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REVIEW ARTICLE

Glutathione transferases in bacteria

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Bacterial glutathione transferases (GSTs) are part of a superfamily of enzymes that play a key role in cellular detoxification. GSTs are widely distributed in prokaryotes and are grouped into several classes. Bacterial GSTs are implicated in a variety of distinct processes such as the biodegradation of xenobiotics, protection against chemical and oxidative stresses and antimicrobial drug resistance. In addition to their role in detoxification, bacterial GSTs are also involved in a variety of distinct metabolic processes such as the biotransformation of dichloromethane, the degradation of lignin and atrazine, and the reductive dechlorination of pentachlorophenol. This review article summarizes the current status of knowledge regarding the functional and structural properties of bacterial GSTs.

Introduction

Glutathione transferases (GSTs; EC 2.5.1.18) constitute a protein superfamily that is involved in cellular detoxification against harmful xenobiotics and endobiotics [1–3]. One common feature of all GSTs is their ability to catalyse nucleophilic attack by the tripeptide glutathione (GSH) on the electrophilic groups of a wide range of hydrophobic toxic compounds, thus promoting their excretion from the cell [1]. GSTs are also involved in several other cell functions, and are capable of binding a large number of endogenous and exogenous compounds non-catalytically [1]. GSTs are widely distributed in nature and are found in both eukaryotes and prokaryotes. GSTs are divided into at least four major families of proteins, namely cytosolic GSTs, mitochondrial GSTs, microsomal GSTs and bacterial fosfomycin-resistance proteins [1,4]. The cytosolic GSTs (cGSTs) have been subgrouped into numerous divergent classes on the basis of their chemical, physical and structural properties [1,2]. The mitochondrial GSTs, also known as kappa class GSTs, are soluble enzymes that have been characterized in eukaryotes [5]. The third GST family comprises membrane-bound transferases called membrane-associated proteins involved in ecosanoid and glutathione metabolism (MAPEG), but these bear no similarity to soluble GSTs [6]. Representatives of all three families are also present in prokaryotes. The fourth family is found exclusively in bacteria.

Abbreviations
BxGST, *Burkholderia xenovorans* GST; CDNB, 1-chloro-2,4-dinitrobenzene; cGST, cytosolic GST; DCM, dichloromethane; EcGST, *Escherichia coli* GST; GSH, glutathione; GST, glutathione transferase; HCCA, 2-hydroxychromene-2-carboxylic acid; MAPEG, membrane-associated proteins involved in ecosanoid and glutathione metabolism; OaGST, *Ochrobactrum anthropi* GST; PmGST, *Proteus mirabilis* GST; TCHQ, tetrachlorohydroquinone.

The first evidence for the presence of GSTs in bacteria was reported more 25 years ago by Takashi Shishido who showed the presence of GST activity in a strain of *Escherichia coli* [7]. Since then, GSTs have been found to be broadly distributed in aerobic prokaryotes, including human and plant pathogens and soil bacteria. Conversely, GSTs have not been identified to date in anaerobic bacteria [8] or in Archaea, the other domain of prokaryotes. The absence of the enzyme in these microorganisms is consistent with the lack of GSH [9].

This review summarizes current knowledge on the functional and structural properties of bacterial GSTs and the potential biotechnological applications of these enzymes.

Classification and phylogenetic relationships

cGSTs, also termed canonical GSTs, are homo- or heterodimeric enzymes found in aerobic forms of life. cGSTs metabolize a broad range of electrophilic substrates via GSH conjugation. They are involved in protecting cells against oxidative stresses, have peroxidase and isomerase activities and are implicated in the development of drug resistance [1,2]. In mammalian species they are well characterized and have been grouped into seven classes: alpha, mu, pi, sigma, theta, omega and zeta [1]. Several other classes are restricted to non-mammalian species: beta, chi, delta, epsilon, lambda, phi and tau [1,2,10]. To assign a cGST to a class, it is generally accepted that proteins with $> 40\%$ sequence identity belong to the same class, whereas GSTs of different classes share $< 25\%$ sequence identity. The identity increases if the N-terminal region only is considered. This region comprises part of the active site, with residues that interact with GSH, and it is evolutionarily conserved [1,2,11]. Besides amino acid sequence identity, immunological properties, kinetic features as well as similarity of the crystal structures provide additional supporting data [1,2].

In bacteria, four different classes of cGSTs have been identified: beta, chi, theta and zeta [2,10,12,13]. Beta class cGSTs have been purified and characterized from several bacteria [14–25]. They are able to conjugate the model substrate 1-chloro-2,4-dinitrobenzene (CDNB) and bind to the GSH–affinity matrix. All beta class enzymes are characterized by the presence of a cysteine residue at the GSH site [12]. The first cGST of this class was characterized from a *Proteus mirabilis* strain (PmGST). PmGST displayed biochemical and structural properties that distinguish it from the GSTs of other families and it was identified as the prototype of the beta class [14,26–36].

Three other orthologues of the beta class were also functionally and structurally characterized, from *E. coli* (EcGST) and from two soil bacteria, *Ochrobactrum anthropi* (OaGST) and *Burkholderia xenovorans* (BxGST, also known as BphK), respectively [20,21,24,37–47].

Theta class enzymes in bacteria are represented by two dichloromethane (DCM) dehalogenases produced by facultative methylotrophic bacteria [48–52]. They exhibit high amino acid sequence similarity to eukaryotic theta class GSTs and also share some properties of these enzymes such as their reactivity with DCM, their lack of activity with CDNB and their inability to bind to GSH affinity matrices [50].

Tetrachlorohydroquinone (TCHQ) dehalogenase was reported by Anandarajah *et al.* [53] to belong to the zeta class on the basis of multiple sequence alignments. In particular, this enzyme is characterized, in the GSH site, by the distinctive motif of zeta class enzymes including two serine and a cysteine residues. TCHQ dehalogenase is involved in two steps of the biodegradation of pentachlorophenol and it also has isomerase activity [53–56].

Recently a novel class of cGSTs, called chi class, was proposed [10]. Two cyanobacterial cGSTs have been purified and characterized from *Thermosynechoccus elongatus* BP-1 (TeGST) and *Synechoccus elongatus* PCC 6301 (SeGST). Although TeGST and SeGST showed typical structural features of cGSTs, the results presented here argued against their incorporation into the beta class. In particular, these enzymes lack cysteines completely indicating a possible different evolutionary pathway for the cyanobacterial GSTs from the beta class GSTs.

Like eukaryotic organisms, bacteria are characterized by multiple GST genes of widely divergent sequences and unknown function [57]. In the *E. coli* genome, in addition to the beta class GST [37] and to Stringent starvation protein A, a RNA polymerase with fold similarity to cGSTs [3], six GST homologues have been identified [58]. The products of two of these genes, YfcF and YfcG, exhibited GST- and GSH-dependent peroxidase activities and were involved in the defence against oxidative stress [59]. Pseudomonads possess more than 10 GST genes [57]. In *P. mirabilis* as well as in *Proteus vulgaris* three and four different GSTs were identified, respectively [14,60]. Recently, genomic analysis of the Gram-negative *Shewanella oneidensis* revealed the presence of six GST-like genes [61]. Two of these GST products showed high sequence similarities to DCM dehalogenases.

Bacterial 2-hydroxychromene-2-carboxylic acid (HCCA) isomerase is a GSH-dependent enzyme that is impli-

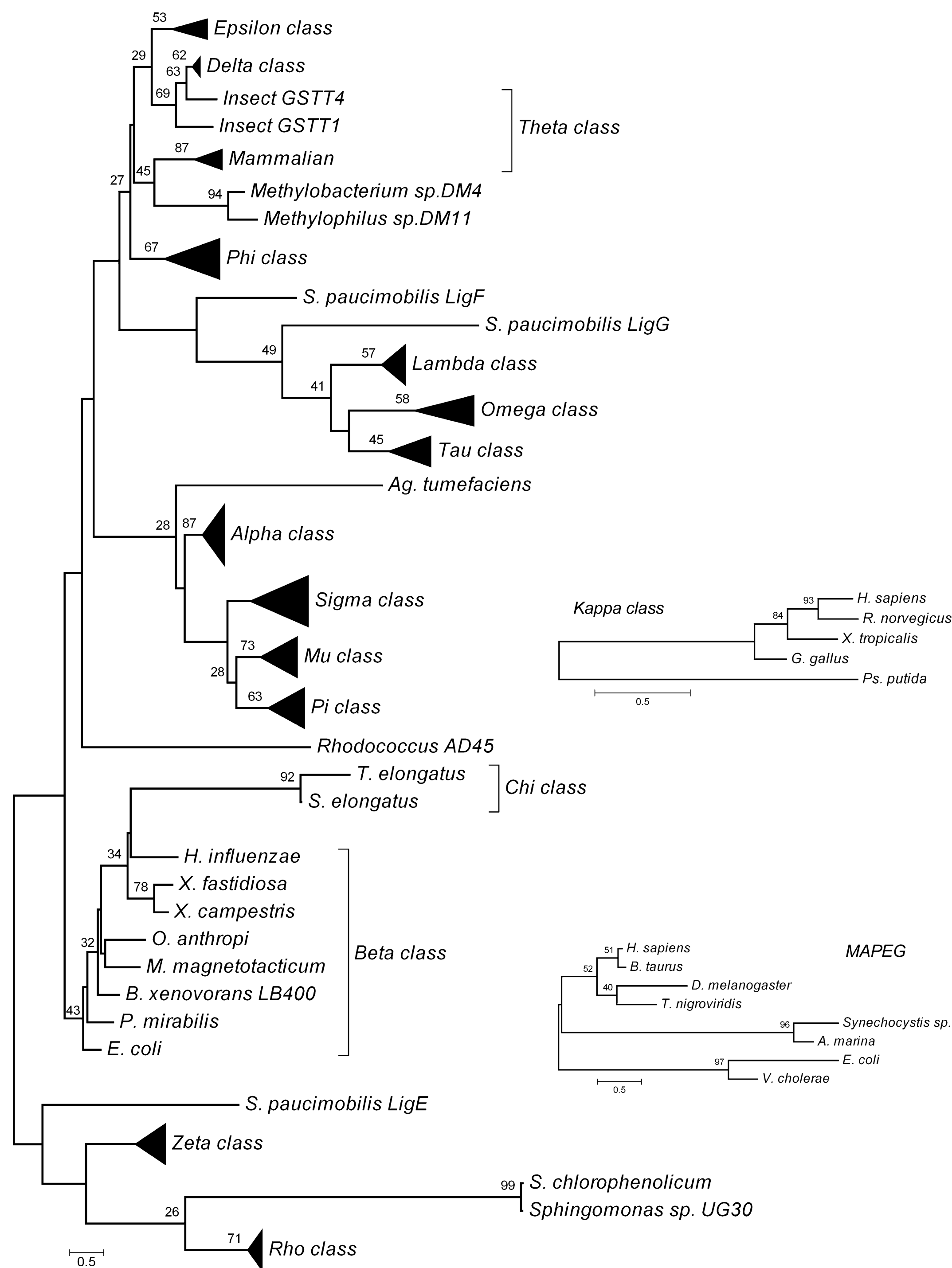
Fig. 1. Evolutionary relationship between representative bacterial and eukaryotic GSTs. Multiple sequence alignment was produced by using CLUSTAL W2 [143] and improved manually. The unrooted phylogenetic trees were constructed by the neighbour-joining method, based on the distances derived from the Dayhoff matrix, with MEGA 4.0 software [144]. The robustness of the branches was assessed by the bootstrap method with 1000 replications. Only nodes with a bootstrap value > 25% are reported. The scale bar represents a distance of 0.5 substitutions per site. The sequences have the following accession numbers: epsilon class: *Anopheles gambiae* (XP_319972), *Drosophila melanogaster* (CG5164); delta class: *An. gambiae* (Q93113), *Anopheles dirus* (AF273039); theta class: *D. melanogaster* (Q9VG96), *An. gambiae* (Q94999), *Bos taurus* (Q2NL00), *Homo sapiens* (P30712); *Methylobacterium* sp. DM4 (P21161); *Methylophilus* sp. DM11 (P43387); phi class: *Arabidopsis thaliana* (P42769), *Zea mays* GSTF3 (Q9ZP62), *Z. mays* GSTF1 (P12653), *Triticum aestivum* (P30111); *Sph. paucimobilis* LigF (P30347); *Sph. paucimobilis* LigG (BAA77216); lambda class: *A. thaliana* (Q9LZ07), *Z. mays* (P49248), *A. thaliana* GSTL2 (Q9M2W2); omega class: *H. sapiens* (P78417), *Rattus norvegicus* (Q9Z339), *Caenorhabditis elegans* (AAA27959); tau class: *Aegilops tauschii* (O04941), *Z. mays* GSTU1 (Y12862), *Z. mays* GSTU2 (AJ010439); *Agrobacterium tumefaciens* (Q8UJG9); alpha class: *H. sapiens* GSTA1 (P08263), *Mus musculus*, GSTA2 (P10648); *R. norvegicus* (P04904), *Gallus gallus* GSTA1 (Q08392), *G. gallus* GSTA2 (Q08393), *Bos taurus* (Q5E9G0); sigma class: *H. sapiens* (O60760), *R. norvegicus* (O35543), *C. elegans* (O16116), *Manduca sexta* (P46429), *Ommastrephes sloanei* (P46088); mu class: *H. sapiens* (P09488), *M. musculus* (P10649), *G. gallus* (P20136), *Dermatophagoides pteronyssinus* (P46419); pi class: *Onchocerca volvulus* (P46427), *Bufo bufo* (P81942), *H. sapiens* (P09211), *R. norvegicus* (P04906); *Rhodococcus* AD45 (AJ249207); chi class: *T. elongatus* (NP_680998), *S. elongatus* (YP_171005), beta class: *Haemophilus influenzae* (P44521), *Xylella fastidiosa* (Q9PE18), *Xanthomonas campestris* (P45875), *O. anthropi* (P81065), *Magnetospirillum magnetotacticum* (ZP_00054555), *B. xenovorans* LB400 (Q9RAFO), *P. mirabilis* (P15214), *E. coli* (P39100); *Sph. paucimobilis* LigE (BAA02032); zeta class: *H. sapiens* (O43708), *M. musculus* (Q9WVL0), *T. aestivum* (O04437), *A. thaliana* (Q9ZVQ3); *Sphingobium chlorophenolicum* (Q03520); *Sphingomonas* spUG30 (AY057901); rho class: *Pleuronectes platessa* (X63761), *Pagrus major* (AB158412), *Micropterus salmoides* (AY335905), *Branchiostoma belcheri tsingtaunese* (AY279519); kappa class: *H. sapiens* (Q9Y2Q3), *R. norvegicus* (P24473), *Xenopus tropicalis* (AAH87819), *G. gallus* (XP_416525), *Ps. putida* (Q51948). MAPEG: *H. sapiens* (P10620), *B. taurus* (Q64L89), *D. melanogaster* (AAN85305), *Tetraodon nigroviridis* (CAF97117), *Synechocystis* sp. (P73795), *Acaryochloris marina* (YP_001518348), *E. coli* (P64515), *V. cholerae* (NP_232423).

cated in the naphtahalene degradation pathway [5,62,63]. HCCA isomerase shares strong similarity with kappa class enzymes, has a conserved serine residue in the GSH site and displays catalytic activity against CDNB [63]. Kappa class GSTs are soluble dimeric proteins isolated and characterized from mammalian mitochondria [1,5]. In humans, hGSTK1 was also located in the peroxisomal fraction suggesting a new role for this class [1]. They are structurally distinct from cGSTs, exhibit GSH activity towards haloarenes and reduce cumene hydroperoxide and *tert*-butyl hydroperoxide [1,5].

The MAPEG family are a wide and ubiquitous group with diverse biological functions [6,64]. The eukaryotic MAPEG family comprises at least six members: 5-lipoxigenase activating protein (FLAP) and leukotriene C_4 synthase (LTC_4) involved in leukotriene biosynthesis, MGST1, MGST2 and MGST3 with GST and peroxidase activity and prostaglandin E synthase (PGES) which catalyses the formation of prostaglandin E_2 from prostaglandin H_2 [6,64,65]. Recently, crystallographic studies on different MAPEG members clearly demonstrated that they are arranged into trimers [66].

MAPEG members were also identified in several bacteria such as *E. coli*, *Vibrio cholerae* and *Synechocystis* sp. [6]. Some of them contained multiple MAPEG paralogues. They showed distant evolutionarily relationships compared with eukaryotic mammalian and non-mammalian forms. Bacterial MAPEG proteins have been grouped in two distinct subfamilies, one of which comprises *E. coli* and *V. cholerae* proteins and the other the *Synechocystis* sp. protein which more closely resembles enzymes belonging to the MGST2/FLAP/LTC_4 subgroup [65]. Genes encoding MAPEG proteins from *E. coli* and *Synechocystis* sp. have been cloned and overexpressed. Membrane fractions from cells overproducing *E. coli* MGST as well as SynMGST showed GST activity with CDNB as second substrate [65]. To date, no information about their physiological role in bacteria is available.

A phylogenetic analysis using representative prokaryotic and eukaryotic members of the GST superfamily is shown in Fig. 1. The phylogenetic relationships among bacterial GSTs are consistent with their known functional and structural features, showing that they are distributed in different classes. The beta class comprises the majority of bacterial GSTs and enzymes belonging to the chi class are closely related to this class. The two DCM dehalogenases are clustered together with mammalian theta class enzymes. TCHQ dehalogenases are closely related to the zeta class. *Agrobacterium tumefaciens* GST shows closer evolutionary relationship with mammalian alpha class than with bacterial GSTs as reported previously [67]. *Sphingomonas paucimobilis* LigF and LigG group together and are distant from the *Sph. paucimobilis* LigE enzyme which is more closely related to the zeta class enzymes. Finally, *Rhodococcus* AD45 form a new branch and it is not grouped with any other class.



Because kappa class and MAPEG proteins are evolutionarily distant from cGSTs two separate trees are presented as insets in the Fig. 1. Bacterial MAPEG proteins group with eukaryotic members, whereas *Pseudomonas putida* HCCA isomerase groups with mammalian kappa class members. Fosfomycin resistance proteins show high divergence in primary sequence and in structure, thus they were not considered.

This classification is in agreement with the evolutionary model proposed by Frova [11]. According to this model, the ancestor from which GSTs originated

is thioredoxin. The first phase of the evolution of GSTs from thioredoxins saw the addition at the C-terminus, or the insertion between the thioredoxin fold, of an all-helical domain to produce two intermediates: bacterial glutaredoxin 2 (GRX2), which evolved into the cGSTs; and bacterial disulfide-bond-forming oxidoreductase A (DsbA), which evolved into the kappa GSTs. In a second phase, cGSTs further diversified into different classes. Beta class enzymes evolved after the dimerization of GRX2. This model is supported by the ability of beta class enzymes to act as thiol disulfide oxidoreductases as well as GSH-conjugating enzymes and by the presence of a cysteine residue in the GSH site [14,68,69]. Finally, a shift from cysteine to serine chemistry resulted in the theta class, followed by the zeta class and then by all others classes. To date, there have are no clear hypotheses about the evolution of MAPEG family members [11].

Structures

Most of the bacterial GSTs identified to date belong to the bacterial-specific beta class and since 1998 the crystal structures of four representatives of this class have been determined, i.e. *P. mirabilis* GST (PmGST) [12], *E. coli* GST (EcGST) [37], *B. xenovorans* BphK (BxGST) [47] and *O. anthropi* GST (OaGST) [41]. This, together with extensive site-directed mutagenesis analysis, has allowed us to dissect in detail the structural and catalytic properties of beta class enzymes.

Beta class GSTs are homodimers with a chain length of $\sim$ 201–203 residues. They display the canonical GST fold with a thioredoxin-like N-terminal domain followed by an all-helical C-terminal domain separated by a short linker (Fig. 2A).

Comparative analysis of the crystal structures reveals that the overall fold in beta class GSTs is remarkably well conserved. Root mean square deviations among equivalent Cαs are generally < 1.5 Å when protein monomers are superimposed, even though sequence identities can be < 35–40%. When beta class monomers are superimposed onto cGSTs belonging to other classes rmsd values increase, ranging from 1.85 to 2.67 Å; these values indicate that the canonical fold has been substantially maintained from bacteria to mammals. Also the monomers' orientation in the different dimers is remarkably conserved (Fig. 2B). For example, when superimposing the OaGST dimer to the PmGST, EcGST and BxGST dimers, rmsd values of 1.614, 1.418 and 1.54 Å, respectively, are obtained [41]. These values are very close to those obtained by superimposing the monomers alone suggesting that strict conservation of the relative orientation of the monomers in the dimer is required for function.

Although the monomer fold in beta class GSTs closely resembles that of other cGSTs, the dimer interface is quite different. In fact, in contrast to other GST classes, there is no open V-shaped interface, although a close-packed arrangement is found, shaped by the presence of stabilizing contacts at both the base and top of the helical pairing that builds the interface [12]. Furthermore, the interface in the majority of GST classes is mainly hydrophobic in nature, whereas in beta class GSTs it is markedly polar. As a consequence, the well-known lock-and-key motif of alpha, pi and mu class GSTs, comprising a protruding aromatic residue of one monomer that fits into a hydrophobic pocket in the other, is absent in beta class GSTs [12,41]. Conversely, other structural motifs originally identified in mammalian GSTs are conserved in beta class GSTs indicating their ancient origin. Among them, an important stabilizing role is played by the so-called N-capping box and hydrophobic staple motifs at the N-terminus of the sixth helix in the C-terminal domain that contribute to the interaction with the N-terminal thioredoxin-like domain [36]. An additional structural motif has been identified in beta class GSTs that appears to be restricted to this class. This motif is formed by a network of hydrogen bonds, located in the proximity of the G-site, which zippers the terminal helix of the C-terminal domain to the starting helix of the thioredoxin-like domain. Partial disruption of this motif has been shown to have dramatic consequences on the both the stability and the functionality of OaGST [41].

GSH binds to beta class GSTs in an extended fashion consistent with what is observed in other classes. Several interactions, both polar and hydrophobic, contribute to its stabilization, including a hydrogen bond with an aspartate residue from the other monomer [12,41].

All beta class GSTs are characterized by the presence of a conserved cysteine residue located close to the GSH sulfydryl group. Notably a mixed disulfide bond was found in the structure of PmGST, with the two sulfurs located at a distance of 2.2 Å (Fig. 2C) [12]. This oxidized state, however, was not found in the structures of OaGST [41] and BxGST [47], where the average distance between sulfur atoms is $\sim$ 3.3 Å, consistent with GSH being in the thiolate form and sharing a hydrogen with the cysteine sulfur of the protein. In PmGST, two other residues are at hydrogen bond distance from the GSH sulfydryl group, a histidine (His106) and a serine (Ser9) (Fig. 2C). The

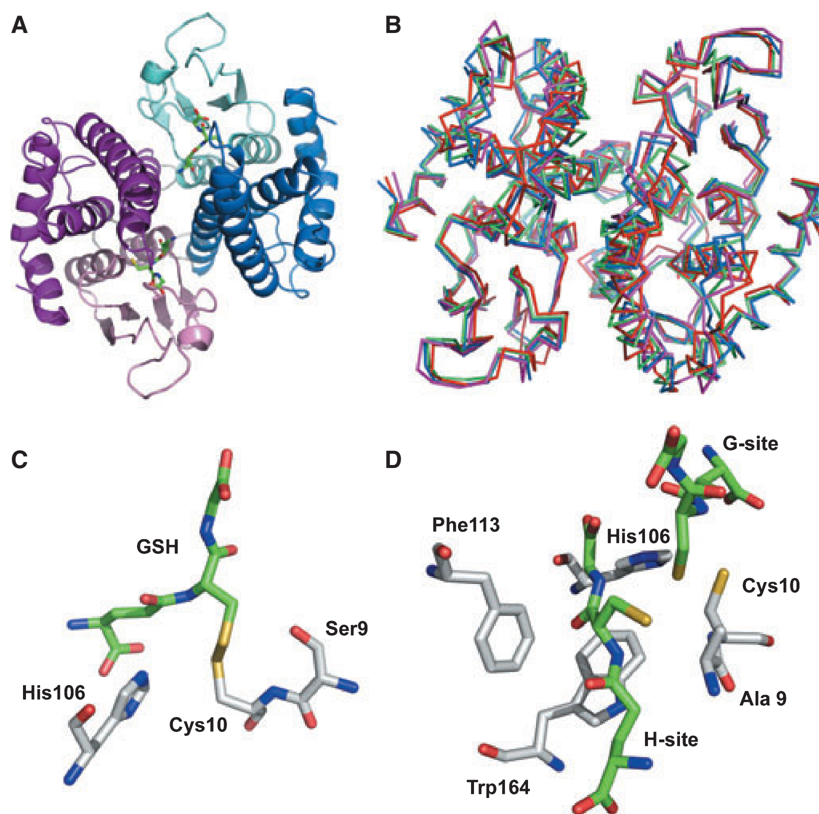
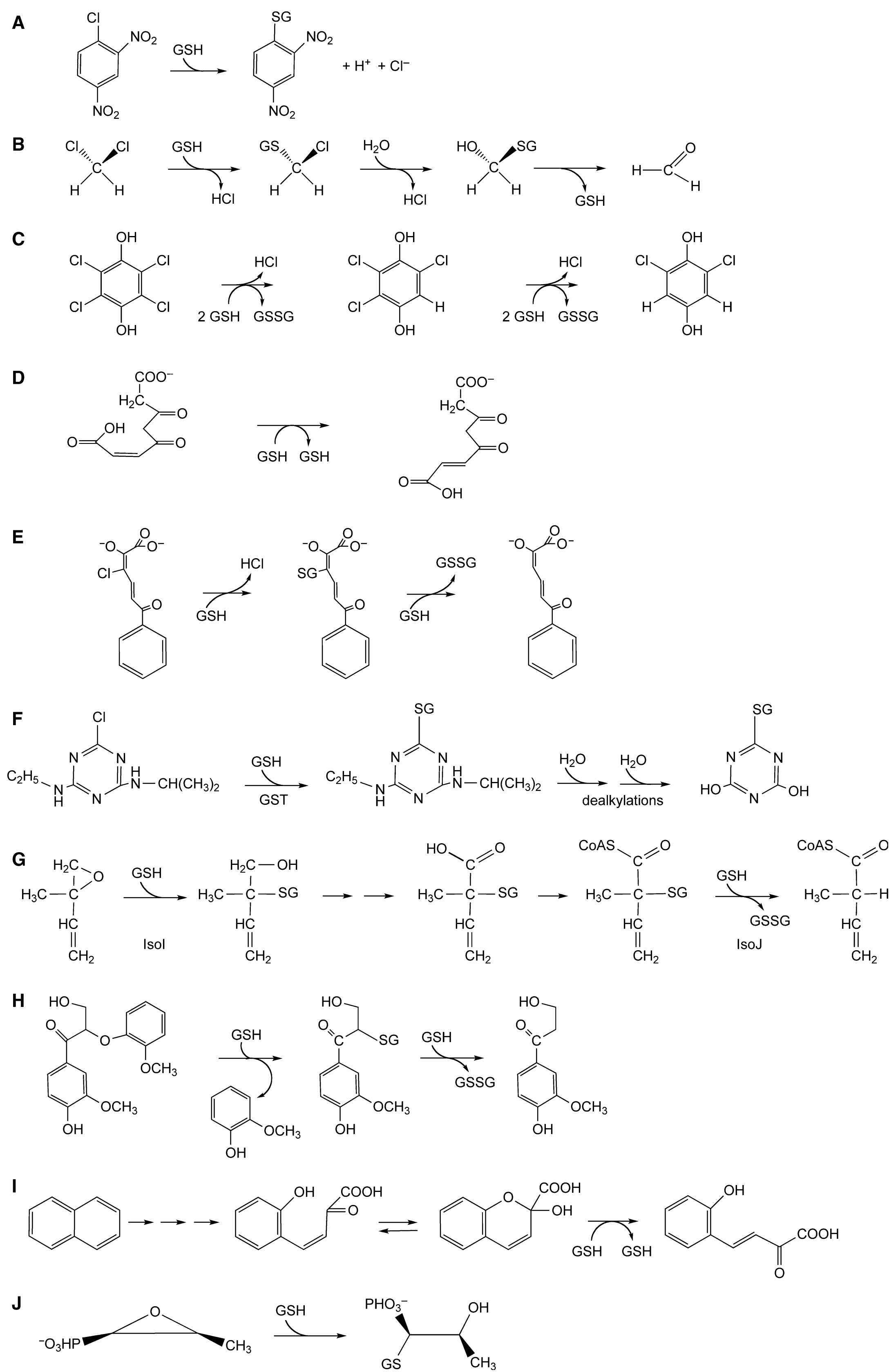


Fig. 2. Structural studies on beta class GSTs. (A) Structure of OaGST shown with the twofold symmetry axis perpendicular to the page (pdb code: 2NTO). The N-terminal thioredoxin domain and the C-terminal all helical domain are highlighted with different tonalities. GSH molecules are shown in sticks. (B) Overlay of four different beta class GSTs dimers shown as Cα traces: OaGST (red), BxGST (green, pdb code: 2GDR), EcGST (magenta, pdb code: 1A0F) and PmGST (cyan, pdb code: 1PMT). This representation highlights the conservation of beta class fold as well as of the monomers' orientation in the dimers. (C) GSH binding site in PmGST. In this crystal structure, GSH (green carbons) forms a mixed disulfide with Cys10. His106 and Ser9 are also at hydrogen bond distance from the GSH sulfur. (D) Structure of BxGST in complex with two GSH molecules (green carbons), one at the canonical G-site and the other bound at the hydrophobic substrates binding site (H-site). The two GSH sulfurs are at close distance from each other. This figure was prepared with PYMOL (DeLano Scientific).

histidine is conserved in other beta class GSTs, whereas Ser9 is not conserved in OaGST, BxGST and EcGST [37,41,47]. Interestingly, mutagenesis data on PmGST and EcGST indicated that none of the above-mentioned residues (Ser9, Cys10 and His106) is strictly required for the classical conjugating activity or is solely responsible for the reduced pK_a of GSH, suggesting that, in contrast with mammalian GSTs, the stabilization of the GSH thiolate is likely the result of a network of interactions [31,33,70]. OaGST deviates partly from this behaviour. In fact, in OaGST the G-site histidine adopts a different orientation with respect to the other enzymes and does not bind GSH [41]. In OaGST, in contrast to what is observed with PmGST and EcGST, mutation of the conserved cysteine to alanine led to a marked decrease in specific activity, due to a dramatic loss in affinity for GSH [42]. Interestingly, it was shown that, in this mutant, GSH preferentially binds in a non-canonical position, occupying the H-site [42]. Another beta class enzyme, BxGST, was also shown to be able to bind a GSH in the H-site [47]. Remarkably this enzyme was crystallized in the presence of two GSH molecules, one with the canonical G-site orientation and the other bound at the H-site (Fig. 2D). In this structure the G- and H-site GSH sulfurs are close ($\sim$ 4.5 Å). These structural data support a mechanism in which once a disulfide bond is formed between the G-site GSH and Cys10 in the reduction of several substrates, a second GSH enters the H-site and a disulfide bond exchange takes place with the formation and release of an oxidized glutathione (GSSG), thus restoring the enzyme's functionality [47].

Two bacterial proteins show significant sequence identity with mitochondrial kappa class GSTs. They are DsbA and the HCCA isomerase. Kappa class enzymes are peculiar in that, although they contain a thioredoxin domain and an all-helical domain, their topology differs from that of cytosolic enzymes because the all-helical domain is inserted into the thioredoxin fold. DsbA is a structural but not a functional homologue of kappa class GSTs because its active site does not bind GSH and it contains a CXXC motif to perform redox catalysis [71]. HCCA isomerase is the fourth enzyme in the naphthalene catabolic pathway of *Ps. putida*. It catalyses the conversion of HCCA, derived from *cis-o*-hydroxybenzeylidene pyruvic acid, to the more stable *trans-o*-hydroxybenzeylidene pyruvic acid (Fig. 3I). Recently, this enzyme has been subjected to extensive structural and functional characterization that has shed light on its peculiarities [63]. HCCA isomerase is a *bona fide*


A
GSH
+ H+ + Cl−
B
GSH
HCl
H2O
HCl
GSH
C
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2 GSH GSSG
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2 GSH GSSG
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kappa class GST: it conserves the same topology as the mammalian enzyme, it is able to bind GSH, which is activated through a conserved serine residue, and it performs the classic conjugating reaction on CDNB. Its structure superimposes well onto the mammalian kappa class enzyme with only minor differences and overall rmsd values among equivalent Cα of ~ 2.2 Å. GSH is bound and stabilized in a similar manner to mammalian kappa class GSTs but more tightly, due to two additional interactions in HCCA isomerase. A possible mechanism for the HCCA isomerase reaction has been proposed in which GSH performs a nucleophilic attack on the HCCA ring and is then released thus behaving as a cofactor (see later).

Structural genomics initiatives are increasing our understanding of the protein-fold evolution and often lead to the functional annotation of proteins whose role was previously undetectable or merely hypothetical, based on sequence alignments alone. This is the case of the *Atu5508* gene product of *Ag. tumefaciens* whose structure has recently been determined [67]. The protein has a dimeric organization, it binds GSH and displays the canonical GST fold even though its sequence identity is < 20% in pairwise comparisons with any previously characterized cGSTs. The protein is also a functional GST because it is able to conjugate GSH to *p*-benzyl chloride, a GST substrate. Although coming from a bacterium, protein-fold analysis and structure superpositions suggest that this protein is more closely related to mammalian GSTs than to other bacterial GSTs. In particular, it lacks the hallmarks of beta class GSTs, including absence of the catalytic cysteine and histidine in the G-site and the presence of a hydrophobic lock-and-key motif at the dimer interface. For these reasons it has been proposed that this protein is the prototype of a new class of bacterial GSTs that includes several close homologues found analysing a large set of environmental sequences in the environmental sequencing project [67].

Functions

Bacterial GSTs are specialized in several detoxification processes. They are able to detoxify a large number of molecules via GSH conjugation (an example with the classic substrate CDNB is shown in Fig. 3A). They have an active role in protecting against oxidative stress and are involved in the detoxification of antimicrobial agents. Some are implicated in the basal metabolism and supply bacterial cells with carbon sources. Bacterial GSTs are also involved in the degradation of several monocyclic aromatic compounds such as toluene, xylenes, phenols and atrazine [72]. They also take part in the degradation of polycyclic aromatic hydrocarbons, a class of hazardous chemicals to both environmental and human health [21,73–78].

Oxidative and xenobiotic stress

Beta class cGSTs are involved in detoxication reactions against toxic effects of several xenobiotics [39,40, 79–81].

In a modulation study it was reported that, in a *P. mirabilis* strain, PmGST contributed to protect the cells against oxidative stress induced by hydrogen peroxide [80]. Increases in the level of mRNA transcription and in protein expression levels were observed when the bacterial cells were exposed to hydrogen peroxide. This result was confirmed by the analysis of a *gst*B gene knock-out in the same *P. mirabilis* strain that was found to be much more sensitive to hydrogen peroxide than the wild-type strain [80]. Similar results were also obtained for *E. coli* GST [59].

The modulation of OaGST in *O. anthropi* in the presence of different xenobiotics was also investigated. Unlike PmGST, hydrogen peroxide did not influence the induction of the enzyme. Atrazine caused an increase in the expression of OaGST at low, non-toxic concentrations, suggesting its involvement in atrazine metabolism [39]. Instead, phenolic compounds induced a marked dose-dependent enhancement of the enzymatic cellular levels correlated to the toxicity of the molecules indicating a role of OaGST in cellular protection [39]. These data were also corroborated by the preponderant presence of the enzyme in the periplasmic space when bacteria were exposed to 4-chlorophenol [40].

However, although OaGST was found in the periplasm, no signal sequence for export by the general secretory (Sec) pathway was found [40]. Recently, an alternative pathway has also been described, the twin-

Fig. 3. Reactions catalysed by bacterial GSTs. (A) Canonical conjugation of CDNB as second substrate; (B) dehalogenation of DCM to formaldehyde; (C) reductive dehalogenation of TCHQ to trichlorohydroquinone and of trichlorohydroquinone to 2,6-dichlorohydroquinone; (D) *cis–trans* isomerization of maleylacetoacetate; (E) dehalogenation of 3-chloro-HOPDA; (F) hypothetical metabolic route for atrazine; (G) opening of epoxide ring and reductive removal of GSH in isoprene metabolism; (H) β-aryl ether cleavage pathway; (I) step 4 of naphthalene catabolic pathway; (J) opening of the epoxide ring of fosfomycin.

arginine translocation (Tat) system, which allows the transfer of folded proteins into periplasmic space [82]. Proteins transported via Tat system possess a distinctive motif (SRRXFLK) at their N-terminus. This motif was also not present in the OaGST sequence [83]. Similarly, no Sec or Tat signals were found in PmGST, EcGST, BxGST and Atu5508 [83,84]. Therefore, the mechanism of OaGST transport (and possibly that of other GSTs) into the periplasm is uncharacterized at present. Moreover, a prokaryotic GSH transporter in *E. coli* has recently been identified [85]. This strongly suggests the presence of GSH in the periplasm, as hypothesized previously [86], and underlines that bacterial GSTs also display activity in this compartment.

PmGST is able to catalyse a glutaredoxin-like reaction using cysteine *S*-sulfate and hydroxyethyl disulfide as substrates [68]. The G-site cysteine residue is essential for this redox activity [68] and the mixed disulfide observed in the PmGST crystal structure has been suggested as an intermediate in glutaredoxin-like reactions [68].

Bacterial GSTs in dehalogenation

Microbial dehalogenases play a key role in the biodegradation of several chlorinated xenobiotics, both aliphatic and aromatic [87,88]. Halogenated compounds are widely used in industry and constitute an important group of environmental pollutants.

Bacterial GSTs catalyse different reactions using GSH as a cofactor, i.e. DCM dehalogenases catalyse the hydrolytic dechlorination of dichloromethane, whereas TCHQ dehalogenase catalyses a reductive dehalogenation reaction.

DCM dehalogenases

DCM dehalogenase is a GSH-dependent enzyme synthesized by a number of facultative methylotrophic bacteria that are able to utilize DCM as a sole carbon and energy source [48]. In the first step of degradation, the enzyme dechlorinates DCM to formaldeyde and inorganic chloride (Fig. 3B). DCM, a significant environmental contaminant, is widely used as an industrial solvent. The properties of two DCMDs are well documented in *Methylobacterium dichloromethanicum* DM4 and in *Methylophilus leisingeri* DM11 [48–52]. Both dehalogenases are closely related to eukaryotic theta class GSTs (Fig. 1). In particular, like theta class GSTs, DCMDs share a conserved serine residue at the N-terminal domain that is essential for catalysis [89]. Nevertheless, on the basis of other criteria such as N-terminal amino acid sequences, kinetic and immunological properties, they have been further subdivided into two classes. One class is formed by group A enzymes, including the DCM dehalogenases of *Methylobacterium dichloromethanicum* DM4. By contrast, *M. leisingeri* DM11 is the prototype for group B enzymes. The most significant difference between the two groups lies in their kinetic properties. Under conditions of substrate saturation, DM11 is significantly faster in dechlorination than DM4.

To date, no structure is known for DCMDs. A 3D homology model of DCMD from the *M. leisingeri* DM11 strain has been presented based on alignments with GST members from the theta and the alpha class [90].

TCHQ dehalogenase

TCHQ dehalogenase catalyses the reduction of TCHQ to trichlorohydroquinone and then to dichlorohydroquinone during the biodegradation of pentachlorophenol, a xenobiotic compound present in the environment, utilized primarily as fungicide for wood preservation [91–94]. The catalytic mechanism of the reaction was exhaustively studied by Copley *et al.* [54,56,95]. TCHQ dehalogenase was purified by *Sphingobium chlorophenolicum*, a soil bacterium that can grow on pentachlorophenol as a sole carbon source [92,96]. TCHQ dehalogenase utilizes two GSH molecules to reductively dechlorinate TCHQ to trichlorohydroquinone and then dichlorohydroquinone (Fig. 3C). First, TCHQ dehalogenase catalyses the nucleophilic attack of GSH on the substrate forming a GSH conjugate, and then converts the conjugate to reduced products. A conserved cysteine is required to this second step of the reaction through the formation of a mixed disulfide with GSH [54,97].

TCHQ dehalogenase also has maleylacetoacetate isomerase activity, and the conserved cysteine is also required for this activity [53]. Maleylacetoacetate isomerases are enzymes that catalyse the GSH-dependent *cis–trans* isomerization of maleylacetoacetate to fumarylacetoacetate during the catabolism of tyrosine and phenylalanine (Fig. 3D). Although the overall sequence identity between TCHQ dehalogenase and maleylacetoacetate isomerase is low, the active site is highly conserved and in both cases contains a catalytic cysteine [53].

Because maleylacetoacetate isomerases are part of an ancient degradation pathway, whereas TCHQ dehalogenase is a more specialized enzyme, it was supposed that TCHQ dehalogenases evolved from maleylacetoacetate isomerases [53].

Biphenyl/polychlorinated biphenyldehalogenation

A cGST characterized from *B. xenovorans* strain LB400, a biphenyl/polychlorinated biphenyl-degrading microorganism, known as BphK or BxGST, is one of the most extensively investigated bacterial GSTs [21,43–47]. This enzyme, which belongs to the beta class, is encoded by a gene (*bphK*) located within the *bph* locus, which specifies the degradation pathway of biphenyl and chlorobiphenyl compounds. *B. xenovorans* LB400 is able to attack a broad spectrum of polychlorinated biphenyl congeners and is able to grow on biphenyl as sole carbon source [21,43]. Mutagenic studies indicated that although the *bphK* gene is not essential for utilization of this carbon source its expression gives advantage to the strain in the utilization of biphenyls [43]. Subsequently, it has been established that BphK catalyses efficiently the dehalogenation of 3-chloro-2-hydroxy-6-oxo-6-phenyl-2,4-dienoate compounds that are produced by the co-metabolism of polychlorinated biphenyls (Fig. 3E) [46]. The proposed reaction mechanism is similar to that described for TCHQ dehalogenase [56]. Although less efficient, BphK enzyme also has dechlorination activity against 4-chlorobenzoate [44]. A *bphK* gene is also present in *Sphingomonas yanoikuyae* B1 and its expression allows *Sph. yanoikuyae* B1 to grow faster on *m*-toluate [98,99].

Atrazine metabolism

GSTs are able to detoxify several classes of herbicides including triazines, a class of compounds to which atrazine, one of the most widely used herbicides, belongs [100,101]. GSTs are involved in the first step of atrazine biodegradation with the removal of the chlorine atom produced by atrazine–GSH conjugation [100,101]. Dechlorination is followed by the stepwise removal of isopropylamine and ethylamine groups by dealkylation [102]. In bacteria, atrazine can be degradated either by a microbial consortium or by a single microorganism [103,104]. For instance, in *Pseudomonas* ADP, atrazine is metabolized to cyanuric acid by three enzymatic steps. The first step is performed by atrazine chlorohydrolase followed by two dealkylations [104].

O. anthropi is a soil bacterium that is able to grow on atrazine utilizing it as source of carbon [105] and which expresses a functional beta class GST [24]. In a modulation study of OaGST in the presence of several xenobiotics, Favaloro and co-workers also showed an increase in the levels of enzyme when atrazine was added to the exponentially growing cells of *O. anthropi*, suggesting an involvement of OaGST in atrazine conjugation with GSH [39]. A proposed atrazine degradation pathway by bacterial GSTs is showed in Fig. 3F.

Isoprene metabolism

Two GSTs were purified from the isoprene-utilizing bacterium *Rhodococcus* sp. strain AD45 and their functional properties were characterized [106–108]. Both enzymes are involved in the metabolism of isoprene, an atmospheric reactive hydrocarbon that plays a role in ozone, organic peroxides and carbonic monoxide formation, and their genes are localized in the isoprene degradation gene cluster [106,108]. The first GST, encoded by the *isoI* gene, catalyses the GSH-dependent ring opening of isoprene monoxide, the primary oxidation product of isoprene (Fig. 3G). The GSH conjugate 1-hydroxy-2-glutathionyl-2-methyl-3-butene is then oxidized in two consecutive steps to 2-glutathionyl-2-methyl-3-butenoic acid by a dehydrogenase (IsoH). The way in which isoprene degradation proceeds has not been fully characterized. A convincing hypothesis is that 2-glutathionyl-2-methyl-3-butenoic acid could be converted to the corresponding CoA-thioester by a racemase expressed by *isoG* gene, to allow the second GST, which is encoded by the *isoJ* gene, to remove the GSH molecule [108,109]. It is thought that IsoJ might catalyse the reductive removal of GSH using a second GSH molecule in a similar fashion as the TCHQ dehalogenase (Fig. 3G) [54,91,108,109]. A relevant difference from TCHQ dehalogenase is that the GSH conjugate is formed several steps before its reduction, because the removal of the GSH from 1-hydroxy-2-glutathionyl-2-methyl-3-butene is energetically unfeasible [109]. Moreover, IsoI/GST is able to degrade halogen epoxides such as 1,2-dichloroepoxyethanes and epichlorohydrin [106,107]. In *Rhodococcus* AD45 a novel GST, namely IsoILR1, was recently characterized with activity towards *cis*-1,2-dichloroethylene epoxide and epoxypropane [110].

Lignin degradation pathway

In *Sph. paucimobilis* SYK-6, the role of three tandemly located genes, *ligE*, *ligF* and *ligG*, involved in the process of lignin degradation, a fundamental step for the earth's carbon cycle, has been described [111–113]. *Sph. paucimobilis* is able to degrade a wide variety of lignin compounds including β-aryl ether. The β-aryl ether cleavage is a fundamental step in lignin degradation, because this intermolecular linkage is the most abundant in lignin. LigE and LigF are enantioselective

GSTs that cleave the β-aryl ether linkage with consumption of GSH [113]. LigG is instead a GSH lyase that catalyses the elimination of GSH from the conjugate produced by LigF (Fig. 3H) [112,113].

Naphthalene metabolism

HCCA isomerase is a GSH-dependent enzyme, structurally related to mammalian kappa class enzymes, involved in the naphthalene degradation pathway [5,62,63]. HCCA isomerase is also involved in naphthalene sulfonates and anthracene catabolism [114,115]. Naphthalene catabolism consists of an upper pathway and a lower pathway. In the upper pathway, naphthalene is metabolized to salycilate in six steps. HCCA isomerase is the fourth enzyme of this pathway and catalyses *cis–trans* isomerization between HCCA and *trans-o*-hydroxybenzylidene pyruvic acid (Fig. 3I). GSH is thought to be involved in this reaction by its covalent addition of HCCA. The addition alters the hybridization state at C7 promoting a rotation around the C7–C8 double bond [63].

Interaction with antibiotics

Several studies on the interaction of PmGST with different classes of antibiotics have been performed and a possible role for the enzyme in antibiotic-resistance proposed [29,116].

First, it was observed that the efficiency of a number of antimicrobial drugs decreased notably in the presence of the purified enzyme in the medium culture, as shown by increased minimal inhibitory concentration values [16,116]. By contrast, the presence of mammalian GSTs had no effect on antibiotic efficiency [16]. Second, studies on the interaction of PmGST with several antibiotics indicated that the enzyme sequesters antimicrobial drugs with avidity [29]. The effect of 18 different antibiotics as inhibitors of PmGST activity using CDNB and GSH as substrates, were also measured. Four of them, namely minocycline, tetracycline, rifamycin and nitrofurantoin, were strong inhibitors with IC_{50} values between 49 and 140 μM. These drugs produced a significant decrease in the k_{cat} values of the bacterial enzyme for both substrates [29]. Furthermore, PmGST displays a protective action against antibiotics also *in vivo* [80,117]. An increase in PmGST levels was observed when bacteria were grown in the presence of drugs [80]. Moreover, viability tests showed that a *gst* null-mutant *P. mirabilis* strain was more sensitive to antibiotics than the wild-type bacterium [80]. Finally, crystallographic data highlighted the presence of a hydrophobic cavity large enough to bind antibacterial molecules located at the dimer interface [12]. These results were strengthened by the preponderant periplasmic location of the enzyme in the periplasmic space [81].

Fosfomycin resistance proteins

Fosfomycin ([1*R*,2*S*]-[1,2-epoxypropyl]-phosphonic acid) is a bactericidal broad-spectrum antibiotic effective against both Gram-negative and Gram-positive bacteria. Fosfomycin inhibits the enzyme MurA (UDP-NAG enolpyruvyl transferase), which catalyses the transfer of enolpyruvate from phospho*enol*pyruvate to uridine diphospho-*N*-acetylglucosamine, the first committed step of bacterial cell-wall biosynthesis. Resistance is mainly chromosomal but resistance genes have also been found on transmissible plasmids [118]. Resistance to fosfomycin can be achieved by several different mechanisms, including decreased uptake of the antibiotic, overexpression or mutation of MurA, and enzymatic modification of the antibiotic [118,119].

FosA, FosB and FosX represent three mechanistically distinct classes of enzymes that confer resistance to fosfomycin by adding GSH, L-cysteine or a hydroxyl group, respectively, to the oxirane ring of the antibiotic, and inactivating it.

FosA was originally identified in strains carrying fosfomycin-resistance plasmids obtained from clinical isolates [120–122]. Suarez and co-workers described a new mechanism of antibiotic resistance due to the enzymatic modification of fosfomycin [121,122]. Inactivation of fosfomycin occurred by the formation of an adduct between its carbon 1 atom and the sulfydryl group of the GSH cysteine resulting in the opening of the epoxide ring of the antibiotic (Fig. 3J). The reaction was catalysed by an enzyme that was referred to as a GST. The enzyme was purified and characterized. It did not bind to the GSH–agarose matrix and did not catalyse the reaction between GSH and CDNB, indicating that this protein had different properties from the canonical GSTs. The enzyme is a homodimer of 32 kDa and its activity is dependent on the addition of the Mn^{2+} cation [122]. In subsequent studies, Armstrong and co-workers demonstrated that FosA is a metalloglutathione transferase related to glyoxalase I and extradiol dioxygenases, members of the vicinal oxygen chelate superfamily [4,123]. In addition, they showed that each subunit of the homodimer contains a mononuclear Mn^{2+} centre that interacts strongly with the antibiotic and also that the enzyme requires a monovalent cation K^{+} for optimal catalytic activity [123]. FosA is also encoded in the bacterial genomes and the 3D structure of a genomically encoded FosA

from the pathogen *Ps. aeruginosa* was described [124]. The protein fold showed similarity to members of vicinal oxygen chelate superfamily and consists of paired βαβββ motifs that form a cupped-shaped cavity for the metal ion-binding site. The K^+ ion is accommodated in a loop located 6.5 Å from the Mn^{2+} cation [124]. The structure of FosA in complex with antiobiotic was also obtained. Plasmid-encoded and genomically encoded enzymes are very similar in structure. The crystal structure of FosA from transposon Tn2921 maintains the same basic molecular arrangement observed in genomically encoded FosA [125]. Recently, based on structural data, the residues involved in the binding of both fosfomycin and GSH substrates have been identified and characterized by mutagenesis [126,127].

FosB was originally identified in fosfomycin-resistant *Staphylococcus* strains and the genes were encoded by plasmids [128–130]. A second type of FosB was identified in the genome of a *Bacillus subtilis* strain and subsequently characterized [131]. The protein is a dimeric metallothiol transferase related to FosA. Unlike FosA, FosB utilizes Mg^{2+} as metal cofactor. In addition, FosB uses L-cysteine rather than GSH as thiol donor and is less efficient than FosA [131]. This is in agreement with previous studies because the GSH molecule was not detectable in most of the Gram-positive bacteria tested, including *Bacillus subtilis* [132].

FosX is a Mn(II)-dependent fosfomycin specific epoxide hydrolase. It catalyses the addition of a water molecule to fosfomycin, thus inactivating it. The enzyme is genomically encoded, it is found in several microorganisms and it has been well characterized in *Mesorhizobium loti* and *Lysteria monocytogenes* [133,134]. The two enzymes showed significant functional differences. *Listeria* FosX is a good catalyst and is responsible for high resistance to fosfomycin [133]. By contrast, *M. loti* FosX produces modest resistance to the antibiotic and, consistently, its kinetic costants are lower than those of *Listeria* FosX. In addition, *M. loti* FosX also catalyses the addition of GSH to the antibiotic even if with low efficiency. It has been suggested that *M. loti* FosX may be an intermediate in the evolution of fosfomycin resistance proteins that plays some yet to be identified role in the catabolism of phosphonates. The structure of FosX was also determined for both enzymes [133,134]. The FosX structures are closely related and the overlay with FosA from *Ps. aeruginosa* [124] shows a large degree of similarity. Unlike FosA, the enzymes do not contain a K^+ ion-binding site near the active site. The most interesting aspect of these structures is the observation that fosfomycin binds to FosX enzymes in a different orientation from that observed in the FosA enzyme.

A new mechanism of fosfomycin inactivation was described by Garcia *et al.* [135] in a fosfomycin-resistant strain of *Pseudomonas syringae*. The bacterium yielded an enzyme, FosC, that inactivated the antibiotic using ATP to phosphorylate fosfomycin in the presence of Mg^{2+}. This finding was corroborated by sequence alignments highlighting a region of partial homology between FosC and the Mg-ATP binding domains of AMP–ATP phosphotransferases. To date, no relationship between FosC and the other fosfomycin resistance proteins is known.

Potential applications of bacterial GSTs

As previously described, bacterial GSTs are involved in several types of chemical transformations and may represent a versatile tool with a variety of biotechnological applications, for example, in the field of bioremediation, an economical alternative to conventional physicochemical methods to clean up environmentally contaminated sites. The relative ease of genetic manipulations in bacteria and their ability to grow rapidly constitute a further advantage.

Several studies have been carried out exploiting the potential of GSTs using both purified proteins and microorganism engineering, some of which are summarized here.

An example of protein engineering using the DNA-shuffling technique was shown by Mannervik *et al.* [136]. They hybridized six alpha class GSTs of differing mammalian origin obtaining chimeric enzymes with improved catalytic properties and altered substrate selectivity towards several noxious iodoalkanes.

Another example is in the engineering of fusion proteins with several distinct enzymatic activities. For example, a trifunctional enzyme with superoxide dismutase, glutathione peroxidase and glutathione transferase activities was recently generated [137]. This recombinant chimeric enzyme was shown to be effective in scavenging reactive oxygen species, thus showing that this approach may have several applications in medicine as well as in environmental field.

Another potential application lies in the preparation of biosensors. These are detection systems widely used to check contaminated environments that combine a biological component with a detector element. Biosensors are competitive systems in comparison to conventional methods being inexpensive, easy to use and characterized by high sensitivity and selectivity. For example, a mammalian GST was used to develop an

optical biosensor for detection of captan in contaminated waters [138]. Captan is used to control a broad spectrum of plant pathogenic microorganisms and it is a strong inhibitor of GSTs [139].

A large number of bacterial species have developed the ability to degrade xenobiotics previously considered to be non-degradable [140]. Examples of microbial consortia combining the ability of two or more bacterial species to metabolize one or more noxious molecules have been described [141]. An alternative route is to engineer a single bacterial strain to carry a complete metabolic pathway that efficiently eliminates environmental toxic compounds [110]. For example, Wood *et al*. [110] engineered in *E. coli* a metabolic pathway to improve the degradation of chlorinated ethenes, which constitute a large group of toxic environmental pollutants [142]. The degradation of these molecules is limited by the accumulation of reactive intermediates epoxides. The authors constructed a recombinant *E. coli* strain in which a toluene *ortho*-monooxygenase from *Burkholderia cepacia* G4, obtained by DNA shuffling, and a GST from *Rhodococcus* AD45 with activity towards *cis*-1,2,-dichloroethylene epoxide and epoxypropane, were co-expressed [110]. The ability of GST to transform epoxides in *E. coli* strain increased the mineralization of *cis*-1,2,-dichloroethylene.

The examples provided above highlight the potential biotechnological applications of using engineered proteins and bacterial strains. In this respect, bacterial GSTs, which are characterized by high stability and by a wide variety of catalysed reactions, undoubtedly, constitute an effective resource for the future.

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