Proteus mirabilis glutathione S-transferase B1-1 is involved in protective mechanisms against oxidative and chemical stresses

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We investigated the effects of several xenobiotics, including antimicrobial agents and general stress factors such as starvation, heat and osmotic shock, on the modulation of expression of *Proteus mirabilis* glutathione S-transferase B1-1 (PmGST B1-1). The level of expression of PmGST B1-1 was established by both Western- and Northern-blot experiments. Our results show that several compounds can modulate expression of PmGST B1-1. The level of PmGST B1-1 increased when bacterial cells were exposed to a variety of stresses such as 1-chloro-2,4-dinitrobenzene, H_2O_2, fosfomycin or tetracycline. A knock-out *gst*B gene was also constructed using the suicide vector pKNOCKlox-Ap. Successful inactivation of the wild-type gene was confirmed by PCR, DNA sequence analysis and Western blotting. Under normal culture conditions, this mutant was viable and displayed no significant phenotypic differences compared with the wild-type. However, viability tests revealed that the null mutant was more sensitive to oxidative stress in the form of H_2O_2 and to several antimicrobial drugs when compared with the wild-type. These results suggest that PmGST B1-1 has an active role in the protection against oxidative stress generated by H_2O_2 and it appears to be involved in the detoxification of antimicrobial agents.

Key words: antimicrobial agent, bacterial glutathione S-transferase, knock-out gene, *Proteus mirabilis* glutathione S-transferase, stress factor, suicide vector.

INTRODUCTION

The detoxification of harmful compounds is a common problem in all prokaryotic and eukaryotic cells, which are constantly under the pressure of multiple chemical stresses. Organisms have developed an elaborate defence system to protect themselves against injurious compounds [1,2].

Some major enzymes that play a key role in cellular detoxification are glutathione S-transferases (GSTs; EC 2.5.1.18), which constitute a superfamily of dimeric multifunctional enzymes, and are involved in xenobiotic detoxification [1–3]. GSTs metabolize a wide variety of electrophilic compounds via glutathione (GSH) conjugation [1–4]. This reaction is the first step in mercapturic acid formation, a pathway through which harmful xenobiotics and endobiotics are inactivated and eliminated from an organism [1–4]. Reactive oxygen species, such as the superoxide radical, H_2O_2 and the hydroxyl radical are generated continuously during aerobic respiration. These species react with DNA, protein and lipid membranes, producing chemicals, which in turn are highly reactive. GSTs contribute to protection of cells from these secondary products of oxidative stress [1,2]. Recently, a direct link between GST induction and oxidative stress has been observed in eukaryotic cells [5]. GSTs are also involved in other cell functions, such as peroxidase and isomerase activities, and are capable of binding a wide range of endogenous and exogenous ligands non-catalytically [1,2].

In eukaryotes, the large number of cytosolic GSTs investigated so far has been grouped into at least ten gene-independent classes on the basis of their physical, chemical, immunological and structural properties [6]. An additional GST family comprises membrane-bound transferases, but these bear no similarity to soluble GSTs [7].

Although several studies were conducted on bacterial GSTs, little is known about their structural and functional properties [8]. Bacterial GSTs seem to be involved in a variety of distinct processes such as biotransformation of dichloromethane [9], degradation of lignin [10] and atrazine [11], and reductive dechlorination of pentachlorophenol [12].

Previously, we have characterized an isoform of GST produced by *Proteus mirabilis* strain, namely *P. mirabilis* glutathione S-transferase B1-1 (PmGST B1-1) [13–21].

PmGST B1-1 displays biochemical and structural properties that distinguish it from the GSTs of other families and it has been identified as the prototype of a new class, namely Beta class [18]. A distinctive feature of this enzyme is the presence of a mixed disulphide between one glutathione molecule and the thiol group of Cys-10, which resides in the GSH-binding site [18,22]. Recent advances revealed that PmGST B1-1 behaves as an intermediate enzyme between thiol-disulphide oxidoreductase and the GST superfamilies [23]. Of particular interest is the proposed role of this enzyme in the detoxification of antibiotics. In fact, PmGST B1-1 is able to bind several antimicrobial drugs *in vitro* and *in vivo* [14,16].

To understand the role of bacterial GSTs better, we investigated the modulation of PmGST B1-1 expression in the presence of several toxic chemicals, including antimicrobial drugs. Typical general stress conditions were also tested. Finally, we constructed a *gst*B null-mutant gene to establish the *in vivo* function of this enzyme.

Results of the present study suggest a role for PmGST B1-1 in the protection against oxidative stress. In addition, the contribution to the detoxification of antibiotics is also reported. (A preliminary account of the present study has appeared elsewhere [24]).

Abbreviations used: as-PCR, asymmetric PCR; CDNB, 1-chloro-2,4-dinitrobenzene; CHP, cumene hydroperoxide; FOS, fosfomycin; GST, glutathione S-transferase; LB, Luria–Bertani; MIC, minimum inhibitory concentration; MINO, minocycline; PmGST B1-1, *Proteus mirabilis* glutathione S-transferase B1-1; RIF, rifamycin; TET, tetracycline.

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MATERIALS AND METHODS

Chemicals

Isopropyl β-D-thiogalactoside and antibiotics were purchased from Sigma–Aldrich (Milano, Italy). All other reagents used were of the highest grade commercially available.

Bacterial strains and growth conditions

The bacterial strains used in conjugations were *P. mirabilis* AF2924 [13] and *Escherichia coli* DH5α as recipients, and *E. coli* S17-1/λpir as a donor.

All strains were cultured in Luria–Bertani (LB) broth or in M9 minimal medium supplemented with 0.4 % glucose as the sole carbon source [25]. Agar was added at a final concentration of 15 g/l to solidify the broth media. To prevent swarming motility of *P. mirabilis*, a non-swarming agar was used (LSW agar, containing per litre: 10 g of tryptone, 5 g of yeast extract, 5 ml of glycerol, 0.4 g of NaCl and 20 g of agar) [26]. Ampicillin (100 μg/ml) and 8 μg/ml tetracycline (TET) were added to the medium as needed. *P. mirabilis* strains were tested using the API-20E identification system (bio-Merieux Italia, Roma, Italy).

To investigate the response of *P. mirabilis* to different stress conditions, single colonies were inoculated into the LB medium and were grown overnight at 37 °C using a heated orbital-shaking bath. After a 1 : 200 dilution, cells were grown in LB medium at 37 °C to an absorbance value A_{600} of 0.4, and aliquots of the cultures were then exposed to 250 μM 1-chloro-2,4-dinitrobenzene (CDNB), 0.5 mM H_2O_2, 0.078 mM cumene hydroperoxide (CHP), 0.5 mM paraquat, 12.5 μg/ml minocycline (MINO), 6.25 μg/ml rifamycin (RIF), 12.5 μg/ml TET or 12.5 μg/ml fosfomycin (FOS). After 3 h of incubation, cells were harvested by centrifugation and processed for further analysis.

To avoid the toxic effects of the compounds tested, sub-inhibitory concentrations equal to half of the minimum inhibitory concentration (MIC) were chosen. The MIC was previously determined by a standard broth microdilution technique [27].

For heat-shock treatment, cells were shifted from 37 to 42 °C. Osmotic stress was achieved by the addition of 0.3 M NaCl. Cells were starved by growing them to the late stationary phase.

For viability studies, bacterial cells were grown as described above. Xenobiotics were added to the growing cells of *P. mirabilis* at various concentrations equal to 0.1 MIC, 0.2 MIC, 0.5 MIC and 0.8 MIC. After the addition of stimuli, aliquots of each culture were taken at 60 min post-inoculation, serially diluted, spread on to LSW agar and then incubated overnight at 37 °C. Bacterial viability was expressed in terms of colony-forming units (cfu)/ml.

Western-blot analysis

Cells cultivated in the presence of different stimulants (as described above) were harvested by centrifugation (10 000 ***g***, for 15 min), washed in 10 mM potassium phosphate buffer (pH 7.0) and disrupted by cold sonication. The particulate material was removed by centrifugation at 100 000 ***g*** for 60 min at 4 °C, and the protein concentration of the supernatant was determined by the method of Bradford [28] using γ-globulin as standard.

Crude extracts (15 μg) were analysed by SDS/PAGE on discontinuous slab gel by the method of Laemmli [29]. Proteins were electrophoretically transferred to a PVDF membrane by using a Mini Trans-Blot unit (Bio-Rad Laboratories, Milano, Italy) for 1 h at 100 V. After transfer, the membrane was incubated for 1 h at room temperature (25 °C) in 3 % (w/v) gelatin in 20 mM Tris/500 mM NaCl (TBS, pH 7.5) to block non-specific binding. The blot was incubated for 2 h with 1 % gelatin in TBS plus 0.05 % Tween 20 (TTBS, pH 7.5) containing antiserum anti-(PmGST B1-1) [13] at a dilution of 1 : 500. After washing with TTBS twice for 5 min, the blot was incubated for 1 h with horseradish peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad Laboratories) diluted to 1 : 3000 in 1 % gelatin in TTBS. The blot was washed three times with TBS and then immersed in development solution [100 ml of TBS containing 60 mg of 4-chloro-1-naphthol and 60 μl of 30 % (w/v) H_2O_2]. The blot was then washed once with distilled water, air-dried and photographed. Quantitative densitometric assays of blot analysis of proteins were performed by NIH Image using laser scanning (Umax Data System Inc., Image S.r.l., Piacenza, Italy). Each experiment was repeated at least three times and the data shown represent typical results.

RNA preparation and Northern blotting

Total RNA was isolated as reported previously [11] from late exponential-phase cells grown under the specific conditions described above. Total isolated RNA (15 μg) was separated on 1.3 % (w/v) formaldehyde agarose gel and transferred to a nitrocellulose membrane by capillary blotting. The membranes were dried at 80 °C for 2 h. Filters were prehybridized at 42 °C for 3–5 h, then hybridized at the same temperature overnight in 50 % (v/v) formamide, 5 × SSC, 0.1 M sodium phosphate and 1 × Denhardt's solution (0.02 % Ficoll/0.02 % polyvinylpyrrolidone/0.02 % BSA). The probe was radiolabelled by asymmetric PCR (as-PCR). The *gst*B gene was obtained by digestion of pGPT1 [16] using *Eco*RI: the gene was recovered from a low-melting agarose gel and used as a template for the as-PCR. The primer was designed as follows: primer Rev-9, 5′-CCGTGACTAGTGCACTATGCA-3′, mapping at the nucleotide position 592–572 of the *gst*B gene. In addition to the template (100 ng) and the antisense Rev-9 primer (500 ng), the 10 μl reaction mixture contained 1 mM $MgCl_2$, dNTPs (dATP, dTTP and dGTP at a concentration of 20 μM; 10 μM dCTP), 25 μCi of [α-^{32}P]dCTP (3000 Ci/mmol; Amersham Biosciences), *Taq* DNA polymerase buffer and 2.5 units of *Taq* DNA polymerase (Promega, Milano, Italy). The as-PCR was subjected to 30 cycles of amplification (60 s at 94 °C, 120 s at 54 °C and 120 s at 72 °C). Blots were exposed to Kodak X-Omat film at − 80 °C.

Construction of *gst*B-null mutant

A *gst*B null-mutant strain was constructed by partial duplication using the suicide vector pKNOCKlox-Ap [30]. Internal fragment (lacking sequences on 5′- and 3′-ends) of the *gst*B gene was amplified from pGPB1 DNA [16] by PCR using the following primers: ForINT, 5′-CGGAATTCCTTTTCTATTGAGCGCATTG-3′, and RevINT, 5′-CGGAATTCCTAGATAGTCTTGTAAATGG-3′. Additional bases were added to the 5′-end of each primer to introduce a recognition sequence for *Eco*RI restriction endonuclease (underlined).

The amplification conditions were 95 °C for 1 min, 47 °C for 1 min, 55 °C for 25 s and 72 °C for 1 min, for 6 cycles followed by 30 cycles of amplification (95 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min).

The PCR product was purified from 1 % agarose gel, digested with *Eco*RI, ligated into pBluescript vector and the ligation mixture was electroporated in *E. coli* DH5α. Internal sequence of *gst*B was extracted with enzymes *Bam*HI and *Xho*I and subcloned into *Bam*HI–*Xho*I sites of the pKNOCKlox-Ap suicide vector, thus creating pKNOCKlox-Ap/Δ*gst*B. The resulting plasmid was

introduced into *E. coli* S17-1/λpir donor cells. Electroporation was performed in a Gene Pulser apparatus according to the manufacturer's instructions (Bio-Rad Laboratories, Richmond, CA, U.S.A.). Recombinant clones were selected based on restriction enzyme analysis of the plasmid DNA extracted from ampicillin-resistant colonies.

The suicide vector construct pKNOCKlox-Ap/Δ*gst*B was mobilized from donor *E. coli* S17-1/λpir into *P. mirabilis* by conjugation. *P. mirabilis* exconjugants were selected on ampicillin/TET plates. Several exconjugant colonies were selected and analysed by PCR using pairs of primers constructed on the basis of the chromosomal DNA sequence located just upstream and downstream of the *gst*B gene: ForNull, 5′-ATCTTCACGGGTATCACCACTC-3′, and RevNull, 5′-CCATAACTTCTGTGCAACGCAG-3′. The PCR product was purified from 1 % agarose gel and its identity was confirmed by DNA sequencing. The positive clones were further analysed by Western blotting.

Determination of GSH concentration

GSH concentration was determined as reported previously [11]. Briefly, the GSH standard was prepared quantitatively using 25 mM KH_2PO_4 adjusted to pH 3.0. Crude extracts of bacterial cells, cultivated in the presence of different compounds after the addition of an equal volume of acetone, were harvested by centrifugation (10 000 ***g*** for 5 min). The supernatants were freeze-dried, resuspended in the same buffer used to prepare the GSH standard and analysed by capillary electrophoresis.

Enzyme assay

GST activity was measured at 30 °C by the spectrophotometric method of Habig and Jakoby [31], using 1 mM GSH and 1 mM CDNB as substrates.

RESULTS

Modulation of PmGST B1-1 protein and *gst*B mRNA

To test the effects of xenobiotics on the expression of PmGST B1-1, the toxic compounds were added during exponential growth. The level of enzyme was examined by Western-blot analysis using the corresponding polyclonal antibodies [13].

Several compounds were capable of increasing the level of enzyme (Figure 1). Treatment of *P. mirabilis* with 0.5 mM H_2O_2 caused a 2-fold increase in the expression of PmGST B1-1. TET (12.5 μg/ml), FOS (12.5 μg/ml) and RIF (6.25 μg/ml) produced 1.5-, 2.4- and 1.5-fold increases respectively. It is interesting to note that 250 μM CDNB, the most common substrate for GSTs, leads to a 4-fold increase in the protein level. Consequently, we tested the effect of different concentrations of CDNB on bacterial growth as well as on the expression of the enzyme. Figure 2 shows the dose-dependent effect on bacterial growth (Figure 2A) and this correlates with a marked increase in the enzyme level (Figure 2B).

In contrast, when cells were exposed to 0.5 mM paraquat or 0.078 mM CHP, the enzyme level was not affected, although the concentrations used were sufficiently high to slow down the bacterial growth rate (results not shown). Similar results were obtained when *P. mirabilis* cells were grown with 12.5 μg/ml MINO (results not shown). Moreover, several stress conditions such as osmotic shock, heat shock and starvation had no effect on the PmGST B1-1 expression (results not shown).

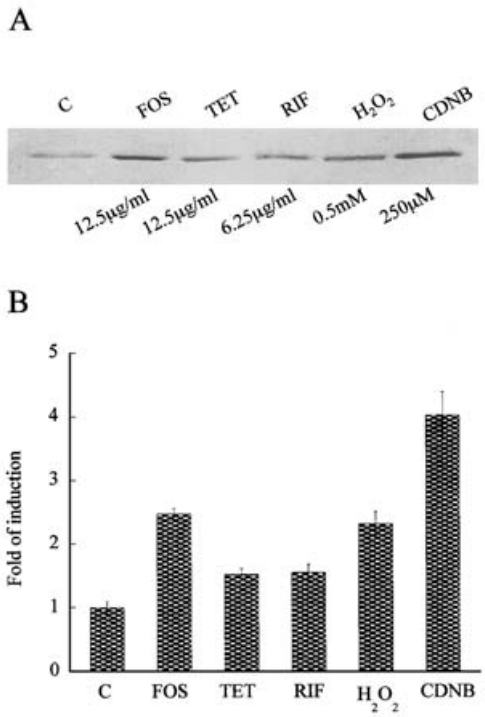

Figure 1 Effect of several compounds on the expression of PmGST B1-1

(**A**) Western-blot analysis of PmGST B1-1; 15 μg of crude extracts were loaded per lane. (**B**) Bar graph showing induction of PmGST B1-1 as measured by densitometry. Cells of each culture were harvested 3 h after the addition of stimuli. The graph shows the means and S.D. for three independent experiments.

To confirm these increases in the GST level promoted by several compounds tested, we investigated the transcriptional response in *P. mirabilis* grown under xenobiotic stress conditions using Northern-blot analysis.

Hybridization of RNA to a specific labelled antisense *gst*B probe resulted in the detection of a single transcript of approx. 800 nucleotides (Figure 3). This size is consistent with the size of the *gst*B gene reported previously [16] and indicates that the gene is monocistronically transcribed. The transcription was induced by stress with H_2O_2, FOS, TET, RIF and CDNB (Figure 3). Thus the results obtained are consistent with the increase observed in the Western blot (Figure 1).

Construction of *P. mirabilis gst*B-null mutant

To evaluate a possible protective role of PmGST B1-1 during xenobiotic stress conditions, we have constructed a *gstB*-null mutant strain. The *gst*B-null mutant strain was generated by inactivation of the respective gene through homologous-recombination-mediated partial duplication of the chromosomal *gst*B gene, as described in the Materials and methods section. The disruption of the chromosomal *gst*B gene in *P. mirabilis* exconjugants was confirmed by PCR using specific primers that flanked the *gst*B gene (Figure 4A). As expected, a 3240 bp PCR DNA fragment was detected in null-mutant strain, indicating the integration of the pKNOCKlox-Ap/Δ*gst*B vector into the chromosome. On the contrary, a 767 bp PCR DNA fragment containing the whole wild-type gene was obtained in the control reaction performed using DNA of a wild-type strain as a template. The identity of both PCR products was confirmed by DNA sequencing (results not shown). The absence of PmGST B1-1

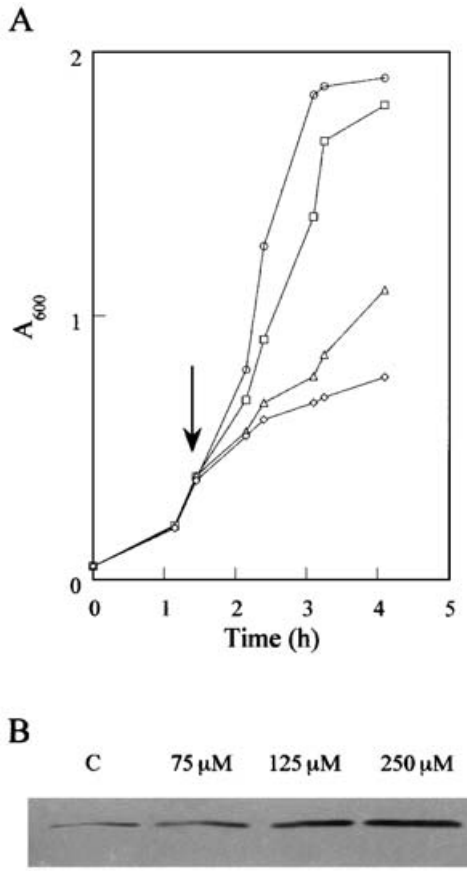

Figure 2 Effect of different concentrations of CDNB on the growth of *P. mirabilis* and on the expression of PmGST B1-1

(**A**) Growth rate of *P. mirabilis* in the presence of the following concentrations of CDNB: Control (C, -○-); 75 μM (-□-); 125 μM (-△-); 250 μM (-◇-). (**B**) Western-blot analysis of PmGST B1-1; 15 μg of crude extracts were loaded per lane. Cells of each culture were harvested 3 h after the addition of stimuli. The pattern shown is representative of three replicate experiments.

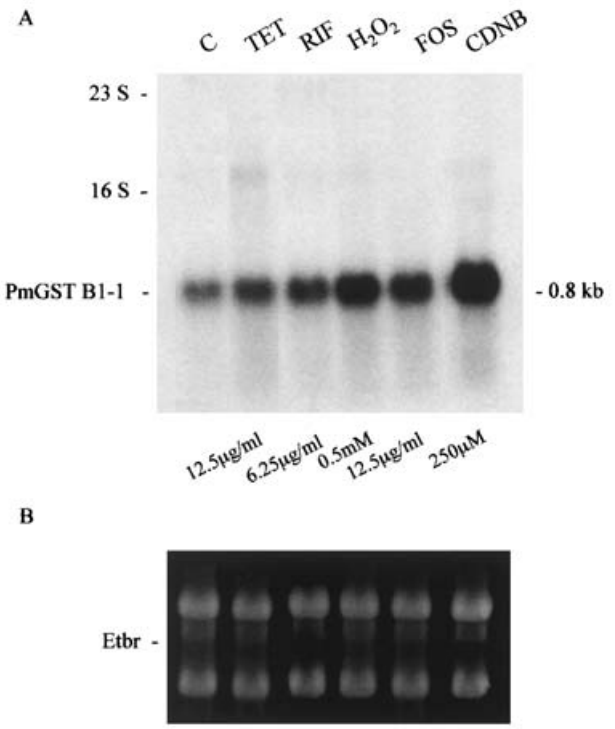

Figure 3 Northern-blot analysis of total RNA from *P. mirabilis*

Modulation of PmGST B1-1 transcript (**A**); 23 and 16 S rRNA, stained with ethidium bromide (Etbr) (**B**). Cells of each culture were harvested 3 h after the addition of stimuli.

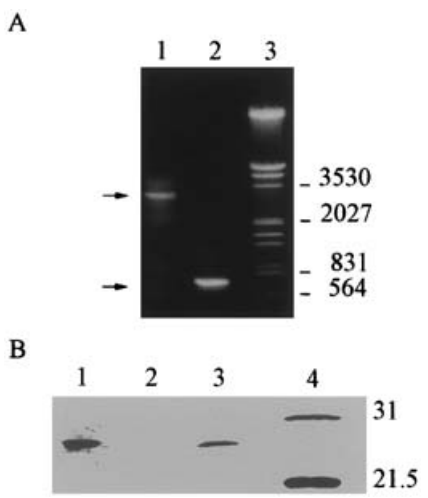

Figure 4 PCR amplification of chromosomal DNA and Western-blot analysis of wild-type and *gst*B-null mutant strains

(**A**) Lane 1, the *gst*B-null mutant; lane 2, the wild-type; lane 3, Lambda DNA molecular-mass markers in kb. Arrows indicate the 3240 bp *gst*B-null mutant fragment and the 767 bp wild-type fragment. (**B**) Lane 1, PmGST B1-1; lane 2, total cellular extract from the *gst*B-null mutant; lane 3, total cellular extract from the wild-type; lane 4, molecular-mass markers in kDa.

from the mutant was shown by Western blotting (Figure 4B). The mutation induced by the pKNOCKlox-Ap plasmid was structurally stable. Mutant strains were capable of growing on ampicillin/TET LSW agar plates after 100 serial passages on LSW agar without antibiotics, and the stability was further confirmed by PCR.

Characteristics of *gst*B-null mutant *P. mirabilis*

The effect of mutation on the growth rate of *P. mirabilis* cells was tested to investigate whether the *gst*B gene is essential for the growth of the bacterium.

The growth rates of the null mutant and wild-type cells were determined in the LB medium. The mutant and wild-type strains were cultured overnight; the cultures were diluted to 1 : 200 in the LB medium, and growth was monitored by reading A_{600}. No significant differences in growth rate were observed between wild-type and null-mutant cells (results not shown). Both strains grew similarly in the minimal nutritional medium. Also, both wild-type and mutant *P. mirabilis* were tested by API-20E substrate profile analysis and were found to have identical patterns.

The results of genome sequencing projects indicate that bacteria, like eukaryotes, contain several GST genes of widely divergent sequences and the precise physiological role of these genes remains to be identified [32]. Furthermore, in a previous report [13], we resolved three GST forms from *P. mirabilis* that appeared quite similar in many respects. This was indicated by similarities in subunits, amino acid composition, substrate specificities and immunological properties. To verify if there was another GST isoform in *P. mirabilis* and considering that the GST activity is not detected in the crude extract of the bacterium [13],

Table 1 Effect of H_2O_2, CDNB, MINO and FOS on the viability of wild-type and *gst*B null-mutant strains

Bacteria were treated with various concentrations equal to 0.1 MIC, 0.2 MIC, 0.5 MIC and 0.8 MIC. Aliquots of each culture were taken at 60 min after the addition of stimuli. The results are means for three independent experiments; S.D. values never exceed 10 % of the means.

	Cell viability	
Chemicals (MIC*)	Wild-type (10^6 cfu/ml) (%)	*gst*B null mutant (10^6 cfu/ml) (%)
None	701 (100)	689 (100)
H_2O_2		
0.1	547 (78)	395 (57)
0.2	534 (76)	420 (61)
0.5	368 (52)	171 (24)
0.8	333 (47)	118 (17)
CDNB		
0.1	686 (98)	649 (93)
0.2	672 (96)	405 (59)
0.5	435 (62)	212 (31)
0.8	294 (42)	196 (28)
FOS		
0.1	301 (43)	189 (27)
0.2	155 (22)	75 (11)
0.5	61 (9)	27 (4)
0.8	32 (5)	13 (2)
MINO		
0.1	596 (85)	551 (80)
0.2	553 (79)	385 (56)
0.5	311 (44)	75 (11)
0.8	133 (19)	34 (5)

* MIC values: 1 mM H_2O_2; 500 μM CDNB; 25 μg/ml FOS; 25 μg/ml MINO.

we attempted purification of the enzyme from the null-mutant strain by GSH-affinity chromatography as reported previously [13]. The eluate did not contain enzymic activity and it did not show any band on silver-stained SDS/polyacrylamide gels, indicating that no additional canonical GST is present in *P. mirabilis*. These conflicting results could be explained considering that the isoforms may have arisen by post-translational modification of a single precursor.

Susceptibility of null-mutant cells to xenobiotics

Viability of a null-mutant strain was measured in comparison with the wild-type after treatment with several compounds (Table 1). The compounds were added to the growing cells (A_{600} 0.4) at various concentrations equal to 0.1 MIC, 0.2 MIC, 0.5 MIC and 0.8 MIC. As shown in Table 1, the effect of the xenobiotics was dose-dependent and lower doses were required to cause death of the null-mutant cells. When null-mutant and wild-type cells were exposed to H_2O_2, they showed remarkable differences in their susceptibility to stress. Unlike the wild-type, only a small number of null-mutant cells were able to survive H_2O_2 treatment. In contrast, in the presence of CHP and paraquat, there was no marked difference between wild-type and null-mutant cells that were little affected by these compounds (results not shown). The effect of CDNB on the bacterial viability was also examined. As shown in Table 1, when bacteria were subjected to this xenobiotic, null-mutant cells were more susceptible when compared with wild-type cells.

A different susceptibility was observed when the bacterial cells were subjected to antimicrobial drugs. In the presence of phosphomycin, although both strains were affected by the antimicrobial drug, null-mutant cells showed lower viability. Moreover, RIF and TET did not significantly affect the viability of both null-mutant and wild-type *P. mirabilis* (results not shown). In contrast with what was observed in Western-blot analysis for MINO, the viability of null-mutant cells was more influenced by this molecule than that of wild-type cells. To verify whether the increased sensitivity of the *gst*B null-mutant strain was dependent on GSH, we determined the intracellular GSH level in both *gst*B-null mutant and wild-type strains growing in the presence or absence of H_2O_2, CDNB, FOS and MINO. Non-appreciable differences in the cellular GSH content were observed, indicating that these compounds did not affect the thiol content (results not shown).

DISCUSSION

GSTs are found in most organisms, and they play a key role in eukaryotic cellular-detoxification processes [1–3].

To elucidate the role of bacterial GSTs in the detoxification, the effect of several compounds on modulation of PmGST B1-1 in *P. mirabilis* was examined. In addition, a *gst*B null-mutant strain was constructed and compared with the wild-type.

It was demonstrated that GSTs are required to protect eukaryotic cells against peroxide-induced cell death [5]. In particular, these enzymes are involved in the detoxification reactions against secondary reactive oxygen species. To evaluate whether PmGST B1-1 contributed to protect the cells against oxidative stress, *P. mirabilis* cells were exposed to H_2O_2, paraquat and CHP. The modulation results suggest that PmGST B1-1 was involved in the bacterial response to oxidative stress induced by H_2O_2. In fact, when the bacterial cells were exposed to H_2O_2, an induction of PmGST B1-1 at both the RNA and protein expression level was observed. On the other hand, when oxidative stress was induced by paraquat or CHP treatment, the GST level remained unchanged. New evidence on the involvement of PmGST B1-1 in protecting cells from oxidative stress were obtained by testing the same oxidative stress inducers on the bacterial viability of both wild-type and null-mutant *P. mirabilis*. The results obtained are in agreement with modulation data. In fact, the null mutants were very sensitive to H_2O_2 when compared with wild-type, unlike CHP and paraquat.

It is known that the expression of many H_2O_2-inducible genes is regulated by the OxyR transcription factor [33]. In the presence of H_2O_2, OxyR becomes oxidized and activates the genes by binding to a DNA region near the coding sequence. A computational approach [34] was utilized to identify several putative OxyR DNA-binding sites and it used previously identified OxyR-binding site sequences as a model to search the *E. coli* genome for new OxyR-binding sites [35]. In our previous work [16], the search for similarities to promoter regions revealed several putative promoter sequences. One of them is located approx. 200 bp upstream of *gst*B ATG codon in accordance with the mRNA transcript of approx. 800 bp (Figure 3). Comparison of this sequence with 32 OxyR-binding site models available online (http://www.lecb.ncifcrf.gov/~toms/paper/zheng.storz2001/supplement) showed a similar OxyR-binding site motif. Another transcriptional factor SoxS regulates the expression of several genes coding for proteins involved in the protection of superoxide generators, such as paraquat [33]. Comparison of the DNA region, located upstream of the gstB gene, with SoxS models (http://bayesweb.wadsworth.org/binding_sites_20020802/ecoli_binding_sites) [36] did not show any similarity. These findings together with our results strongly suggest that PmGST B1-1 participates in bacterial defence against oxidative damage induced by H_2O_2.

CDNB is usually utilized as a model substrate in the conjugation reaction with GSH to characterize the GSTs. When the cells were grown in the presence of CDNB, the level of enzyme was 4-fold higher than that in the control culture. This increase was confirmed by Northern-blot analysis. To quantify this effect on the regulation of PmGST B1-1, CDNB was added to the growing cells of *P. mirabilis* at different concentrations. The presence of CDNB led to a concentration-dependent inhibition of bacterial growth and at the same time to a corresponding increase in the enzyme level. Viability tests confirmed that cells lacking the *gst*B gene were more susceptible when compared with the wild-type strain. It is possible that, similar to membrane-active aromatic compounds, CDNB causes toxicity at the membrane level and PmGST B1-1 helps to protect the cells from this damage.

A possible role for PmGST B1-1 in the protection against the toxic effect of the antimicrobial agents has been suggested previously [14,16]. Studies on the interaction of PmGST B1-1 with a number of antimicrobial agents indicated that this enzyme was able to sequester antibiotics with avidity [14,16]. Indeed, our previous studies demonstrated the presence of PmGST B1-1 not only in the cytoplasm but also in the periplasmic compartment [37]. In addition, structural data indicate that there is a hydrophobic cavity large enough to bind an antibiotic molecule located at the dimer interface of the enzyme [18]. To understand whether this interaction has a protective action *in vivo*, we have tested the effects of several antimicrobial agents on the expression of the enzyme in *P. mirabilis*. Consequently, we have chosen the most representative molecules utilized previously [16]. Among the antimicrobial drugs tested, TET and RIF increased the level of enzyme, although similar susceptibility was observed on the growth rate of the wild-type and null-mutant strains. The increase in PmGST B1-1 protein was comparable with that observed in the PmGST B1-1 mRNA for the same drugs. It is interesting to note that TET and RIF were found to be strong inhibitors for catalytic activity of PmGST B1-1 [16].

Moreover, in contrast with what was observed in Western-blot analysis, null-mutant strains displayed the greatest sensitivity to MINO. This discrepancy could be explained considering that the basal level of the enzyme is sufficient to protect bacteria against this drug. These results suggest the involvement of PmGST B1-1 in antibiotic detoxification.

The effect of other stress factors on the expression of PmGST B1-1 was also explored. None of the general stresses tested influenced the level of the enzyme.

Taken together, these results clearly suggest that PmGST B1-1 has an active role in the protection against oxidative stress generated by H_2O_2, but it also appears that the enzyme has a wider physiological role. In particular, this enzyme appears to be also involved in the detoxification of antimicrobial agents.

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