Molecular cloning, expression and site-directed mutagenesis of glutathione S-transferase from *Ochrobactrum anthropi*

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The gene coding for a novel glutathione S-transferase (GST) has been isolated from the bacterium *Ochrobactrum anthropi*. A PCR fragment of 230 bp was obtained using oligonucleotide primers deduced from N-terminal and 'internal' sequences of the purified enzyme. The gene was obtained by screening of a genomic DNA partial library from *O. anthropi* constructed in pBluescript with a PCR fragment probe. The gene encodes a protein (OaGST) of 201 amino acids with a calculated molecular mass of 21 738 Da. The product of the gene was expressed and characterized; it showed GST activity with substrates 1-chloro-2,4-dinitrobenzene (CDNB), *p*-nitrobenzyl chloride and 4-nitroquinoline 1-oxide, and glutathione-dependent peroxidase activity towards cumene hydroperoxide. The overexpressed product of the gene was also confirmed to have *in vivo* GST activity towards CDNB. The interaction of the recombinant GST with several antibiotics indicated that the enzyme is involved in the binding of rifamycin and tetracycline. The OaGST amino acid sequence showed the greatest identity (45 %) with a GST from *Pseudomonas* sp. strain LB400. A serine residue in the N-terminal region is conserved in almost all known bacterial GSTs, and it appears to be the counterpart of the catalytic serine residue present in Theta-class GSTs. Substitution of the Ser-11 residue resulted in a mutant OaGST protein lacking CDNB-conjugating activity; moreover the mutant enzyme was not able to bind Sepharose–GSH affinity matrices.

INTRODUCTION

The glutathione S-transferases (GSTs; EC 2.5.1.18) are a family of multifunctional dimeric proteins that catalyse the conjugation of the sulphur atom of glutathione with a large variety of electrophilic compounds of both endobiotic and xenobiotic origin [1–4]. Cytosolic GSTs are subdivided into at least six main classes, Alpha, Mu, Pi [5], Theta [6,7], Sigma [8,9] and Zeta [10], based on substrate specificity and primary sequences. The inter-class sequence identity is generally lower than 30 %. GSTs are involved in the intracellular binding and transport of hydrophobic compounds, may participate in the synthesis of prostaglandins and leukotrienes, and might play a key role in the elimination of organic hydroperoxides [1–3].

In prokaryotes, GST activity is very low and hence it has only recently been discovered. Although very little is known about the properties of GSTs in prokaryotes, they seem to be implicated in the biodegradation of xenobiotics [11,12], including antibiotics [13–15]. In addition to the GSTs of *Escherichia coli* [16] and *Proteus mirabilis* [17], the prokaryotic members of the GST family also include the dichloromethane dehalogenase of *Methylophylus* sp. [18] and the *pcpC*-encoded enzyme involved in the reductive dechlorination of pentachlorophenol by *Sphingomonas paucimobilis* [19]. In *Pseudomonas* sp. strain LB400, a GST-encoding gene, with an unclear function, was found within the *pbh* locus, where the genes encoding polypeptides of polychlorinated-biphenyl-degrading enzymes are located [20].

Crystallography studies of GSTs from several species [9,21–23], including bacteria [24], have elucidated the subunit architecture and quaternary structure of these enzymes. Despite their low inter-class sequence identity, the GST monomers show a basically similar structural organization, i.e. two domains joined by a short linker of six or seven amino acid residues. Domain I (GSH-binding site), which is mainly located in the N-terminal region of the protein, is an α/β structure. GST domain II provides most of the hydrophobic binding site (H-site), and is formed by α-helices.

In Alpha, Mu, Pi and Sigma GSTs, a conserved tyrosine residue in the N-terminal region is essential for catalysis by activation of the thiol group of GSH [25–27]. This tyrosine residue is also conserved in several bacterial GSTs, but mutagenesis experiments with the GSTs from *E. coli* and *P. mirabilis* demonstrate that it is not essential for catalysis [16,28]. Theta-class GSTs from the plant *Arabidopsis thaliana* [29] and the Australian sheep blowfly *Lucilia cuprina* [23] lack the tyrosine residue in the active site, and site-directed mutagenesis experiments demonstrated that a serine residue present in the N-terminal portion is involved in catalysis [30].

We have previously reported the purification and partial characterization of a novel GST from *Ochrobactrum anthropi* [31], a bacterium isolated from soil [32]. Here we report the molecular cloning, overexpression and characterization of the GST from *O. anthropi*, termed OaGST. An OaGST mutant obtained by site-directed mutagenesis of Ser-11 showed a dramatic loss of catalytic activity using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate, indicating that, in contrast with the GST from *P. mirabilis* [28], OaGST may be included in the Theta class.

Abbreviations used: GST, glutathione S-transferase; OaGST, GST from *Ochrobactrum anthropi*; CDNB, 1-chloro-2,4-dinitrobenzene; IPTG, isopropyl β-D-thiogalactopyranoside; TNS, 2-*p*-toluidinylnaphthalene-6-sulphonate.

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The amino acid and nucleotide sequences reported in this paper have been submitted to the EMBL Data Bank with the accession number Y17279.

EXPERIMENTAL

Organisms and growth conditions

O. anthropi was used in this study [32]. *E. coli* XL1-Blue was used as the host strain for cloning and expression. *Epicurian coli* XL1-Blue Supercompetent Cells (Stratagene) were used for mutagenesis. Bacterial strains were grown in LB medium in a rotary shaker at 37 °C. For *E. coli* strains, ampicillin was added to LB medium at a final concentration of 50 μg/ml. Isopropyl β-D-thiogalactopyranoside (IPTG; Sigma) was added at a final concentration of 1 mM for the induction of gene expression.

DNA techniques

Cells of *O. anthropi* were lysed with lysozyme and proteinase K, and genomic DNA was prepared as described elsewhere [33]. Recombinant plasmid DNA from *E. coli* clones was isolated by the alkaline lysis method [33]. Agarose gel electrophoresis, DNA restriction, ligase reactions and treatment with alkaline phosphatase were carried out using standard procedures.

Amino acid sequencing

GST from *O. anthropi* was purified as previously described [31]. About 30 μg of purified GST was subjected to tryptic digestion. After 30 min of digestion, the fragments were separated by SDS/12.5 %-PAGE. After electrophoresis, the polypeptide bands on the gel were transferred to a Pro-Blott membrane (Applied Biosystems) in 10 mM Caps buffer containing 10 % (v/v) methanol. The membrane was briefly stained with 0.1 % (w/v) Coomassie Blue in 50 % (v/v) methanol. Stained bands were excised and then subjected to automated Edman degradation analysis on a 476-A gas-phase sequencer (Applied Biosystems).

Molecular cloning

Two degenerate oligonucleotides were designed on the basis of the amino acid sequences derived from OaGST: a 27-mer corresponding to N-terminal amino acid residues [5′-GCGAATTCATGAA(A/G)(C/T)TITA(C/T)TA(C/T)AA(A/G)G-3′; sense] and a 26-mer corresponding to the C-terminal residues of an internal amino acid sequence [5′-CGGGATCC(G/A)AT(G/A)TA(C/T)TGCAG(G/A)ATIGC-3′; antisense]. Both primers had 5′ recognition sites for the restriction enzymes *Eco*RI and *Bam*HI (underlined). Primers were designed to amplify a portion of *OaGST* gene by PCR on a DNA Thermal Cycler (MJR Research). In addition to the template (genomic DNA from *O. anthropi*) and primers, the 50 μl reaction mixture contained 2 mM $MgCl_2$, 0.2 mM dNTPs, *Taq* DNA polymerase buffer and 5 units of *Taq* DNA polymerase (Promega), and was subjected to 30 cycles of amplification (60 s at 94 °C, 60 s at 45 °C, 25 s at 55 °C and 60 s at 72 °C). A PCR product (230 bp) was recovered from low-melting agarose gel (Pharmacia), digested with the restriction enzymes *Eco*RI/*Bam*HI and cloned into the *Eco*RI/*Bam*HI sites of the *E. coli* vector pBluescript SK; the resulting plasmid was designated pBsk230. The PCR product was confirmed by DNA sequencing. A DNA probe was prepared by digestion of pBsk230 with *Eco*RI/*Bam*HI. This probe was random-prime-labelled with [α-^{32}P]dCTP (3000 Ci/mmol; Amersham) using the Multiprime DNA labelling system (Amersham).

Genomic DNA (10 μg) was digested with *Eco*RI, and the digest was fractionated by 1 % (w/v) agarose gel electrophoresis by the standard method [33]. DNA was transferred from the agarose gel to nylon membranes (Hybond N; Amersham) as described by the manufacturer. Hybridization using the PCR probe was performed in 5 × SSC (1 × SSC = 0.15 M NaCl/0.05 M sodium citrate) containing 1 % SDS, 5 × Denhardt's solution and 0.1 mg/ml denatured salmon sperm DNA at 42 °C for 16 h. After hybridization, the membrane was washed for 30 min with 2 × SSC/0.1 % SDS at 65 °C, and for 20 min with 0.2 × SSC/0.1 % SDS at 65 °C. Hybridization with a PCR probe to Southern blots of the *Eco*RI digest showed a unique hybridization band corresponding to a 5.0 kbp fragment. In order to clone the fragment containing the *OaGST* gene, a digest was prepared with 10 μg of genomic DNA from *O. anthropi*. Restriction fragments of genomic DNA were fractionated by preparative 1 % agarose gel electrophoresis. DNA fragments between 4.0 and 6.0 kbp in size were extracted from the gel using the Geneclean II Kit (BIO 101 Inc.) as described by the manufacturer. The *Eco*RI fragments were ligated into a pBluescript vector that had been digested with *Eco*RI and dephosphorylated. The recombinant plasmids obtained were used to transform *E. coli* XL1-Blue, used in the preparation of a partial gene library. Colony hybridization using nitrocellulose membranes (Sartorius) was carried out using the standard procedure [33], and hybridized clones were detected with a PCR probe under the same conditions as for Southern blotting. One clone was selected, and the plasmid in the clone was designated pB-OaGST.

DNA sequencing

DNA sequence analysis was performed on double-stranded DNA using the dideoxynucleotide chain-termination method (Sequencing Kit; Pharmacia) with [α-^{35}S]dATP (Amersham).

Construction of an expression plasmid containing the *OaGST* gene

On the basis of the open reading frame coding for OaGST, two oligonucleotide primers were designed as follows: primer F, 5′-GCGAATTCATGAAGCTTTACTACAAGG-3′ (sense); primer R, 5′-GCGAATTCTTAGTTCAGGCCTTCTTC-3′ (antisense). Both primers contain a 5′ recognition site for the restriction enzyme *Eco*RI (underlined). PCR was performed to obtain the *OaGST* gene; in addition to the template (pB-OaGST) and the primers (F and R), the 50 μl reaction mixture contained 2 mM $MgCl_2$, 0.2 mM dNTPs, *Pfu* DNA polymerase buffer and 2.5 units of *Pfu* DNA polymerase (Stratagene). The PCR reaction was subjected to six cycles of amplification (60 s at 94 °C, 60 s at 45 °C, 25 s at 55 °C and 60 s at 68 °C) followed by 24 cycles of amplification (60 s at 94 °C, 60 s at 55 °C, 60 s at 68 °C). The *OaGST* gene was recovered from a low-melting agarose gel, digested with the restriction enzyme *Eco*RI and cloned into the *Eco*RI site of the expression vector pBTac1 (Boehringer Mannheim) under the *Tac* promoter; the resulting plasmid was designated pT-OaGST. The presence of the *OaGST* gene and the correct orientation were verified by DNA sequencing.

Expression and purification of recombinant OaGST

An overnight culture of *E. coli* XL1-Blue transformed with pT-OaGST was diluted 1:10 and grown until the A_{600} reached 0.4. To induce *OaGST* gene transcription, IPTG was added to a final concentration of 1 mM and the incubation was prolonged for a further 5 h. Cells were harvested by centrifugation (10000 ***g*** for 15 min), resuspended in 10 mM potassium phosphate buffer, pH 7, and disrupted by cold sonication using a Vibra cell (Sonics and Materials Inc.). The particulate material was removed by centrifugation at 100000 ***g***, and the supernatant was loaded on to a glutathione–Sepharose 4B column (Pharmacia). The column

was washed with PBS, and the overexpressed protein, which was bound to the affinity column, was eluted with 10 mM GSH in 50 mM Tris/HCl buffer, pH 8.0. The OaGST fractions were dialysed and concentrated using Centriprep-10 (Amicon) with 10 mM potassium phosphate buffer (pH 7.0) and stored at −80 °C.

Mutagenesis of the *OaGST* gene

The plasmid pT-OaGST, carrying the *OaGST* gene, was used as a template for mutagenesis using the QuickChange Site-Directed Mutagenesis Kit (Stratagene). The two mutagenic primers containing the mutation (shown in bold type) for the replacement of Ser-11 with an alanine residue (S11A) were as follows: 5′-GT-GGGGTGCGAGCG**C**ACAAGCGCCGACC-3′ and 5′-GG-TCGGCGCTTGT**G**CGCTCGCACCCCAC-3′. Both primers annealed to the same target sequence on opposite strands of pT-OaGST. The site-directed mutagenesis was performed as described by the manufacturer. The clone used for production of the S11A mutant was confirmed by DNA sequencing. *E. coli* XL1-Blue cells transformed by the pT-OaGST mutant were induced by IPTG under the same conditions as for cells transformed by pT-OaGST wild-type. The crude extracts were loaded on GSH–Sepharose 4B (Pharmacia) and GSH–Sepharose 6B (Sigma) columns.

Enzyme assay and kinetic studies

GST activity towards CDNB (Aldrich), 1,2-dichloro-4-nitrobenzene (Fluka), *p*-nitrobenzyl chloride (Aldrich), ethacrynic acid (Sigma), 1,2-epoxy-3-(*p*-nitrophenoxy)propane (Eastman Kodak), Δ^5-androstene-3,17-dione (Sigma), *trans*-4-phenyl-3-buten-2-one (Aldrich), *p*-nitrophenyl acetate and bromosulphthalein (Serva) was assayed at 30 °C as described by Habig and Jakoby [34]. The selenium-independent GSH peroxidase activity of GST was measured with cumene hydroperoxide (Sigma), as previously reported [35]. GST activity towards 4-nitroquinoline 1-oxide (Fluka) was measured as reported by Stanley and Benson [36]. The inhibition of OaGST was determined by using different concentrations of each antibiotic. The IC_{50} value represents the concentration of drug causing 50 % inhibition of GST activity. The inhibition of OaGST was determined using herbicides under the same conditions.

Binding studies with 2-*p*-toluidinylnaphthalene-6-sulphonate (TNS; Sigma) were performed with a Spex spectrofluorimeter (model Floromax) equipped with a temperature-controlled cell compartment maintained at 25 °C, according to procedures previously described [37,38].

RESULTS

Molecular cloning and sequencing of the *OaGST* gene

The N-terminal amino acid sequence of OaGST has been reported previously [31]. An internal peptide, P1, was purified from a tryptic digestion and sequenced (Table 1). On the basis of the N-terminal and P1 sequences, two degenerate oligonucleotides were designed; by PCR, using genomic DNA from *O. anthropi*, a 230 bp DNA fragment was obtained. Southern blotting with a PCR fragment probe revealed a 5.0 kbp band in the hybridization pattern of the genomic DNA digested with *Eco*RI. By using the PCR fragment probe, a single positive clone was obtained from an *O. anthropi* genomic partial library. Figure 1 shows the restriction map of the 5.0 kbp fragment present in the positive clone, pB-OaGST, and the strategy for sequencing. The open reading frame of 603 bp and a Shine–Dalgarno sequence upstream from the initiation codon are shown in Figure 2. The deduced amino acid sequence contains the N-terminal and P1 sequences determined from the purified enzyme. The calculated molecular mass of the encoded protein was 21738 Da, which is compatible with the molecular mass (24000 Da) of the purified protein estimated by SDS/PAGE [31].

Table 1 Amino acid sequences from purified GST from *O. anthropi*

The N-terminal sequence was reported previously [31], and the internal sequence (P1) was obtained by tryptic digestion.

Peptide	Amino acid sequence
N-terminal	MKLYYKVGACSLAPHIILSEAGLPY
P1	GAVPALEVKPGTVITQNAAILQYIG

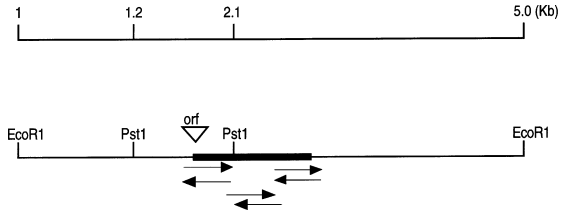

Figure 1 Restriction map of the 5.0 kb fragment and summary of the sequencing strategy

The arrows indicate the direction of sequencing. The solid bar in the *Eco*RI fragment indicates the open reading frame (orf).

Expression and characterization of OaGST

In *O. anthropi*, GST is present in very low amounts [31], setting a limit to the study of the physical/chemical properties of this enzyme. Therefore the *OaGST* gene was amplified by PCR from pB-OaGST and cloned into an expression vector. Induced *E. coli* XL1-Blue cells bearing pT-OaGST overexpressed a protein with the same electrophoretic mobility as the native enzyme on SDS/PAGE (Figure 3). The relative molecular mass of recombinant OaGST was estimated by size-exclusion chromatography to be about 45000 Da (results not shown), thus confirming the dimeric structure of this enzyme. A similar value was obtained with other bacterial GSTs [16,17].

The yield of cloned OaGST was about 100 mg per litre of culture. Substrate specificities toward several compounds are summarized in Table 2. Similar to other GSTs of bacterial origin, CDNB was the preferred substrate of OaGST, and cumene hydroperoxide was the second best substrate. Relatively low activity was detected with *p*-nitrobenzyl chloride and other known substrates for mammalian GSTs. The kinetic parameters for CDNB, cumene hydroperoxide and *p*-nitrobenzyl chloride are reported in Table 3.

TNS has been used previously to investigate the nature of the hydrophobic binding site of mammalian GSTs. The results presented in Figure 4 indicate that TNS binds OaGST in a positive co-operative manner (Hill coefficient of 1.4), with a K_d value of 114 μM.

OaGST was tested for sensitivity *in vitro* to the action of several classes of antibiotics. It was found that tetracycline and rifamycin were the strongest inhibitors, having IC_{50} values of

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  1  ATCGAAACTGATGGGAGATTTTT ATG AAG CTT TAC TAC AAG GTC GGC GCT TGT TCG CTC   59
  1                 SD       Met Lys Leu Tyr Tyr Lys Val Gly Ala Cys Ser Leu   12

 60  GCA CCC CAC ATC ATT CTG AGC GAG GCG GGC CTG CCT TAT GAG CTG GAG GCC GTG  113
 13  Ala Pro His Ile Ile Leu Ser Glu Ala Gly Leu Pro Tyr Glu Leu Glu Ala Val   30

114  GAT CTC AAG GCC AAG AAG ACA GCG GAC GGT GGC GAT TAT TTC GCA GTC AAT CCG  167
 31  Asp Leu Lys Ala Lys Lys Thr Ala Asp Gly Gly Asp Tyr Phe Ala Val Asn Pro   48

168  CGC GGT GCG GTC CCG GCG CTG GAA GTG AAG CCC GGC ACT GTC ATC ACG CAG AAT  221
 49  Arg Gly Ala Val Pro Ala Leu Glu Val Lys Pro Gly Thr Val Ile Thr Gln Asn   66

222  GCG GCA ATT CTC CAA TAT ATC GGT GAT CAT TCC GAT GTT GCA GCA TTC AAG CCC  275
 67  Ala Ala Ile Leu Gln Tyr Ile Gly Asp His Ser Asp Val Ala Ala Phe Lys Pro   84

276  GCC TAT GGT TCA ATC GAA CGC GCA CGC CTG CAG GAA GCG TTG GGC TTC TGT TCG  329
 85  Ala Tyr Gly Ser Ile Glu Arg Ala Arg Leu Gln Glu Ala Leu Gly Phe Cys Ser  102

330  GAT TTG CAT GCG GCC TTT AGC GGC CTG TTC GCG CCC AAC CTG AGC GAG GAA GCG  383
103  Asp Leu His Ala Ala Phe Ser Gly Leu Phe Ala Pro Asn Leu Ser Glu Glu Ala  120

384  AGG GCT GGC GTC ATC GCC AAC ATC AAT CGT CGT CTG GGT CAG CTC GAA GCC ATG  437
121  Arg Ala Gly Val Ile Ala Asn Ile Asn Arg Arg Leu Gly Gln Leu Glu Ala Met  138

438  CTG TCG GAC AAG AAC GCC TAC TGG CTT GGC GAT GAC TTC ACT CAA CCA GAT GCC  491
139  Leu Ser Asp Lys Asn Ala Tyr Trp Leu Gly Asp Asp Phe Thr Gln Pro Asp Ala  156

492  TAT GCG TCG GTG ATC ATC GGT TGG GGC GTT GGT CAA AAG CTC GAT TTG AGC GCC  545
157  Tyr Ala Ser Val Ile Ile Gly Trp Gly Val Gly Gln Lys Leu Asp Leu Ser Ala  174

546  TAT CCC AAG GCG CTG AAA CTG CGC GAA CGT GTG CTG GCC CGC CCG AAC GTG CAG  599
175  Tyr Pro Lys Ala Leu Lys Leu Arg Glu Arg Val Leu Ala Arg Pro Asn Val Gln  192

600  AAG GCA TTC AAG GAA GAA GGC CTG AAC TAA AAATACAGCCGGAGGTCCAAACCTCCGGCTG  660
193  Lys Ala Phe Lys Glu Glu Gly Leu Asn stop                                 201
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Figure 2 Nucleotide sequence and deduced amino acid sequence of OaGST

The amino acid sequence and residue numbers are given in italics. The underlined sequences perfectly match those determined by amino acid sequencing of the purified GST. The Shine–Dalgarno sequence (SD) is double-underlined.

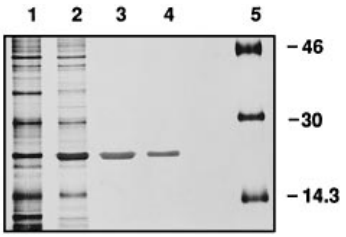

Figure 3 SDS/PAGE analysis of GST from *O. anthropi*

Lane 1, total cellular extracts of *E. coli* XL1-Blue (pT-OaGST) before induction; lane 2, same as lane 1 but after induction; lane 3, affinity-purified cloned OaGST; lane 4, native-purified GST from *O. anthropi*; lane 5, molecular mass standards (from top to bottom): ovalbumin (46 kDa), carbonic anhydrase (30 kDa) and lysozyme (14.3 kDa).

118 μM and 230 μM respectively. No inhibition was seen with ampicillin, streptomycin or kanamycin when used at concentrations up to 1 mM. Moreover, at a concentration of 1 mM, the following pesticides had no inhibitory effect on OaGST: atrazine, captan, captafol and dichlorofen.

Primary structure comparison

Comparison of the deduced primary structure of OaGST with those of proteins present in the GenBank Data Base indicated that the greatest similarity was with bacterial GSTs (Figure 5). The highest score of 45% identity was found with *Pseudomonas* LB400 GST. Multiple alignments of the deduced amino acid sequences of OaGST and other GSTs showed many conserved amino acids, including Ser-11.

Table 2 Substrate specificity of OaGST

The values shown are means ± S.D., calculated from at least three replicates. ND, not detectable.

Substrate	Specific activity (μmol/min per mg)
CDNB	5.6 ± 0.138
1,2-Dichloro-4-nitrobenzene	0.003 ± 0.0001
Ethacrynic acid	0.015 ± 0.0008
Cumene hydroperoxide	0.45 ± 0.010
p-Nitrobenzyl chloride	0.15 ± 0.005
1,2-Epoxy-3-(*p*-nitrophenoxy)propane	0.007 ± 0.0002
trans-4-Phenyl-3-buten-2-one	0.001 ± 0.0001
Δ^5-Androstene-3,17-dione	0.003 ± 0.0002
p-Nitrophenyl acetate	0.002 ± 0.0001
Bromosulphothalein	ND
4-Nitroquinoline 1-oxide	0.019 ± 0.0009
trans-2-Nonenal	ND

Site-directed mutagenesis

To investigate whether the conserved Ser-11 residue is important for catalysis, a mutated *OaGST* gene was constructed. Ser-11 was replaced with an alanine residue by site-directed mutagenesis. The cytosolic fractions containing either wild-type OaGST or the mutant enzyme were examined by immunoblot analysis using an antiserum raised against purified GST from *O. anthropi* [31]. As shown in Figure 6, the mutant OaGST co-migrated with the wild-type protein, with comparable levels of expression.

Table 3 Kinetic parameters for OaGST determined with CDNB, cumene hydroperoxide (CuOOH) and *p*-nitrobenzyl chloride (*p*NBC)

K_m (mM)		V_{max} (μmol/min per mg)		k_{cat} (min^{-1})		k_{cat}/K_m ($mM^{-1} \cdot min^{-1}$)	
GSH	CDNB	GSH	CDNB	GSH	CDNB	GSH	CDNB
0.32 ± 0.02	2.7 ± 0.21	5.19 ± 0.45	20.6 ± 1.74	249 ± 30	992 ± 90	778	367
GSH	CuOOH	GSH	CuOOH	GSH	CuOOH	GSH	CuOOH
0.065 ± 0.006	1.32 ± 0.09	0.63 ± 0.04	1.66 ± 0.09	30.2 ± 2.5	79.6 ± 8.3	464	60
GSH	*p*NBC	GSH	*p*NBC	GSH	*p*NBC	GSH	*p*NBC
0.11 ± 0.08	0.91 ± 0.07	0.14 ± 0.09	0.27 ± 0.02	6.86 ± 0.52	13.3 ± 0.98	62	14

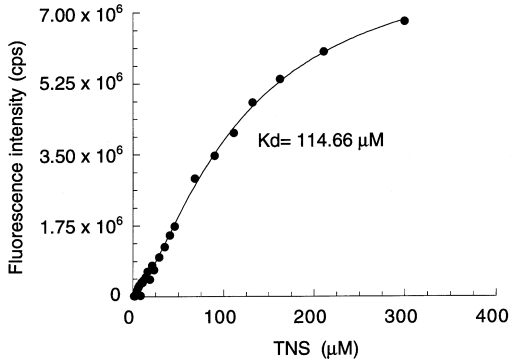

Figure 4 Binding of TNS to OaGST

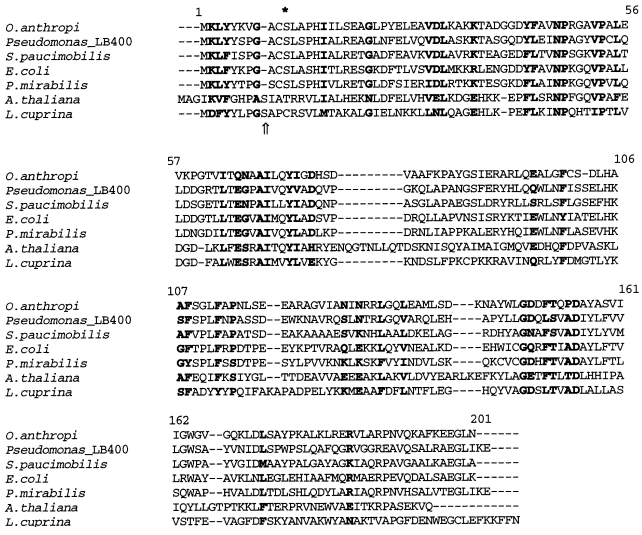

Figure 5 Alignment of *O. anthropi* GST, other bacterial GSTs and eukaryotic Theta-class GSTs

Sequences were obtained with the following accession numbers: bacterial GST sequences from *Pseudomonas* LB400 (EMBL gene database entry X76500), *S. paucimobilis* (EMBL gene database entry AF001779), *E. coli* (P39100; GT_ECOLI) and *P. mirabilis* (P15214; GT_PROMI). Eukaryotic Theta-class GST sequences are from *A. thaliana* (P46422; GTH4_ARATH) and *L. cuprina* (P42860; GTT1_LUCCU). Invariant or conservatively replaced residues in the GST sequences are indicated in boldface. The numbering of the *O. anthropi* GST is indicated. The asterisk indicates the conserved serine residue in bacterial GSTs; the arrow indicates the 'catalytic serine' in Theta-class GSTs.

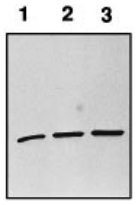

Figure 6 Immunochemical analysis of the cytosolic fraction of wild-type and mutant OaGSTs

The cytosolic fractions from individual cultures harbouring the wild-type and mutant enzymes were immunoblotted with antiserum raised against native GST from *O. anthropi*. Lane 1, affinity-purified cloned OaGST; lane 2, total cellular extract from *E. coli* XL1-Blue harbouring OaGST wild type; lane 3, total cellular extracts of *E. coli* XL1-Blue harbouring mutant OaGST.

Table 4 Comparison of specific activities of wild-type and mutant OaGST crude extracts

Values are means ± S.D. calculated from at least three replicates.

Substrate	Specific activity (μmol/min per mg)	
	OaGST wild type	OaGST mutant
CDNB	0.0901 ± 0.0018	0.0029 ± 0.0002
Cumene hydroperoxide	0.0108 ± 0.0020	0.0100 ± 0.0015
p-Nitrobenzyl chloride	0.0016 ± 0.0001	0.0016 ± 0.0001

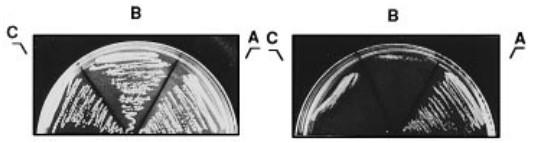

Figure 7 Activity of wild-type and mutant OaGSTs *in vivo*

Bacteria (*E. coli* XL1-Blue) expressing wild-type or mutant enzymes are indicated by different letters: A, pTac vector alone; B, pT-OaGST wild type; C, pT-OaGST mutant. On the left, one loopful of overnight culture was evenly streaked on to an LB agar plate containing 50 μg/ml ampicillin to serve as a control for normal growth. On the right, another loopful of the same culture was streaked on to an LB agar plate containing 50 μg/ml ampicillin and 20 μg/ml CDNB.

Specific activities of the wild-type and mutant OaGSTs towards CDNB and several substrates were tested in the crude extracts (Table 4). The replacement of Ser-11 with alanine resulted in a marked decrease in enzyme activity with CDNB, whereas no difference was observed with cumene hydroperoxide or *p*-nitrobenzyl chloride. Furthermore, the mutant OaGST was unable to bind glutathione–Sepharose 4B and 6B affinity matrices, and

only a very small amount of enzyme was retained in the column; neither the eluant nor the unbound material showed any CDNB-conjugating activity (results not shown).

Analysis of wild-type and mutant OaGST activity *in vivo*

OaGST activity *in vivo* was tested using the method of Lee et al. The molecular basis of this test relies on the fact that CDNB, the common substrate for GSTs, has antibiotic activity [39]. This activity is enhanced when *E. coli* expresses a functional GST [40].

E. coli colonies carrying either wild-type or mutant pT-OaGST were grown overnight in LB medium supplemented with 50 μg/ml ampicillin. A loopful of bacterial colony was evenly streaked across a fixed area on agar plates containing 20 μg/ml CDNB. The results shown in Figure 7 indicate that, in the presence of CDNB, the growth of *E. coli* transformed with wild-type OaGST was inhibited, whereas *E. coli* carrying the OaGST mutant showed active growth. These results confirm the low activity of the OaGST mutant measured *in vitro*. This activity could be comparable with the plating efficiency observed *in vivo*.

DISCUSSION

We have previously purified a GST from *O. anthropi* [31], a bacterium isolated from soil contaminated by pesticides [32]. The yield of purified protein was very low, setting a limit to the complete characterization of the enzyme. In this paper, we describe the molecular cloning, overexpression and site-directed mutagenesis of a novel GST from *O. anthropi*.

The structural *OaGST* gene was isolated from the genomic DNA of *O. anthropi*. The amino acid sequence, deduced from the gene structure, contained N-terminal and internal sequences that perfectly matched the sequences deduced from the purified GST. Moreover, native and recombinant OaGSTs showed equivalent molecular masses on SDS/PAGE.

The yield of cloned OaGST was about 100 mg per litre of culture, allowing the characterization of the enzyme. It has been shown that CDNB has an antibiotic activity on *E. coli* [39]. Furthermore, experiments performed with *E. coli* DH5α transformed with an expression vector containing human GST in the presence of CDNB showed increased growth inhibition [40]. To assess whether our GST behaved similarly, *E. coli* XL1-Blue expressing OaGST were grown in plates containing CDNB. In our case, the bacteria showed a very low plating efficiency, confirming the OaGST activity *in vivo*.

Several GST-family marker substrates were tested. OaGST showed the highest activity towards CDNB, the most highly reactive substrate of mammalian GSTs [1]. Detectable activity was also measured with other substrates. Futhermore, OaGST binds TNS with a K_d higher than that found for mammalian GSTs, suggesting that the hydrophobic binding sites of mammalian and bacterial GSTs are markedly different in structure and function. A possible binding role for bacterial GSTs in antibiotic resistance has been suggested [13–15]. GSTB1-1 from *P. mirabilis* is able to avidly sequester antibiotics, in particular tetracycline, minocycline and rifamycin [41]. Similar binding properties have also been found for OaGST.

It is suggested that bacterial GSTs could be involved in the degradation of herbicides, in view of the involvement of plant GSTs in the conjugation of herbicides to GSH [42–44]. GST-encoding genes were found in the degradation pathway of biphenyl/polychlorobiphenyl in *Pseudomonas* sp. [20], in the aromatic hydrocarbon degradation pathway in *Cyclocasticus oligotrophus* RB1 [45], and in the degradation pathway of fluoranthene in *Sphingomonas paucimobilis* EPA505 [19]. Although *O. anthropi* is a micro-organism that is able to grow on agar plates containing atrazine as the only source of carbon [32], atrazine and other herbicides did not inhibit OaGST conjugation activity *in vitro*. This suggests that, if a GST is involved in the enzymic pathway for the degradation of this pesticide, it may be involved in the detoxification of atrazine metabolites.

An important aspect of the study of GSTs is to understand the mechanisms involved in catalysis. The GST amino acid sequences in the literature provide the database for identifying highly conserved residues considered to be important in catalysis. The deduced amino acid sequence of OaGST was compared with those of GSTs from other species, and showed greatest identity with *Pseudomonas* LB400 GST (45%) and a low identity with non-bacterial GSTs (about 20%). Nishida et al. [16] reported that many amino acid residues important in the structure and function of the mammalian enzymes are highly conserved in eukaryotic and bacterial GSTs. These amino acids are also conserved in OaGST; this therefore supports the hypothesis that bacterial GSTs and eukaryotic GSTs diverged from the same ancestral gene.

Concerning catalytic mechanism, it has been demonstrated that, in the Alpha, Mu and Pi GST classes, the conserved tyrosine residue in the N-terminal portion is essential for catalysis [25–27] and has been proposed to activate the bound GSH by stabilizing its thiolate form. The conserved tyrosine residue, also present in some bacterial GSTs, was mutated in *E. coli* GST by site-directed mutagenesis [16]. That study demonstrated that this tyrosine residue was not essential for catalysis. A recent study carried out on *P. mirabilis* GST demonstrated that neither tyrosine nor serine residues present in the N-terminus of the enzyme are implicated in catalysis [28].

In a previous paper, we proposed that serine residues, highly conserved in the N-terminal sequence of bacterial GSTs, could be a good candidate for involvement in catalysis [31]. To confirm this hypothesis, we prepared an OaGST mutant obtained by replacing Ser-11 with alanine. This mutation led to a marked decrease in GSH-conjugating activity with CDNB, thus confirming the importance of Ser-11 in this enzyme-catalysed reaction. It is interesting to note that *E. coli* carrying the mutated OaGST, when cultivated in the presence of CDNB, showed less growth inhibition compared with *E. coli* carrying wild-type OaGST. This effect, as reported by Lee et al. [40], could be related to the low GST-conjugating activity observed *in vitro*. It has been reported that GSH metabolism is involved in the enhancement of growth inhibition by the presence of an active heterologous GST [40]. Although the enzyme activity of mutated OaGST was only 3% of wild type, *E. coli* bearing mutated OaGST, in the presence of CDNB, also showed inhibited plating efficiency.

Thus, from a catalytic point of view, OaGST is different from other bacterial GSTs, and much more similar to the eukaryotic 'serine-dependent' Theta GST class. It has been reported that the serine residue in human GSTT2-2 is important for reactivity with some substrates [46]; we tested the specific activity of the OaGST mutant with cumene hydroperoxide and *p*-nitrobenzyl chloride, obtaining values similar to those found using wild-type OaGST. The role performed by Ser-11 in catalysis by this bacterial GST seems to be important only for some particular second substrates. These results suggest that the enzyme structure may be modified by the second substrate used, thus underlining that other amino acids could be involved in catalysis.

In conclusion, elucidation of the three-dimensional structure of OaGST by X-ray crystallography will be essential in order to better understand how this enzyme interacts with and activates GSH.

This work was partially supported by the Regione Abruzzo (P.O.M. 1994/1996, Sottoprogramma 3; Misura 3.1, Ricerca e Sperimentazione; A.R.S.S.A.). We thank M. P. De Simone for corrections to the English, A. Piccoli for gel filtration analyses, and R. Bertazzi, P. Di Nardo and the Gustavus A. Pfeiffer Memorial Library staff for their valuable contributions in editing the manuscript before submission.

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Received 16 June 1998/30 July 1998; accepted 1 September 1998