

Heterologous expression of four glutathione transferase genes genetically linked to a major insecticide-resistance locus from the malaria vector *Anopheles gambiae*

Federica ORTELLI*, Louise C. ROSSITER*, John VONTAS†, Hilary RANSON*¹ and Janet HEMINGWAY*

*Liverpool School of Tropical Medicine, Vector Biology Research Group, Pembroke Place, Liverpool L3 5QA, U.K., and †IMBB-FORTH Vassilika Vouton, P.O. Box 1527, 71110 Heraklion, Crete, Greece

A cluster of eight genes encoding glutathione transferases (GSTs) are located on division 33B of polytene chromosome arm 3R of the African malaria mosquito, *Anopheles gambiae*. This region of the genome contains a major 1,1,1-trichloro-2,2-bis-(*p*-chlorophenyl)ethane (DDT)-resistance locus, *rtDI*. These GSTs belong to the insect-specific Epsilon class and share between 22.6 and 65.2% identity at the amino acid level. Two distinct allelic variants of the Epsilon GST, *GSTe1*, differing at 12 out of 224 amino acid residues, are present in laboratory and field populations of *A. gambiae*. To investigate the possible role of these GSTs in conferring resistance to the insecticide DDT, both *GSTe1* alleles, plus three additional members of this gene cluster, were expressed in *Escherichia coli* and the recom-

binant proteins biochemically characterized. The five putative glutathione transferases encoded catalytically active subunits with variable biochemical properties. For example, the two allelic variants of GSTE1-1 encoded proteins with over 100-fold variation in peroxidase activity, while the three remaining GSTs had no detectable peroxidase activity. Only GSTE2-2 was able to metabolize DDT. Western blots using antibodies raised against these GSTs indicated that the expression of GSTE2-2 is elevated in a DDT-resistant strain of *A. gambiae*.

Key words: *Anopheles gambiae*, DDT, glutathione transferase (GST), insecticide resistance, mosquito.

INTRODUCTION

Glutathione transferases (GSTs) are soluble dimeric proteins that are ubiquitous in Nature. They are involved in the metabolism, detoxification and excretion of a large number of endogenous and exogenous compounds from the cell. A typical GST-catalysed reaction involves the transfer of the tripeptide glutathione to an electrophilic substrate. To facilitate this reaction, each GST monomer has two distinct binding sites, a G site, which binds glutathione, and an H site, which binds the substrate. Variability in the structure of the H site largely accounts for the wide range of substrate specificities of the GSTs (for a recent review see [1]). This variability has been achieved by extensive duplication and diversification of this supergene family. Over 40 GST genes have been detected in the genomes of most higher eukaryotes for which full genome sequence data are currently available [2]. These have been classified into at least 13 different classes based on their amino acid sequence identities, immunological properties and, where known, substrate specificities. Some of these classes are found across multiple eukaryotic phyla, for example the Zeta and Omega classes [3,4], whereas others, such as the insect-specific Delta and Epsilon classes, are more restricted in their distribution [5].

Insect GSTs are of particular interest because of their potential to cause resistance to all the major families of insecticide. For example, in the diamond back moth, *Plutella xylostella*, elevated levels of an Epsilon-class GST confers resistance to organophosphate insecticides [6,7], whereas in the brown plant-

hopper, *Nilaparvata lugens*, permethrin resistance is associated with the over-expression of *nlGST1-1*, a class-Delta GST [8,9]. GSTs also catalyse the dehydrochlorination of 1,1,1-trichloro-2,2-bis-(*p*-chlorophenyl)ethane (DDT) to the non-toxic metabolite, 1,1-dichloro-2,2-bis-(*p*-chlorophenyl)ethane (DDE) [10].

We are interested in the role of GSTs in conferring DDT resistance in the malaria vector, *Anopheles gambiae*. This mosquito is responsible for over 1 million malaria-associated deaths in Africa each year [11]. Spraying the insides of houses with DDT is widely used to control the transmission of the disease, but these efforts are often disrupted by the emergence of insecticide resistance. Earlier biochemical studies had indicated that DDT resistance is associated with both qualitative and quantitative changes in multiple GST enzymes [12]. Genetic mapping studies showed that chromosome 3 division 33B contains a major locus controlling DDT resistance [13], and subsequent analysis of the draft genome sequence of *A. gambiae* located a cluster of eight sequentially arranged GST genes within this genomic region [14] (Figure 1). Previously we implicated one member of this GST gene cluster, the Epsilon GST gene, *GSTe2*, in conferring DDT resistance on the basis that this gene was over-transcribed in a DDT-resistant strain of *A. gambiae* and the recombinant protein from *GSTe2* possessed DDT dehydrochlorinase activity [5]. However, the role of the other members of this GST cluster in DDT resistance was unknown. In this study we analysed the amino acid sequence of all members of the gene cluster from insecticide-susceptible and -resistant strains and report on the *in vitro* expression and characterization of four of these GST proteins.

Abbreviations used: CDNB, 1-chloro-2,4-dinitrobenzene; DCNB, 1,2-dichloro-4-nitrobenzene; DDE, 1,1-dichloro-2,2-bis-(*p*-chlorophenyl)ethane; DDT, 1,1,1-trichloro-2,2-bis-(*p*-chlorophenyl)ethane; GST, glutathione transferase.

¹ To whom correspondence should be addressed (e-mail Hranson@liverpool.ac.uk).

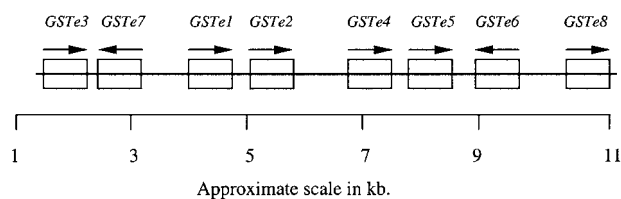


Figure 1 Schematic diagram showing the organization and orientation of eight GST genes clustered on division 33B of polytene chromosome arm 3R of *A. gambiae*

The arrows indicate the direction of transcription.

EXPERIMENTAL

Mosquito strains

The ZAN/U strain of *A. gambiae* was colonized from a DDT-resistant field population from Zanzibar, Tanzania, in 1982. Adults of this strain have been maintained under regular DDT selection pressure. Kisumu is a laboratory insecticide-susceptible strain colonized originally from Kisumu in Western Kenya. The RSP strain was colonized from the same geographical area of Kenya, but individuals in this strain exhibit the phenotype of reduced susceptibility to permethrin [15]. Field samples of *A. gambiae* s.s. were collected in 2002 from Accra, Ghana, and Gamba, Gabon.

Sequencing

Preliminary partial sequences of the Epsilon GST cluster on chromosome 3R, division 33B, were obtained from sequencing of multiple clones of an *A. gambiae* bacterial artificial chromosome library as described previously [5]. These data were supplemented by sequence data from the *A. gambiae* trace data set as it became available online (www.ncbi.nlm.nih.gov/blast/mmtrace.html). As putative GST genes were identified, primers were designed to enable the amplification of the full-length coding sequence of each gene (Table 1). Each gene in the cluster was amplified from genomic DNA and cDNA extracted from individual *A. gambiae* mosquitoes using techniques described previously [5]. The resultant products were cloned into the pGEM T-easy vector (Promega) and, after transformation into *Escherichia coli*, plasmids were extracted and purified using Qiagen miniprep

kits. Sequencing was performed using ABI BigDye terminator chemistry and, after electrophoresis using an ABI 3100 sequencer, contigs were assembled and the sequences annotated using the LASERGENE software package (DNASTar, Madison, WI, U.S.A.). Amino acid sequences were aligned using ClustalW [16].

Expression

The coding regions of *GSTe1*, *GSTe2*, *GSTe4* and *GSTe8* were amplified using ZAN/U or Kisumu cDNA as a template, *Pfu* polymerase (Stratagene) and primers that contained the initiation and termination codons of the genes preceded by *Bam*HI sites (Table 1). The products were subcloned into the pGEM T-easy vector and sequenced to ensure that no errors had been introduced during PCR amplification. The PCR inserts were excised from the vectors using *Bam*HI and ligated into the *Bam*HI site of the pET3a vector (Novagen). After confirmation of correct orientation, the expression constructs were used to transform *E. coli* BL21(DE3)pLysS competent cells. Expression was induced by the addition of 0.4 mM isopropyl β -D-thiogalactoside, and the recombinant proteins were purified with a T7-tag immunoaffinity column (Novagen) as described in [5]. Enzyme purity and subunit size were determined by overloading SDS/PAGE gels and staining with Coomassie Brilliant Blue. Proteins were concentrated with Centrprep and Centricon units (Amicon), and stored at -20°C after the addition of glycerol (50%) and dithiothreitol to a final concentration of 15 mM.

Antibody production and Western blots

Polyclonal antibodies against the purified recombinant *GSTe1* and *GSTe2* proteins were raised in New Zealand White rabbits by standard procedures.

Recombinant proteins and total protein, extracted from mass homogenates of ZAN/U and Kisumu individuals, were resolved by SDS/PAGE, and transferred on to an Hybond ECL nitrocellulose membrane (Amersham Bioscience) using a Trans-Blot Semi Dry Transfer Cell (Bio-Rad) according to manufacturer's instructions but with an extended transfer time of 90 min. The filters were probed with the GST polyclonal antibodies (1:5000 dilution) for 1 h, followed by incubation with anti-rabbit secondary antibody (1:2000 dilution) for a further 1 h. Detection was performed with the enhanced chemiluminescence detection system (Amersham Biosciences).

Table 1 Sequence of primers used in the study

*Bam*HI sites are shown in italics.

Primer name	Primer sequence	Target
e2R2	5'-AGCCCAATGCTTTGGCCGTC-3'	Amplify intergenic region between <i>GSTe1</i> and <i>GSTe2</i>
e1kF	5'-CATCTTCTACAAACACGTGC-3'	
e1zF	5'-GCATCGAGTACGTTTCGTACG-3'	
e1dF	5'-GTCAATGAGGCACTGCATTC-3'	Allele-specific PCR for <i>GSTe1</i>
e1dR	5'-GTGATCCGGCTACGTAATCG-3'	
e1BamF	5'-GGATCCATGCCGAAACCTGTGCTA-3'	Generation of expression construct for <i>GSTe1</i>
e1BamR	5'-GGATCCTTACAGTTTTGCATTCTTCGC-3'	
e2BamF	5'-GGATCCATGTCCAACCTTGACTGTA-3'	Generation of expression construct for <i>GSTe2</i>
e2BamR	5'-GGATCCTTAAGCCTTAGCATTCTCCTC-3'	
e4BamF	5'-GGATCCATGCCAAACATTAAGC-3'	Generation of expression construct for <i>GSTe4</i>
e4BamR	5'-GGATCCTCACTTGGCGTTGG-3'	
e8BamF	5'-GGATCCATGATTCTGTACTACG-3'	Generation of expression construct for <i>GSTe8</i>
e8BamR	5'-GGATCCCTACAGCTTAATCTTTTC-3'	

Biochemical assays

GST activities using the model substrates 1-chloro-2,4-dinitrobenzene (CDNB), 1,2-dichloro-4-nitrobenzene (DCNB) and *p*-nitrophenol acetate ('pNPA') were determined using the methods of [17]. Kinetic parameters (V_{\max} and K_m) were estimated for CDNB-conjugating activity by non-linear regression using the Micromath Scientist program (Micromath Scientific, Salt Lake City, UT, U.S.A.). Selenium-independent glutathione peroxidase ('GPOX') activity was determined with cumene hydroperoxide by the method of [18] using 1.8 μg of recombinant GST. DDT dehydrochlorinase activity and HPLC analysis and quantification of the DDT metabolites were performed as described previously [19]. Protein concentration was determined using the Bio-Rad protein reagent with BSA as the standard protein [20].

Each of the purified recombinant GST proteins was applied to a *S*-hexylglutathione-agarose affinity column equilibrated with 25 mM Bis-Tris/propane buffer, pH 6.5, containing 15 mM dithiothreitol and 25 μM PMSF (buffer A). The column was washed with buffer A containing 0.2 M NaCl and the bound protein was eluted with 5 mM *S*-hexylglutathione. The unbound and bound fractions were assayed for GST activity using CDNB as described above.

GSTe1-allele-specific PCR

PCR and restriction digestion were used to distinguish between allelic variants of the *GSTe1* gene. The allele found in the ZAN/U strain contains an additional *AluI* site, at position 496 bp (where ATG is position 1). PCR amplification followed by *AluI* restriction digestion is a rapid method of genotyping at this locus. Field-sampled *A. gambiae* individuals as well as standard laboratory strains were used to assess the presence of the two *GSTe1* alleles reported in this study. The PCR was performed with primers e1dF and e1dR (Table 1) with an annealing temperature of 54 °C.

RESULTS

Sequencing of the *A. gambiae* Epsilon GST class

The full-length coding sequence of each of the eight GST genes on chromosome 3R division 33B was amplified from multiple individuals of the ZAN/U strain of *A. gambiae*. An alignment of the predicted amino acid sequences of these genes is shown in Figure 2. The percentage identity at the amino acid levels ranges from 22.6 to 65.2% (Table 2). Two members of this gene cluster, *GSTe1* and *GSTe2*, have been described previously and assigned to the insect-specific Epsilon class of GSTs [5]. Five additional genes within this cluster encode putative proteins with >40% sequence identity with the products of these two *A. gambiae* genes or members of the Epsilon GST class in *Drosophila melanogaster* [5], and these were therefore also classified as Epsilon GSTs and assigned the names *GSTe3*–*GSTe7*. Phylogenetic studies of insect GSTs support this nomenclature: the seven *A. gambiae* Epsilon GSTs form a single monophyletic clade with Epsilon GSTs identified in other insect species [14]. The classification of the remaining GST in the cluster is less clear. The predicted amino acid sequence of this gene shares less than 40% identity with other Epsilon GSTs, and therefore would be excluded from membership of this class according to widely used criteria [21]. Nevertheless, for reasons discussed below, this GST was classified tentatively as a member of the Epsilon class and assigned the name *GSTe8*.

The eight GST genes were also PCR amplified from cDNA and genomic DNA extracted from the insecticide-susceptible

Table 2 Pairwise percentage identities between derived amino acid sequences of *A. gambiae* GSTs

	Identity (%)							
	GSTE1	GSTE2	GSTE3	GSTE4	GSTE5	GSTE6	GSTE7	GSTE8
GSTE1	–	65.2	38.6	53.1	49.1	45.1	43.3	27.6
GSTE2		–	38.5	53.4	48.9	43.4	52.2	28.1
GSTE3			–	43.9	42.6	31.8	34.5	22.6
GSTE4				–	58.7	37.3	43.3	27.6
GSTE5					–	35.2	42.4	27.6
GSTE6						–	38.8	22.6
GSTE7							–	24.4

Kisumu strain. Comparison of the nucleotide sequences of these genes with those from the ZAN/U strain identified multiple substitutions. These polymorphisms appeared repeatedly in different clones from the same strain and thus presumably reflect allelic variants of the genes that have been maintained in the laboratory colonies. A total of 35 nucleotide substitutions resulted in alterations in the combined putative protein sequences of the eight genes (total amino acid length, 1802). The variable residues are shown in italics in Figure 2.

Of the eight GST genes described, one, *GSTe1*, showed an exceptionally high level of sequence variation between the DDT-susceptible and -resistant *A. gambiae* strains. A total of 37 nucleotide substitutions were identified within the coding sequence of *GSTe1* alleles from the ZAN/U and Kisumu strains (henceforth referred to as *GSTe1z* and *GSTe1k*, respectively). Twelve of these resulted in amino acid substitutions (Figure 2), five of which were non-conservative.

To verify that *GSTe1z* and *GSTe1k* were allelic variants of the same gene, we designed allele-specific primers, e1zF and e1kF (Table 1) to use in PCR reactions with a primer, e2R2, complementary to the neighbouring gene, *GSTe2*, to amplify the intergenic region between these two genes. A band of identical size was obtained when using ZAN/U or Kisumu genomic DNA as a template, the sequence of which showed less than 2% variation within the intergenic region between the two genes (compared with 5.5% within the coding region of *GSTe1*), suggesting that *GSTe1z* and *GSTe1k* are indeed products of the same gene.

To determine whether the allelic variants of *GSTe1* were present in natural populations, we designed primers e1dF and e1dR (Table 1), complementary to conserved regions of *GSTe1* but flanking an *AluI* polymorphism introduced by a T-to-C substitution in the ZAN/U strain. These were used to amplify a 253 bp fragment of the *GSTe1* gene from genomic DNA extracted from individual *A. gambiae* mosquitoes collected from Accra, Ghana, and Gamba, Gabon. Digestion of this fragment of the *GSTe1z* allele with *AluI* generates two products of 141 and 112 bp that can be distinguished clearly from the undigested 253 bp fragment generated from the *GSTe1k* allele (Figure 3). Three of the four individuals from Ghana were heterozygous at the *GSTe1* locus, and the remaining individual was homozygous for *GSTe1k*. In the six individuals collected from Gabon, all three genotypes were represented (Figure 3).

Expression and characterization of recombinant GSTE1-1, GSTE2-2, GSTE4-4 and GSTE8-8

Earlier studies had shown that at least one member of the Epsilon GST class, GSTE2-2, efficiently catalysed the dehydrochlorination of DDT to DDE [5], but it was not known if this was

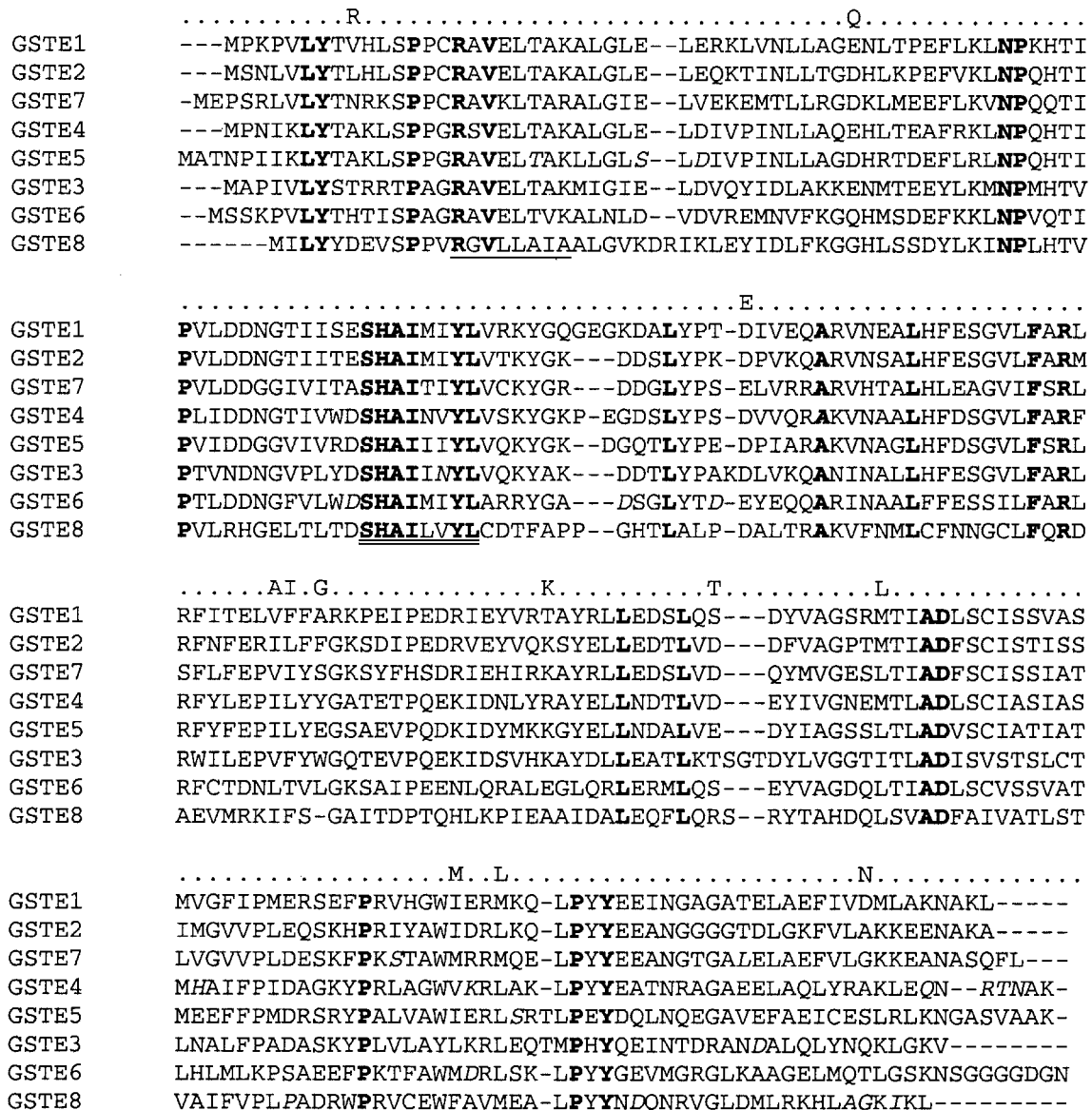


Figure 2 Clustal W alignment of predicted amino acid sequences of *A. gambiae* GST cluster on chromosome 3R, division 33B

Sequences shown are from the ZAN/U strain. Amino acid substitutions found in GSTE1 from the Kisumu strain are shown in line 1 of the alignment. Variant residues within the remaining proteins are shown in italics. Residues in bold are conserved in all eight proteins. The two underlined regions are motifs characteristic of Epsilon GSTs (single underline) or most GST classes (double underline). Accession numbers for the sequences are: GSTe1, AY063776; GSTe2, AF316636; GSTe3, AY070234; GSTe4, AY070254; GSTe5, AY070255; GSTe6, AY070256; GSTe7, AF491816; GSTe8, AY070257.

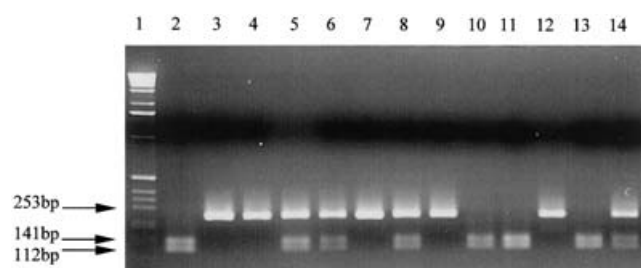
a trait specific to GSTE2-2 or a general property of all Epsilon GSTs. As a first step to resolving this question, we expressed four of the genes in the GST cluster described in this report in *E. coli*, and purified the recombinant proteins produced. Both allelic variants of the *GSTe1* gene were expressed, as we hypothesized that the presence of the *GSTe1z* allele may contribute to the DDT-resistance phenotype observed in the ZAN/U strain.

All five *A. gambiae* GSTs were expressed at high levels in *E. coli* and produced catalytically active recombinant GST homodimers. All of the recombinant purified enzymes were capable of conjugating the GST model substrate CDNB. Specific activities with this model substrate are lower than those reported for insect Delta-class GSTs. Activity of GSTE1-1 and GSTE2-2 with DCNB was among the highest reported for insect GSTs and comparable with the DCNB activity of *P. xylostella* GST-3

[7]. The kinetic properties of the enzymes varied, but the rate of turnover of CDNB (k_{cat}/K_m) was similar for all five proteins (Table 3). Peroxidase activity, measured using the model organic peroxide substrate, cumene hydroperoxide, was undetectable in GSTE2-2, GSTE4-4 or GSTE8-8, even when the amount of recombinant enzyme added was increased 7-fold above the standard assay conditions. Both allelic variants of GSTE1-1 displayed peroxidase activity, but the activity of the Kisumu protein was > 100-fold higher than the ZAN/U protein (Table 3). The level of DDT dehydrochlorinase activity (2.77 nmol of DDE formed/ μ g of protein) detected in GSTE2-2 exceeds the levels reported previously for any GST enzyme, and is > 350-fold higher than the DDT activity of the Delta-class *A. gambiae* GSTs, GSTD1-5 and GSTD1-6. None of the remaining GSTs tested in this study possessed detectable DDT dehydrochlorinase activity.

Table 3 Substrate specificities and kinetic parameters for recombinant *A. gambiae* GSTsAll data are means \pm S.D. ($n = 3$). n.d., activity was not detected; pNPA, *p*-nitrophenol acetate; CHP, cumene hydroperoxide; DDTase, DDT dehydrochlorinase.

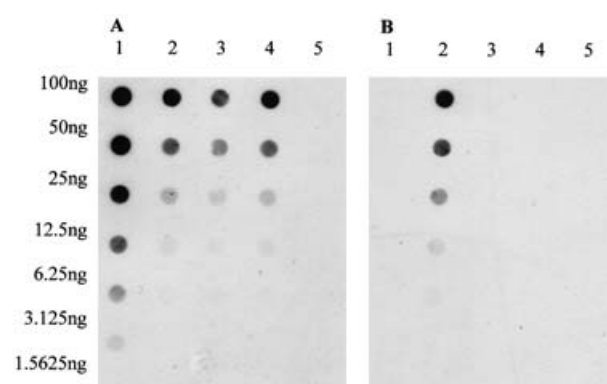
	GSTE1-1z	GSTE1-1k	GSTE2-2	GSTE4-4	GSTE8-8
Substrate-specific activity					
CDNB (mol/mg per min)	3.5 \pm 0.492	28.2 \pm 4.67	12.5 \pm 0.58	16.5 \pm 1.02	7.4 \pm 0.50
DCNB (μ mol/mg per min)	8.615 \pm 0.825	4.48 \pm 0.047	5.87 \pm 0.24	0.07 \pm 0.01	0.1 \pm 0.01
pNPA (μ mol/mg per min)	14.1 \pm 1.11	3.51 \pm 1.53	2.37 \pm 0.14	n.d.	n.d.
CHP (nmol/mg per min)	1.33 \pm 0.015	174.9 \pm 36.7	n.d.	n.d.	n.d.
DDTase activity (nmol of DDE/ μ g)	n.d.	n.d.	2.77	n.d.	n.d.
Kinetic parameters					
K_m for GSH (mM)	1.58 \pm 0.345	5.77 \pm 1.055	6.72 \pm 1.705	5.38 \pm 0.79	3.51 \pm 0.726
K_m for CDNB (mM)	0.037 \pm 0.006	0.458 \pm 0.22	0.069 \pm 0.01	0.131 \pm 0.026	0.082 \pm 0.015
V_{max} (μ mol/mg per min)	3.9 \pm 0.182	30.85 \pm 4.29	13.1 \pm 0.4	16.3 \pm 1.59	7.9 \pm 0.517
k_{cat} (s^{-1})	3.28 \pm 0.15	25.98 \pm 361	10.84 \pm 0.33	13.74 \pm 1.34	6.42 \pm 0.42
k_{cat}/K_m ($mM^{-1} \cdot s^{-1}$)	88.6	56.7	157.1	104.9	78.3
Binding to <i>S</i> -hexylglutathione affinity column	Unbound	Unbound	Unbound	Bound	Bound

**Figure 3** Diagnostic PCR to distinguish between two alleles of *GSTE1* from laboratory and field collections of *A. gambiae* (see text for details)

Lane 1, 1-kb DNA marker; lane 2, ZAN/U laboratory strain; lane 3, Kisumu laboratory strain; lane 4, RSP laboratory strain; lanes 5–8; field-sampled individuals from Accra, Ghana; lanes 9–14, field-sampled individuals from Gamba, Gabon.

Immunological cross reactivity and Western blotting

Antibodies raised against GSTE1 and GSTE2 were used to probe dot blots containing serial dilutions of the four recombinant GST proteins described in this study. As shown in Figure 4, the anti-GSTE1 and anti-GSTE2 sera differed in their specificity towards other members of this gene cluster. Anti-GSTE1 serum detected all four recombinant proteins, with varying sensitivities, whereas anti-GSTE2 was specific to GSTE2-2 under the conditions of the assay. Having ascertained that the GSTE1 antiserum, which was raised against recombinant protein derived from the ZAN/U strain, detected both GSTE1-1k and GSTE1-1z with equal intensity (results not shown), we used this antisera to probe an SDS/PAGE gel containing equal amounts of total protein from 1-day-old adults from the Kisumu and ZAN/U strains. Figure 5 shows that a band of approx. 25 kDa is present at higher levels in the DDT-resistant ZAN/U strain, suggesting that one or more of the genes described in the present report are overexpressed in this strain. To determine if the elevation in protein was due to an increase in the level of GSTE2-2, the only protein of the four characterized with detectable DDT dehydrochlorinase activity, we used the GSTE2 antiserum to probe an SDS/PAGE gel containing protein extracts from larval, pupal and adult Kisumu and ZAN/U mosquitoes. A stronger signal was detected in the ZAN/U strain in all three life stages. As this antiserum does not detect GSTE1-1, GSTE4-4 or GSTE8-8, this result suggests strongly that the

**Figure 4** Dot blot of four purified recombinant *A. gambiae* GSTs probed with antisera raised against mosquito Epsilon GSTs

Duplicate nitrocellulose membranes containing serial dilutions of the purified recombinant proteins GSTE1-1 (lanes 1), GSTE2-2 (lanes 2), GSTE4-4 (lanes 3) and GSTE8-8 (lanes 4), or PBS (lanes 5), were probed with a 1:5000 dilution of antisera raised against GSTE1 (A) or GSTE2 (B).

GSTE2-2 protein is present at elevated levels throughout the life span of the resistant mosquitoes (Figure 6).

DISCUSSION

The observation that the genomic location of a cluster of eight GST genes coincides with a region of the genome containing a major locus conferring DDT resistance [13] prompted this study on the biochemical and immunological properties of members of this GST cluster. As earlier biochemical studies on partially purified GST fractions from DDT-resistant and -susceptible *A. gambiae* had indicated that resistance was associated with both qualitative and quantitative changes in multiple GST enzymes [12], we predicted that the substitution of alternative allelic variants of these genes and/or the over-production of multiple members of this GST cluster could account for the resistant phenotype. To test these hypotheses we first compared the putative amino acid sequences of these GSTs in insecticide-susceptible and -resistant strains. The number of amino acid polymorphisms between the resistant and susceptible strains was highly variable for each putative GST protein. The majority of amino acid

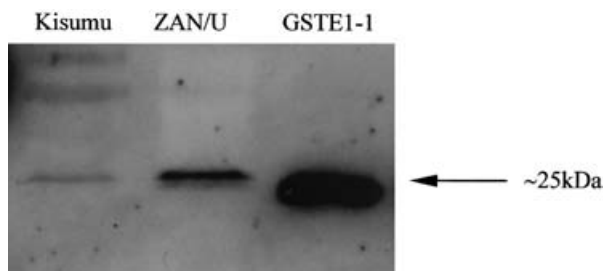


Figure 5 Western blot of crude homogenates from Kisumu and ZAN/U *A. gambiae* probed with GSTE1 antiserum

Protein (10 μ g) from 1-day-old Kisumu and ZAN/U adults was resolved on a 5% stacking/12% resolving gel. Purified recombinant GSTE1-1 (100 ng) was also loaded as a positive control. The gel was transferred to a nitrocellulose membrane and probed with a 1:5000 dilution of antiserum raised against GSTE1.

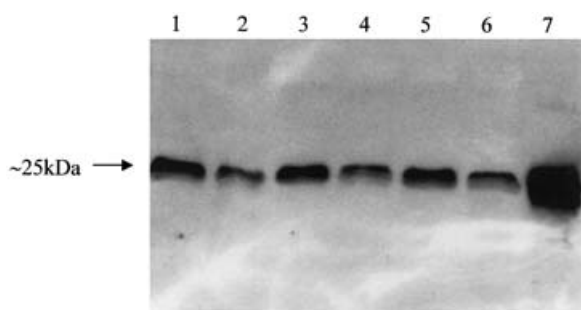


Figure 6 Western blot of crude homogenates from different life stages of Kisumu and ZAN/U strains of *A. gambiae* probed with GSTE2 antiserum

Protein (10 μ g) from 1-day-old Kisumu and ZAN/U adults, pupae and fourth-instar larvae were resolved on a 5% stacking/12% resolving gel. Purified recombinant GSTE2-2 (100 ng) was also loaded as a positive control. The gel was transferred to a nitrocellulose membrane and probed with a 1:5000 dilution of antiserum raised against GSTE2. Lane 1, ZAN/U adults; lane 2, Kisumu adults; lane 3, ZAN/U pupae; lane 4, Kisumu pupae; lane 5, ZAN/U fourth-instar larvae; lane 6, Kisumu fourth-instar larvae; lane 7, purified recombinant GSTE2-2.

substitutions in the eight GSTs (28 out of 35) are located in the C-terminal domain of the proteins. This domain contributes most of the residues that form the H-site, involved in substrate binding, and substitutions in this domain can affect the substrate specificity of the GSTs [1]. The GST gene showing the most interstrain substitutions was *GSTe1*. Two alleles of this strain, *GSTe1k* and *GSTe1z*, differing at 12 amino acid positions, were isolated from the insecticide-susceptible Kisumu strain and the DDT-resistant ZAN/U strain, respectively.

To determine whether these differences in the primary sequence of the GSTE1z subunit contribute to the DDT-resistant phenotype of the ZAN/U strain and to establish if either of these *GSTe1* alleles encoded proteins able to detoxify DDT, we expressed both allelic variants *in vitro*. Both encoded catalytically active subunits with markedly different biochemical properties, but neither was able to catalyse the dehydrochlorination of DDT. Further sequencing of *GSTe1* from additional laboratory strains suggested initially that a bottleneck in the Kisumu strain may have caused the fixation of a rare allele at the *GSTe1* locus, as neither the pyrethroid-resistant RSP strain, nor the G3 strain from the Gambia, nor the PEST strain, a laboratory hybrid strain [22] used for the *A. gambiae* genome-sequencing project [2], contained the *GSTe1k* allele (results not shown). However, when we assayed

randomly selected field specimens of *A. gambiae* we found that both allelic variants were present in equal frequencies.

The physiological role of the Epsilon GST class in mosquitoes is unknown, but it is intriguing that the two allelic variants are maintained in natural populations. The *GSTe1k* allele confers high levels of peroxidase activity. In the rice brown planthopper, elevated peroxidase activity has been shown to be protective against damage caused by lipid peroxidation products induced by exposure to pyrethroid insecticides [9]. This resistance mechanism has not been found in *Anopheles*, as yet, but it is possible that the presence of a GST allele with high peroxidase activity may provide a fitness advantage in the presence of insecticide exposure, thus explaining its persistence in *A. gambiae* populations.

Of the four GST proteins described in this report, DDT dehydrochlorinase activity was confined to the recombinant GSTE2-2 protein. This protein has the highest level of DDT dehydrochlorinase activity reported for any GST. Several studies have linked DCNB activity with insecticide resistance (e.g. [10,23]). GSTE2-2 has a high specific activity with this substrate, comparable with that of *P. xylostella* GST-3 and *Aedes aegypti* GST-2 (10.8 and 16.4 μ mol/min per mg respectively), both of which have been implicated in insecticide resistance. However, within the *A. gambiae* Epsilon GST class, the GSTE1-1z protein has the highest specific activity with the substrate DCNB and yet does not appear to confer insecticide resistance.

It is possible that one or more of the remaining four genes in this cluster may encode proteins capable of metabolizing DDT, but preliminary studies on crude *E. coli* homogenates expressing recombinant *GSTe6* and *GSTe7* failed to detect any DDT dehydrochlorinase activity. Our earlier studies on the Delta class of GSTs in *A. gambiae* identified enzymes that had low levels of activity against DDT, but expression studies failed to implicate members of the Delta class in DDT resistance [24].

We reported previously that transcripts of *GSTe2* were present at significantly higher levels in DDT-resistant mosquitoes [5]. In the present study we demonstrate that this correlation also applies at the protein level. DDT resistance in worldwide populations of *D. melanogaster* is caused by over-transcription of a single P450 allele [25], and it is conceivable that the overproduction of GSTE2 may be both necessary and sufficient to cause DDT resistance in *A. gambiae*. Comparing the differences in signal intensity obtained when probing Kisumu and ZAN/U adult crude protein homogenates with the non-specific antibody raised against GSTE1, with the results from probing with the specific antibody raised against GSTE2, however, suggests that additional proteins within this GST class are present at higher amounts in the DDT-resistant strain. Antibodies specific to each of the members of this gene cluster will be needed to fully resolve this issue but, if this proves to be true, it would support earlier biochemical data suggesting that multiple GST enzymes are present at higher amounts in resistant individuals [19].

The classification of the eighth gene, *GSTe8*, in the GST cluster described in this report remains ambiguous. This GST has less than 40% identity with other members of the Epsilon class, the cut off widely used to classify GSTs [21]. However, a database search using BLASTp [26] and the putative translation of *GSTe8* as a query, identified the *P. xylostella* Epsilon GST, GST-3, as the characterized protein with the highest similarity (39% identity over 205 amino acids). Furthermore, the protein product of *GSTe8* reacts with antisera raised against an Epsilon GST. This highlights the limitations of the present classification system for GST classes lacking data on their three-dimensional structure or physiological role. GSTs are dimeric proteins, and a useful criterion for classification is the ability to form heterodimers only

with members of the same class [1]. The *in vitro* studies described in this report do not address issues relating to the prevalence and properties of homodimers versus heterodimers. To investigate this we plan to co-express different combinations of subunits in *E. coli* and detect the presence of the respective homo- and heterodimers using analogous methods to those used for characterization of recombinant plant GST heterodimers [27].

We acknowledge the *Anopheles gambiae* genome consortium for generating data referred to in this article. We thank Dr Eryl Lyddell (Cardiff University, Cardiff, Wales, U.K.) for the polyclonal antibody production, John Morgan for the sequencing service and Peter Mohloai and Kofi Adasi for providing field mosquito specimens.

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Received 27 January 2003/15 April 2003; accepted 28 April 2003

Published as BJ Immediate Publication 28 April 2003, DOI 10.1042/BJ20030169