Identification of a novel class of insect glutathione S-transferases involved in resistance to DDT in the malaria vector *Anopheles gambiae*

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The sequence and cytological location of five *Anopheles gambiae* glutathione S-transferase (GST) genes are described. Three of these genes, *aggst1-8*, *aggst1-9* and *aggst1-10*, belong to the insect class I family and are located on chromosome 2R, in close proximity to previously described members of this gene family. The remaining two genes, *aggst3-1* and *aggst3-2*, have a low sequence similarity to either of the two previously recognized classes of insect GSTs and this prompted a re-evaluation of the classification of insect GST enzymes. We provide evidence for seven possible classes of insect protein with GST-like subunits. Four of these contain sequences with significant similarities to mammalian GSTs. The largest novel insect GST class, class III, contains functional GST enzymes including two of the *A. gambiae* GSTs described in this report and GSTs from *Drosophila melanogaster*, *Musca domestica*, *Manduca sexta* and *Plutella xylostella*. The genes encoding the class III GST of *A. gambiae* map to a region of the genome on chromosome 3R that contains a major DDT [1,1,1-trichloro-2,2-bis-(*p*-chlorophenyl)ethane] resistance gene, suggesting that this gene family is involved in GST-based resistance in this important malaria vector. In further support of their role in resistance, we show that the mRNA levels of *aggst3-2* are approx. 5-fold higher in a DDT resistant strain than in the susceptible strain and demonstrate that recombinant AgGST3-2 has very high DDT dehydrochlorinase activity.

Key words: classification of insect GST enzymes, *Drosophila* genome, insecticide resistance.

INTRODUCTION

Glutathione S-transferases (GSTs) are a major family of detoxification enzymes found in most organisms. They help to protect cells from oxidative stress and chemical toxicants by aiding the excretion of electrophilic and lipophilic compounds from the cell (reviewed in [1]). Eukaryotes contain multiple GSTs with differing catalytic activities to accommodate the wide range of functions of this enzyme family. Mammalian GSTs have been classified into eight cytosolic classes (Alpha, Mu, Pi, Theta, Sigma, Zeta, Kappa and Omega) and a microsomal class [2–7], whereas only two classes of insect GSTs (classes I and II) have so far been described [8]. (An alternative nomenclature in which the insect classes are assigned Greek letters in line with the mammalian GST classification system has been proposed [9] and is discussed in the present paper). The insect class I GSTs are encoded by a large complex gene family. Additional heterogeneity within this class is introduced by alternative splicing in *Anopheles gambiae* and the presence of fusion genes in *Musca domestica* [10,11]. In *Drosophila melanogaster* and *A. gambiae* this gene family is tightly clustered [10,12], in contrast with the family in *M. domestica*, in which the class I GSTs are dispersed throughout the genome [13]. The class II insect GST family consists of a single gene in all three species [14,15].

Interest in insect GSTs is focused on the role of these enzymes in insecticide resistance. Elevated GST activity has been detected in strains of insects resistant to organophosphates [8] and organochlorines [16] and this enzyme family has recently been implicated in resistance to pyrethroid insecticides [17,18]. *A. gambiae* GSTs are of particular interest because of their involvement in resistance to DDT [1,1,1-trichloro-2,2-bis-(*p*-chlorophenyl)ethane] in this important malaria vector. In the 1950s and 1960s house spraying with DDT was the primary line of defence against malaria and, although the advent of DDT-resistant strains of mosquitoes has decreased the effectiveness of this control measure, this insecticide is still used today for malaria control in many parts of the world [19]. In *A. gambiae*, an increased rate of DDT dehydrochlorination in the resistant strain is associated with quantitative increases in multiple GST enzymes [20].

We have studied the *A. gambiae* class I and class II GST genes to ascertain their role in conferring DDT resistance. The single class II GST, *aggst2-1* [15] is highly expressed in *A. gambiae* larvae but is barely detectable in adult insects. Because DDT resistance in this species is life-stage specific and the insecticides are used as adulticides both in the field and for selection of the resistant strain in the laboratory, the developmental expression profile discounted a prominent role for *aggst2-1* in conferring

Abbreviations used: BAC, bacterial artificial chromosome; 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 S-transferase; RACE, rapid amplification of cDNA ends.

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The nucleotide sequence data reported will appear in DDBJ, EMBL and GenBank® Nucleotide Sequence Databases under the accession numbers AF316635 to AF316638.

DDT resistance. The class I GSTs are expressed at high levels in both larvae and adults [10]. Several recombinant *A. gambiae* GST enzymes are able to metabolize DDT but, by using antibodies raised against these class I GSTs, we have demonstrated that these enzymes are not the most important family in DDT resistance [21]. Furthermore, we have no evidence to suggest that any of these genes are overexpressed in resistant mosquitoes (N. Roberts and J. Hemingway, unpublished work).

Hence either *A. gambiae* contains additional genes encoding class I GSTs, or further classes of insect GSTs exist. We now present evidence to support both these hypotheses. We report the cloning of five novel *A. gambiae* genes encoding GSTs. Three of these have been classified as class I GSTs, whereas the remaining two genes belong to a previously undescribed class, which we have named class III. We propose that the class III GSTs represent the major enzyme family conferring resistance to DDT in the malaria mosquito, *A. gambiae*.

EXPERIMENTAL

Mosquito strains

The ZAN/U strain of *A. gambiae* was colonized from a DDT-resistant field population from Zanzibar, Tanzania, in 1982. This strain has been maintained under regular adult selection pressure with DDT. Kisumu is a laboratory insecticide-susceptible strain originally colonized from Kisumu, western Kenya. The PEST strain is fixed for the standard chromosome arrangement [22] and was used to construct the *A. gambiae* bacterial artificial chromosome (BAC) library (X. Wang, Z. Ke, A. J. Cornel, D. Smoller and F. H. Collins, unpublished work).

DNA extraction and sequencing

BAC DNA was isolated with Qiagen Plasmid maxi kits. BAC sequencing reactions were performed with 1 μg of BAC DNA as a template and ABI BigDye Terminator chemistry. After electrophoresis on an ABI 377 automatic sequencer, contigs were assembled and the sequences annotated with the LASERGENE software package (DNAstar, Madison, WI, U.S.A.).

Total RNA was extracted from individual mosquitoes with the TRI reagent (Sigma), in accordance with the manufacturer's instructions. The RNA was treated with DNase to remove any contaminating genomic DNA and the mRNA was reverse-transcribed into cDNA by using Superscript II (Gibco BRL) and an oligo(dT) adapter primer (5′-GACTCGAGTCGACATCGA-$(dT)_{17}$-3′).

Genomic DNA was extracted from individual adult mosquitoes as described previously [23].

In situ hybridization

BAC clones were physically mapped to polytene chromosomes prepared from half-gravid ovaries of the PEST strain of *A. gambiae* as described previously [24].

Quantification of *aggst3-2* mRNA levels

Incorporation of the fluorescent dye SYBR GreenI (Molecular Probes) into double-stranded PCR products was used to determine the mRNA copy number of *aggst3-2* in individual mosquitoes. An *aggst3-2* standard plasmid was constructed by inserting a 353 bp fragment from the coding region of the *aggst3-2* gene, amplified from ZAN/U cDNA with the primers 3-2f (5′-GTACGATCATCACCGAGAGC-3′) and 3-2r (5′-CTTCGACTGCTCCAACGGC-3′), into *p*GEM T-easy vector (Promega). A control plasmid was constructed by inserting a partial fragment from the gene encoding ribosomal S7 protein [25], amplified with primers SPC (5′-GTGCCGGTGCCGA-AACAGAA-3′) and SPD (5′-AGCACAAACACTCCAATA-ATCAAG-3′), into the *p*GEM T-easy vector. These plasmids were used as template DNA at concentrations ranging from 1 ng to 10 fg to produce standard curves using a Roche Lightcycler in accordance with the manufacturer's recommended protocols. For quantification of the copy number, approx. 2% of the cDNA from an individual mosquito was used as a template for the control (*S7*) primers and 6% was used for quantifying *aggst3-2* expression; 40 rounds of amplification were performed in glass capillaries containing 5 pmol of each primer, 1 × SYBR GreenI mix and a final concentration of 3 mM $MgCl_2$. The amplification cycle was as follows: 95 °C for 1 s, 62 °C for 3 s and 72 °C for 15 s, with incorporation of fluorescence measured at 87 °C for *aggst3-2* quantification, and 95 °C for 1 s, 60 °C for 3 s and 72 °C for 10 s, with incorporation of fluorescence measured at 86 °C for *S7* quantification. Each sample was analysed in duplicate in each experiment and the results are means for two separate experiments. The data were quantified with LightCycler Software V3 (Roche) and converted into copy number as described in [26].

Expression of *aggst3-2 in vitro*

The coding region of *aggst3-2* was amplified in a PCR reaction with ZAN/U cDNA as a template, *Pfu* polymerase (Stratagene) and primers that contained the initiation and termination codons of the gene preceded by *Bam*H1 sites. The single product of approx. 680 bp was subcloned into T-easy (Promega) and sequenced to ensure that no errors had been introduced during amplification. The insert was then isolated by digestion with *Bam*H1, ligated into the *Bam*H1 site of the pET3a vector (Novagen) and the resultant expression construct was used to transform *Escherichia coli* Origami (DE3)pLysS cells. The orientations of the inserts were determined by restriction digestion; colonies containing the insert in both the forward and reverse orientations were grown at 37 °C to an attenuance (D_{600}) of 0.6. Expression of the recombinant protein was induced by the addition of isopropyl β-D-thiogalactoside to 0.4 mM and, after incubation for a further 3 h, the cells were harvested by centrifugation for 10 min at 5000 ***g***. After a single round of freeze–thawing, the cells were resuspended in 50 mM Tris/HCl (pH 8.0)/2 mM EDTA/0.1 M NaCl. Protein concentration was determined with Bio-Rad protein reagent [27]; GST activity was assayed spectrophotometrically by measuring the conjugation of GSH to the standard GST substrates 1-chloro-2,4-dinitrobenzene (CDNB) and 1,2-dichloro-4-nitrobenzene (DCNB) [28].

DDT dehydrochlorinase activity was assayed by incubating the crude cell extract with 0.1 mM DDT and 10 mM GSH in 0.1 M sodium phosphate buffer, pH 6.5, for 2 h at 30 °C. The samples were extracted twice with chloroform, air-dried and then resuspended in propan-2-ol. HPLC analysis of DDT metabolites was performed as described by Prapanthadara et al. [20], with a flow rate of 0.6 ml/min. Constructs containing the insert in the negative orientation were assayed to control for non-enzymic DDT metabolism. Controls omitting GSH in the incubation mixture were also included to verify the dependence of the reaction on GSH.

Phylogenetic analysis of insect GSTs

A search of the GenBank® database located 21 non-*Drosophila* insect GST sequences including seven *A. gambiae* GSTs. These were retrieved and the putative amino acid sequences were aligned

against the *A. gambiae* GST sequences described here, with the CLUSTAL W program [29] A total of 42 *Drosophila* sequences predicted to contain GST protein domains are present in the *D. melanogaster* genome [30]. These sequences were retrieved from FlyBase (http://flybase.bio.indiana.edu) to enable us to incorporate them into our phylogenetic analysis. Ten of these sequences are identical to previously submitted *Drosophila* GST sequences and were included in our analysis as they appear in GenBank® (with the exception of *DmGST26* and *DmGST22*, which are reported to be pseudogenes [12] and were therefore excluded). Of the remaining 32 putative *Drosophila* genes for GST, four sequences (CG15100, CG4623, CG12304 and CG11901) were considered unlikely to encode functional GST enzymes on the basis of their transcript length and/or low degree of similarity to genes encoding GST; they were therefore discarded. The annotations of the remaining genes for GST were studied and most of the amino acid translations were accepted as published with the following exceptions. (1) Two putative full-length transcripts were derived from the annotations for CG12930 and CG6673. The derived amino acid sequences of both of these were included in the study (denoted by A and B). (2) The translation of CG1681 seems to be lacking 37 residues at the N-terminus. A search of the nucleotide sequence of this annotation identified a putative 5′ exon, approx. 3 kb upstream from the 3′ end of the gene, which was joined to the amino acid translation to produce a putative full-length gene. (3) Annotations CG1702, CG10065 and CG17639 predicted very large translation products of which only approx. 220 residues showed significant similarity to GSTs. These translations were therefore trimmed manually.

After updating the alignment to contain these *Drosophila* sequences, evolutionary distances were calculated by using the Jukes–Cantor algorithm [31]; phylogenetic trees were determined by the neighbour-joining method [32] with TREECON for Windows [33]. The amino acid translation of a Rat Kappa GST (rGSTTK1-1 [5]) was used as an outgroup to root the tree.

RESULTS

Cloning of GSTs

As part of the *A. gambiae* genome initiative, the insert ends of each clone from a BAC library have been determined by single-pass sequencing at Genoscope and the Institut Pasteur (www.genoscope.cns.fr/externe/English/Projets/Projet_AK/AK.html). The resultant sequences were queried against the GenBank® database and two BAC clones were identified in which end sequences had significant similarity to GSTs (04H09 and 28I09). The end sequence of clone 04H09 (sequenced with primer SP6) was predicted to encode the carboxy region of a GST. Primers were designed to amplify this partial gene encoding a GST and used to screen the BAC library for overlapping clones. Four positive clones were identified (05C11, 06I12, 12H09 and 28A19) and primers designed against the 3′ GST sequence in 04H09 were used for partial sequencing of clone 06I12. The results of this sequencing not only completed the genomic sequence of the GST gene present in the end sequence of clone 04H09, but also identified an additional gene encoding a GST, approx. 350 bp downstream from this gene. These *A. gambiae* GST genes have low levels of similarity to the two insect GST classes previously recognized (class I and class II) and were therefore tentatively assigned to a third class of insect GSTs and named *aggst3-1* and *aggst3-2* (see the Discussion).

The end sequence of BAC clone 28I09 (sequenced with primer SP6) contained the 3′ end of one gene for GST (later named *aggst1-9*) and the 5′ end of a second gene encoding a GST

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                                                          ↓
AgGST3-2    MSNLVLYTLHLSPPCRAVELTAKALGLELEQKTINLLTGDHLKPEFVKLNPQHTIPVLDD
AgGST3-1    MPKPVLYTVHLSPPCRAVELTAKALGLELERKLVNLLAGENLTPEFLKLNPKHTIPVLDD
AgGST1-8    ---MDFYYHPASPYCRSVMLVAKALKLSLNLQFVDLMKDEQLRPTFTVLNPFHCVPTLVD
AgGST1-9    ---MDLYYNILSPPSRAILLLGEALQLKFNLISLDVHRKDYVNPAFKKINPQHTVPTLVV
AgGST1-10   ---MDLYYHIRSPPCQPVVFLARHLGLEFNHIVTSIY-DPADFEVLKKVNPQHTIPTLVD
                                                  ↑         *   ↓
AgGST3-2    NGTIITESHAIMIYLVTKYGKD---DSLYPKDPVKQARVNSALHFESGVLFARMRFNPER
AgGST3-1    NGTIISESHAIMIYLVRKYGQGEGKDALYPTDIVEQARVNEALHFESGVLFARLRFITEL
AgGST1-8    NDLTMWESRAILVYLVDKYGRTN--SRLYPKDAKTRAIINQRLFFDHGTLGTRLEDYYYP
AgGST1-9    DGVAICEPGAILIYLAEQYAPAG--TTYYPPDPLRRAIVNQRLLFECGTLYKCIFVYYSP
AgGST1-10   NGHILWESYAILIYLAEKYALD---DSLYPKDVCERSIVHQRLFFDSGMFQN--TTLQAV

AgGST3-2    ILFFGKSDIPEDRVEYVQKSYELLEDTLVDD-FVAGPTMTIADFSCISTISSIMGVVPLE
AgGST3-1    VFFARKPEIPEDRIEYVRTAYRLLEDSLQSD-YVAGSRMTIADLSCISSVASMVGFIPME
AgGST1-8    LYFEGATPGGE-KLEKLEEALAVLNGYLINNPYAAGPNITLADYSLVSTVTSLE-VVQHD
AgGST1-9    VVLERATPVET-DRQKLIEAVAVLDGILQHSAFVAGDCLTVADYSLVCTVSMLV-VLKPE
AgGST1-10   LSHLRNNPITDEHLAKVKRGVEIVEMYLTDSPYVAGQKLTIADFSIFVSFCSLD-MMKYD
                                          *     *     *

AgGST3-2    QSKHPRIYAWIDRLKQ-LPYYEEAN--GGGGTDLGKFVLAKKEENAKA
AgGST3-1    RSEPPRVHGWIERMKQ-LPYYEEIN--GAGATELAEFIVDMLAKNAKL
AgGST1-8    LSKYPAISAWYEGCKATMADFQEINESGMQQYRLTSSLVPHLQLLHMPFAEM
AgGST1-9    LAPYVAVRRWYERCKEGDCGLHRPD--TACRYNVPKMDGAGKQQRLTHS
AgGST1-10   LTAYPNVQRWFAKMGTHIPDLEPTR--KTIEEELRALLQSMNK
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Figure 1 Alignment of deduced amino acid sequences of the five *A. gambiae* genes encoding GST described in this report

Gaps introduced to maximize sequence identity are shown by a horizontal dash. Residues shown in bold are shared by all class I and class III *A. gambiae* GSTs. The residues denoted by an asterisk are shared by all known GSTs [37]. The arrows indicate intron positions (see the text for further details).

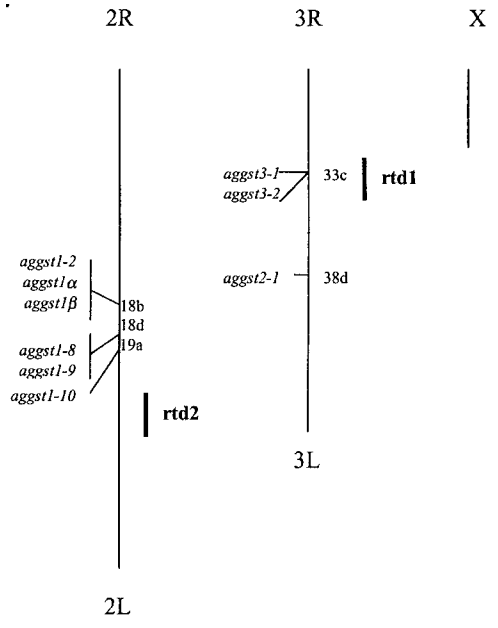

Figure 2 Schematic representation of the *A. gambiae* genome showing the location of genes for GSTs and loci associated with resistance to DDT (*rtd1* and *rtd2*)

Cytological positions are shown to the right of the chromosome and the two major regions of the genome associated with DDT resistance are shown as solid bars.

(*aggst1-8*). Primers designed against the end sequence of 28I09 were used to search the BAC library for overlapping clones and a single positive, 10O16, was identified and used as a template for obtaining the full length-genomic sequences of genes *aggst1-8* and *aggst1-9*.

3′-Rapid amplification of cDNA ends (RACE) reactions with primers incorporating the initiator methionine codon of the putative genes encoding GST were used to verify that these genes were expressed in *A. gambiae*. Transcripts of *aggst3-1*, *aggst3-2*

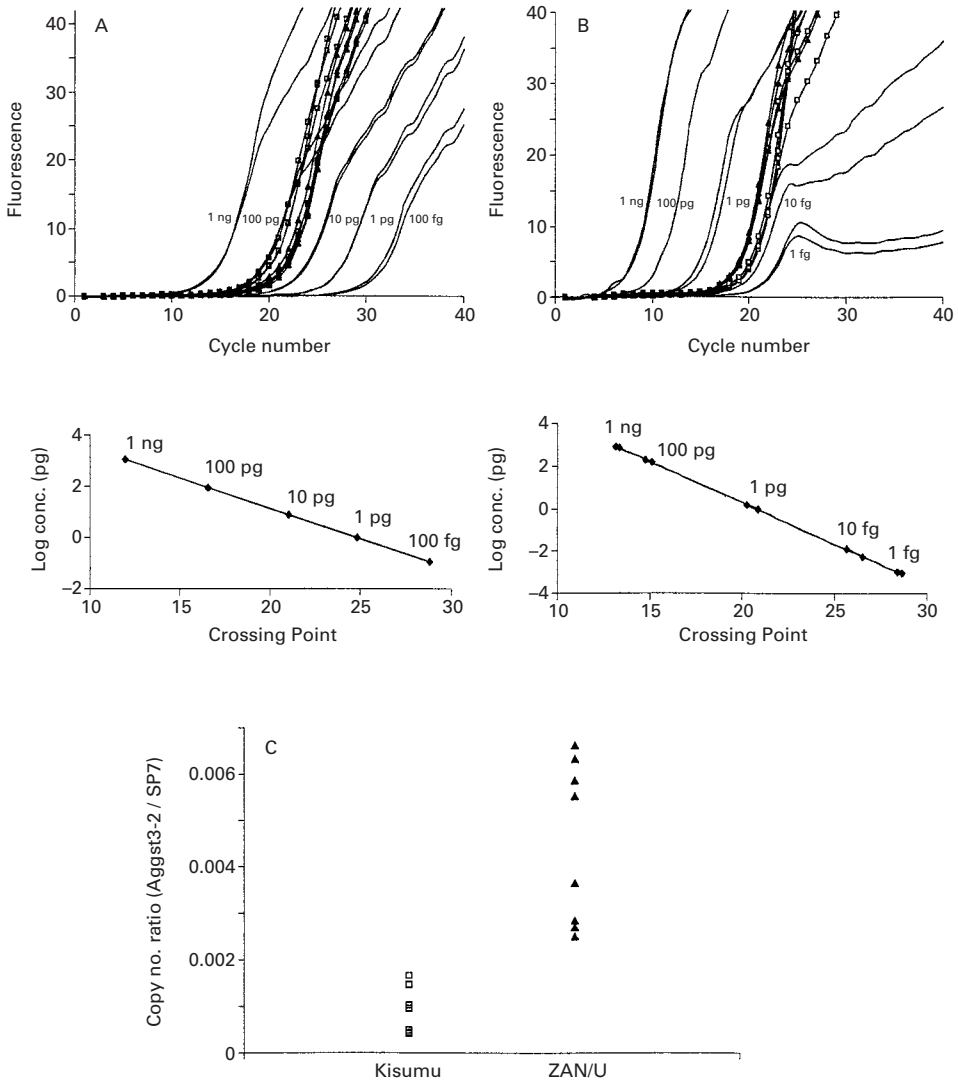

Figure 3 Quantification of *aggst3-2* mRNA expression levels in individual *A. gambiae* adults

(**A**, **B**) Upper panels: SYBR GreenI fluorescence acquisition by PCR products from serially diluted (1 ng to 1 fg) standard plasmids and individual adult mosquito cDNA against cycle number. Lower panels: standard curves derived from plotting the crossing points against the logarithm of copy number for plasmids *S7* and *aggst3-2*. (**A**) *SP7* standardization control; (**B**) *aggst3-2*. (**C**) mRNA copy number of *aggst3-2* transcript relative to the number of *SP7* transcripts for each individual mosquito.

and *aggst1-8* were detected in adult mosquitoes. 3′-RACE with a primer complementary to *aggst1-9* gave a product of the expected size but with only 36.8 % sequence identity to *aggst1-9*. This gene transcript has relatively high similarity to *aggst1-5* and *aggst1-6* (42.9 % and 42.4 % similarity at the nucleotide level) and was therefore provisionally classified as a class I GST and named *aggst1-10*. The BAC library was screened with primers specific to *aggst1-10* to enable the genomic organization and physical location of *aggst1-10* to be established. As attempts at 3′-RACE for *aggst1-9* were unsuccessful we designed PCR primer pairs specific to this gene and used these in PCR reactions with fourth-instar larvae, pupae and adult mosquito cDNA as templates; however, we were unable to detect a transcript in any of these life stages. This gene might be expressed in earlier life stages but the possibility that *aggst1-9* is a pseudogene cannot be discounted at this stage.

An amino acid alignment of the five *A. gambiae* genes for GSTs identified in this study is shown in Figure 1. The four invariant residues proposed to be crucial for the correct folding of GST enzymes [34] are conserved in these, and in all previously identified, *A. gambiae* GST sequences (indicated by an asterisk in Figure 1). In addition to these, a further eight residues are constant in all *A. gambiae* GSTs. *aggst1-8* and *aggst1-10* contain a single intron within the 5′ coding region at an identical position to the intron in the alternately spliced *A. gambiae* gene for GST, *aggst1α* [10]. *aggst3-1* and *aggst3-2* also contained an intron at this position and an additional intron at position 119 (numbers according to AgGST3-1 sequence) (Figure 1). *aggst1-9* is unique among the newly described *A. gambiae* GSTs in being intronless.

Physical mapping

The cytological location of the *A. gambiae* genes encoding GSTs was determined by *in situ* hybridization. Figure 2 shows the positions of all these genes on the polytene chromosomes. Six genes for GSTs are located on chromosome 2R within divisions

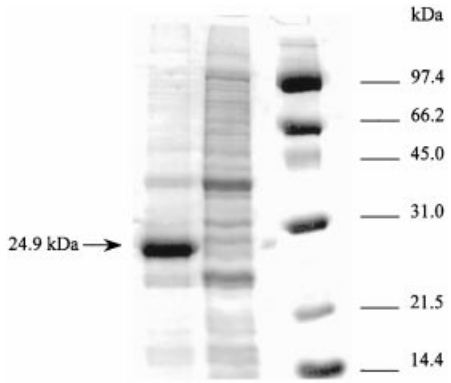

Figure 4 Heterologous expression of AgGST3-2

E. coli cultures containing *aggst3-2* expression constructs, prepared with the insert in either the correct or the reverse orientation within the pET3a vector, were induced with isopropyl β-D-thiogalactoside. A 10 μl sample of whole cells was separated on a 12% (w/v) resolving, 5% (w/v) stacking SDS/polyacrylamide gel and stained with Coomassie Blue R250. Left lane, an *E. coli* culture containing *aggst3-2* in the correct orientation; middle lane, an *E. coli* culture containing *aggst3-2* in reverse orientation (negative control); right lane, molecular mass standards (molecular masses indicated at the right). The subunit size of *aggst3-2* is predicted to be 24.9 kDa on the basis of its amino acid translation. A band of this approximate size is clearly visible in the left lane.

18-19. These include the *aggst1*α, *aggst1*β and *aggst1-2* genes described previously [10,35] and the three class I GSTs described here, namely *aggst1-8*, *aggst1-9* and *aggst1-10*. Two possible sites of hybridization for the single class II GST have been reported [15] but only one of these, on division 38d, was confirmed by further experiments. The class III GSTs are located on division 33c on chromosome 3R. This position coincides with the location of one of two major quantitative trait loci associated with DDT resistance in the ZAN/U strain of *A. gambiae* [36] (Figure 2).

Quantitative analysis of *aggst3-2* expression

The co-localization of the *A. gambiae* class III genes with a major gene conferring DDT resistance prompted us to study the relative expression levels of members of this insect class in susceptible and resistant insects by using real-time PCR technology. From the standard curves shown in Figures 3(A) and 3(B) it was possible to extrapolate the fluorescence values obtained with Kisumu and ZAN/U cDNA and calculate the initial template copy number. By dividing the copy number of *aggst3-2* by the copy number of *S7* the values for GST expression in each individual mosquito were standardized for variations in initial cDNA concentrations so that the relative expression of *aggst3-2* between the susceptible and resistant strains could be compared (Figure 3C). The average ratio of *aggst3-2* copy number to *S7* copy number in the ZAN/U strain was $(4.6 \pm 1.72) \times 10^{-3}$ compared with $(9.5 \pm 4.75) \times 10^{-4}$ in the Kisumu strain, representing an approximate 5-fold overexpression in the resistant strain.

Expression *in vitro*

To verify that the *A. gambiae* class III GSTs encoded catalytically active enzymes, we expressed *aggst3-2* in *E. coli* (Figure 4) and measured the CDNB- and DCNB-conjugating activity of the crude protein homogenates. No CDNB-conjugating activity was detectable in the control cultures but replicate crude cell extracts from two separate *E. coli* cultures expressing recombinant AgGST3-2 had a mean CDNB-conjugating activity of 2.879 ± 0.8 μmol/min per mg of crude protein and a mean DCNB-conjugating activity of 5.74 ± 2.7 μmol/min per mg of crude protein.

DDT dehydrochlorinase activity of recombinant AgGST3-2 was measured as nmol of 1,1-dichloro-2,2-bis-(*p*-chlorophenyl) ethane (DDE) detected by HPLC analysis after incubation of the crude cell extract with 100 nmol of DDT as described in the Experimental section. The recovery of DDT/DDE after extraction and analysis ranged from 43% to 58%. Representative results are shown in Figure 5. DDE was undetectable in the control reactions containing the insert in the negative orientation

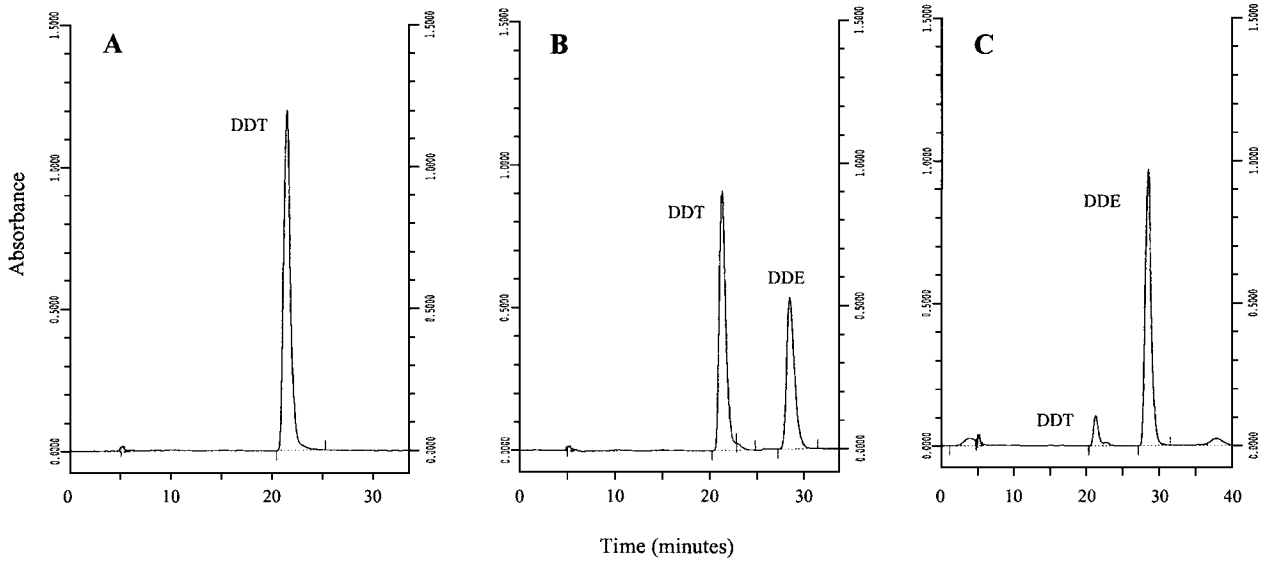

Figure 5 HPLC analysis of DDT dehydrochlorinase activity by crude cell extracts expressing recombinant AgGST3-2

(**A**) Control insert in reverse orientation. (**B**) Insert in correct orientation; 50 μl of cell extract. (**C**) Insert in correct orientation; 200 μl of cell extract.

Table 1 Pairwise percentage similarities between derived amino acid sequences of *A. gambiae* GSTs

Sequences shown in **bold** are described here for the first time.

	Similarity (%)										
Sequence	agGST1-3	agGST1-4	agGST1-5	agGST1-6	agGST1-7	**agGST1-8**	**agGST1-9**	**agGST1-10**	agGST2-1	agGST3-1	agGST3-2
agGST1-2	47.4	46.4	49.3	49.8	36.4	**39.2**	**37.3**	**27.8**	12.9	**27.8**	**30.1**
agGST1-3		58.4	61.2	60.8	33.5	**34.2**	**27.9**	**29.4**	11.0	**27.9**	**29.2**
agGST1-4			63.2	65.6	39.6	**42.4**	**34.6**	**29.4**	11.1	**30.9**	**32.7**
agGST1-5				82.8	43.1	**41.1**	**35.9**	**35.4**	14.8	**33.5**	**37.3**
agGST1-6					45.5	**43.1**	**35.4**	**35.9**	14.4	**34.9**	**38.8**
agGST1-7						**39.9**	**32.1**	**29.4**	11.5	**30.3**	**34.4**
agGST1-8							**37.7**	**29.9**	**11.9**	**26.8**	**29.9**
agGST1-9								**28.9**	**16.1**	**24.5**	**26.4**
agGST1-10									**12.8**	**28.0**	**27.5**
agGST2-1										**10.1**	**11.9**
agGST3-1											**63.8**

Figure 6 Dendrogram illustrating the relationship between insect GSTs

Amino acid sequences were aligned by using CLUSTAL W and the tree was constructed with the neighbour-joining method program from a similarity matrix of pairwise comparisons made by using the Jukes–Cantor algorithm. Selected bootstrap values from 500 replicate trees are shown (as percentage values) at the dendrogram nodes. The sequences denoted by CG were obtained from the *Drosophila* genome annotations (http://flybase.bio.indiana.edu) as described in the text. All other sequences were retrieved from GenBank® or are described in this study. The tree was rooted with the rat gene encoding Kappa GST (GenBank® accession number S83436). Abbreviations: Dm, *D. melanogaster*; Md, *M. domestica*; Lucil, *Lucilia cuprina*; Ag, *A. gambiae*; Cv, *Culicodies variipennis*; Ae, *Ae. aegypti*; Plx, *P. xylostella*; Msex, *Ma. sexta*.

(Figure 5A) and in the absence of GSH, indicating that DDT dehydrochlorinase activity in the assay was dependent on both enzyme and GSH. In the experimental assays expressing recombinant AgGST3-2, the percentage conversion of DDT to DDE was dependent on the amount of crude extract used in the assay. For example, in the experiment shown in Figure 5(B), 50 μl of extract was used; 24 nmol of DDE and 33 nmol of DDT were detected by HPLC. When the volume of cell extract was increased to 200 μl (Figure 5C) 43 nmol of DDE was recovered, representing a 92% conversion of DDT to DDE. If these values are expressed as nmol of DDE/μg of protein, values of 12.5 and 5.5 are obtained for the experiments shown in Figures 5(B) and 5(C) respectively, suggesting that the concentration of DDT was rate-limiting in the experiment shown in Figure 5(C).

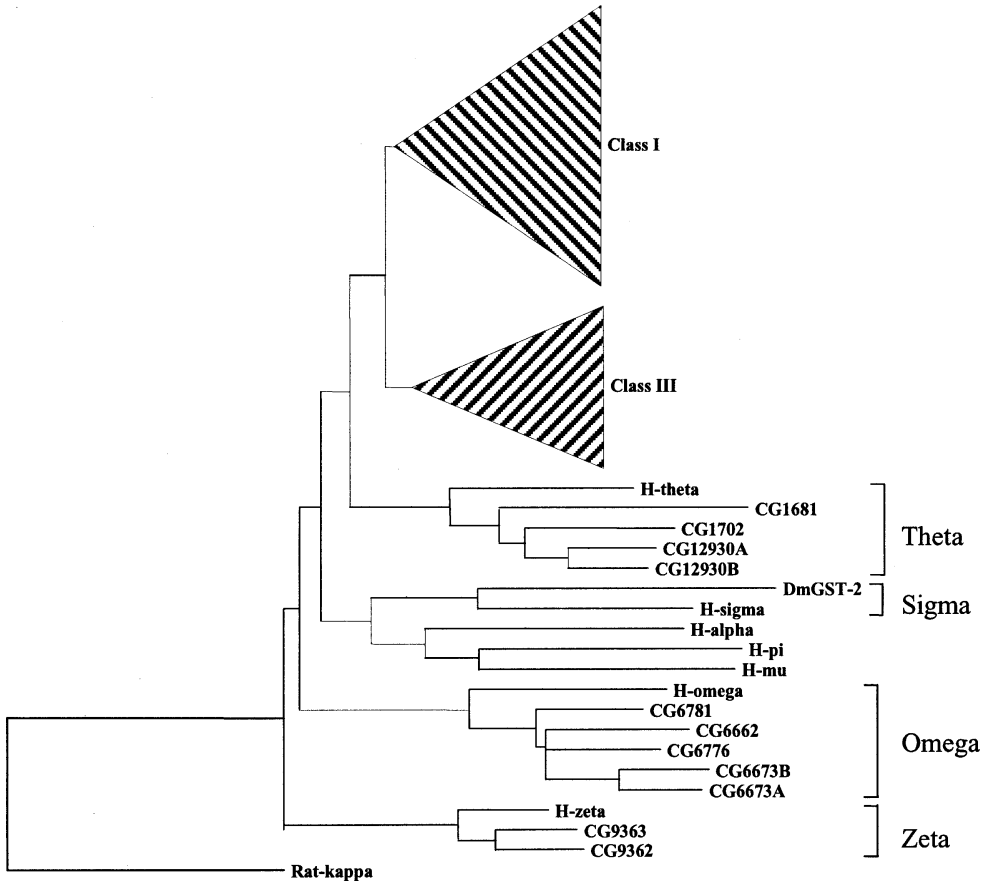

Figure 7 Dendrogram illustrating the relationship between *Drosophila* GSTs and mammalian representatives from each of the evolutionarily distinct GST classes

See the legend to Figure 6 for details.

Phylogenetic analysis

Table 1 shows the percentage similarity between the deduced amino acid sequences of all known *A. gambiae* GSTs. Previous classifications have designated GSTs as being members of the same class if their amino acid sequences are more than 40% identical [1]. By this criterion, only *aggst1-8* of the five newly described *A. gambiae* GSTs would be classified as belonging to the insect class I family and none of these genes would be classified as class II. This suggests that the present classification of insect GSTs into only two classes might need re-evaluating. We therefore conducted a phylogenetic analysis of all the known insect GST sequences, including 28 sequences retrieved from the *Drosophila* genome database.

Figure 6 shows a phylogenetic tree illustrating the relationships between these sequences, based on a CLUSTAL W amino acid alignment. The available insect GST sequences can be split into at least seven subdivisions on the basis of this phylogeny. Only three of these contain sequences with confirmed GST activity. These are the pre-existing classes I and class II plus a third clade, which we have called class III, in line with the existing nomenclature for insect GSTs, containing 20 sequences including *aggst3-1* and *aggst3-2* and the published genes DmGST-3 from *D. melanogaster*, GST-3 from *Plutella xylostella* and MsGST1 from *Manduca sexta* [37–39]. The low support for the monophyly of class I and of class III, indicated by the bootstrap values in Figure 6, perhaps suggests that these classes should be further subdivided. We therefore used amino acid distance matrices (results not shown) to examine the support for this classification. In this approach, we classified a gene for GST as belonging to a particular class if it satisfied the following two criteria: (1) at least 40% sequence similarity to a member of this class from a different species, and (2) less than 40% sequence similarity to all other classes of insect GST. The assignment of genes to classes II and III is supported by these criteria but ambiguities arose in the classification of class I. In Figure 6, and in the choice of nomenclature, we have classified *aggst1-9* and *aggst1-10* (plus the putative *Drosophila* GST, cg10065) as class I GSTs. However, these sequences show less than 40% sequence similarity to other members of this group and therefore do not belong to this class on the basis of previously published criteria [1]. Nevertheless, because these genes show the greatest levels of identity with class I GSTs out of all currently known insect GSTs, we propose to assign these GSTs to class I at present.

Relationship between mammalian and insect GSTs

The remaining four subdivisions shown in Figure 6 consist solely of sequences with GST-like domains, retrieved from the *Drosophila* genome database, and have not been experimentally verified as functional GST enzymes. CG4688 and CG11784 have the greatest similarity to MdGST6A (31.5% and 35.1% respectively) but, because these levels of similarity are below the arbitrary cut-off value, they have been classified as belonging to a separate

subdivision in Figure 6. CG9362 and CG9363 are closely related (62.6 % similarity to each other) but have less than 20 % similarity to any other known insect GST sequence. Similarly, the amino acid similarities support the existence of a distinct subdivision containing CG6773A, CG6773B, CG6781, CG6662 and CG6776 and a separate subdivision containing CG1702, CG12930A and CG12930B; however, the members of both these subdivisions have very low similarity to previously characterized insect GSTs.

To investigate the relationship between the insect and mammalian GST classes, we took a representative member of each of the eight cytosolic classes of mammalian GST and used CLUSTAL W to align these sequences with the putative *Drosophila* GST sequences. This alignment was used to generate the phylogenetic tree shown in Figure 7. Previous classifications have denoted all insect GSTs as belonging to the Theta class [34] but the phylogeny shown in Figure 7 does not support this conclusion. Although this tree is not intended to resolve the true phylogenetic relationship between GST families, it is interesting to note that representatives from the Theta, Sigma, Omega and Zeta class are present in *Drosophila*. For example, the *Drosophila* sequences CG9362 and CG9363 possess 56–58 % identity with the human Zeta GST, GSTZ1-1, and these putative insect GSTs contain N-terminal motifs closely related to the SSCXWRVRIAL (single-letter amino acid codes) motif found in Zeta class GSTs from plants, nematodes and mammals [4] (the predicted translations for CG9362 and CG9363 contain the motifs SSCSWRVRVAL and SSCSWRVRIAM respectively). A further subdivision of insect GSTs shows high similarity to the Omega-class human GST GST O1-1. All five putative *Drosophila* sequences within this subdivision, which are clustered on chromosome 3L, also contain a cysteine residue flanked by phenylalanine and proline residues at the proposed active site [7]. Finally, as noted recently [40], the insect class II enzymes are phylogenetically related to the Sigma class.

DISCUSSION

Evolutionary relationship between insect GSTs

We have identified five *A. gambiae* genes encoding GSTs, at least four of which are actively transcribed in adult mosquitoes. The fifth gene, *aggst1-9*, seems to be transcriptionally silent in adult and larval mosquitoes. It is not known whether this gene is expressed during earlier life stages or whether it is a pseudogene. A comparison of amino acid sequence similarities found that four of the genes described here were below the threshold for inclusion in either insect class I or class II, suggesting that the current classification system for insect GSTs is inadequate. This observation is supported by biochemical data from *A. gambiae* that identified at least eight fractions with GST activity, only one of which was immunologically related to the class I GST family [20]. The publication of the first draft of the *Drosophila* genome [41] prompted us to re-examine the classification of insect GSTs. Our analysis supported the existence of seven possible classes of protein with GST-like subunits. The existing class I and II families were resolved by our analysis and five additional classes were proposed. Of these five classes, only the largest, which we have named class III, includes insect proteins with confirmed GST activity ([38] and the present study). In our choice of *Drosophila* sequences to include in the analysis we selected only those encoding peptides of the approximate size of GST subunits (approx. 25 kDa) and showing significant similarity throughout the entire sequence rather than just at the N-terminus. Nevertheless, we acknowledge that some genes might have GST-like domains but not possess GST activity and likewise that some proteins might have acquired GST activity as a result of convergent evolution [42]. Hence we might have inadvertently included sequences that are not part of the same phylogenetic gene tree.

A preliminary investigation into the relationship between the insect GST classes and the previously characterized mammalian cytosolic GST classes revealed that four of the subgroups of insect GSTs have significant sequence similarity to confirmed GST classes (Theta, Sigma, Omega and Zeta). The class I insect GST subgroup has also been referred to as the Delta class and the insect class II as Sigma [9], in line with the nomenclature of the existing GST classes. The insect class III family, described for the first time here, does not belong to the Delta class or to any of the other existing GST families on the basis of established criteria. Therefore, to maintain consistency with the proposed nomenclature for GST classes [9], the insect class III would perhaps be more appropriately denoted by a Greek character. We suggest that hereafter the insect class III family be referred to as Epsilon (ϵ).

Of all known *A. gambiae* genes encoding GSTs, *aggst1-2* and *aggst1-9* are unique in that their open reading frames are uninterrupted by introns, although the presence of introns in the 5′ non-coding sequence, as found in *aggst1β*, MdGST1 and DmGST1 [10,43], cannot be discounted. The remaining class I and class III GSTs in *A. gambiae* all possess an intron at the identical position in the 5′ coding region and *aggst3-1* and *aggst3-2* also have a second intron within the centre of the open reading frame. This contrasts with the situation in *Drosophila*, in which none of the class I GST*D* genes clustered on chromosome 3R division 87B [12] have introns within their open reading frames, and furthermore an analysis of the 10 putative class III GSTs on chromosome 2R division 55C retrieved from the *Drosophila* genome database predicts that these genes are also intronless [41]. However, it is not true that all *Drosophila* GST genes are intronless. For example, the class II *Drosophila* GST DmGST-2 is interrupted by two introns [14].

The conservation of intron/exon boundaries across the class I and class III genes in *A. gambiae* and the absence of introns in the homologous gene families in *Drosophila* might suggest that the duplication events that occurred to produce the class I and class III lineages occurred after the divergence of the Nematocera and Brachycera Dipteran suborders. This hypothesis, however, is not supported when all members of the class I and class III families are considered. For example, a homologue of the *A. gambiae* class I gene *aggst1-7* has been identified in *Drosophila* (CG17639) and both of these genes possess two introns at identical sites (H. Ranson and N. Roberts, unpublished work), perhaps suggesting a common ancestor for these two genes.

Role of *A. gambiae* GSTs in insecticide resistance

An association between elevated GST activity and insecticide resistance has been observed in many insect species but there have been very few reports describing the individual enzymes involved. GST-2 from the mosquito *Aedes aegypti* is overexpressed in a DDT-resistant strain [16] but the ability of this enzyme to metabolize DDT has not been established and therefore the significance of this result is not clear. In addition, there have been reports that expression of housefly MdGST-3 is positively correlated with resistance and that recombinant MdGST-3 is able to degrade the insecticide dimethylparathion [11,44]. However, the genomic organization of this gene seems to be extremely complex with a variant gene copy number in different strains and hence the exact role of this enzyme in insecticide resistance is difficult to ascertain [11]. To our knowledge there have been only two substantiated reports of a direct

relationship between GST overexpression and resistance. The first is in the diamondback moth, *P. xylostella*. In this insect, increased expression of the PxGST3 gene, which encodes an enzyme capable of degrading organophosphorous insecticides, is strongly correlated with resistance [38]. The second example is in *D. melanogaster*, in which a recombinant GST enzyme, GST D1, exhibiting DDTase activity was found at elevated levels in a DDT-resistant strain [45].

With our results from *A. gambiae* we have shown that members of the class I family of insect GSTs are not of major importance in DDT resistance [21] and the expression profile of class II also discounts a major role for this gene in resistance [15]. To establish the identity of the GST enzymes responsible for resistance, we conducted a genome-wide scan to identify regions of the genome associated with resistance to DDT. We identified two major loci, the first, *rtd1*, on chromosome 3R between divisions 32c and 34c, and the second, *rtd2*, on chromosome 2L in close proximity to division 21 [36]. Two of the genes encoding GST described in this study map to chromosome 3R, division 33c, i.e. in the exact midpoint of the boundaries defined by *rtd1*, invoking the hypothesis that this resistance locus is a *cis*-acting regulatory element controlling the expression of these class III GSTs. In support of this we have now shown that *aggst3-2* is overexpressed in the resistant strain and that recombinant *aggst3-2* is very efficient at metabolizing DDT. DDT dehydrochlorinase activity has previously been reported for recombinant GSTs from *A. gambiae* [21] but this is the first definitive demonstration that a GST with DDT dehydrochlorinase activity is overexpressed in a DDT-resistant strain of mosquitoes. By analogy with *Drosophila*, in which 10 class III genes for GST are tightly clustered within approx. 14 kb of DNA [41], it is likely that the class III family in *A. gambiae* extends beyond the two members described here. If multiple members of this gene family are under the control of a common regulatory factor, a mutation in this factor could account for the elevated activity of several different GST enzymes observed in our earlier biochemical studies [21,46].

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