Modulation of the glutathione S-transferase in *Ochrobactrum anthropi*: function of xenobiotic substrates and other forms of stress

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The glutathione S-transferase gene of the atrazine-degrading bacterium *Ochrobactrum anthropi* (OaGST) encodes a single-subunit polypeptide of 201 amino acid residues (Favaloro et al. 1998, *Biochem. J.* **335**, 573–579). RNA blot analysis showed that the gene is transcribed into an mRNA of about 800 nucleotides, indicating a monocistronic transcription of the OaGST gene. The modulation of OaGST in this bacterium, in the presence of different stimulants, was investigated. The level of expression of OaGST was detected both by measuring the mRNA level and by immunoblotting experiments. OaGST is a constitutive enzyme which is also inducible by several stimulants. In fact, atrazine caused an increase in the expression of OaGST even at concentrations which had no effect on growth rates of the bacteria. Moreover, the presence of other aromatic substrates of this bacterium, such as phenol and chlorophenols, leads to a marked

enhancement in OaGST expression. In this case, the expression of OaGST was related to growth inhibition and membrane damage caused by these hydrophobic compounds, and to the adaptive responses of the cell membranes. On the other hand, toluene and xylene, two aromatic compounds not degradable by this bacterium, did not induce the OaGST expression. The same was observed for other stress conditions such as low pH, heat shock, hydrogen peroxide, osmotic stress, starvation, the presence of aliphatic alcohols or heavy metals. These results suggest a co-regulation of the OaGST gene by the catabolic pathways of phenols and chlorophenols in this bacterium. Therefore, OaGST could function as a detoxifying agent within the catabolism of these xenobiotics.

Key words: GST modulation, xenobiotics, stress factors.

INTRODUCTION

Glutathione S-transferases (GSTs; EC 2.5.1.18) are a family of multi-functional dimeric proteins involved in a variety of stress conditions, and in particular in detoxification reactions. They catalyse the conjugation of glutathione (GSH) to a number of hydrophobic electrophilic substrates, and glutathione-dependent isomerizations, as well. Furthermore, they show peroxidase activity [1–4]. GSTs, using GSH as co-enzyme and/or substrate, are involved in degradation reactions, such as epoxide hydrolases [5,6] and dehalogenases [7,8]. In addition, several GSTs have the capacity to bind to a range of lipophilic compounds acting as ligands but not as substrates. Nevertheless, the binding mechanism of hydrophobic substrates to GSTs *in situ* is not entirely understood. Crystallographic studies of GSTs from eukaryotes [9–12] and prokaryotes [13,14] have elucidated the subunit architecture and quaternary structure of these enzymes. Generally, the GSH binding site is located at the N-terminal domain, whereas the binding site for toxic electrophilic compounds is located at the C-terminal domain of the protein. Although GSTs have also been isolated from many bacteria [15] their functions are still not completely understood. Bacterial GSTs seem to be implicated in the biodegradation of xenobiotics [8,16,17]. In fact, the prokaryotic members of the GST family include the dichloromethane dehalogenase of *Methylophilus* sp. [18] and the *pcpC*-encoded enzyme involved in the reductive dechlorination of pentachlorophenol by *Sphingomonas paucimobilis* [19]. More-

over, in *Pseudomonas* LB400 a GST gene was found to be included in a gene cluster of the degradation pathway of biphenyls but its function within this degradation pathway has not been described in detail [20]. It was postulated that it could be involved in the dehalogenation/detoxification of biodegradation intermediates [20].

Recently, we were able to isolate [21] and genetically describe [22] a GST from a bacterium, *Ochrobactrum anthropi*, which was able to degrade atrazine, using it as the sole carbon source [23]. This GST, like most of the described bacterial GSTs, could be included in the class Beta [13,14].

In this paper, the modulation of the *O. anthropi* GST (OaGST), in the presence of different organic compounds and under different stress conditions was investigated to better understand the function of this enzyme in prokaryotic organisms.

MATERIALS AND METHODS

Microorganism and culture conditions

All experiments were carried out with *Ochrobactrum anthropi* [23]. The strain was cultivated in M9 minimal medium supplemented with 20 mM sodium succinate as the sole carbon source. To stimulate growth, 0.3 g yeast extract/l (Difco) was added. Cells were grown in 150 ml Erlenmeyer flasks containing 40 ml of medium in a horizontal shaker at 30 °C. The aromatic substrate spectrum of *O. anthropi* was examined by testing the growth of

Abbreviations used: OaGST, glutathione S-transferase gene of atrazine-degrading bacterium *Ochrobactrum anthropi*; CDNB, 1-chloro-2,4-dinitrobenzene; GST, glutathione S-transferase; FAME, fatty acid methyl esters; as-PCR, asymmetric PCR; ARE, antioxidant response element.

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the bacterium on solid and liquid mineral medium containing the tested aromatic compounds as the only carbon and energy source. To avoid the toxic effects of aromatic compounds, 50 % growth-inhibiting concentrations (LD_{50}) were chosen. This was previously measured by growth tests on *O. anthropi* according to methods described by Heipieper et al. [24].

E. coli XL1-Blue was used as the host strain for OaGST expression and was grown in LB medium in a rotary shaker at 37 °C. For the *E. coli* strain, ampicillin was added to LB medium at a final concentration of 100 µg/ml. Isopropyl β-D-thiogalactopyranoside (IPTG; Sigma) was added to a final concentration of 1 mM for the induction of gene expression.

Measurement of growth and growth inhibition

An inoculum from an overnight culture was transferred to 40 ml fresh medium leading to a starting attenuation (*D*) of 0.05. After 4 h of exponential growth the toxins were added. Cell growth was measured by monitoring the $D_{560\text{ nm}}$ of the cell suspensions with a spectrophotometer. Growth inhibition caused by toxins was measured by comparing the difference in growth rates µ (/h) between the toxified cultures and the control culture, as described by Heipieper et al. [24]. The different concentrations of growth inhibiting toxins were defined as the percentages of the growth rates (/h) of cultures grown with toxins and that of a control culture without toxins.

Preparation of anti-OaGST antibodies

Recombinant OaGST (r-OaGST) was expressed and purified according to procedures previously described [22]. One volume of PBS containing 100 µg of r-OaGST was emulsified with 9 vol. of Freund's complete adjuvant and subsequently injected intraperitoneally into a Balb/c mouse. After primary immunization, the animal was given 500 µl of emulsion containing 100 µg of antigen on days 7, 14, and 21. Abdominal swelling occurred after the third or fourth injection. The ascitic fluid was then collected and used for immunoblotting.

Western blot analysis

O. anthropi cells cultivated in the presence of different stimulants (as described above) 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 protein concentration of the supernatant was determined by the bicinchoninic acid assay (BCA, Pierce), with BSA as standard. Crude extracts (25 µg) were analysed by SDS/PAGE in 12.5 % polyacrylamide gels. Proteins were electrophoretically transferred to nitrocellulose (for 3 h at 1 A) using an immunoblot transfer apparatus (Hoefer). After transfer, the nitrocellulose was incubated for 1 h at room temperature in 3 % (w/v) BSA in Tris 20 mM/NaCl 500 mM, pH 7.5 (TBS) to block non-specific binding. The blot was incubated overnight at 4 °C with 3 % BSA in TBS containing antiserum at a dilution of 1:500. After three 15-min washes with TBS, containing 0.1 % BSA and 0.2 % (v/v) NP-40, the blot was incubated for 1 h at room temperature with a peroxidase-conjugated goat anti-mouse immunoglobulin (Sigma) diluted at 1:1000 in 3 % BSA in TBS. The blot was again washed three times with TBS containing 0.1 % BSA, 0.2 % NP-40. Antibodies were visualized using the chemiluminescence detection system (ECL, Amersham).

RNA isolation

Total RNA was isolated from late log phase cells grown under specific conditions as described above. Samples (10 ml) were centrifuged (15000 *g* for 15 min), the cell pellet was then resuspended in 800 µl of buffer A (20 mM sodium acetate, pH 5.5, 1 mM EDTA) on ice. After addition of 40 µl of 10 % SDS and 800 µl of hot acidic phenol (pH 5.5, 65 °C), the samples were mixed for 1 min and incubated for 10 min at 65 °C. The aqueous phase was extracted with phenol/dichloromethane (1:1; pH 7) and dichloromethane. RNA was precipitated with 1:10 of 3 M sodium acetate and ethanol.

Northern blot analysis

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 pre-hybridized 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 and 0.02 % BSA). The probe was radiolabelled by asymmetric polymerase chain reaction (as-PCR). OaGST gene was obtained by digestion of pT-OaGST [22] using the *Eco*RI enzyme: the gene was recovered from a low-melting agarose gel and used as template for the as-PCR. The primer was designed as follows: primer Rev-O 5'-ACCGATGATCACCGACGC-3' mapping at the nucleotide positions 512–495 of the OaGST gene. In addition to the template (100 ng) and the Rev-O primer (500 ng), the 10 µl reaction mixture contained 1 mM MgCl₂, dNTPs (dATP, dTTP and dGTP at a concentration of 20 µM; dCTP 10 µM), 25 µCi [α -³²P]dCTP (3000 Ci/mmol, Amersham), Taq DNA polymerase buffer and 2.5 units of Taq DNA polymerase (Promega). The as-PCR reaction was subjected to 30 cycles of amplification (60 s at 94 °C, 120 s at 54 °C, 120 s at 72 °C). Blots were exposed to Kodak X-Omat film at –80 °C. Quantitative densitometric assays of chromatographic blot analysis of protein and mRNA were performed using laser scanning (Umax Data System Inc.) by NIH Image. Each blot was repeated at least three times and the data shown represent typical results.

Lipid extraction and transesterification

Cells of 30-ml suspensions (about 3×10^{10} cells) were centrifuged 3 h after addition of the toxic agents and washed with phosphate buffer (50 mM, pH 7.0). The lipids were extracted with chloroform/methanol/water as described by Bligh and Dyer [25]. Fatty acid methyl esters (FAME) were prepared by 15 min incubation at 95 °C in boron trifluoride/methanol using the method of Morrison and Smith [26]. The FAME were extracted with hexane.

Determination of fatty acid composition

Analysis of FAME was performed using a 8520 gas chromatograph (Dani, Italy – capillary column: Chrompack-Sil 5 CB; 25 m; temperature programme from 150 to 250 °C; flame ionization detector). The FAME were identified with the aid of standards. The relative amounts of the FAME were determined from their peak areas using a Chromatopac C-R6A integrator (Shimadzu, Kyoto, Japan). Replicate determinations indicated that the relative error (S.D./mean) × 100 % of the values was less than 5 %.

Assay of GST activity

The activity was assayed by the spectrophotometric method described by Habig and Jakoby [27], using 1 mM GSH and 1 mM 1-chloro-2,4-dinitrobenzene (CDNB) as substrates. Protein concentration was determined by the bicinchoninic acid assay ('BCA', Pierce) with bovine serum albumin as standard.

Measurement of cellular K⁺ content

One-millilitre samples were withdrawn before and after addition of toxins. Separation of cells from supernatant was carried out by rapid centrifugation. The cell pellets were disrupted in 5% (w/v) trichloroacetic acid, and debris was removed by centrifugation. The released K⁺ of the supernatant was measured by flame photometry in a Unicam atomic absorption spectrometer (Unicam). All experiments were repeated 3 times. The average data of these results are shown. The standard deviation was less than 5%.

Glutathione determination

The GSH standard was prepared quantitatively using 25 mM KH₂PO₄ adjusted to a pH of 3. Crude extracts of *Ochrobactrum anthropi* cells cultivated in the presence of different stimulants (as described above in Western blot analysis), after the addition of an equal volume of acetone, were harvested by centrifugation (10000 *g* for 5 min). The supernatants were lyophilized and resuspended in the same buffer used to prepare the GSH standard. The samples were analysed by capillary electrophoresis (Capillary electrophoresis) analysis. The results are expressed as µg of GSH per protein concentration.

Capillary electrophoresis was performed using a P/ACE 2100 system (Beckman Instruments, Fullerton, CA, U.S.A.), equipped with a UV detector, and elaborated with a Gold 6.0 software acquisition system on an IBM 55SX personal computer. The column used was an uncoated fused-silica capillary tube (Polymicron) of 50 µm ID, 50 cm length to detector and with a total length of 57 cm. Electrophoretic analysis was performed using the UV detector set at a wavelength of 200 nm, and at an applied voltage of 15 kV at 25 °C. Moreover, the silica column was pre-rinsed with bi-distilled water (1 min) and separation buffer (1.5 min); the capillary was washed with 0.1 M NaOH (10 min), double-distilled water (5 min), and buffer (60 min). The electrolyte buffer was 25 mM NaH₂PO₄, 15 mM Na₂B₄O₇ and 50 mM SDS, pH 8.3.

RESULTS

Catabolism of aromatic compounds by *O. anthropi*

In a screening test for consumption of aromatic compounds it was shown that besides atrazine [23], the cells were also able to grow with phenol and several chlorophenols as carbon and energy source (data not shown). This was also reported for other strains of *O. anthropi* [28,29]. Non-hydroxylated aromatic compounds, like toluene or xylene, were not metabolized by this bacterium (data not shown).

Transcriptional analysis of the OaGST gene

To determine the transcript size of the OaGST gene and the modulation of its mRNA, total RNA from *O. anthropi* cultivated in minimal medium with succinate as carbon source and in the presence of several stimulants was subjected to Northern analysis. The RNAs were hybridized to a specific antisense probe obtained by an asPCR (see Material and methods). An RNA transcript of

Figure 1 Northern blot analysis of total RNA from *O. anthropi*

Modulation of the OaGST transcript (A); 23S and 16S rRNA ethidium bromide stained (B); C, control; ATR, atrazine; PHE, phenol; 4-CP, 4-chlorophenol.

approx. 800 nucleotides was found, indicating that the OaGST gene appears to be monocistronically transcribed. Transcription was induced under xenobiotic stress conditions by atrazine, CDNB, phenol, and 4-chlorophenol, but not by osmotic stress caused by the addition of 0.4 M NaCl (Figure 1).

Moreover, the increased level of the transcript found in cells grown in the presence of atrazine and aromatic compounds is consistent with the increase observed in Western blot (Figure 2).

Modulation of OaGST in the presence of different stimulants

To quantify the effects of different stimulants on the regulation of OaGST expression, the different stimulants were added to the exponentially growing cells of *O. anthropi* at different growth-inhibiting concentrations. The expression of OaGST level was measured by immunoblot analysis (Western blot) using an OaGST antiserum.

Figure 2 is representative of an immunoblot analysis of the OaGST level in the crude extracts of cells grown either in the presence of atrazine or of several aromatic compounds. The quantification of this increase due to selected aromatic compounds, is shown in Figures 3–6 (lower panels). Atrazine was slightly toxic to the bacteria. Even at concentrations above the water solubility of this compound (30 mg/l), the cells were not completely inhibited in their growth. Even at very low concen-

Figure 2 Immunoblot analysis of the cytosolic fraction of *O. anthropi*

Twenty-five μ g of crude extracts were loaded per lane for Western blot analysis. (A) C, control; 4-CP, 4-chlorophenol. (B) C, control; ATR, atrazine; R-OaGST, recombinant GST of *O. anthropi*.

trations, atrazine caused up to a 4.4-fold increase in the level of OaGST, although there was no effect on bacterial growth (Figures 2, 3). On the other hand, the presence of organic solvents, such as phenol (Figure 4), 4-chlorophenol (Figures 2 and 5), CDNB (Figures 2 and 6), and 2,4-chlorophenol (data not shown) led to a concentration-dependent inhibition of bacterial growth. These compounds also induced a marked increase in cellular OaGST; this effect was correlated to the growth inhibition caused by compounds tested at different concentrations.

Aromatic compounds are known to be toxic, depending on their hydrophobicity, expressed as their log *P* values [30]. The higher the log *P* values of phenols, the higher the increase in the OaGST level. In fact, phenol (log *P*: 1.45) leads to a 9.5-fold increase in OaGST, these values for 4-chlorophenol (log *P*: 2.40) and 2,4-dichlorophenol (log *P*: 3.21) were 14.9-fold and 16.5-fold, respectively.

To evaluate whether the OaGST-inducing agents are all thiol-active, possessing also a common activation mechanism, we detected the intracellular GSH level in bacteria grown either in

Figure 3 Effect of atrazine

Effect on growth (●), K^+ content 1 h after addition of the stimulants (◆); increase in OaGST content measured by Western blot analysis (▲), and degree of saturation of membrane fatty acids (■) of *O. anthropi*.

the presence or absence of stimulants. As shown in Table 1, non-appreciable differences in the GSH content were observed.

Moreover, the co-administration of stimulants and the antioxidant *N*-acetylcysteine did not influence the induction of OaGST (data not shown). This indicates that the oxidative metabolism of phenol did not contribute to the induction of OaGST.

Effect of the stimulants on OaGST enzyme activity

None of the cell extracts investigated, not even the ones with the highest cellular concentration of OaGST, showed an increase in the enzymatic GST activity using CDNB as substrate (data not shown).

Effect of different organic compounds on the cell membranes

Organic solvents are known to be toxic to cells, mainly due to their action on the membranes as so-called 'membrane-active compounds' [30]. Therefore, it was interesting to test whether the OaGST induction showed a correlation with the toxic actions of these compounds to the cells and the adaptive membrane reactions, respectively. We investigated possible relations between the effects of aromatic compounds on the modulation of OaGST and their actions on membranes. One of the first indications of membrane damage in bacteria caused by organic

Figure 4 Effect of phenol

Effect on growth (●), K⁺ content 1 h after addition of the stimulants (◆); increase in OaGST content measured by Western blot analysis (▲), and degree of saturation of membrane fatty acids (■) of *O. anthropi*.

Figure 6 Effect of CDNB

Effect on growth (●), K⁺ content 1 h after addition of the stimulants (◆); increase in OaGST content measured by Western blot analysis (▲), and degree of saturation of membrane fatty acids (■) of *O. anthropi*.

Figure 5 Effect of 4-chlorophenol

Effect on growth (●), K⁺ content 1 h after addition of the stimulants (◆); increase in OaGST content measured by Western blot analysis (▲), and degree of saturation of membrane fatty acids (■) of *O. anthropi*.

Table 1 Effect of different stimulants on GSH concentrations measured by capillary electrophoresis

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

GSH (μg/mg crude extract)	
Control	8.61 ± 0.83
Atrazine	8.36 ± 0.91
Phenol	8.66 ± 0.87
4-Chlorophenol	9.12 ± 0.89
CDNB	8.02 ± 0.78

solvents is the efflux of potassium ions [31,32]. Figures 3–6 (upper panels) show the cellular potassium concentrations, 1 h after the addition of toxins. After this time, all possible cellular reactions had taken place in order to compensate the loss of K⁺, e.g. by an enhanced uptake. A dose-dependent correlation was observed between the growth inhibition caused by the investigated compounds, and the loss of cellular K⁺ content.

Other aspects demonstrating the toxicity of membrane-active compounds are the membrane-adaptive responses of the bacteria to these chemicals. Hereby, the modification of the fatty acids represents the main adaptive response of bacteria to membrane-active compounds [33,34]. Figures 3–6 show that there is indeed a correlation between an increase in the saturation of membrane fatty acids and the level of expression of OaGST.

Table 2 Effect of different compounds and stress factors on the expression of OaGST measured by Western blot with polyclonal antibodies

Compound/stress factor	Induction of OaGST	Concentration (mM) causing the highest OaGST expression
Phenol	+	5.00
4-Chlorophenol	+	0.60
2,4-Dichlorophenol	+	0.12
Toluene	—	—
Xylene	—	—
Atrazine	+	0.50
1-Chloro-2,4-dinitrobenzene	+	0.05
Methanol	—	—
Ethanol	—	—
1-Octanol	—	—
Osmotic stress (NaCl)	—	—
Zn ²⁺	—	—
Heat shock	—	—
Low pH	—	—
Starvation	—	—
Hydrogen peroxide	—	—

Effect of other stress conditions on the expression of OaGST

The results and correlation obtained in the former, posed the question whether the modulation of OaGST expression was caused not only by the aromatic compounds but also by other stress conditions which had been shown to also cause adaptive responses [35–37], other stress factors were applied to exponentially growing cells. For all stress factors (heat shock, low pH, osmotic stress, hydrogen peroxide, heavy metals or aliphatic alcohols), were chosen conditions that reduced cell growth up to 50 % and caused the greatest effects in adaptive responses [38]. The results are shown in Table 2.

None of the general stress factors affected the level of OaGST. Furthermore, although toluene and xylene are aromatic compounds, they did not affect OaGST expression, possibly because they are not degraded by *Ochrobactrum anthropi*.

DISCUSSION

Here we demonstrate, for the first time in bacteria, the physiological regulation mechanisms of a bacterial GST, at both the RNA and protein expression level. Membrane-active toxins, like phenol and chlorinated phenols, cause a dose-dependent increase in the expression of OaGST, directly correlated to the toxicity of the compounds, while atrazine causes an increase in OaGST expression at very low, non-toxic concentrations.

As discussed recently by Hayes et al. [39] many of the GST inducing agents in mammalian cells require to be converted to thiol-active metabolites in order to be effective, and the GSH depletion seems to play a role on GST activation. Since the GSH concentration in *O. anthropi* cells grown in the presence of stimulants is not affected, the concentration of thiols does not seem to play a role in the OaGST expression, suggesting a different way of GST induction in bacteria.

The induction mechanism in *O. anthropi* appears to be very different compared to eukaryotic cells, indeed, also the oxidative stress (i.e. hydrogen peroxide) is not involved in OaGST modulation.

In eukaryotic cells, the phenolic compounds which form reactive oxygen species and oxidants, such as hydrogen peroxide,

activate the expression of the GST through the antioxidant-responsive element (ARE). The ARE represents a regulatory element which activates genes encoding proteins e.g. glutathione S-transferases that protect eukaryotic cells against oxidative stress [40–42]. In the phenol-degrading bacteria the first metabolic step is the formation of catechol. This oxidative metabolism could suggest an OaGST induction mechanism similar to the eukaryotic system. Although the OaGST level was enhanced by the presence of phenol, the co-administration of the antioxidant *N*-acetylcysteine did not modulate the OaGST induction, suggesting a different mechanism compared to the eukaryotic ARE-mediated transcriptional activation. Thus, the expression of OaGST is strictly related to growth inhibition and to membrane damage (K⁺ efflux) caused by hydrophobic membrane-active compounds. The fact that induction of OaGST is also correlated to adaptive cell responses to membrane damage (e.g. increase in fatty acid saturation) could give rise to the hypothesis that OaGST is involved in the general response mechanisms of cells to toxins. On the other hand, typical stress factors such as starvation, heat shock, osmotic stress, low pH, heavy metals or aliphatic alcohols showed no effect. This demonstrates that the increase of OaGST level caused by phenols is not due to regulation via the stress response described for enzymes involved in detoxification and/or adaptation of/to toxic compounds [38]. This also excludes genetic regulation exerted by the sigma factor global regulator necessary for general stress response (σ^S) [43].

Only aromatic compounds which are a potential substrates for OaGST (like atrazine and CDNB) or which are degraded by this bacterium (like atrazine, phenol and chlorophenols) induce the OaGST gene. Thus, the hydrophobicity of the compounds seems to play a role in the increase of OaGST expression. The phenols tested showed that the higher hydrophobicity was correlated to the higher expression level of OaGST. The penetration of these compounds into the membrane could play an important role in the regulation of OaGST expression. On the other hand, compounds which are also known to be membrane active, causing the same effects as phenols, such as toluene and xylene [34], cannot be degraded by this bacterium and caused no increase in OaGST expression.

This leads to the hypothesis of a possible co-regulation of the (unclustered) OaGST, which seems to be monocistronically transcribed by mechanisms leading to an induction of catabolic pathways for aromatic compounds, especially phenols, in *O. anthropi*.

Up to now, the involvement of GST in bacterial degradation of phenols or chlorophenols has never been reported. GST could help protect the cells from damage caused by toxic intermediates, produced during the action of either monooxygenases (phenols, catechols) or dioxygenases (catechols, *cis*, *cis* muconic acid-derivatives) involved in the degradation of phenol and chlorophenols.

The regulation of OaGST in the presence of aromatic substrates seems to be very complex. Only compounds which are potential substrates for this bacterium induce OaGST expression. In the case of phenols this expression also seems to depend on the toxicity caused by these membrane-active aromatic compounds.

It has been suggested that bacterial GSTs could be implicated in the degradation of herbicides in view of the involvement of plant GSTs in the conjugation of herbicides to glutathione [44–46]. Previously, we observed that atrazine and other herbicides did not inhibit OaGST conjugation activity *in vitro* [22]. However, atrazine also causes an increase in the expression of OaGST at a very low concentration, independent from any toxic action, thus suggesting an involvement of this enzyme in atrazine metabolism.

None of the samples, not even the ones showing the highest increase in the cellular level of OaGST, showed an increase in the enzymatic GST activity using CDNB as substrate, known to have the highest specificity for OaGST [22]. Hzuka et al. [47] showed no effect on GST activity in growing *E. coli* B cells in the presence of CDNB. The increase in OaGST protein, as detected by Western blot analysis, was comparable to that observed in the OaGST mRNA. The absence of a corresponding increase in enzyme activity suggests either the presence of an inhibitor of the enzyme, or the need for additional modifications of the protein to generate enzyme activity.

Further experiments will attempt to verify the hypothesis concerning the co-regulation of OaGST associated with the catabolic pathway(s) for aromatic substrates of this bacterium and the function of GST in this catabolism.

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