the manufacturer's instructions. Double-stranded DNA was sequenced using the Sanger *et al.* (1977) dideoxy chain-termination method. Data were analysed using programs in the DNA Star package. DNA searches of the EMBL database were carried out using BLAST (part of the GCG program; Wisconsin Sequence Analysis Package, University of Wisconsin). PCR amplifications were performed on an Omnigene thermal cycler. The oligonucleotide sequences of the PCR primers used to amplify *gstA* were as follows: 5' CTGCATATGAACTCTACCATCAC 3' (start codon bold) and 5' GTCAAGCTTCA TGCGGCAAGGCCG 3' (stop codon bold).

Transposon mutagenesis. Plasmids were transferred to *R. leguminosarum* by conjugation (Buchanan-Wollaston *et al.*, 1980) using the mobilizing plasmid pRK2013 (Figurski & Helinski, 1979). *Tnlac* mutagenesis of pBIO1000, which contains the *gstA* and *gstR* genes cloned in the wide-host-range plasmid pLAFR1, was performed as follows. pBIO1000 was transformed into *E. coli* strain A118, which contains a chromosomally located copy of *Tnlac*. The resultant strain was used as the donor in subsequent triparental matings with *R. leguminosarum* bv. *viciae* strain 8401pRL1JI as the recipient. Transposon insertions into 8401pRL1JI were selected on complete TY medium containing 400 μ g streptomycin ml⁻¹ (8401pRL1JI), 5 μ g tetracycline ml⁻¹ (pLAFR1) and 250 μ g kanamycin ml⁻¹ (*Tnlac*). Transconjugants containing *Tnlac* in pBIO1000 arose at a frequency of approximately 10⁻⁶ per recipient. Plasmid DNA was isolated from transconjugants and using appropriate restriction enzymes the approximate sites and orientations of the *Tnlac* insertions were determined. For precise mapping of *Tnlac* insertions, the junctions between the ends of IS50L and the pBIO1000 DNA were sequenced. This was achieved using a fluorescently labelled 25-mer oligonucleotide primer designed to bind to part of the *Tn5* inverted repeat. The primer had the sequence: 5' TTCC-CGTTTTCCAGGATCTGACTTC 3'.

Tnlac insertions in *gstA* and *gstR* of pBIO1000 were incorporated into the 8401pRL1JI genome by marker exchange (Ruvkun & Ausubel, 1981) using the IncP1 plasmid pPH1JI to force the elimination of pBIO1000, as described by Downie *et al.* (1983).

Pea seedlings (*Pisum sativum* cv. Wisconsin Perfection) were inoculated with mutant *R. leguminosarum*, then grown and assayed for nodule formation and nitrogen fixation (Beynon *et al.*, 1980).

Construction of *gstA/gstR* promoter *lacZ* fusions. The *gstA/gstR* promoter region was cloned into the *lac* promoter probe plasmid pMP220 as follows. A 465 bp *Ava*II fragment from pBIO860 (spanning the entire intergenic region between *gstA* and *gstR*, from nucleotides 1669 to 2134) was cloned into the *Sma*I site of pUC18 (see Fig. 1). The orientation of the *Ava*II insert was determined using a *Pst*I digest. The resultant clone was digested with either (1) *Eco*RI and *Bam*HI and the resulting *Eco*RI-*Bam*HI fragment cloned into *Bgl*II-*Eco*RI-digested pMP220 to yield pBIO1016 or (2) *Eco*RI and *Xba*I and the resulting *Eco*RI-*Xba*I fragment cloned into *Eco*RI-*Xba*I-digested pMP220 to form pBIO1017. In plasmids pBIO1016 and pBIO1017, the *lacZ* gene of pMP220 would be under the control of the *gstA* and the *gstR* promoter, respectively.

Enzyme assays. β -Galactosidase assays in *Rhizobium* were done as described by Rossen *et al.* (1985). GST activity was assayed essentially by the method of Habig *et al.* (1974), modified as follows. Cultures of *R. leguminosarum* were

Table 1. Strains and plasmids used in this study

Strain/plasmid	Relevant properties	Reference/source
<i>R. leguminosarum</i> bv. <i>viciae</i>		
8401(pRL1JI)	Derivative of 8401 carrying pRL1JI; Str ^r	Downie <i>et al.</i> (1983)
J102	Derivative of 8401pRL1JI carrying <i>gstA</i> ::Tn5 <i>lac</i>	This work
J106	Derivative of 8401pRL1JI carrying <i>gstR</i> ::Tn5 <i>lac</i>	This work
<i>E. coli</i>		
JM101	Host used for transformation	Messing (1979)
DH5 α	Host used for transformation	Hanahan (1983)
A118	CC118 containing Tn5 <i>lacZ</i> in its chromosome; Km ^r	Simon <i>et al.</i> (1989)
BL21(DE3)	Host used for expression of <i>gstA</i>	Novagen
Plasmids		
pKN16	Cosmid carrying 30 kb <i>R. leguminosarum</i> bv. <i>viciae</i> B155 DNA, including <i>gstA</i> and <i>gstR</i>	Nadler <i>et al.</i> (1990)
pMP220	Broad-host-range, promoter probe cloning vector, Tc ^r	Spaink <i>et al.</i> (1985)
pPH1JI	Inc P-1 plasmid used for marker exchange; Gm ^r	Beringer <i>et al.</i> (1979)
pRK2013	Helper plasmid; contains RK2 <i>tra</i> genes; Kan ^r	Figurski & Helinski (1979)
pET-21a	Used for high-level gene expression; Amp ^r	Novagen
pUC18	<i>E. coli</i> cloning vector; Amp ^r	Yanisch-Perron <i>et al.</i> (1985)
pBIO1000	Contains <i>gstA</i> and <i>gstR</i> cloned in pLAFR1	This work
pBIO1016	465 bp <i>Ava</i> II fragment containing predicted <i>gstA/R</i> promoter (in <i>gstA</i> orientation) cloned in pMP220	This work
pBIO1017	465 bp <i>Ava</i> II fragment containing predicted <i>gstA/R</i> promoter (in <i>gstR</i> orientation) cloned in pMP220	This work
pBIO860	Derivative of pKN16 containing <i>gstA</i> and <i>gstR</i> cloned in pUC18	This work
pBIO872	612 bp <i>gstA</i> PCR fragment cloned in pET-21a	This work

grown overnight at 28 °C to a cell density of approximately 10^9 ml⁻¹. Cells were harvested by centrifugation and washed twice in buffer K (0.1 M potassium phosphate buffer, pH 6.8; 1.0 mM EDTA). The final pellet was resuspended in 10 ml buffer K and disrupted using a French press. Disrupted cells were centrifuged (48000 g, 40 min) at 4 °C. Cell-free extracts were maintained at 4 °C until assayed, within 1–4 h. The reaction mixture consisted of 1.0 mM CDNB, 1.0 mM GSH and cell-free extract (200 μ l) in a total volume of 1.0 ml. The reaction was initiated by addition of GSH and enzyme activity was calculated using a molar extinction coefficient of 9.6 mM⁻¹ cm⁻¹ at 340 nm and 25 °C. Control reactions (without enzyme) were included to determine non-enzymic CDNB–GSH conjugation, and the background reaction was monitored at 340 nm for 2 min before addition of GSH. A Philips PU8730 UV/VIS spectrophotometer was used. One unit of enzyme activity was defined as the amount of the enzyme catalysing the formation of 1 μ mol product min⁻¹ (Habig *et al.*, 1974). Specific activity was defined as the units of enzyme activity (mg protein)⁻¹ as measured according to the method of Bradford (1976) with bovine serum albumin as a standard.

Over-expression of *gstA* in *E. coli*. The *gstA* gene was amplified using the two 24-mer oligonucleotide primers listed above. One of the primers contained a *Nde*I site (CATATG) that overlapped the translational start site and the other had a *Hind*III site (AAGCTT) that overlapped the *gstA* translational stop codon. The 612 bp PCR product was cloned into *Nde*I–*Hind*III sites of the pET-21a expression vector as described by Novagen, and the resulting plasmid, pBIO872, was transformed into *E. coli* B strain BL21(DE3). To confirm

that the fragment had been correctly cloned, the junctions of the vector and the insert were sequenced. An overnight culture (0.2 ml) of strain BL21(pBIO872) containing 100 μ g ampicillin ml⁻¹ was inoculated into 20 ml of the same medium, and incubated with shaking at 37 °C until the OD₆₀₀ reached 0.6–1.0. The *lac* gene inducer IPTG was added to a final concentration of 1 mM and the incubation was continued for 3 h until the OD₆₀₀ reached about 2.0. Cells were harvested and resuspended in SDS-PAGE sample buffer and analysed on SDS-polyacrylamide gels (15%, w/v, acrylamide). Proteins were detected by staining with Coomassie Blue R-250.

Purification of GstA protein. Cells from a 1 litre culture of *E. coli* BL21(pBIO872) which had been grown in LB in the presence of IPTG were harvested and washed in 40 ml PBS (150 mM NaCl, 4 mM NaH₂PO₄, 16 mM Na₂HPO₄, pH 7.4) containing 1% (v/v) Triton X-100. The cells were centrifuged, then resuspended in 15 ml PBS, and disrupted in a French press. Disrupted cells were centrifuged (48000 g, 40 min). The supernatant was applied to a GSH-Sepharose 4B column (2 ml) equilibrated with PBS plus 1% (v/v) Triton X-100, and the column was washed with 10 ml PBS. The enzyme was eluted with 20 ml 50 mM Tris/HCl (pH 8.0) containing 5 mM GSH. The GstA fractions were pooled, dialysed against water, concentrated by ultrafiltration with a Diaflo YM3 membrane (Amicon) and stored at –80 °C. All operations in the purification procedure were performed at 4 °C.

Electrospray mass spectrometry. Mass spectrometry was performed with a Fisons VG form (quadrupole) mass spectrometer fitted with a pneumatically assisted electrospray (ion spray) source and controlled by VG Mass Lynx software (VG

Biotech). The carrier solvent was acetonitrile/water (1:1, v/v); and the solution flow was at the rate of 10 $\mu\text{l min}^{-1}$ using a Hewlett Packard 1090 LC. Protein samples were dissolved in carrier solvent at a concentration of 20 $\text{pmol } \mu\text{l}^{-1}$ containing formic acid (0.1%, v/v), centrifuged at 5000 g for 2 min and then 20 μl samples were injected directly into the carrier stream. Horse heart myoglobin was used as a standard.

RESULTS AND DISCUSSION

Identification of *gstA* and a putative transcriptional regulator, *gstR*

A wide-host-range cosmid, pKN16, containing approximately 30 kb cloned *R. leguminosarum* bv. *viciae* DNA, was isolated by Nadler *et al.* (1990). pKN16 contains 10 *EcoRI* fragments. One of these, 3.7 kb in size, lies approximately 5 kb upstream of the previously identified *feuP* gene (Yeoman *et al.*, 1997). This fragment was cloned into pUC18 to form pBIO860. Various restriction fragments from pBIO860 were subcloned into pUC18 and their sequences were determined. The sequence of the entire 3.7 kb fragment was then completed, using appropriate primers designed to extend the contigs that had already been obtained.

The sequenced region was analysed for ORFs; it was clear that it contained two genes with homology to genes of known functions. One of these, which we term *gstA*, has a product that is similar to the GST enzyme family (see below). The other, termed *gstR*, is transcribed divergently from *gstA*, the translational starts of the two genes being separated by 143 bp (Fig. 1). The deduced GStR protein was similar to members of the LysR family of bacterial transcriptional regulators (see below).

In addition to these two genes, a DNA homology search (FASTA) showed that immediately downstream of the *gstR* stop codon there are 62 bp with 87% identity to a region of DNA in another strain (VF39) of *R. leguminosarum* bv. *viciae*. This is downstream of a gene, *pss4*, which specifies a glycosyltransferase that is required for one of the earliest steps in the synthesis of the acidic exopolysaccharide (Ivashina *et al.*, 1994). Interestingly, another gene, *pssA*, which corresponds in sequence and function to *pss4*, has also been identified in a strain of *R.*

Fig. 1. Restriction map of the *gstA/gstR* region of *R. leguminosarum* cloned in pBIO860. Numbers refer to the bp positions in the DNA sequence. The triangles represent the position of the *Tn5lacZ* insertions, the black box represents the promoter region and the dotted box shows the region of homology to the non-coding region of the *pss4* gene of *R. leguminosarum* bv. *viciae* VF39.

leguminosarum bv. *phaseoli* (Borthakur *et al.*, 1988). However, the DNA 3' of *pssA* has no similarity to that downstream of *pss4* and no similarity was found between the sequences in the vicinity of *pssA* and *gstR*.

Comparison of *R. leguminosarum* GStA with other GSTs

The deduced polypeptide product of *gstA* has a calculated molecular mass of 21989 Da. A database search of its predicted amino acid sequence showed it to be homologous to a number of GST or GST-like sequences from plants, animals and other bacteria (Fig. 2). The similarity in sequence is most striking at the N-terminal end, in the regions of the GSTs that are most highly conserved amongst themselves. It has been proposed that the region between residues 51 and 82 (as shown in the GStA sequence in Fig. 2) is involved in binding the glutathione substrate so a priori would be expected to be well-conserved (Ketterer *et al.*, 1988; Telakowski-Hopkins *et al.*, 1986; Tu & Qian, 1986). However, the region of highly conserved sequence extends further towards the N-terminus and there are other short patches of homology at other positions towards the C-terminus of the other GST proteins and GStA.

As indicated in the legend to Fig. 2, the overall incidence of similar or identical amino acids in GStA is greatest with the proteins of higher plants, rather than to those of animals or other bacteria. The evolutionary significance, if any, of these levels of similarity is not clear.

Comparison of GStR and other proteins of the LysR family

Comparison of the deduced protein sequence of the product of *gstR* with those in databases indicated that it was a member of the large family of bacterial transcriptional regulators of the so-called LysR family (see Schell, 1993). Generally, any two members of this family have approximately 20% identity with each other, and this was the overall value in pairwise comparisons of GStR with other individual proteins (not shown). The most highly conserved region is towards the N-terminus and comprises a helix–turn–helix domain that is responsible for the binding of the proteins to regulatory DNA sequences (Schell, 1993). As shown in Fig. 3, the deduced sequence of GStR shares several of the consensus residues in this motif.

Members of the family also possess intermittent regions of conserved sequences throughout the length of the protein, these being involved in the recognition of the particular inducer molecule and for both the binding to DNA and the response to the inducer (Schell, 1993). The GStR protein shared several of these conserved sequences (not shown), but there were no extended regions of striking identity in the C-terminal region of GStR compared to the other regulators. This indicates that GStR is a novel member of the family and is likely to respond to a compound that does not act as an inducer with any other known member.

As a control, purified GST from equine liver was confirmed to generate a condensation product between GSH and CDNB with a major peak of absorbance at 340 nm and a minor one at 411 nm. In contrast, when *R. leguminosarum* cell-free extract was used in the reaction, the 411 nm peak was the major one in comparison with that at 340 nm. The band at 340 nm is due to the accumulation of the dinitrophenyl–glutathione (DNP–SG) conjugate, while the band at 411 nm is probably due to a further metabolism of the DNP–SG conjugate. Neither peak was observed if GSH or CDNB was omitted from the reaction mixture with the cell-free extract nor if the cell-free extracts were boiled prior to adding the substrates. Thus, using CDNB as a substrate, we conclude that *R. leguminosarum* bv. *viciae* strain 8401pRL1JI does contain GSH-dependent GST activity but, as with other bacteria (Zablotowicz *et al.*, 1995), the activity was low [0.052×10^{-3} units (mg protein) $^{-1}$].

Two maize GSTs have very little or no activity with CDNB as a substrate (Timmerman, 1989) but did have activity towards the herbicide atrazine. This indicates the importance of assaying with a variety of substrates when characterizing GST. Accordingly, *R. leguminosarum* strain 8401pRL1JI was examined for its ability to condense GSH with the two herbicides atrazine and Paraquat. In each case, the concentration of substrate in the reaction mixture was 1 mM. There was no detectable GST activity when either molecule was used as substrate (not shown).

Fig. 3. Comparison of the N-terminus of GstR and selected members of the LysR family of bacterial transcriptional activators (see Goldberg *et al.*, 1991, IrgB; Wek & Hatfield, 1986, IlvY; Henikoff *et al.*, 1988, LysR; Honore *et al.*, 1986, AmpR; Györgypal & Kondorosi, 1991, NodD; LaRoche & Leisinger, 1991, DcmR). Amino acids in GstR that are present in the corresponding position of two or more of the other regulators are indicated by asterisks. The numbers in parentheses refer to the distance of the amino acids from the N-termini of the proteins. The conserved helix-turn-helix domain of these proteins is also indicated.

Table 2. Effect of *gstR* mutation on the expression of *gstA-lacZ* and *gstR-lacZ*

Plasmids pBIO1016 and pBIO1017, containing, respectively, the *gstA-lacZ* and *gstR-lacZ* fusions, were mobilized into wild-type strain 8401pRL1JI and its *gstR* mutant derivative strain J106; β -galactosidase activities were measured as described by Rossen *et al.* (1985). As a control, the vector pMP220 was also introduced into each strain.

<i>R. leguminosarum</i> strain	β -Galactosidase activity (Miller units)
8401pRL1JI(pMP220)	60 $\pm$ 10
8401pRL1JI(pBIO1016)	72 $\pm$ 12
8401pRL1JI(pBIO1017)	305 $\pm$ 18
J106(pMP220)	69 $\pm$ 10
J106(pBIO1016)	75 $\pm$ 8
J106(pBIO1017)	280 $\pm$ 12

Induction of *gstA*

It has been shown that the transcription of the *dcmA* gene (whose product has similarity to GST) was stimulated by growing the cells in the presence of the substrate dichloromethane (LaRoche & Leisinger, 1991). This regulation depended upon the presence of the *dcmR* gene that is transcribed divergently from the 'structural' gene *dcmA*. In an attempt to induce GST activity in *R. leguminosarum*, cells of strain 8401pRL1JI were grown in media supplemented with CDNB (10 μ M), Paraquat (50 μ M) or atrazine (500 μ M) (these concentrations are not toxic to the cells). After 24 h growth in these supplemented media, cells were harvested and assayed for GST activity, using CDNB as substrate. There was no increase in activity compared to the unsupplemented control, showing that if *gstA* is inducible, these three compounds were ineffective as inducers.

Effects of mutations in *gstA* and *gstR*

To test if the *gst* genes that we had identified were responsible for the low level of GST activity in *R. leguminosarum*, we set out to isolate and characterize mutations in *gstA* and *gstR*. Following mutagenesis of pBIO1000 with Tn5lac (see Methods), one insertion into *gstA* and one into *gstR* were isolated, their locations being shown in Fig. 1. In both mutants, the *lacZ* gene of Tn5lac was in the opposite orientation to that of the *gst* genes into which the transposon had inserted.

The *gstA::Tn5lac* and the *gstR::Tn5lac* mutations were each introduced by marker exchange into the genome of *R. leguminosarum* strain 8401pRL1JI; for each allele, two potential homogenates were checked by Southern blotting using the 3.7 kb *EcoRI* fragment from pBIO860 as a probe to genomic DNA isolated from the mutants. In all cases examined, the transposon had inserted into the corresponding position in the genomic DNA.

The *gstA::Tn5lac* and *gstR::Tn5lac* mutant strains were found to be prototrophic, have normal colony morphology and induced normal numbers of N₂-fixing nodules on peas.

The two mutants were assayed for their GST activity, using CDNB as the substrate. In both cases, they had the same low-level activity as did the wild-type strain, whether or not the cells had been pregrown in the presence of CDNB. Thus it would seem that this low level of activity seen with CDNB as a substrate is not due to the *gstA* and *gstR* genes identified here. It therefore appears that *R. leguminosarum* possesses another gene encoding an enzyme, as yet uncharacterized, that is capable of a low level of GST activity with CDNB as the substrate.

Effect of a *gstR* mutation on the transcription of *gstR* and *gstA*

The *gstR* gene is transcribed divergently from *gstA*, suggesting that *gstR* might be involved in the regulation of *gstA* (see Schell, 1993). To test this, we examined the expression of both *gstA* and *gstR* in the wild-type and in the *gstR::Tn5lac* mutant strain that we had isolated. Note that in this *gstR* mutant, the *lacZ* gene was in the opposite orientation to *gstR* itself and the levels of β -galactosidase in the *gstR::Tn5lac* mutant were essentially the same as in the control strain that lacked the transposon (Table 2).

To examine the expression of *gstA* and *gstR*, we constructed transcriptional *lacZ* fusions to the proposed promoter regions of both these genes. The *gstA* and *gstR* genes each contain a single *AvaII* site (Fig. 1). The DNA between these two sites spans the intergenic region between *gstA* and *gstR* and so, presumably, contains the promoters for both these genes. This *AvaII* fragment was cloned in both orientations into the wide-host-range *lacZ* promoter probe vector pMP220 to form pBIO1016 (*gstA* promoter fusion) and pBIO1017 (*gstR* promoter fusion).

These two plasmids were each mobilized into the wild-type *R. leguminosarum* strain 8401pRL1JI and into 8401pRL1JI containing the Tn5lac insertion in *gstR* (strain J106). These sets of transconjugants were grown in liquid minimal Y medium and assayed for β -galactosidase activity. As shown in Table 2, in the wild-type background, expression of *gstA-lacZ* was at a low level whereas that of *gstR-lacZ* was relatively high. This is consistent with most other examples of the LysR family, in which the regulatory gene is expressed constitutively but the regulated genes are only transcribed at high levels when bacteria are exposed to an appropriate inducer (Schell, 1993). Essentially the same levels of transcription of both the *gstA-lacZ* and *gstR-lacZ* fusions were found in the *gstR* mutant J106 as in the wild-type, 8401pRL1JI. Therefore, under the conditions used here, there is no evidence to show that *gstR* regulates itself or the adjacent *gstA* 'structural' gene.

Fig. 4. Purification of *R. leguminosarum* GstA protein. Proteins were separated by PAGE. Lanes: 1, standard marker proteins; 2, total proteins from extracts of *E. coli* pET-21a; 3, over-expressed *gstA* in *E. coli*; 4, purified protein after passage through the glutathione affinity column.

In most examples of the LysR family, the regulatory gene represses its own transcription independently of the presence of an inducer molecule (see Schell, 1993), although there are exceptions to this (Smit *et al.*, 1992; Taira *et al.*, 1991). Activation of transcription of any 'target' genes normally requires an inducer; it remains to be seen if *gstR* does in fact regulate *gstA* but that this only occurs in the presence of some as yet unidentified inducer molecule.

Purification of the product of *R. leguminosarum* *gstA*

To characterize some of the properties of the purified *R. leguminosarum* *gstA* gene product, *gstA* was cloned into the expression vector pET-21a to form pBIO872 as described in Methods. Following induction of expression by the addition of IPTG to *E. coli* strain BL21(pBIO872), the most predominant single protein seen on a Coomassie-Blue-stained gel had an estimated size of approximately 22 kDa, consistent with that predicted from the DNA sequence of *gstA* (Fig. 4). This prominent protein band did not occur in the control strain containing only pET-21a or in extracts of cells containing pBIO872 to which no IPTG had been added.

To purify the GstA protein, cell-free extracts of *E. coli* containing the over-expressed *gstA* gene were loaded onto a GSH-Sepharose affinity column. After elution

with GSH, samples were electrophoretically separated on a denaturing acrylamide gel. A single band was eluted with the expected molecular mass of approximately 22 kDa for the GstA; this GSH-dependent elution of this protein clearly demonstrates that it does bind to GSH. To confirm that this protein corresponds to *R. leguminosarum* GstA, it was subjected to electrospray mass spectrometry to determine its exact molecular mass. This analysis identified two products, a major one (approximately 92% of the total signal) with a molecular mass of 22290 Da and a minor one with a molecular mass of 21990 Da. The latter corresponds exactly in size to that predicted for the monomeric form of the GstA; the former is larger by 300 Da, which corresponds to the molecular mass of GSH. Thus each monomer of the *R. leguminosarum* GstA protein apparently binds covalently to a single molecule of the GSH that had been used to elute it from the column. This binding of GSH to the protein is most likely to be via a disulphide link. To confirm this, the protein sample that had been eluted from the GSH affinity column was treated with 1 mM DTT, dialysed excessively with water and its molecular mass was measured again. It was found that more than 95% of the protein had a molecular mass of 21990 Da, consistent with the DTT having reduced the disulphide bond which rendered the enzyme free of glutathione. Cell-free extracts from *E. coli* containing the over-expressed *gstA* product and the purified protein were assayed for GST activity using CDNB plus GSH as substrates. Induction of *gstA* expression with IPTG in the strain containing pBIO872 gave no detectable increase in activity above that from *E. coli* containing the plasmid vector alone. Likewise, assaying the purified GstA yielded no detectable GST activity, confirming that CDNB cannot act as a substrate for the product of *R. leguminosarum* *gstA*. Indeed, our finding is similar to that of a GSH-affinity binding protein obtained from *Bacteroides fragilis*, which binds to a GSH column but has no glutathione transferase activity (Piccolomini *et al.*, 1991). Note further that CDNB has also been shown to be a poor substrate for certain GSTs from mammals (Jakoby & Habig, 1980; Ketterer *et al.*, 1988) and plants (Gronwald *et al.*, 1987; Timmerman, 1989; Dean *et al.*, 1990; Edwards & Dixon, 1991; Meyer, D. J. *et al.*, 1991), so this failure to catalyse the condensation of CDNB and GSH would not be unique.

The inability of the over-expressed *gstA* to catalyse the condensation between GSH and CDNB is unlikely to be due to the need for some post-translational modification of the protein by *E. coli*. Most prokaryotic enzymes do not require any such modifications for their function. Further, it has been shown that eukaryotic *gst* genes specify functional GST enzymes, with the appropriate substrate specificity, when cloned in *E. coli* (Bernasconi *et al.*, 1995).

Furthermore, immunoblotting analysis showed that none of the polyclonal antibodies raised against human GST α 1-1 or rat GST YC₂ cross-reacted with the purified *Rhizobium* *gstA* gene product, but they did

recognize equine GST (N. K. Tawfiq Alkafar, unpublished results). This indicates that the antigenic domain of bacterial GSTs is different from those of mammals, a property that has been noted before in bacterial GSTs (Di Ilio *et al.*, 1991).

Concluding remarks

The role of most bacterial GSTs remains something of an enigma. In some bacteria that have particular catabolic pathways that allow them to detoxify certain xenobiotics such as dichloromethane, the proteins responsible have some sequence similarity to eukaryotic GSTs (Hofer *et al.*, 1994; Masai *et al.*, 1991, 1993; LaRoche & Leisinger, 1991). However, the ability to condense GSH to substrates such as CDNB appears to be quite widespread in prokaryotes (Zablotowicz *et al.*, 1995) and there have been reports of GSH-binding proteins from other bacteria (Piccolomini *et al.*, 1991). However, other than these particular cases in which GST-like proteins are involved in the catabolism of unusual compounds, there is no clear biological role for this enzyme in bacteria such as *E. coli*, *Haemophilus influenzae* (where a *gstA*-like gene has been identified from sequencing; Fleischmann *et al.*, 1995) or, as described here, *R. leguminosarum*.

Future work will involve attempts to identify active substrates for the *gstA* gene product of *R. leguminosarum* and to examine if there are any inducer molecules that might activate transcription of *gstA* through the action of *gstR*. Since an insertion mutation in *gstA* did not abolish the low levels of GST activity, there is also scope to characterize the protein and the gene responsible for this activity.

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