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MINIREVIEW

Bacterial Glutathione *S*-Transferases: What Are They Good for?

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INTRODUCTION

Glutathione *S*-transferases (GSTs) play an important role in higher eukaryotes in the binding, transformation, and detoxification of a wide variety of both endogenous and exogenous electrophilic compounds. Despite early reports describing GST activity in bacteria, such enzymes failed to attract the interest of microbiologists and molecular biologists until fairly recently. The last few years, however, have seen the discovery of bacterial proteins and genes that encompass a wide variety of enzyme activities and can be ascribed to the large superfamily of GSTs. Studies of these enzymes and genes have raised a variety of questions for microbiologists interested in microbial evolution, biotechnology, and metabolism alike. How related are these enzymes? How much catalytic versatility can we expect in this enzyme superfamily, and how important are these enzymes to the metabolism of the bacteria which carry them?

This article presents an overview of what is known about the biochemistry of GSTs in bacteria. The sequences of these proteins are then examined in the context of the abundant information available on the sequences and structures of GSTs in eukaryotes. Finally, potential applications of bacterial GSTs suggested by recent research are discussed.

MANY FUNCTIONS FOR A UNIQUE COFACTOR

Cyanobacteria, proteobacteria, phototrophs (19), and a few gram-positive bacteria (63) are the only bacteria known to contain glutathione. Nevertheless, several bacterial genera have not been tested for the presence of this tripeptide, and significant variations in glutathione content have been observed between different species of the same genus (63). The high levels of glutathione found in cyanobacteria, which produce oxygen by photosynthesis, and in purple bacteria, which use oxygen as a terminal electron acceptor, point to a bacterial origin for glutathione metabolism around the time when aerobic conditions became widespread on earth. Accordingly, glutathione metabolism would have subsequently spread to eukaryotes by endosymbiotic events (19, 69). Glutathione metabolism may have begun prior to the appearance of oxygenic photosynthesis and originally served purposes other than protecting cells against oxygen. High levels of glutathione amide were recently found in anaerobic green sulfur bacteria from the genus *Chromatium* (7), and evidence that this compound is involved in anaerobic sulfide metabolism was provided. In *Escherichia coli* and other proteobacteria, glutathione itself and glutathione-dependent enzymes, among which are GSTs, are involved in a stunning variety of metabolic processes and afford protection against oxidative stress but also ensure the correct folding, synthesis, regulation, and degradation of enzymes and multienzyme complexes (39, 69).

GSTs: FUNCTIONAL VERSATILITY WITHIN A CONSERVED FRAMEWORK

GST enzymes have been studied extensively in eukaryotes since their discovery in 1961 (see references 28, 55, and 77 for recent reviews). These enzymes are usually active as dimers, and examples of both homodimeric and heterodimeric GSTs are known. GST enzymes can either be expressed constitutively or be induced by a wide range of compounds of both natural origin and xenobiotic origin. The known substrates of GSTs are most often xenobiotic synthetic chemicals. The elucidation of physiological GST substrates has often proven more difficult, but a number of natural products have also been shown to be substrates for different types of GSTs. Some of these represent compounds that may arise during oxidative damage to cell components, such as endogenous lipids, DNA hydroperoxides, and hydroxyalkenals (8). Other natural GST substrates include steroids, leukotrienes, anthocyanines (54), and organic isothiocyanates (38). In addition, several GST enzymes have the capacity to bind lipophilic compounds that act as ligands but not as substrates. Eukaryotic GST enzymes were first divided into alpha, mu, and pi classes on the basis of their distinct but broad and overlapping substrate specificities for model substrates (52; see Fig. 1). This classification was confirmed as protein sequence data became available, with sequence identities of 60 to 90% at the protein level within a given class and around 30% between classes. The discovery of GST enzymes with properties at odds with those of the previously defined classes, including poor activity with 1-chloro-2,4-dinitrobenzene (CDNB; Fig. 1) or lack of binding to glutathione-derivatized affinity supports, led to the definition of a new class of GST enzymes, theta (58). The theta class of GSTs includes all of the known bacterial GSTs, as well as representatives from plants, mammals, fish, birds, insects, yeasts, and fungi. It was thus proposed that class theta was the progenitor of the other classes of GST enzymes (11, 68, 82). Somewhat paradoxically, considering our limited knowledge of bacterial GSTs compared to their eukaryotic homologs, this suggests that GST genes have their primordial origin in bacteria. What were the functions and the substrate range of ancestral GSTs? Elements of answers to these questions may be uncovered as bacterial GST enzymes are characterized in more detail.

Structural studies on GST enzymes. One key unifying principle towards understanding the functional versatility and sequence variability of GST enzymes may be the conservation of structural features observed across GST enzymes of all classes.

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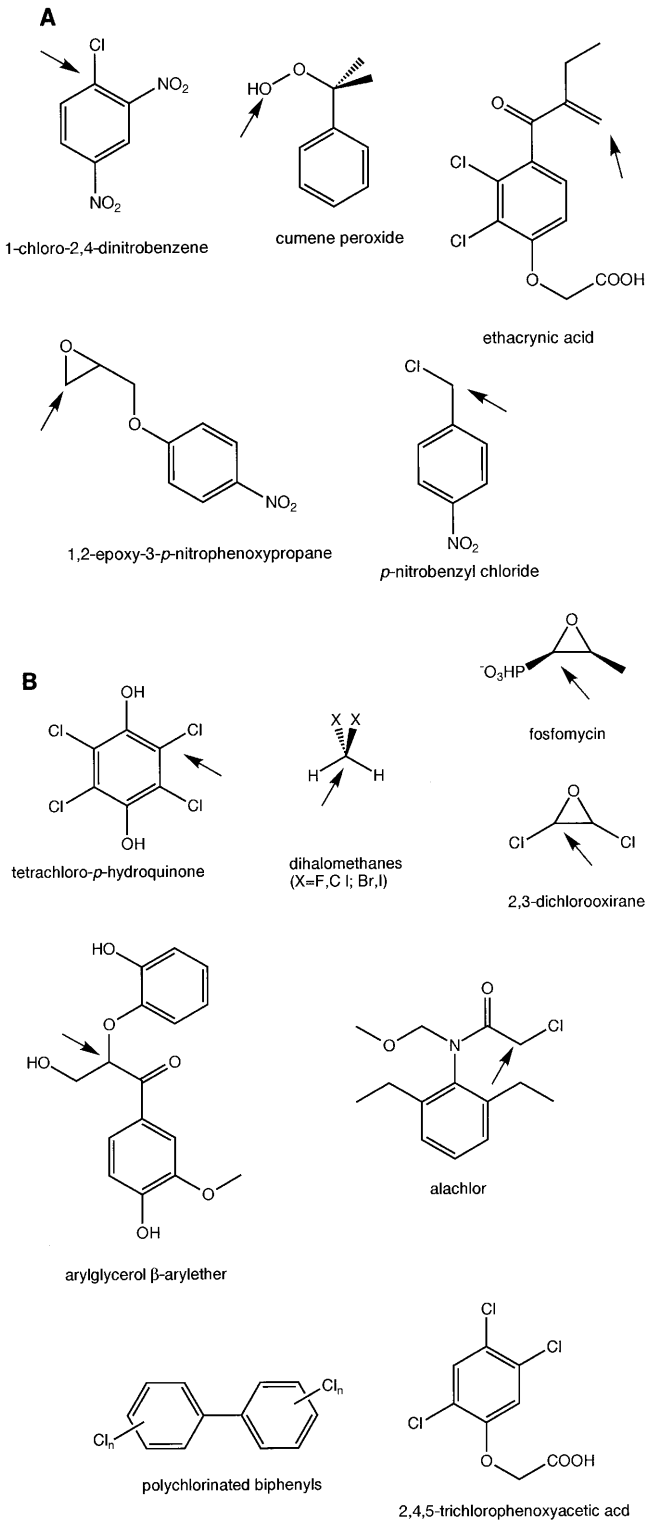


FIG. 1. (A) Standard substrates used to measure the activity of GST enzymes. (B) Physiological substrates of various bacterial GST enzymes. The arrows point to the electrophilic carbon atom at which glutathione addition takes place. The two compounds without an arrow are shown to indicate sets of genes involved in the degradation of these compounds that include a putative GST gene of unknown function.

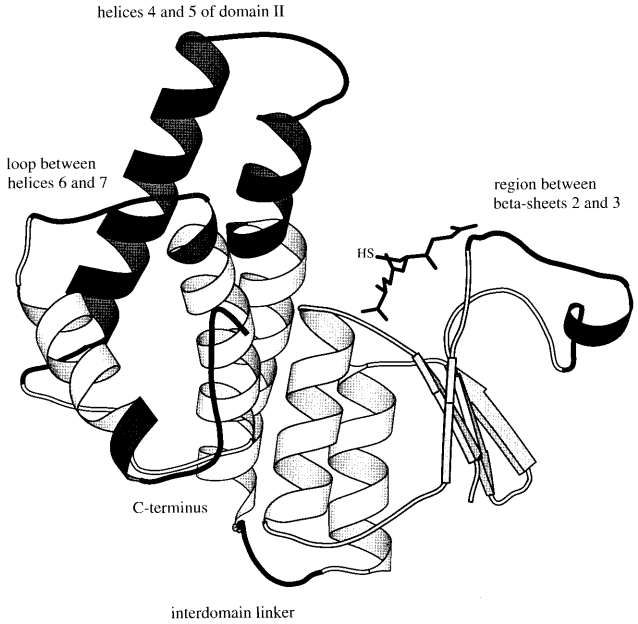


FIG. 2. MOLSCRIPT (40) representation of the structure of the theta class GST enzyme from *L. cuprina* (90), with N-terminal domain I on the right and C-terminal all-helical domain II on the left. Specific structural elements of the *Lucilia* enzyme differing from those of the X-ray structure of another theta class enzyme, that from the plant *A. thaliana* (72), are highlighted in black, and the thiol moiety (HS) of the glutathione cofactor is indicated.

Structures of many enzymes of all classes have been reported since the first GST structure was published in 1991 (5, 18, 72, 89, and references therein), including the structures of theta class enzymes from the Australian sheep blowfly *Lucilia cuprina* (90) and the plant *Arabidopsis thaliana* (72). No structure of a bacterial GST is available, although the crystallization and preliminary structural characterization of a GST enzyme from *Proteus mirabilis* (20) have recently been reported.

GSTs with known structures from all classes are homodimeric enzymes in which each monomer folds into a two-domain conformation (Fig. 2). The N-terminal domain comprises most of the glutathione-binding site and consists of a βαβαββα module, while the predominantly α-helical C-terminal domain is involved primarily in the binding of hydrophobic substrates. Nevertheless, inspection of multiple alignments of GST sequences (see below and Fig. 3) indicates that most of the residues that are highly conserved in enzymes of the alpha, mu, and pi classes are not retained in bacterial and other theta class GSTs (72, 73). This underlines the tolerance of the GST framework to considerable protein sequence variation, which allows different GSTs to carry out a wide variety of glutathione-dependent conjugation functions. Although no other proteins are known to adopt the same overall topology as GST enzymes, domain I of human liver GST displays structural similarities to glutathione peroxidase, thioredoxin, and glutaredoxin (78). The N-terminal domain of GSTs can therefore be described as a canonical glutathione-binding domain.

Despite the observed conservation of structural features in crystallized GST enzymes of all classes, several differences between the two available structures of GST enzymes of the theta class are apparent (72, 90; Fig. 2 and 3). These differences are reflected both in the low level of sequence identity between the two GSTs from *L. cuprina* and *A. thaliana* (21.6%) and in the distinct biochemical and kinetic properties of these enzymes. For instance, the *L. cuprina* enzyme is highly active

with CDNB, whereas the plant enzyme is not. In addition, a unique second, nonproductive glutathione-binding site was observed in the plant enzyme (72). This suggests that relatively large structural differences may be expected in bacterial theta class GST enzymes as well.

YET MORE VERSATILITY: BACTERIAL GST ENZYMES

Early screening studies focussed on the search for GST activity in bacteria using CDNB as a substrate. Such investigations led to the conclusion that GST activity is rare in bacteria and that the levels of activity, where found, were quite low compared to those found in eukaryotes (reviewed in references 19, 69, and 77). The discovery of dichloromethane (DCM) dehalogenase (DCMD)/GST enzymes in methylotrophic bacteria (37, 49, 75) and the purification and detailed characterization of GST enzymes from *P. mirabilis* (15) and *E. coli* B (32) changed this belief somewhat. The present explosive development in gene sequencing technology, along with the characterization of bacterial GST enzymes with novel functions, provides us with a new perspective on the occurrence of GST in bacteria (Table 1). On the one hand, the gene sequences of previously characterized enzymes shown by their function to be GSTs have confirmed these assignments. On the other hand, many recently discovered GSTs now being investigated at the protein level were first detected, in some cases even by chance, as open reading frames similar to already known GST sequences.

CDNB-active GSTs from bacteria. Because it allows easy spectrophotometric detection of the glutathione conjugate, CDNB has been the most widely used substrate for assaying GST activity in crude cell extracts in eukaryotic systems and during GST purification involving glutathione affinity supports (52, 60). Several CDNB-active GST enzymes have also been obtained from bacteria by these methods (Table 1). The best-characterized such bacterial GST is the major one of three isoforms with different pIs isolated from *P. mirabilis* (15). This homodimeric enzyme is also active with the typical eukaryotic GST substrates cumene peroxide, ethacrynic acid, and 1,2-epoxy-3-*p*-nitrophenoxypropane (Fig. 1 and Table 1). The sequence of this enzyme was first determined at the protein level (61), but the corresponding gene sequence is now also available (70). Since a preliminary account of the crystallization of the enzyme was recently published (20), detailed structural information on this enzyme may soon be forthcoming.

The physiological role of the CDNB-active enzymes from bacteria purified by affinity chromatography remains elusive. They are not quantitatively prominent proteins in the cell, judging from the purification factors reported (0.002 to 0.1%) (32, 64, 71). The presence of CDNB-active GST enzymes was associated with increased resistance of the bacterial host to several antibiotics (70, 71). In addition, some of these enzymes were shown to have alkyl peroxidase activity (64, 71). The physiological significance of this reaction in bacteria, for example, in protection against oxidative damage, remains to be investigated.

It is well recognized that many GST enzymes assigned to the theta class, some of which are described in more detail below, may not be detected with CDNB as a substrate or purified by glutathione affinity chromatography. Indeed, the distinct emphasis on these methods in the field of GST research so far may help to explain why so little is known about bacterial members of the GST family of proteins.

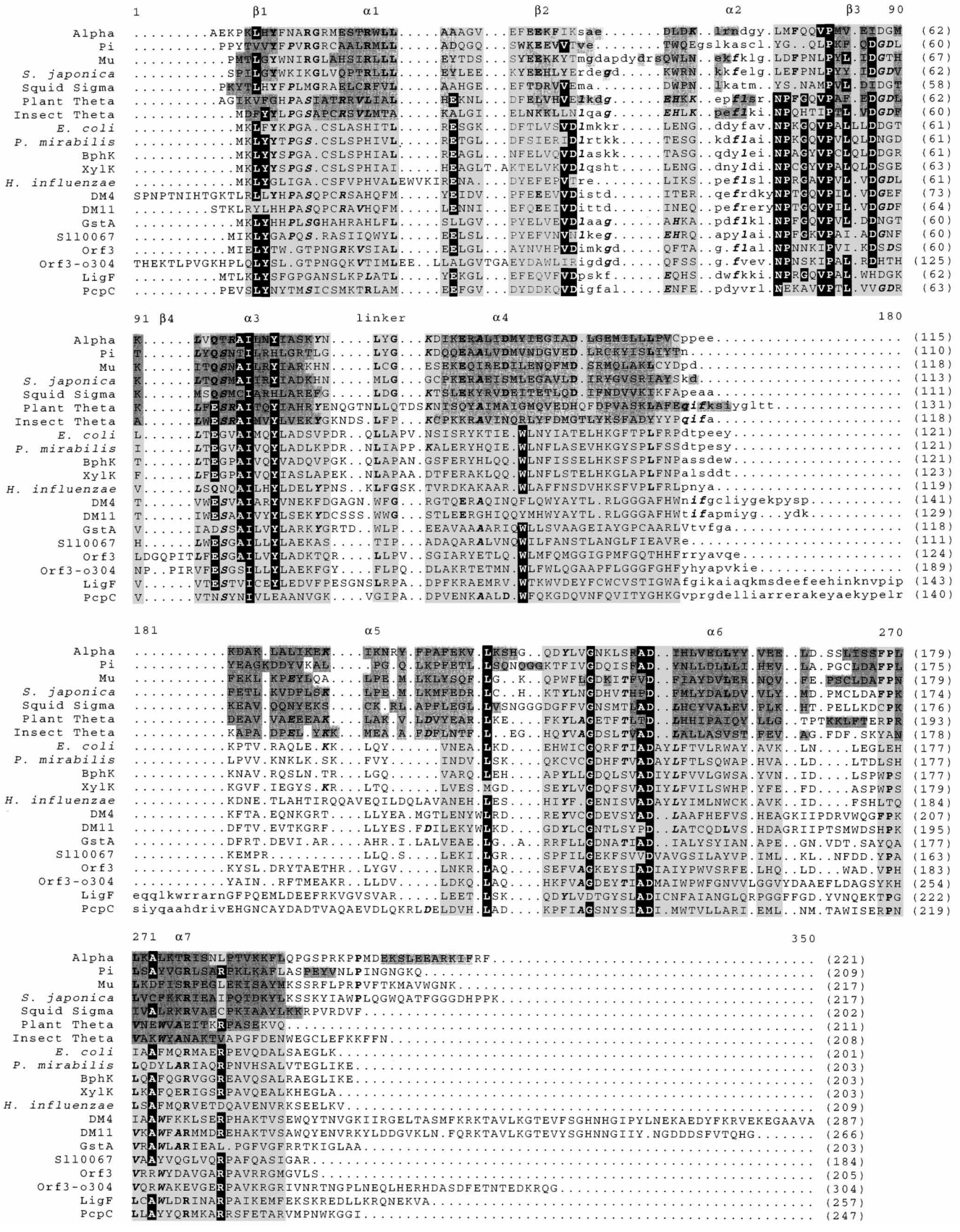
GST genes associated with the metabolism of aromatic compounds. Several bacterial operons and ensembles of genes implicated in the metabolism of aromatic compounds were recently characterized, in which a putative GST gene was detected by analysis of the DNA sequence, but no physiological role could be determined for the corresponding protein. For example, a gene encoding a putative GST subsequently shown to be active with CDNB was located in the *bph* locus of *Burkholderia* (31) (previously *Pseudomonas*) strain LB400, as the 7th gene (*bphK*) in an operon of 11 tightly spaced genes involved in the degradation of biphenyl and chlorinated biphenyl compounds (30). A function in biphenyl degradation was found for all gene products in the operon except BphK. It was suggested that BphK might be involved in the dehalogenation of halogenated biphenyls, but this hypothesis has not been confirmed. It has meanwhile become apparent that several similar operons in different *Burkholderia*, *Pseudomonas*, or *Sphingomonas* strains contain a *bphK* gene homolog encoding a polypeptide with high sequence identity to BphK (41). In addition, XylK, another CDNB-active GST with 61% protein sequence identity to BphK, was reported recently from *Cycloclasticus oligotrophus* RB1, a marine methylotrophic bacterium involved in the metabolism of polycyclic aromatic hydrocarbons (88). In contrast to the situation in the *bph* operon, the divergent orientation of both genes upstream and downstream of *xylK* relative to *xylK* itself implies the existence of several transcription units. Preliminary experiments, however, did not support a role in dehalogenation for this CDNB-active GST, and the function of XylK remains unknown.

The *orf3* gene from *Burkholderia cepacia* AC1100 is another example of a putative GST gene with an unknown function (13, 14). *orf3* was detected as the third open reading frame in a group of six genes involved in the mineralization of 2,4,5-trichlorophenoxyacetic acid. These genes are found in the same orientation, in the order *tftE* (maleyl acetate reductase), *tftF* (glutathione reductase), *orf3* (the putative GST gene), *orf4*, a partially overlapping gene with homology to *orf3* for which no protein product was detected, *tftG* (5-chlorohydroxyquinol dehydrogenase), and *tftH* (hydroxyquinol-1,2-dioxygenase). Gene knockout experiments demonstrated that *orf3* and *orf4* are the only genes in this cluster not required for bacterial growth on 2,4,5-trichlorophenoxyacetic acid. The *orf3* gene expressed in *E. coli* did not show activity with CDNB (13).

The location of apparently nonessential GST genes in operons or gene clusters involved in the ring cleavage and further metabolism of aromatic compounds suggests a possible role for the corresponding proteins in these metabolic pathways: some GST enzymes are known to function as double-bond isomerases with maleyl acetate or maleyl acetoacetate as the substrate (36, 76), and glutathione-dependent isomerases have long been described in bacterial catabolic pathways of aromatic compounds (12). However, isomerase activity has not been described for any bacterial GST.

GST enzymes using glutathione as a reductant. Other bacterial GST enzymes involved in the metabolism of aromatic compounds, but for which a physiological function has been demonstrated, oxidize reduced glutathione to glutathione disulfide during the catalytic cycle.

The reductive dehalogenation of the 2,3,5,6-tetrachloro-*p*-hydroxyquinone intermediate in pentachlorophenol mineralization by *Sphingomonas chlorophenolica* (65; formerly identified as a *Flavobacterium* strain) was shown to depend on glutathione as the reducing agent (91). Tetrachloro-*p*-hydroquinone dehydrogenase (PcpC), the enzyme catalyzing the stepwise reduction of 2,3,5,6-tetrachloro-*p*-hydroquinone to 2,3,6-trichloro-*p*-hydroquinone and then to 2,6-dichloro-*p*-hydroquinone, was obtained in pure form after 50-fold purification (92). The corresponding gene, *pcpC*, was subsequently cloned and sequenced (66). The *pcpC* gene is transcribed un-


1 β1 α1 β2 α2 β3 90
Alpha
Pi
Mu
S. japonica
Squid Sigma
Plant Theta
Insect Theta
E. coli
P. mirabilis
BphK
XylK
H. influenzae
DM4
DM11
GstA
Sll0067
Orf3
Orf3-o304
LigF
PcpC
91 β4 α3 linker α4 180
181 α5 α6 270
271 α7 350

der the control of its own promoter as a single constitutive monocistronic message. Whether the PcpC protein, which is not active with CDNB, can be retained on glutathione-derivatized affinity supports was not reported.

Two genes encoding β-etherase enzymes catalyzing the reductive cleavage of ether linkages in arylglycerol-β-arylethers typical of lignin (Fig. 1) were cloned and sequenced from *Pseudomonas paucimobilis* SYK-6 (57). Although the immediately consecutive *ligF* and *ligE* genes are similar to each other, only LigF shows significant similarity to GST protein sequences. LigF is the only bacterial GST which has been suggested to be membrane associated. Glutathione enhanced β-etherase activity, but not CDNB activity, in crude extracts of *E. coli* carrying the *ligF* gene on a plasmid (57).

DCMDs. DCMDs catalyze the glutathione-dependent hydrolysis of DCM to formaldehyde and allow methylotrophic bacteria to grow on DCM as the sole source of carbon and energy (49). Glutathione acts as a true cofactor and is regenerated during the catalyzed reaction. Dihalomethanes also act as inducers of DCMD expression at low concentrations. DCMD enzymes constitute up to 7% of the cell protein in *Methylophilus* sp. strain DM11 and up to 15% of that in *Hyphomicrobium* sp. strain DM2 and *Methylobacterium* sp. strain DM4 (75). The enzyme from strain DM11 has approximately sixfold higher specific activity than the enzymes from strains DM2 and DM4 (49, 75, 87). DCMDs lack significant activity with CDNB (43, 87) and cannot be purified on glutathione-derivatized affinity supports (86).

Canonical CDNB-active GST enzymes which can be retained on affinity supports have not been characterized in DCM-degrading strains. Two such GST isozymes purified from another methylotrophic bacterium, *Methylobacterium organophilum* XX, appear unusual in that they were reported to be monomers with molecular masses of 38 and 43 kDa (Table 1, 80).

DCMD genes of strains DM4 and DM11 have been cloned and sequenced (6, 43), and the corresponding proteins are each other's closest relatives in sequence databases (56% identity). DCMD genes from more than a dozen methylotrophic strains originally isolated from sites contaminated with DCM were shown to be located on a 4.2-kb *Bam*HI fragment (50, 74), suggesting that propagation of DCM utilization genes may have occurred by horizontal transfer. A recent sequence analysis of a 10-kb gene region including the *dcmA* gene from strain DM4 revealed the presence of three different insertion elements upstream and downstream of the gene for DCMD/GST (50, 74).

The regulation of GST expression in bacteria is still an essentially uninvestigated field, in contrast to the wealth of information available on the subject of plant and mammalian GSTs (28, 55). The *dcmR* gene found in a divergent orientation upstream of the structural gene *dcmA* in strain DM4 constitutes an exception in this respect (44). DcmR apparently acts by repressing the transcription of the *dcmA* structural gene coding for the DCM dehalogenase in the absence of DCM, but several other factors also appear to be involved in regulating the expression of the *dcmA* gene (74).

Thiol-dependent epoxidases. Several GST enzymes from all classes are known to be active with various model epoxides (15, 52, 64). Two types of bacterial glutathione-dependent enzymes have been described for which such a function is known to be physiologically significant. Plasmid-encoded genes responsible for fosfomycin resistance (reviewed in reference 79) encode short polypeptides of about 140 amino acids with GST activity (2, 3). Closely related genes were cloned from transposon Tn*2921* (62, 79) and from *Staphylococcus epidermidis* and sequenced (94). These genes may have originated in a gram-positive bacterium and then spread by horizontal transfer (79), an interesting proposal given that glutathione has rarely been found in high concentrations in gram-positive bacteria (63). In *Serratia marcescens*, the fosfomycin resistance gene is located close to another as yet unpublished gene with similarity to mammalian and bacterial GST enzymes (79). No similarity to known glutathione-binding domains or to any other sequences in the databases was detected when the fosfomycin resistance gene sequences were reported, but the corresponding protein sequences have been shown (86) to display a low but significant level of sequence similarity to recently characterized bacterial extradiol dioxygenases involved in biphenyl metabolism (27, 29). Sequence alignments reveal that the three conserved residues defining the iron(II) ion-binding site in these dioxygenases are also present in fosfomycin-inactivating enzymes. This may have physiological relevance, since the activity of the purified fosfomycin resistance protein is stimulated about threefold by divalent iron or manganese (3). The weak sequence similarity of fosfomycin-inactivating GST enzymes to extradiol dioxygenases also raises the intriguing possibility that these enzymes are structurally related. However, the structure of extradiol dioxygenase (27) appears to be very different from that of GSTs (5, 18, 89). This implies that quite similar glutathione-dependent conjugation reactions could be performed by enzymes with completely different structural features.

Very recently, the glutathione-dependent ring opening of 2,3-dichlorooxirane (*cis*-1,2-dichloroethene epoxide in Fig. 1) was reported in cell extracts of *Rhodococcus* sp. strain AD45 (85). This strain thus represents a further example of a gram-positive bacterium containing glutathione. Glutathione was shown to be the physiological cofactor involved in epoxide ring opening. An enzyme catalyzing this reaction was identified, purified to homogeneity, and shown to be a single 26-kDa polypeptide in solution. The K_m of this enzyme for glutathione (25 mM) is unusually high, even for GST enzymes of the theta class (59; see below), and no activity was detected with CDNB as the substrate. These last two characteristics are reminiscent

FIG. 3. Alignments of bacterial GST sequences with sequences of representative eukaryotic GST enzymes of all classes for which the structure are known. The X-ray sequences are as follows (accession numbers are in parentheses): Alpha, protein from human liver (P08263); Pi, protein from human placenta (P09211); Mu, protein from human muscle (P28161); *S. japonica* (P08515); Squid Sigma, from *Ommastrephes sloanei* (P46088); Plant Theta, from *A. thaliana* (P46422); Insect Theta, from *L. cuprina* (P42860). The bacterial sequences are as follows: *E. coli*, GST from *E. coli* K-12 strain JM105; *P. mirabilis*, GSTB1-1 from *P. mirabilis*; BphK, protein from *Burkholderia* sp. strain LB400; XylK, protein from *C. oligotrophus*; *H. influenzae*, open reading frame HI0111 from *H. influenzae*; DM4, DCMD from *Methylobacterium* sp. strain DM4; DM11, DCMD from *Methylophilus* sp. strain DM11; GstA, putative GST from *R. leguminosarum*; Sll0067, putative GST from *Synechocystis* sp. strain PCC6803; Orf3, putative GST from *B. cepacia* AC1100; Orf_o304, putative GST from *E. coli* K-12 strain MG1655; LigF, β-etherase from *P. paucimobilis* SYK-6; PcpC, tetrahydroquinone reductase from *S. chlorophenolica*. Residues in lowercase denote regions of low structural similarity between enzymes with known structures. The conserved regions in the alignment used to construct the phylogenetic tree in Fig. 4 are shaded in light grey. The positions of secondary-structure elements of GST enzymes with known structures are shaded in dark grey. Secondary-structure elements in the *L. cuprina* enzyme are labelled above the alignment. Positions with a black background are those which are conserved in at least 10 of the 13 bacterial GST sequences shown in the alignment, while residues in bold italics are positions of identity in both sequences of the theta class with known structures and in other sequences shown in the alignment. Residues in boldface roman type are highly conserved in enzymes of the alpha, mu, and pi classes (18) and also, if present, in those of the theta class.

TABLE 1. Identification and properties of bacterial GST proteins and genes

Strain(s)	Protein denomination	Database accession no.[a]	Length[b]	Activity (nmol/min/mg) on CDNB[c,d]	Other substrate(s)[c]	Reference(s)
Proteus mirabilis	GSTB1-1 (Pm-GST6.0)[e]	P15214, *U38482*	203 (22.5)	3,000	EA, CPO, EPNP	15, 61, 70
Klebsiella oxytoca, *Enterobacter cloacae*, *Proteus vulgaris*, *Pseudomonas aeruginosa*	GST	No sequence	(22–23)	160–4,800		71
Escherichia coli B	GST	No sequence	(25)	3,200	NBC	32
Escherichia coli K-12 strain JM83	GST	P39100[f]	(24)	2,550		4
Escherichia coli K-12 strain JM105	GST	P39100, *D38497*	201 (25)	10,000	EA, CPO	64
Serratia marcescens	Sm-GST-7.3[d]	P22416[f]	(22)	160	CPO	16
Xanthomonas campestris	Xc-GST-4.5[d]	P45875[f]	(22)	650	EA, CPO	17
Burkholderia[g] sp. strain LB400	BphK	Q59721, *X76500*	203 (22.4)	Active		30
Haemophilus influenzae	HI0111	P44251, *L42023*	209			24[h]
Cycloclasticus oligotrophus	XylK	Q46153, *U51165*	203 (22.5)	Active		88
Hyphomicrobium sp. strain DM2	DcmA	No sequence	(35)	ND	Dihalomethanes	37
Methylobacterium sp. strain DM4	DcmA	P21161, *M32346*	288 (35)	ND	Dihalomethanes	43, 75
Methylophilus sp. strain DM11	DcmA	P43387, *L26544*	267 (34)	<10	Dihalomethanes	6, 75, 87
Methylobacterium organophilum	GST[d]	No sequence	(45/38)[d]	500–800	DCNB	80
Pseudomonas paucimobilis SYK-6	LigF	P30347, *D11473*	257	ND	β-Aryl ethers	57
Sphingomonas chlorophenolica[i]	PcpC	Q03520, *M98559*	248 (30)	ND	TeCH, TrCH	66, 91, 92
Burkholderia cepacia AC1100	Orf3	Q45073, *U19883*	205	ND		13
Escherichia coli K-12 strain MG1655	Orf-o304	*U28377*[h]	304			
Synechocystis sp. strain PCC6803	Sll0067	Q55139, *D64001*	184			35[h]
Rhizobium leguminosarum	GstA	Q52828, *X89816*	203			81
Serratia marcescens	FosA	A60631 (pir), *M31685*	141 (16)	ND	Fosfomycin	3, 62, 79
Staphylococcus epidermidis	FosB	Q03377, *X54227*	139 (15)	ND	Fosfomycin	94
Rhodococcus sp. strain AD45	GST	No sequence	(26)	ND	2,3-Dichloroxirane, alkane epoxides	85

[a] Swissprot protein database numbers are in roman type, and DNA database numbers are in italics.
[b] Protein length is in amino acids. Where known, the subunit molecular mass in kilodaltons, determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, is given in parentheses.
[c] Abbreviations for substrates used: CDNB: 1-chloro-2,4-dinitrobenzene; DCNB: 1,2-dichloro-4-nitrobenzene; EA: ethacrynic acid; CPO: cumene peroxide; EPNP: 1,2-epoxy-3-*p*-nitrophenyl phosphate, NBC: *p*-nitrobenzyl chloride; TeCH, tetrachloro-*p*-hydroquinone; TrCH, trichloro-*p*-hydroquinone (Fig. 1).
[d] ND, not detected.
[e] Isoforms detected.
[f] N-terminal protein sequence only.
[g] New assignment (31). Originally described as *Pseudomonas* sp. strain LB400 (30).
[h] Sequence data only.
[i] New assignment (65). Originally published as a *Flavobacterium* sp. strain (66, 91, 92).

of the fosfomycin-inactivating GST enzymes (3, 79), but several differences between the two systems were noted.

Protein engineering studies on bacterial GST enzymes. Numerous protein engineering studies on eukaryotic GST enzymes of the alpha, mu, and pi classes have already delineated the roles of many residues in the structural and functional integrity of these enzymes. In stark contrast, reports of analogous studies with enzymes of the theta class have been scarce and have focussed on the importance of a tyrosine residue at the N terminus of the protein known to be essential for GST function in enzymes of the alpha, mu, and pi classes (Fig. 3). In the latter enzymes, the hydroxyl group of the N-terminal tyrosine residue is located near the glutathione thiol and contributes to the enhancement of its nucleophilicity by H bonding (5, 18, 89). Although a tyrosine residue is present at the corresponding position in the majority of theta class GST sequences, its exact location is more variable than in GST sequences of the alpha, mu, and pi classes, and some theta class GSTs even lack this residue (Fig. 3). Indeed, the replacement of N-terminal tyrosine residues in a *Drosophila* theta class GST (48) and in an *E. coli* GST (64) had no significant effect on the activity of these enzymes. More recently, X-ray studies showed that the N-terminal tyrosine residue is too far away in the structure of the theta class *L. cuprina* enzyme to be able to make contact with the glutathione thiol (90). These studies rather suggested a nearby serine residue as the nucleophilicity-enhancing residue. Protein engineering analysis of the *L. cuprina* enzyme and the DCMD from strain DM11 demonstrated that the N-terminal tyrosine is dispensable (87) and that the N-terminal serine residue is essential (9, 87) for catalytic activity in these proteins. Nevertheless, inspection of sequence alignments (Fig. 3) suggests that several bacterial GST enzymes may lack such a serine residue. Considering the large differences in the sequences and catalytic properties of bacte-

rial GSTs, protein engineering investigations will probably be the technique of choice for detailed investigations of the function of bacterial GST enzymes in the near future.

Implications of the functional and biochemical diversity of bacterial GST enzymes. In contrast to known CDNB-conjugating enzymes, bacterial GST enzymes for which a physiologically relevant substrate has been demonstrated, such as DCMDs and the aromatic reductive dehalogenase PcpC, are expressed at quite high levels in the cell and yield metabolites and energy for bacterial growth. This type of GST enzyme may be quite specific to bacteria, since GST enzymes from eukaryotes appear not to be active in central metabolism but rather to specialize primarily in detoxification reactions. Other striking differences in enzymatic properties are apparent within the group of bacterial GSTs. For example, K_m values for glutathione in the millimolar range (59) are often thought to be characteristic of theta class enzymes. However, the DCMD from DM11 (K_m, 66 μM [87]) and the GST from *E. coli* (K_m, 40 μM [64]) have a higher affinity for glutathione. Interestingly, both the *E. coli* enzyme (64) and the *P. mirabilis* enzyme, which shows a rather low affinity for glutathione (K_m, 686 μM [70]), could be purified by glutathione affinity chromatography, but the DCMD from strain DM11 could not (86). Thus, some uncharacterized features of GST enzymes of the theta class appear to be responsible for their glutathione-derivatized support-binding properties. It is possible that the theta class of GST enzymes will have to be split further as details of the catalytic and structural properties of these enzymes become known. The analysis of sequence relationships among bacterial GST enzymes presented below also supports this hypothesis.

HOW RELATED ARE BACTERIAL GSTs? INFERENCES FROM SEQUENCE COMPARISONS

In the absence of a detailed structure of any bacterial GST, sequence comparisons with GST proteins for which the X-ray structure has been solved are of great importance in obtaining models of these enzymes to try to understand how catalysis is achieved at the level of individual amino acids. Construction of models of bacterial GSTs is made more difficult by the important sequence variation observed within the theta class of GST sequences (sequence identity between 13 and 61%, with an average of about 25%). This low level of sequence identity is perhaps to be expected between homologous enzymes catalyzing a wide range of reactions on different types of substrates. Fortunately, it seems clear that these reactions are performed within the structurally conserved GST framework, which can be used as a guide for construction of sequence alignments of bacterial GST enzymes (Fig. 3).

The regions corresponding to secondary-structure elements in the sequences of GST enzymes with known structures can be aligned with confidence to the ensemble of bacterial GST sequences, with the exception of the second helix in the C-terminal domain and the C-terminal end of these proteins (Fig. 3). All bacterial GST sequences have higher pairwise sequence identity to the sequences of theta class GST enzymes with known structures from the insect *L. cuprina* (90) or the plant *A. thaliana* (72) than to protein sequences of GST enzymes of other classes. Also, the N-terminal domain of GST enzymes involved in glutathione binding is more strongly conserved than the C-terminal domain (Fig. 3). Indeed, the sequences of the C-terminal domain are often too different (below 20% identity) to be detected as being similar in automated searches of sequence databases. This lends support to the idea that the C-terminal domain of GST enzymes plays a crucial role in determining their functional specificity. The region corresponding to the first two helices of domain II is the most variable part in alignments of GST sequences, and both PcpC and LigF proteins appear to contain large sequence insertions in this region (Fig. 3). Interestingly, the lack of GST activity in squid crystallins homologous to squid GST enzymes is thought to have been initiated by the insertion of a loop between these two helices in the functional GST ancestor (84).

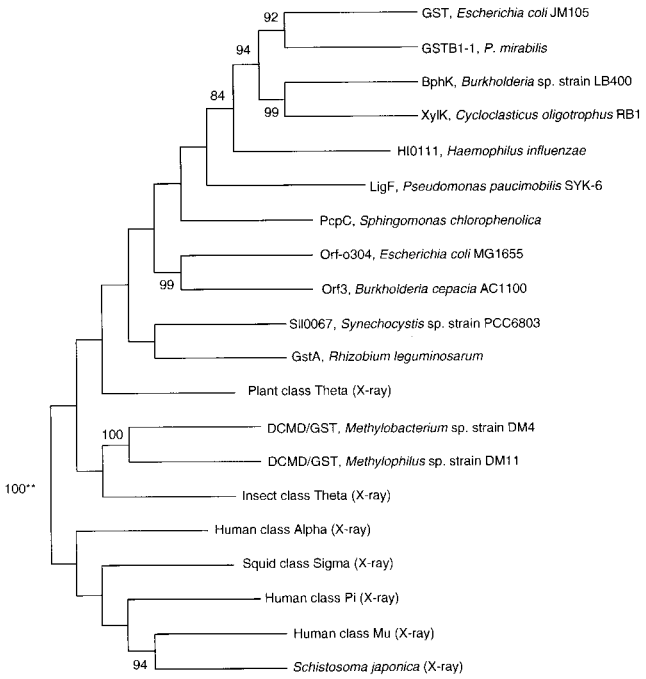


FIG. 4. Phylogenetic representation of the sequence relationships between known bacterial GST sequences and selected representatives of all known classes of GST enzymes generated from 153 conserved amino acid positions from the alignment presented in Fig. 3. The depicted tree is the most parsimonious consensus tree obtained by bootstrap analysis of the alignment (100 replicates) with the PROTPARS program from the PHYLIP package (21). Nodes which are well supported by phylogenetic analysis are indicated by the percentages of replicates for which those particular nodes were recovered. The root (indicated by stars) was arbitrarily set to the node separating theta class GST sequences from sequences of other classes.

The better-conserved regions in the multiple sequence alignment shown in Fig. 3 were used to generate a tree representation of sequence relationships of bacterial GST sequences with each other and with GST sequences from enzymes with known structures (Fig. 4). Due to the low level of identity between sequences, only a few nodes in the tree are statistically well supported in bootstrapping analyses. This caveat notwithstanding, it seems clear that bacterial GST sequences can be classified in several different groups. As already mentioned, DCMDs (6, 43) are each other's closest relatives in sequence databases. They are also more closely related to theta class human, rat, and mouse GST enzymes that hydrolyze DCM and to some insect GST enzymes than to other bacterial GSTs. In contrast, the sequences most closely related to the PcpC protein from *S. chlorophenolica* (66) are GST enzymes of plant origin. The group of CDNB-active GSTs clearly cluster together and also include HI0111 from *Haemophilus influenzae* (24), an open reading frame encoding a putative GST similar to the CDNB-active BphK protein. The protein sequence closest to the *B. cepacia orf3* gene product in sequence databases is encoded by *orf-o304* from *E. coli*, an unusually long polypeptide (304 residues) by GST standards. Although the function of

orf-o304 is unknown, it is perhaps of interest to note that it lies just downstream of *gsp*, which encodes another glutathione-dependent enzyme, glutathionyl-spermidine synthetase/amidase (10). Gene sequences encoding putative GSTs from *Synechocystis* sp. strain PCC6803 obtained in the course of the cyanobacterial genome sequencing project (35) and from *Rhizobium leguminosarum* (81) are also the most closely related bacterial genes in sequence databases. Finally, eukaryotic GST enzymes of all other classes are clearly recovered as the outgroup to theta class GSTs.

It is evident from the above that bacterial, plant, insect, and mammal GST enzymes cannot be clustered according to established phylogenetic groups. This perhaps reflects the parallel and independent evolution of GST genes from very ancient ancestors dating back to endosymbiotic events, although the possibility of horizontal transfer of GST genes specialized in detoxification reactions should be kept in mind. In addition, some of the uncharacterized bacterial putative GST genes may turn out to encode not GST enzymes but rather proteins with a glutathione-binding module with sequence similarities to theta class GSTs (39, 67).

EXPLORING THE REPERTOIRE: POTENTIAL APPLICATIONS OF BACTERIAL GSTs

The wide range of compounds which can interact with members of the GST family within the same structural framework suggests that GST enzymes may be recruited and harnessed to catalyze a given conjugation reaction of interest. The variety of the small but rapidly increasing number of bacterial GSTs characterized so far also indicates that a large repertoire of such enzymes remains to be uncovered in the bacterial world.

At the protein level, two types of GST can be distinguished. On the one hand, some GSTs, such as the bacterial DCMD, lignin β-etherase, and reductive dehalogenase enzymes which have already been characterized, catalyze reactions that yield products which can be used for bacterial growth. In principle, the selection in bacteria of mutant GST enzymes of this type with the ability to react with a given chemical can be envisaged if the target substrate can be used for growth by the bacterial host. However, no example of a successful application of such an approach is known.

In contrast, GST enzymes of the other, more common type react with electrophilic compounds to yield stable glutathione conjugates. The toxicity and persistence of these conjugates in bacteria and the details of the routes by which they are further metabolized or excreted have only recently begun to be investigated. Nevertheless, promising examples of the detoxification of xenobiotic compounds, carcinogens, and pollutants involving GSTs in bacterial systems some of which are presented below, have already been documented.

Use of GSTs in detoxification studies of chemical compounds in bacteria. GST enzymes feature prominently in recent developments of toxicological methods in bacterial systems (25), for which the Ames test is the classical example (53). The Ames test relies on the detection of mutations causing phenotypic reversion in *Salmonella typhimurium* tester strains auxotrophic for histidine. Mutations are induced by metabolites of potentially mutagenic chemicals generated by exogenously added rat liver cytosolic enzymes, which contain large quantities of GST enzymes. Newer variants of this test feature plasmid-encoded, cloned eukaryotic theta class GSTs instead of rat liver cytosol fractions (25, 83). This may avoid some of the problems associated with potentially mutagenic compounds which are too short-lived to show an effect in the standard Ames assay or are not able to cross biological membranes. In addition, it allows the study of the mechanisms of GST-dependent activation and inactivation of electrophilic chemicals.

Similar bacterial systems can also be used for the selection of GST enzyme mutants with altered substrate specificities that effect improved detoxification of a given compound. For example, random mutagenesis of a rat alpha class GST gene yielded mutants encoding enzymes with increased resistance to mechlorethamine, a DNA-alkylating drug used in chemotherapy, after selection in *E. coli* (26). A somewhat related strategy was used to characterize structure-activity relationships of a human alpha class GST gene. The known inhibitory effect of CDNB on the growth of *E. coli* and the increase in this inhibition after conjugation with glutathione were used to select for null mutants of the plasmid-encoded GST gene conferring increased resistance of *E. coli* to CDNB (47). The mutations found in the GST genes after selection encoded amino acid changes in the protein which would not have been easily predicted to be important for the stability and activity of the enzyme.

Degradation of herbicides by bacterial GST enzymes and further metabolism of glutathione conjugates. The conjugation and detoxification of herbicides and pesticides by GSTs is an area where the interests of plant scientists and microbiologists increasingly overlap. In plants, GSTs are involved in the detoxification and transport of pesticides and herbicides, in the metabolism of endogenous compounds, and in protection from pathogenic infection, often in the course of stress-related responses (55). Most efforts have concentrated on pesticide uptake and degradation by crop plants (42), but little is known about biodegradation by native plants and the sharing of tasks with rhizosphere bacteria (1).

A recent reappraisal of GST activity in bacteria aimed at identifying pesticide-conjugating microorganisms confirmed that such activity may be widespread in rhizosphere gram-negative bacteria (93). Our knowledge of the bacterial metabolism associated with glutathione conjugation, however, is still rudimentary compared to what is known for plants and mammals, in which the corresponding metabolic routes have been investigated in detail. Glutathione conjugates of pesticides and herbicides can be metabolized to cysteine conjugates by the action of soil microorganisms (reviewed in reference 23). These conjugates can be transformed to the corresponding thiols, pyruvate, and ammonia by cysteine β-lyases, enzymes which have been detected and characterized in several bacteria (45). Further, soil microorganisms are known to methylate and oxidize thiolated metabolites of pesticides and herbicides resulting from the β-lyase to their methylsulfinyl ($-SOCH_3$) or methylsulfonyl ($-SO_2CH_3$) derivatives. Unlike in mammals and plants, however, the direct oxidative route from thiols to sulfonates may prevail in soil microorganisms (see reference 23 for further references). Indeed, water-soluble, sulfonated metabolites of alachlor and metolachlor were detected and identified in groundwater and soil, and it was recently shown that a gram-negative bacterial isolate was able to use the sulfonate derivative of a related chloroacetanilide herbicide, metazachlor, as the sole source of sulfur for growth (46).

Alternatively, glutathione conjugates produced by bacteria from electrophilic compounds might also be disposed of by excretion, although little is known about such pathways. ATP-dependent pumps specific for glutathione conjugates are known in mammals (33) and plants (51, 54, 56). In plants, glutathione conjugates are accumulated in the vacuole rather than excreted as in mammals. The best-described such system in bacteria is from *E. coli*, in which potassium efflux glutathione-gated channels participate in the detoxification of electro-

philic compounds such as CDNB and methylglyoxal (22). The role of bacteria and the involvement of GST enzymes in the biological processes featuring sulfur-containing metabolites of xenobiotics thus represent an important and exciting area for research.

BACTERIAL GSTs: ANCIENT ENZYMES WITH A PROMISING FUTURE

As the putative direct descendents of ancestral GSTs, bacterial GSTs already have a rich and diverse history. Nevertheless, their future in our technological world also appears to be assured. The combination of molecular biological and protein chemical techniques available today will doubtless result in bacterial GST enzymes being characterized at an increasing rate. The development of screening and selection programs using bacterial systems based on GSTs may yield new catalysts for the detoxification of harmful or persistent chemicals. Suitable GST genes may be either recruited by screening or engineered by site-directed or random mutagenesis for applications in biodegradation (34) or toxicology (25). Finally, such investigations may provide new insights into the basic genetics and biochemistry of glutathione conjugate metabolism and the importance of GSTs in bacteria.

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