Molecular cloning and overexpression of a glutathione transferase gene from *Proteus mirabilis*

Brunella PERITO*†, Nerino ALLOCATI‡, Enrico CASALONE*†, Michele MASULLI‡, Beatrice DRAGANI†, Mario POLSINELLI*, Antonio ACETO† and Carmine DI ILIO†§

*Dipartimento di Biologia Animale e Genetica, Università di Firenze, via Romana 17, I-50125 Firenze, Italy, and Istituti di †Scienze Biochimiche e ‡Medicina Sperimentale, Università 'G. D'Annunzio', via dei Vestini, 31, I-66100 Chieti, Italy

The structural gene of the *Proteus mirabilis* glutathione transferase GSTB1-1 (*gstB*) has been isolated from genomic DNA. A nucleotide sequence determination of *gstB* predicted a translational product of 203 amino acid residues, perfectly matching the sequence of the previously purified protein [Mignogna, Allocati, Aceto, Piccolomini, Di Ilio, Barra and Martini (1993) Eur. J. Biochem. **211**, 421–425]. The *P. mirabilis* GST sequence revealed 56% identity with the *Escherichia coli* GST at DNA level and 54% amino acid identity. Similarity has been revealed also with the translation products of the recently cloned gene *bphH* from *Haemophilus influenzae* (28% identity) and ORF3 of *Burkholderia cepacia* (27% identity). Putative promoter sequences with high similarity to the *E. coli* σ^{70} consensus promoter

and to promoters of *P. mirabilis* *cat* and *glnA* genes preceded the ATG of the *gstB* open reading frame (ORF). *gstB* was brought under control of the *tac* promoter and overexpressed in *E. coli* by induction with isopropyl- β -D-thiogalactopyranoside and growth at 37 °C. The physicochemical and catalytic properties of overexpressed protein were indistinguishable from those of the enzyme purified from *P. mirabilis* extract. Unlike the GST belonging to Mu and Theta classes, GSTB1-1 was unable to metabolize dichloromethane. The study of the interaction of cloned GSTB1-1 with a number of antibiotics indicates that this enzyme actively participates in the binding of tetracyclines and rifamycin.

INTRODUCTION

Glutathione transferases (GSTs; EC 2.5.1.18) are a family of multifunctional dimeric proteins that catalyse the conjugation of the sulphur atom of glutathione to a large variety of electrophilic compounds of both endobiotic and xenobiotic origin [1–4]. GSTs are also involved in the intracellular binding and transport of hydrophobic compounds, participate in the synthesis of prostaglandins and leukotrienes, and might play a key role in the elimination of organic hydroperoxides [1–3]. Most cytosolic GSTs so far characterized have been classified into at least five classes: Alpha, Mu, Pi, Theta and Sigma [5–7]. GSTs have also been isolated from micro-organisms [8–14], but little is known about their biological functions, structures and regulation. In our laboratory we have previously purified a GST from *Proteus mirabilis* (GSTB1-1) that did not react with antisera against mammalian GST [9]. The complete amino acid sequence of GSTB1-1 has been recently resolved, indicating low sequence similarity to mammalian and plant GST [15]. However, despite their low sequence similarity, limited proteolysis studies [16] have indicated a common three-dimensional organization to mammalian and bacterial GSTs. By immunolabelling techniques we found that the GSTB1-1 content in the periplasmic space is much higher than in the cytoplasm [17]. By assuming that GSTB1-1 has a role in detoxification, its predominantly periplasmic location could represent a boundary against the toxic effect of xenobiotics, including antibiotics [18]. In *P. mirabilis*, as in other bacteria, GSTB1-1 is present in very low amounts, about 0.06% of the total protein extract [9,18], setting a limit to the study of the physico-chemical properties of this enzyme. Thus the availability of a system for high-yield heterologous expression is

needed. Such a system is also necessary to clarify the role of bacterial GST in the detoxification of endobiotics as well as xenobiotics. Of particular interest is the proposed role of bacterial GST in the binding of antibiotics [18].

Here we describe the molecular cloning and overexpression of GSTB1-1 in *Escherichia coli* to yield high levels of the active enzyme. In addition, to understand better the relationship of bacterial GST with antimicrobial agents, the interactions of cloned GSTB1-1 with several classes of antibiotics have also been investigated.

MATERIALS AND METHODS

Bacterial strains, plasmids and culture conditions

To clone and express GSTB1-1 the *Escherichia coli* XL1-Blue strain was used [19]. It was grown on Luria–Bertani (LB) medium [20], supplemented with tetracycline (12.5 μ g/ml) and ampicillin (50 μ g/ml) when appropriate. pBluescript II KS+ (Stratagene, La Jolla, CA, U.S.A.) and pBTac1 (Boehringer Mannheim, Germany) were used as cloning and expression vectors respectively.

Chemicals

Isopropyl- β -D-thiogalactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside, as well as all antibiotics used in the present work, were purchased from Sigma–Aldrich (Milan, Italy). All other reagents used were of the highest grade commercially available.

Abbreviations used: CDNB, 1-chloro-2,4-dinitrobenzene; GSH, glutathione; GST, glutathione transferase; IPTG, isopropyl- β -D-thiogalactopyranoside; LB, Luria–Bertani; MIC, minimum inhibitory concentration; ORF, open reading frame.

§ To whom correspondence should be addressed.

The nucleotide sequence reported will appear in DDBJ, EMBL and GenBank Nucleotide Sequence Databases under the accession number U38482.

DNA techniques

Genomic DNA isolation, transformation and other cloning procedures were done by standard techniques [20]. Preparative amounts of plasmid DNA were obtained by using Qiagen columns (Qiagen Inc., Chatsworth, CA, U.S.A.). Transformations and other cloning procedures were done by standard techniques [20]. Restriction enzymes were obtained from Boehringer and digestions were performed as recommended by the manufacturers. DNA amplification was performed by PCR by using GeneAmp PCR Reagent Kit with AmpliTaq DNA Polymerase or UITma DNA Polymerase (Perkin Elmer Cetus, Norwalk, CT, U.S.A.). Southern hybridization was performed with Hybond-N membrane filters (Amersham, Bucks, U.K.), the non-radioactive DIG DNA labelling kit and the DIG Luminescent Detection kit (Boehringer) in accordance with the suppliers' instructions. DNA sequences were determined by the dideoxy-chain termination method [21] from double-stranded DNA templates, with [³²S]dATP (Amersham) and Sequenase Version 2.0 DNA Sequencing Kit (U.S. Biochemical Corp., Cleveland, OH, U.S.A.). Either commercial primers or primers designed for the sequence were used.

Sequence analysis

Nucleic acid sequences were analysed by using the MacVector program, version 4.1.4 (IBI, New Haven, CT, U.S.A.).

Enzyme purification

To induce the *gstB* gene transcription, IPTG was added to a final concentration of 1 mM when *E. coli* strains, grown at 37 °C in LB medium supplemented with tetracycline and ampicillin, reached an approximate A_{550} of 0.4, and the incubation was prolonged for a further 4 h. The cells were collected by centrifugation, washed twice and resuspended in 10 mM potassium phosphate buffer, pH 7.0, containing 1 mM EDTA (buffer A), and disrupted by cold sonication (five bursts of 3 min each at 300 W) with a Labsonic 1510 (Braun) sonicator. The particulate material was removed by centrifugation at 105 000 *g* for 1 h at 4 °C and the supernatant loaded on a glutathione-Sepharose affinity column [22] that had been pre-equilibrated with buffer A. The column was washed with buffer A supplemented with 50 mM KCl. The enzyme was eluted with 50 mM Tris/HCl buffer, pH 9.6, supplemented with 5 mM glutathione (GSH). The fractions containing GST activity were pooled, concentrated and dialysed by ultrafiltration and used for further studies.

Analytical procedures

SDS/PAGE in discontinuous slab gel was performed by the method of Laemmli [23]. The SDS concentration was 0.1 % and the spacer and the separating gels contained 3 % and 12.5 % (w/v) acrylamide respectively. Western blots were made essentially by the protocol described by Towbin et al. [24] with a slight modification as described previously [18]. Isoelectric focusing was performed in accordance with the manufacturer's instructions on a 5 % (w/v) polyacrylamide horizontal slab gel with 2.2 % (w/v) Ampholine, pH 3.5–9.5 (Ampholine PAG plate; Pharmacia). HPLC analysis was performed by the method described by Ostlund-Farrants et al. [25] with a Vydak 201 TP5 column (Crompack, Middelburg, The Netherlands) equilibrated with 30 % (v/v) acetonitrile in 0.1 % (v/v) trifluoroacetic acid. The column was developed at 1 ml/min by a 60 min linear gradient from 30 % to 60 % acetonitrile in 0.1 % trifluoroacetic acid. Protein contents were determined by the method of Bradford [26] with γ -globulin as standard.

Enzyme assay

GST activity towards 1-chloro-2,4-dinitrobenzene (CDNB), 1,2-dichloro-4-nitrobenzene, ethacrynic acid, *p*-nitrobenzyl chloride, 1,2-epoxy-3-(*p*-nitrophenoxy)propane, *trans*-4-phenyl-3-buten-2-one, Δ^5 -androstene 3,17-dione, *p*-nitrophenyl acetate and bromosulphthalein was assayed at 30 °C by the methods of Habig and Jakoby [27]. The selenium-independent glutathione peroxidase activity of GST was measured with cumene hydroperoxide as previously reported [28]. GST activity towards 4-nitroquinoline 1-oxide was measured as reported by Stanley and Benson [29]. Dichloromethane dehalogenase activity was determined as described by Staub and Leisinger [30].

Inhibition studies

The effects of antibiotics as inhibitors were measured by using 1 mM CDNB and 1 mM GSH in 10 mM potassium phosphate buffer, pH 6.5, plus 1 mM EDTA. Stock solutions of antibiotics were freshly prepared immediately before use by the method of Anhalt and Washington [31]. The enzyme was preincubated with antibiotics for at least 1 min before the addition of substrates. The inhibition of GSTB1-1 was determined by using at least ten different concentrations of each antibiotic. The IC_{50} value represents the concentration of drug causing 50 % inhibition of activity.

For the enzyme kinetic determinations either CDNB or GSH was held constant at 1 and 5 mM respectively, while the concentration of the other substrate varied (from 0.1 to 5 mM for GSH and from 0.1 to 1.6 mM for CDNB). To study the nature of antibiotic inhibition the kinetic parameters of GSTB1-1 were also determined in the presence of the drugs. In all cases the data fitted well to the equation for a rectangular hyperbola. Fitting was carried out with the computer program ENZFITTER based on an iterative Gauss–Newton procedure [32].

Growth curve

A single colony of *E. coli* XL1Blue (pGPT1) was inoculated into LB medium and grown overnight in a water bath shaker. LB medium (150 ml) containing 3 ml of overnight culture and 1 mM IPTG was incubated at 37 °C in a water bath shaker, monitoring the absorbance at 600 nm. When an absorbance of 0.250 was reached, the cells were exposed to one-eighth, one-quarter and one-half of the minimum inhibitory concentration (MIC) of antibiotic. A sample without IPTG was used as control. MIC values of 50, 100 and 3.2 μ g/ml, found for rifamycin, tetracycline and minocycline respectively, were determined by a standard broth microdilution technique [33].

RESULTS AND DISCUSSION

Cloning and nucleotide sequence of the *P. mirabilis* *gstB* gene

On the basis of the amino acid sequence of GSTB1-1 [15] two degenerate oligonucleotides were designed: a 25-mer corresponding to the N-terminal amino acid residues 1–6, 5'-GCGAATTC ATG AA(A/G) (C/T)TI TA(C/T) TA(C/T) AC-3' (primer 1), and a 29-mer corresponding to the C-terminal residues 198–203 with an additional stop codon, 5'-GCGAATTC TTA (C/T)TC (C/T)TT IAT IA(A/G) ICC (C/T)TC-3' (primer 2). Both primers had a 5' recognizing site for the restriction enzyme *Eco*RI (underlined) and a CG tail to facilitate the cloning procedure. PCR was performed with the two oligonucleotides to amplify a fragment from *P. mirabilis* genomic DNA corresponding to the entire *gstB* coding region. A PCR product of

>ORF 1
 1 AAGCTTCTCTAGAGCAACAGCTGATTTATGCAATGCTATGGTCTGAAGGTATTCGTGTAAAGGATCTCTGAGGTCCAAATC
 91 GTACCTTAGCAGCATCTGGTATCAAGATTTCCTGAAAATGTTGGACCATGCGAATCTGTACCCCTTTAGCTGACAGTCACTACTG
 181 AAGATGGGTAAATCTGCACTGCTTCTTATGCTCTGACTTCTGCGGGATCACCGTGAAATCACTCAGCTAGACGGTGGTTTAGCA
 271 TTGACGCAATGAATGAATGAGTGGAAATTAATTTCCCTTTATTCATTATCTTCTGAAACCTCGAATATCTCTGGAGTTTCTT
 361 TTTTCAATGAATGATGATCTGTCACAGATTACAAAAGCTTATTTATTTCCAGATTGCGTTTGTCAAACTTTATAAGCCCTTAT
 451 TATCTAATATTAGTGGGGTATTTCTTATCTTCAGGGTATCACCACATCATGATAGGAGTATTTAAGAGTAAATTTGCTACACGCC
 541 GGTAGTGTCTGCTGCTTCTCTCATATGTTTACGTGAAACGGTCTGACTTTCTTATTAGCGCATGATTATAGCACCAAAAAAC
 631 AGAGTCAGGAAAGATTTTCTGCAATCAACCTTAAGGCAAGTCCGGTCTTCAATAGATTAATGTTGATATTTAAACGGAGGTGT
 721 TGCTATTGTGAGTATCTTGGCAGTCTGAAGCAGATGAAATCTTATGCCCACCAAAAGCATAGAACGTTATCATCAAAATGAGTG
 811 GCTAACTTTCTTGGCAGTGAAGTCTAATAAGGCTACAGCCCTTATTTTCACTGATAGCCGTGAAAGTTATCTCCTGTGTAATAA
 901 CAACTAAAAAGATTTTGTATTAATGATGATCTAGCAACAAAAATGTTTGTGATGATCACTTACTTACTGGCGGATGGCA
 991 TCTGTTTACGTTAAGTCAATGGGACCTCATGTGGCCTAGATTAACGACATTACAGCACTTACAGCACTGATTCACACA
 1081 AGTCTTAATGCTGATGCTAGCTAGTACGAGGATTAAGAGTAAAGTTCATATAAGGCGAGTCTCATTATAAATCAATAAAA
 1171 AGCGAAATTAATGACTTCCGCTTATATTAACATATGATTAATCTAATCGTGTGACAGAAATTTGGTGGTGCACATTTGAT
 1261 CTTGAGCGGACGAGTTGTACTCTGACTCATCTCTTTAGTTTAACTCACTCTATTAACAGCAGCAGCACTGCTTAATG
 1351 CGGTTTTAGCTCAACACTTTTGTAAATCCACGACATTAACCATGTTAGTATCCCGACCAACAGCTGCTGACAAAAAT
 1441 CAACAGCGTGGCTGACGCTGCACTGATCTGACGTCACGATCAATTTCAAGGATCATGACGAAGCGTCTGCTGATAAAT
 1531 GTTTTACTAATCAATTTTAGGCGCTTCTGCGCAATTAATGACGGCGCTTTACACATGCTCAACATATGACGCGTCTGACACAG
 1621 CTAATGTTCAAGTCTAATAGTTGGGTGATCAATATCACTGATGGCAAGGATCTTCAAAAGTACCGCAAAATTTCTGGAGGA
 1711 CAATCAACCTTTTTCAGGATGCTCCATAACCGGATCAGCAATGATGCTGATGATGATGATGATGATGATGATGATGATGATGATGAT
 1801 CAATGCTGGCGCTTCTGCTGCGGACGCAATTAACCACTTAATACGCTACACATGCTGATTAATTTACCAATGACGATCATCAA
 1891 CGATCTCAATGATGCTGCTGCGGACATCACACACCGCTCATTTTCTGATGATGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT
 1981 GGCCAAACATTTACCCCATACGGGATTTGGAATTCAGCAGNCGTATTACCTGCGCAAAACATCATGAGATGATGATGAGAGT
 2071 ATATTTTTCATGACATAACCGATTATTTGTAATGACACACACCCCAAGCTT

Figure 1 Nucleotide sequence of the 2123 bp *Hind* III fragment containing the *gstB* gene and its flanking regions of *Proteus mirabilis*

The predicted translation of *gstB* and the putative initiation and termination codons are given in bold. The putative ribosome binding site, the sequences similar to the *E. coli* σ^{70} and *Proteus* promoter sequences are underlined. Arrows indicate inverted repeats with perfect or imperfect dyad symmetry.

approx. 600 bp was obtained. It was eluted from agarose gel, digested with the restriction enzyme *Eco*RI and cloned in the unique *Eco*RI site of the *E. coli* vector pBluescript II KS+. The analysis of the nucleotide sequence of four recombinant plasmids revealed that they carried 609 bp fragments coding for 203 amino acids, agreeing with the size of the previously sequenced protein, but none of them coded a protein perfectly matching GSTB1-1. Conservative and non-conservative nucleotide substitutions were found in two regions corresponding to the annealing sites of the primers, as a foreseeable consequence of their degenerate nature, and also in other regions (at a rate of about 0.25%), presumably due to the use of an error-prone *Taq* polymerase in the PCR reaction. These latter results confirmed the inadequacy of *Taq* polymerases in obtaining a correct amplification product in polymerization reactions of few hundred nucleotides, as in our case.

To obtain the cloning of the correct sequence, the *Eco*RI fragment corresponding to the *gstB* gene was labelled and used to probe, by Southern hybridization, the *P. mirabilis* genomic DNA digested with different restriction enzymes. The *Hind*III digestion gave a unique hybridization band corresponding to a 2.1 kb fragment. Pooled fragments of a *Hind*III digestion of *P. mirabilis* genomic DNA between 1.8 and 2.4 kb in size were cloned in pBluescript II KS+. Screening of 300 recombinant *E. coli* XL1Blue clones by colony hybridization with the *gstB* probe led to the identification of two positive clones. The foreign fragments of the two recombinant plasmids were sequenced in their terminal regions, yielding identical results. The nucleotide sequence of the 2123 bp *Hind*III fragment in one of the two plasmids, referred to as pGPB1, is shown in Figure 1. Three putative coding regions with a size greater than 300 nt were

identified: the first, ORF1, at position 1–299 is the 3' segment of a truncated open reading frame (ORF), 75% identical with the 3' region of the *E. coli* *envM* gene [34]; the second, at position 521–1129, is *gstB*; the third, ORF2, is transcribed in the opposite direction from nt 1738 to nt 1214, its entire putative translation product being 41% identical with the C-terminal region of a hypothetical unidentified protein (GenBank accession number U11243-1) of *Haemophilus influenzae* [35].

The *gstB* starts with an ATG codon (nt 521), ends with a TAA codon (nt 1130) and is preceded by a potential ribosome binding site (Figure 1). The G+C content of *gstB* is 39.7 mol%, in agreement with the G+C content of *Proteus* [36]. Its predicted translational product of 203 amino acids matched the sequence of the purified protein exactly [15].

The search for similarities with promoting regions from enteric bacteria revealed: a hexamer at position 294 identical with the putative –35 sequence of the *P. mirabilis* *cat* gene [37]; two sequences similar in five out of six bases to the –10 putative region of the *P. vulgaris* *glnA* gene [38] at positions 309 and 314; and two hexamers, one at position 411 and the other at position 438, similar in five out of six bases to the –35 and –10 regions respectively of the *E. coli* σ^{70} consensus promoter [39]. Three regions with a thorough dyad symmetry are present downstream of the *gstB* TAA stop codon and could be involved in transcription termination. A structure with a similar function is downstream of the TAA of the putative ORF1. Two imperfect complementary inverted sequences were found internal to the *gstB* coding region, at positions 798–838 and 1084–1117.

The *P. mirabilis* *gstB* ORF product was 56% identical with the *E. coli* *gst* product [13], the only prokaryotic gene encoding a product with a canonical GST activity. The region between the *envM*-similar ORF1 and *gstB* did not show similarity to the region downstream of the *E. coli* *envM*, nor with the region upstream of the *E. coli* *gst* gene, consistent with the different map positions of *envM* and *gst* genes in *E. coli* [13,34]. However, the ORF2 C-terminal fragment (residues 137–174) of *P. mirabilis* was 63% identical with the translation of the 3' region downstream of the *E. coli* *gst* gene, running in the opposite direction, and nucleotides previously identified as a putative truncated ORF, indicating a similar organization of this region in both bacteria.

The similarity of GSTB1-1 to eukaryotic GSTs has been discussed previously [13,15,40,41]. The similarity of GSTB1-1 to bacterial GST and GST-like proteins has been reported by some authors [13,15,40,41]. During this work a further sequence analysis revealed a 28% identity of GSTB1-1 with the putative *bphH* gene from *H. influenzae* [35] and 27% identity with the ORF3 of *Burkholderia cepacia* involved in the degradation of 2,4,5-trichlorophenoxyacetic acid [42]. The identity in both cases increased to 35% if the N-terminal region up to position 80 was considered, as already observed by Mignogna et al. [15].

Expression and characterization of GSTB1-1 in *E. coli*

The *E. coli* XL1Blue (pGPB1) clone was tested for the production of recombinant GST. Immunoblot analysis revealed that the protein was expressed but quantification, after the affinity chromatography step, indicated a low production of enzyme. However, the recombinant GST was, by analytical procedures, identical with GSTB1-1 isolated from *P. mirabilis* [9]. With the aim of obtaining an *E. coli* clone overproducing the recombinant enzyme, *gstB* was amplified by PCR from pGPB1 plasmid by using primer 1 and primer 2 and the proofreading DNA polymerase UITma. The PCR products were cloned after *Eco*RI digestion in the *Eco*RI site of pBluescript and sequenced. Out of

Figure 2 The pGPT1 expression plasmid

Figure 3 SDS/PAGE analysis of total cellular extracts of *E. coli* XL1Blue (pGPT1)

Lane 1, standards and their molecular masses, from top to bottom [phosphorylase *b* (94 kDa), BSA (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa) and α -lactalbumin (14.4 kDa)]; lane 2, affinity-purified cloned GSTB1-1; lane 3, total cellular protein (80 μ g) before induction; lane 4, total cellular protein (80 μ g) after induction.

three amplified products, one showed a nucleotide sequence perfectly matching the amino acid sequence despite conservative nucleotide substitutions at positions 7 (T $\rightarrow$ C), 15 (C $\rightarrow$ T), 600 (A $\rightarrow$ G) and 603 (A $\rightarrow$ C) owing to primer degeneration. *gstB* was cloned in the *EcoRI* site of pBTac1 vector under the *tac* promoter, obtaining the pGPT1 plasmid (Figure 2). Induced *E. coli* XL1Blue cells bearing pGPT1 overexpressed a protein with an apparent subunit molecular mass of 22.5 kDa (Figure 3), which cross-reacted with the antisera raised against native GSTB1-1. The yield of cloned GSTB1-1 was about 45 mg per litre of culture, which is about 240-fold higher than the yield from *P. mirabilis* lysate [9]. The HPLC elution profiles of both native and cloned GSTB1-1 were essentially identical, showing a single peak eluting respectively at 37.84 ± 0.03 min and 37.95 ± 0.025 (mean $\pm$ S.D.) min. A single band centred at pH 6.1 was obtained when both native and cloned GSTB1-1 were submitted to isoelectrofocusing analysis (Figure 4).

Figure 4 Analytical isoelectrofocusing of native and cloned GSTB1-1

Lane 1, pI marker proteins; lane 2, native GSTB1-1; lane 3, cloned GSTB1-1.

Table 1 Substrate specificity of cloned GSTB1-1

Abbreviation: n.d., no detectable activity.

Substrate	Specific activity (μ M/min per mg)
1-Chloro-2,4-dinitrobenzene	1.1
1,2-Dichloro-4-nitrobenzene	n.d.
Ethacrynic acid	0.022
Cumene hydroperoxide	0.38
<i>p</i> -Nitrobenzyl chloride	0.004
1,2-Epoxy-3-(<i>p</i> -nitrophenoxy)propane	n.d.
<i>trans</i> -4-Phenyl-3-buten-2-one	n.d.
Δ^5 -Androstene 3,17-dione	n.d.
<i>p</i> -Nitrophenyl acetate	n.d.
Bromosulphthalein	n.d.
4-Nitroquinoline 1-oxide	n.d.
Dichloromethane	n.d.

Similar to the result obtained with native GSTB1-1, the cloned enzyme has a narrow range of substrate specificities [9]. In fact, as can be seen in Table 1, the recombinant GSTB1-1, in addition to its capacity to reduce cumene hydroperoxide, is able to conjugate GSH only to CDNB and ethacrynic acid.

Collectively, these results clearly indicate that the recombinant GSTB1-1 is structurally, kinetically and immunologically indistinguishable from native GSTB1-1.

It has been demonstrated that different species of methylotrophic bacteria contain dichloromethane dehalogenase, an enzyme that is able to catalyse the degradation of dichloromethane to formaldehyde, using GSH as co-substrate [43,44]. This enzyme, although structurally different from the mammalian GST, is now considered to be a member of the Theta class of the GST family [43]. Furthermore Meyer et al. [7] and Blocki et al. [43] have demonstrated that Theta class GSTs, and to a smaller extent Mu class GSTs, have the capacity to transform dichloromethane into formaldehyde. When GSTB1-1 was tested for its capacity to metabolize dichloromethane no significant activity was found.

This finding seems to support the idea that mammalian Theta class GST is more closely related to dichloromethane dehalogenase than to bacterial GST.

Table 2 IC₅₀ values of different antibiotics for cloned GSTB1-1 activity with CDNB as substrate

The values are means $\pm$ S.D. for at least six determinations with three different enzyme preparations.

Antibiotic	IC ₅₀ (μ M)
Cefotaxime	652 $\pm$ 60
Moxalactam	729 $\pm$ 80
Cephalothin	1929 $\pm$ 150
Tetracycline	49 $\pm$ 3
Minocycline	54 $\pm$ 5
Rifamycin	85 $\pm$ 9
Nitrofurantoin	140 $\pm$ 13
Chloramphenicol	941 $\pm$ 90

Table 3 Effect of several antibiotics on kinetic parameters of cloned GSTB1-1

The values are means $\pm$ S.D. for at least four determinations of three different enzyme preparations.

Antibiotic	K_m (μ M)		k_{cat} (min ⁻¹)		$10^{-3} \times k_{cat}/K_m$ (μ M ⁻¹ · min ⁻¹)	
	GSH	CDNB	GSH	CDNB	GSH	CDNB
None	686 $\pm$ 80	730 $\pm$ 75	58.1 $\pm$ 6	69.3 $\pm$ 6	84	95
Cefotaxime	989 $\pm$ 90	503 $\pm$ 50	32.1 $\pm$ 3	39.7 $\pm$ 4	32	79
Tetracycline	619 $\pm$ 70	428 $\pm$ 45	25.4 $\pm$ 3	31.5 $\pm$ 4	41	74
Rifamycin	869 $\pm$ 85	378 $\pm$ 40	33.0 $\pm$ 3	29.0 $\pm$ 3	38	77
Chloramphenicol	807 $\pm$ 70	412 $\pm$ 35	25.1 $\pm$ 4	31.5 $\pm$ 3	31	76
Nitrofurantoin	532 $\pm$ 80	267 $\pm$ 25	24.2 $\pm$ 3	24.5 $\pm$ 3	45	92

Interaction with antibiotics

A possible binding role for bacterial GST in antibiotic resistance has been suggested previously [18]. The interaction of a range of antibiotics with GSTB1-1 was investigated. In the first series of experiments GSTB1-1 was tested for sensitivity *in vitro* to the action of several classes of antibiotics. In Table 2 eight antibiotics and their IC₅₀ values for GSTB1-1 catalytic activity are reported. Tetracycline and minocycline were found to be the strongest inhibitors, having IC₅₀ values of 49 and 54 μ M respectively. Relatively low IC₅₀ values were also found for rifamycin (85 μ M) and nitrofurantoin (140 μ M). GSTB1-1 proved to be less susceptible to inhibition by cefotaxime, moxalactam, cephalothin and chloramphenicol, showing IC₅₀ values of 652, 729, 1929 and 941 μ M respectively. Nalidixic acid, norfloxacin, oxolinic acid, ampicillin, amoxacillin, piperacillin, erythromycin, troleandomycin, tobramycin and kanamycin up to a concentration of 5 mM were ineffective in inhibiting GSTB1-1 activity. To understand better the nature of the interaction, GSTB1-1 kinetic parameters in the presence and in the absence of the classes representing the antibiotics cefotaxime, tetracycline, rifamycin, chloramphenicol and nitrofurantoin were determined (Table 3). All drugs tested produced a significant decrease (to approx. one-half to one-third) in the k_{cat} values of GSTB1-1 for both substrates. These results indicate that GSTB1-1 binds the antibiotics listed in Table 3 at a site or sites remote from the catalytic site (GSH site) or the CDNB binding site (H site). Furthermore the fact that the drugs, in addition to decreasing the k_{cat} values, also caused a significant decrease in the K_m value for CDNB

Figure 5 Effect of rifamycin, minocycline and tetracycline on the growth rate of cells overexpressing GSTB1-1

Symbols: $\square$, 1/2 $\times$ MIC plus IPTG; $\blacksquare$, 1/2 $\times$ MIC without IPTG; $\circ$, 1/4 $\times$ MIC plus IPTG; $\bullet$, 1/4 $\times$ MIC without IPTG; $\triangle$, 1/8 $\times$ MIC plus IPTG; $\blacktriangle$, 1/8 $\times$ MIC without IPTG. MIC values are: 50 μ g/ml for rifamycin, 3.2 μ g/ml for minocycline and 100 μ g/ml for tetracycline.

suggests that the binding of antibiotics induces a conformational change in the protein, affecting the CDNB binding site.

In view of the binding capacity of the bacterial GST towards antibiotics, the growth rate of GSTB1-1-overexpressed cells, in the presence of different concentrations of rifamycin and tetracyclines, was measured and compared with cells not overexpressed for GSTB1-1. As can be seen in Figure 5, rifamycin, but not tetracyclines, differentially affected the growth rate of cells overexpressed and not overexpressed for GSTB1-1. In fact in the presence of 6.25–25 μ g/ml rifamycin the cells overexpressing GSTB1-1 continued to grow, whereas the growth of cells not overexpressing GSTB1-1 was substantially inhibited.

In conclusion, these results clearly indicate that GSTB1-1 is able to sequester antibiotics with avidity. This suggests that GSTB1-1 might be involved in resistance to several classes of antibiotic, particularly rifamycin. Alternatively, in view of the role of GST in protecting the cells from toxic insult, it is possible that tetracyclines and rifamycin, as well as other antibiotics, might impair the detoxication of other xenobiotics or endobiotics that are metabolized through the mercapturic acid pathway. Studies based on X-ray crystallography and site-directed mutagenesis are under way to improve our understanding of the relationship between GSTB1-1 and antibiotics.

This work was supported by CNR Progetto Finalizzato 'Prevenzione e Controllo dei Fattori di Malattia' (FATMA) and Sottoprogetto 'Qualità dell'Ambiente e Salute', contract no. 91.00146.PF41.

REFERENCES

- 1 Hayes, J. D. and Pulford, D. J. (1995) *Crit. Rev. Biochem. Mol. Biol.* **30**, 445–600
- 2 Ketterer, B., Meyer, D. J. and Clark, A. G. (1988) in *Glutathione Conjugation, Mechanisms and Biological Significance: Soluble Glutathione Transferase Isoenzymes* (Sies, H. and Ketterer, B., eds.), pp. 73–135, Academic Press, London
- 3 Mannervik, B. and Danielson, U. H. (1988) *Crit. Rev. Biochem.* **23**, 283–337
- 4 Pickett, C. B. and Lu, A. Y. H. (1989) *Annu. Rev. Biochem.* **58**, 743–764
- 5 Buetler, T. M. and Eaton, D. L. (1992) *Environ. Carcinogen. Ecotox. Rev.* **10**, 181–203
- 6 Mannervik, B., Alin, P., Guthenberg, C., Jensson, H., Tahir, M. K., Warholm, M. and Jornvall, H. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 7203–7206
- 7 Meyer, D. J., Coles, B., Pemble, S. E., Gilmore, K. S., Fraser, G. M. and Ketterer, B. (1991) *Biochem. J.* **274**, 409–414
- 8 Arca, P., Garcia, P., Hardisson, C. and Suarez, J. E. (1990) *FEBS Lett.* **263**, 77–79
- 9 Di Ilio, C., Aceto, A., Piccolomini, R., Allocati, N., Faraone, A., Cellini, L., Ravagnan, G. and Federici, G. (1988) *Biochem. J.* **255**, 971–975
- 10 Di Ilio, C., Aceto, A., Piccolomini, R., Allocati, N., Faraone, A., Bucciarelli, T., Barra, D. and Federici, G. (1991) *Biochim. Biophys. Acta* **1077**, 141–146
- 11 Di Ilio, C., Aceto, A., Allocati, N., Piccolomini, R., Bucciarelli, T., Dragani, B., Faraone, A., Sacchetta, P., Petruzzelli, R. and Federici, G. (1993) *Arch. Biochem. Biophys.* **305**, 110–114
- 12 Iizuka, M., Inoue, Y., Murata, K. and Kimura, A. (1989) *J. Bacteriol.* **171**, 6039–6042
- 13 Nishida, M., Kong, K. H., Inoue, H. and Takahashi, K. (1994) *J. Biol. Chem.* **269**, 32536–32541
- 14 Zablotowicz, R. M., Hoagland, R. E., Locke, M. A. and Hickey, W. J. (1995) *Appl. Environ. Microbiol.* **61**, 1054–1060
- 15 Mignogna, G., Allocati, N., Aceto, A., Piccolomini, R., Di Ilio, C., Barra, D. and Martini, F. (1993) *Eur. J. Biochem.* **211**, 421–425
- 16 Aceto, A., Dragani, B., Allocati, N., Angelucci, S., Bucciarelli, T., Sacchetta, P., Di Ilio, C. and Martini, F. (1995) *Int. J. Biochem.* **27**, 1033–1041
- 17 Allocati, N., Cellini, L., Aceto, A., Iezzi, T., Angelucci, S., Robuffo, I. and Di Ilio, C. (1994) *FEBS Lett.* **354**, 191–194
- 18 Piccolomini, R., Di Ilio, C., Aceto, A., Allocati, N., Faraone, A., Cellini, L., Ravagnan, G. and Federici, G. (1989) *J. Gen. Microbiol.* **135**, 3119–3125
- 19 Bullock, W. O., Fernandez, J. M. and Short, J. M. (1987) *BioTechniques* **5**, 376–379
- 20 Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) *Molecular Cloning: a Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
- 21 Sanger, F., Nicklen, S. and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5463–5467
- 22 Simons, P. C. and Vander Jagt, D. L. (1977) *Anal. Biochem.* **82**, 334–341
- 23 Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- 24 Towbin, H., Staehelin, T. and Gordon, L. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4350–4354
- 25 Ostlund-Farrants, A. K., Meyer, D. J., Coles, B., Southan, C., Aitken, A., Johnson, P. J. and Ketterer, B. (1987) *Biochem. J.* **245**, 423–428
- 26 Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
- 27 Habig, W. H. and Jakoby, W. B. (1981) *Methods Enzymol.* **77**, 398–405
- 28 Di Ilio, C., Sacchetta, P., Lo Bello, M., Caccuri, A. M. and Federici, G. (1986) *J. Mol. Cell Cardiol.* **18**, 983–991
- 29 Stanley, J. S. and Benson, A. M. (1988) *Biochem. J.* **256**, 303–306
- 30 Staub, D. and Leisinger, T. (1985) *J. Bacteriol.* **162**, 676–681
- 31 Anhalt, J. P. and Washington II, J. A. (1991) in *Manual of Clinical Microbiology* (Balows, A., Hausler, Jr., W. J., Herrmann, K. L., Isenberg, H. D. and Shadomy, H. J., eds.), pp. 1199–1200, American Society for Microbiology, Washington, DC
- 32 Leatherbarrow, R. J. (1987) *ENZFITTER. A Non-linear Regression Data Analysis Program for the IBM PC and True Compatibles* (Leatherbarrow, R. J., ed.), Biosoft, Cambridge, UK
- 33 Sahm, D. F. and Washington, II, J. A. (1991) in *Manual of Clinical Microbiology* (Balows, A., Hausler, Jr., W. J., Herrmann, K. L., Isenberg, H. D. and Shadomy, H. J., eds.), pp. 1105–1116, American Society for Microbiology, Washington, DC
- 34 Bergler, H., Högenauer, G. and Turnowsky, F. (1992) *J. Gen. Microbiol.* **138**, 2093–2100
- 35 Fleischmann, R. D., Adams, M. D., White, O., Clayton, R. A., Kirkness, E. F., Kerlavage, A. R., Bult, C. J., Tomb, J. F., Dougherty, B. A., Merrick, J. M. et al. (1995) *Science* **269**, 496–512
- 36 Wada, K., Wada, Y., Ishibashi, F., Gojibori, T. and Ikemura, T. (1992) *Nucleic Acids Res.* **20**, 2111–2118
- 37 Charles, I. G., Keyte, J. W. and Shaw, W. V. (1985) *J. Bacteriol.* **164**, 123–129
- 38 Steglitz-Mörsdorf, U., Mörsdorf, G. and Kaltwasser, H. (1993) *FEMS Microbiol. Lett.* **106**, 157–164
- 39 Harley, C. B. and Reynolds, R. P. (1987) *Nucleic Acids Res.* **15**, 2343–2361
- 40 Bader, R. and Leisinger, T. (1994) *J. Bacteriol.* **176**, 3466–3473
- 41 Hofer, B., Backhaus, S. and Timmis, K. N. (1994) *Gene* **144**, 9–16
- 42 Daubaras, D. L., Hershberger, C. D., Kitano, K. and Chakrabarty, A. M. (1995) *Appl. Environ. Microbiol.* **61**, 1279–1289
- 43 Blocki, F. A., Logan, M. S. P., Baoli, C. and Wackett, L. P. (1994) *J. Biol. Chem.* **269**, 8826–8830
- 44 La Roche, S. D. and Leisinger, T. (1990) *J. Bacteriol.* **172**, 164–171