The independent gene amplification of electrophoretically indistinguishable B esterases from the insecticide-resistant mosquito *Culex quinquefasciatus*

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Resistance to organophosphates in *Culex* mosquitoes is typically associated with increased activity of non-specific esterases. The commonest phenotype involves two elevated esterases, A_2 and B_2, while some strains have elevation of esterase B_1 alone. Overexpression of the two B esterase electromorphs is due to gene amplification. Full-length cDNAs coding for amplified esterase B genes from a resistant Cuban strain (MRES, with amplified B_1 esterase) and a Sri Lankan strain (PelRR, with amplified B_2 esterase) of *C. quinquefasciatus* have been sequenced. In addition, a partial-length cDNA coding for a B esterase from an insecticide-susceptible Sri Lankan strain (PelSS) has been sequenced. All the nucleotide sequences and the inferred amino acid sequences show a high level of identity (> 95% at the nucleotide and amino acid level), confirming that they are an allelic series. The two B_1 esterase nucleotide sequences {MRES and the previously published TEM-R [Mouches, Pauplin, Agarwal, Lemieux, Herzog, Abadon, Beyssat-Arnaouty, Hyrien, De Saint Vincent, Georghiou and Pasteur (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 2574–2578]} showed the lowest identity, and restriction-fragment-length-polymorphism analysis of the two strains was different. On the basis of these data we suggest that the two electrophoretically identical B_1 esterase isoenzymes from California and Cuba have been amplified independently. Alternatively, if amplification has occurred only once, the original amplification has not occurred recently.

INTRODUCTION

Culex quinquefasciatus mosquitoes are vectors of both filariasis and Japanese encephalitis and are a worldwide biting nuisance. Organophosphorus insecticides (OPs) have been extensively used to control *Culex* populations. Resistance to the OPs is most commonly mediated by the increase in activity of carboxylesterases (Villani et al., 1983; Raymond et al., 1987, 1989; Bisset et al., 1990; Peiris and Hemingway, 1990a,b). A number of different esterase isozymes have been associated with resistance in *Culex*. Raymond et al. (1987) have classified these as 'A' or 'B' esterases with respect to their preference for the substrates α- or β-naphthyl acetate. The different isoenzymes are then given numerical subscripts, which originally denoted their relative electrophoretic mobilities (although as more isoenzymes have been described, this nomenclature has largely broken down). The most common resistance-associated phenotype is elevation of two esterases, namely A_2 and B_2, which occur in complete linkage disequilibrium. These two esterases have been purified (Ketterman et al., 1992; Karunaratne et al., 1993). The resistance mechanism involves overproduction of the esterases, allowing sequestration of, and cross-resistance to, many OPs (Peiris and Hemingway, 1990a,b; Ketterman et al., 1993; Karunaratne et al., 1993). The overproduction of the B esterases is due to gene amplification (Mouches et al., 1986; Raymond et al., 1989) and has been observed in natural populations of *Culex* throughout the world. Esterase B_1 has always initially been found amplified alone, and occurs in the U.S.A., Central America, the Caribbean and in Asia. Esterase B_2 is always elevated with esterase A_2 and has been found in more than 30 countries worldwide. The amplification units on which the B_1 and B_2 esterase genes occur are significantly larger than the genes themselves (Mouches et al., 1990; Raymond et al., 1991). Restriction-fragment-length-polymorphism (RFLP) analysis of the B_1 and B_2 esterase genes and their flanking regions suggests that they are alleles which have been independently amplified. With reference to the B_2 esterase gene, the similarity of RFLPs from numerous *Culex* strains has led to the hypothesis that the initial amplification event occurred once and has rapidly spread worldwide (Raymond et al., 1991). However, significant qualitative differences in the kinetics of OP interaction occur in purified esterases A_2 and B_2 from a number of resistant *C. quinquefasciatus* strains (Ketterman et al., 1993). In this study, PCR techniques and a novel 5′ rapid amplification of cDNA ends (RACE) procedure have been used to isolate cDNA sequences coding for amplified esterases B_1, B_2 and an unamplified esterase B from different strains of *C. quinquefasciatus*. The esterase B_1 was from an OP-resistant Cuban strain, MRES (Bisset et al., 1990). The esterase B_2 was from an OP-resistant Sri Lankan strain, namely PelRR (Peiris and Hemingway 1990b) and the unamplified esterase B from a susceptible Sri Lankan strain, namely PelSS (Amin and Peiris, 1990). Sequence alignments were then carried out between these esterases and those already published to allow us to address the apparent anomaly between the RFLP work and the enzymology.

On the basis of the X-ray structures of *Torpedo californica* (electric fish) acetylcholinesterase and *Geotrichum candidum* (fungus) lipase and their three-dimensional superposition, a large number of esterases have been aligned (Cygler et al., 1993). A number of residues are conserved in all these esterases, including the TEM-R B_1 esterase (Mouches et al., 1990), which are thought

Abbreviations used: OPs, organophosphorus insecticides; RFLP, restriction-fragment-length polymorphism; RACE, rapid amplification of cDNA ends; TE, 10 mM Tris/HCl (pH 8)/1 mM EDTA (pH 8); DEPC, diethyl pyrocarbonate.

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The cDNA sequence data reported in this paper have been deposited in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession numbers Z32694 (PelRR B_2 esterase), Z32695 (MRES B_1 esterase) and Z32696 (susceptible B esterase).

to be important in either catalytic activity or three-dimensional structure. We therefore compared the data from these three new esterase sequences with that alignment.

EXPERIMENTAL

Mosquito strains

A heterozygous population (Pel) of *C. quinquefasciatus* Say was collected from Peliyagoda, Sri Lanka, in 1986. It was selected to give a susceptible strain, PelSS, and a resistant strain, PelRR (Amin and Peiris, 1990; Peiris and Hemingway, 1990a). PelRR was 31-fold more resistant to the OP temephos than PelSS and also showed broad-spectrum cross-resistance to a range of OPs (Peiris and Hemingway, 1990b). Classical genetics indicated that the resistance in this strain was not consistent with a single major gene, although the resistance is entirely esterase-based (Peiris and Hemingway, 1990b, 1993).

The PelSS strain was selected by single-family selection from the Pel strain as described by Amin and Peiris (1990).

The PelRR strain was selected from the same Pel parental strain by mass selection with temephos as described by Peiris and Hemingway (1990a). Since then, insecticide resistance in this strain has been maintained by exposing fourth-instar larvae of every third generation to the LD_{50} concentration of temephos.

The MRES strain of *C. quinquefasciatus* is an OP- and carbamate-resistant strain from Havana, Cuba, and was collected from the Quibu River district in 1986. An altered acetylcholinesterase and an elevated B_1 esterase resulted in high levels (> 1000-fold) of malathion resistance in this strain (Bisset et al., 1991). The strain was selected as fourth-instar larvae at the 85–95% mortality level for 22 generations with malathion. Insecticide resistance has been maintained since by exposing the fourth-instar larvae of every third generation to the LD_{50} concentration of malathion.

Isolation of genomic DNA

The method used was an adaptation of Miller et al. (1988). About 1 g wet weight of fourth-instar larvae were ground in liquid N_2. The homogenate was added to 10 vol. of extraction buffer [10 mM Tris/HCl (pH 8)/0.1 M EDTA/0.5% (w/v) SDS/20 μg/ml pancreatic RNAase]. After incubation for 1 h at 37 °C, proteinase K was added to a final concentration of 100 μg/ml and the homogenate was incubated at 50 °C for 3 h. After cooling on ice for 10 min, 0.35 vol. of saturated NaCl was added to precipitate protein. The homogenate was well mixed and stored on ice for a further 5 min, then centrifuged at 16000 *g* for 20 min. The supernatant was removed and the DNA precipitated by adding an equal volume of propan-2-ol. The DNA was resuspended in 7.5 ml of 10 mM Tris/HCl (pH 8)/1 mM EDTA (pH 8) (TE) containing 20 μg/ml RNAase and incubated at 37 °C for 1 h, then extracted with phenol, phenol/chloroform and finally chloroform. After precipitation with ethanol, the DNA was resuspended in a small volume of TE and stored at 4 °C.

Genomic DNA studies

A PelRR B_2 esterase cDNA fragment was used as a probe to determine the haplotype of the B esterases being studied. A 10 μg portion of genomic DNA was digested to completion with *Eco*RI and separated by gel electrophoresis through 0.8% (w/v) agarose. The DNA was transferred to nylon membranes (Amersham) and hybridized with the ^{32}P-labelled probe (sp. radioactivity > 2×10^6 c.p.m./μg) at 65 °C for 16 h in Hybridization Buffer [5 × Denhardt's solution/6 × SSC (1 × SSC is 0.15 M NaCl/0.015 M sodium citrate)/0.1% (w/v) SDS/0.1% (w/v) sodium pyrophosphate/5% (w/v) polyethylene glycol 8000/100 μg/ml boiled sheared herring sperm DNA]. The final washes were at 65 °C in 0.1 × SSC and 0.1% (w/v) SDS for 20 min.

Isolation of total RNA

The method used was adapted from that described by Sambrook et al. (1989). A 1 g (wet weight) portion of fourth-instar larvae were thoroughly ground in liquid N_2. The larval homogenate was added to 20 vol. of guanidinium thiocyanate buffer [4.0 M guanidinium thiocyanate/0.1 M Tris/HCl (pH 7.5)/1% (v/v) β-mercaptoethanol/0.5% (w/v) sodium dodecyl sarcosinate]. After vortex-mixing, the homogenate was centrifuged at 5000 *g* for 20 min. The supernatant was loaded on to a cushion of diethyl pyrocarbonate (DEPC)-treated 5.7 M CsCl/0.01 M EDTA, pH 7.5. After centrifugation at 20 °C for 20 h at 40000 rev./min (150000 *g*), the sedimented RNA pellet was removed and washed with 70% (v/v) ethanol and resuspended in TE, pH 7.6. After phenol/chloroform and chloroform extraction the RNA was precipitated with the addition of 0.1 vol. of DEPC-treated 3 M sodium acetate, pH 5.2, and 2.5 vol. of ice-cold ethanol. The pellet was collected by centrifugation at 12000 *g* for 10 min and, after being washed with 70% (v/v) ethanol, was resuspended in a small volume of formamide and stored at −70 °C. To precipitate the RNA, 3 vol. of ethanol was added.

Synthesis of cDNA

mRNA was isolated with the PolyATract mRNA Isolation System IV (Promega). cDNA was synthesized with the Riboclone cDNA Synthesis System (Promega) using an oligo-(dT) adaptor primer [5′ GACTCGAGTCGACATCGA-$(dT)_{17}$ 3′]. Both first- and second-strand cDNA were synthesized and purified by ethanol precipitation and dissolved in a small volume of water.

Isolation of and sequencing of B esterase cDNAs

Initially, primers were used to isolate an internal fragment of the B esterase cDNA by PCR. Primers were constructed on the basis of knowledge of the B_1 esterase sequence (Mouches et al., 1990). The primer set 5′ T/CTA/GACC/A/GGTG/C/TCAGAC 3′ and 5′ G/ACAG/ATTT/A/G/CGGA/GTCG/A/T/CCC 3′ were used to amplify a product of 1440 bp. The 50 μl PCR reaction contained 5 ng of double-stranded cDNA, 50 ng of each primer, 0.5 mM dNTPs, 2 mM $MgCl_2$ and was buffered in *Taq* DNA polymerase buffer [50 mM KCl/10 mM Tris/HCl (pH 9.0 at 25 °C)/0.1% Triton X-100]. The reaction mixture was overlaid with mineral oil and heated in a DNA thermal cycler (Hybaid) to 94 °C for 3 min. The mixture was removed and 2.5 units of *Taq* DNA polymerase (Promega) were added to the reaction mixture, which was then heated to 94 °C for a further 3 min. Then 35 cycles of amplification were carried out using a step programme (37 °C, 3 min; 72 °C, 3 min; and 94 °C, 3 min).

The 3′ B esterase cDNA was amplified using an internal forward primer and the adaptor primer sequence (5′ GACTCGAGTCGACATCGA 3′) for the reverse primer. The PCR was carried out with first-strand cDNA. The PCR conditions were the same, except for the addition of 40 ng of first-strand cDNA, an increased annealing temperature of 50 °C and an extension time in the first cycle of 4 min.

We were not able to amplify any of the 5′ B esterase cDNAs by RACE. 5′ RACE uses the addition of a linker at the 5′ end of the first-strand cDNA pool with RNA ligase (Tessier et al., 1986; Troutt et al., 1992). To overcome this, a double-stranded linker

was added to blunted double-stranded cDNA. The 50 μl blunting reaction mixture contained 1 μg of cDNA, 0.5 mM dNTPs and was buffered in T4 DNA polymerase repair buffer [18 mM $(NH_4)_2SO_4$/0.066 M Tris/HCl (pH 8.3)/6.6 mM $MgCl_2$/10 mM β-mercaptoethanol]. Two units of T4 DNA polymerase were added and the reaction mixture was incubated for 15 min at 37 °C. After phenol/chloroform extraction, the cDNA was precipitated, resuspended and ligated to the double-stranded linker. The linker used was made up of two oligonucleotides [5′ TGACCGGCAGCAAAATG 3′ (Oligo A) and its reverse complement 5′ CATTTTGCTGCCGGTCA 3′ (Oligo B). Oligo A was 5′-phosphorylated with T4 polynucleotide kinase (Stratagene) and 3′-blocked with the addition of ddATP using terminal deoxynucleotidyl transferase (Stratagene), following the manufacturer's recommendations. This allowed the direction specific ligation of the linker to the cDNA pool, because only the 5′-phosphorylated Oligo A was able to ligate to the 3′-hydroxy group of the cDNA. Equimolar amounts of the two oligonucleotides were heated to 94 °C for 2 min and annealed by cooling to room temperature. A 100-fold molar excess of linker was ligated to the cDNA at 14 °C for 16 h with T4 DNA ligase (Promega). The 5′ B esterase cDNA was amplified using an internal reverse primer and Oligo A. A 40 ng portion of cDNA was used directly from the ligation reaction for the PCR. The PCR conditions were the same apart from an increased annealing temperature of 50 °C and decreased cycling times of 1 min for each step.

The amplified B esterase cDNA products from the PCR reactions were subcloned into pBluescript (Stratagene). The vector was restricted with *Eco*RV, and single thymidine residues were added at the 3′ end of each fragment (Marchuk et al., 1991) to facilitate the ligation.

DNA inserts from purified plasmid DNA were sequenced with Sequenase Version 2.0 (USB Biochemical Corporation) using the manufacturer's recommendations. For each cDNA product, at least two separate PCR clones were sequenced. Sequencing of both strands of the B esterase cDNA was completed using primers complementary to pBluescript and a large number of primers complementary to the B esterase nucleotide sequence. For each B esterase cDNA, the overlapping PCR products were sequenced to ensure isolation of a single cDNA sequence. Alignments of the nucleotide sequences and the inferred amino acid sequences were carried out using the DNA* package (DNASTAR).

RESULTS

For the present study, only those nucleotides within the open reading frame of the B esterase cDNAs and their inferred amino acids were compared. The novel approach used for the amplification of the 5′ end of the cDNAs relied upon the ligation of a double-stranded linker to the double-stranded-cDNA pool. Oligo A of the double-stranded linker was 5′-phosphorylated in order to allow its ligation to the cDNA pool. The linker will also ligate to the 3′ end of the cDNA pool, which could lead to the amplification of the whole cDNA pool in the PCR (as Oligo A could anneal to both ends of the cDNA). However, the specificity of the internal primer and the short extension time of the PCR cycle kept the amplification of non-specific sequences to a minimum.

As was seen in the TEM-R B_1 esterase gene (Mouches et al., 1990), the MRES B_1 and the PelRR B_2 esterase cDNAs had a short region of 5′ non-coding sequence (results not shown). In the MRES strain, two 5′ non-coding regions, identical apart from a short insert in the longer sequence, were isolated (results not shown). This was followed by an open reading frame of 1623 bp starting with an initial ATG codon and ending with a TGA stop codon. There followed a short 3′ non-coding sequence and a poly(A) tail (results not shown). The open reading frames of both the MRES B_1 and the PelRR B_2 esterase cDNAs coded for proteins of 540 amino acids, as for the TEM-R B_1 esterase. The 5′ region of the PelSS B esterase has not, as yet, been isolated. A 1524 bp open reading frame was sequenced for the PelSS B esterase, ending in a TGA stop codon. This coded for a total of 503 amino acids. All the nucleotide sequences aligned without gaps, as did the inferred amino acid sequences. Figure 1 shows the alignment of the four B esterase nucleotide sequences (PelRR B_2, TEM-R B_1, MRES B_1 and PelSS B). Identities range from 95.2% (TEM-R B_1 with MRES B_1 and PelSS B) to 98.8% (MRES B_1 with PelSS B) corresponding to nucleotide differences ranging from 78 to 18. An alignment of the five inferred amino acid B esterase sequences [PelRR B_2, TEM-R B_1, MRES B_1, PelSS B and a previously sequenced partial B_2(Mouches et al., 1990)] is shown in Figure 2. Identity between all the *Culex* esterases ranges from 95.2% (TEM-R B_1 with PelSS B) to 98.6% [previously published partial amplified B_2 (Mouches et al., 1990) with PelRR B_2] which corresponds to amino acid differences ranging from 24 to 3.

To date, it has been shown that the B_1 esterase gene has a 2.1 kb *Eco*RI RFLP and the B_2 esterase gene a 9 kb *Eco*RI RFLP (Raymond et al., 1991). To determine if the RFLP patterns of the three B esterase genes (from PelRR, PelSS and MRES) conformed to this pattern, a 1350 bp PelRR B_2 esterase cDNA fragment was hybridized to *Eco*RI-digested genomic DNA from the three strains (Figure 3). The band from the PelRR strain, containing B_2 esterase, corresponded to 9 kb, a finding consistent with that already reported. There was also a band at 3.3 kb present in both the PelRR and PelSS strains. The 9 kb band was not seen in the PelSS strain. The *Eco*RI banding pattern from the MRES strain, which has amplified esterase B_1, differed from that reported for TEM-R. There was a doublet of bands at 3.2 and 3.0 kb, which has not previously been reported for any amplified B esterase.

The relationship between the amino acid sequence and the three-dimensional structure of a large family of esterases and related proteins (including TEM-R esterase B_1) has recently been undertaken (Cygler et al., 1993). It was shown that 25 amino acid residues were conserved in all the esterases studied. It is thought that these residues are vital for the structure (residues used for packing, salt bridges, and disulphide bridges) and function (residues in the active site) of these proteins. These are also conserved in all the B esterase amino acid sequences and are shown in Figure 2. On the basis of the three-dimensional structure of the aligned esterases, the Ser-His-Glu active-site triad is seen at amino acid positions 191, 442 and 324 respectively in the MRES B_1, PelRR B_2 and PelSS B protein sequences (Figure 2). Nucleotide changes between gene sequences can lead to changes in the amino acid sequence, but many changes are 'silent', since often a mutation of the nucleotide at the third base of the codon has no effect on the translated amino acid sequence. The ratio of silent to non-silent changes can be used to determine whether a sequence is under active positive selection pressure. The number of silent nucleotide differences between the four B esterase sequences range from 4 (MRES B_1 with PelSS B) to 55 (MRES B_1 with TEM-R B_1), which account for 22.2% and 70.5% of the total nucleotide changes respectively. The 54 silent nucleotide differences between PelRR B_2 and TEM-R B_1 account for 77.1% of all nucleotide changes.

It has yet to be demonstrated whether the B esterases are glycosylated. There are five possible sites of N-linked glyco-

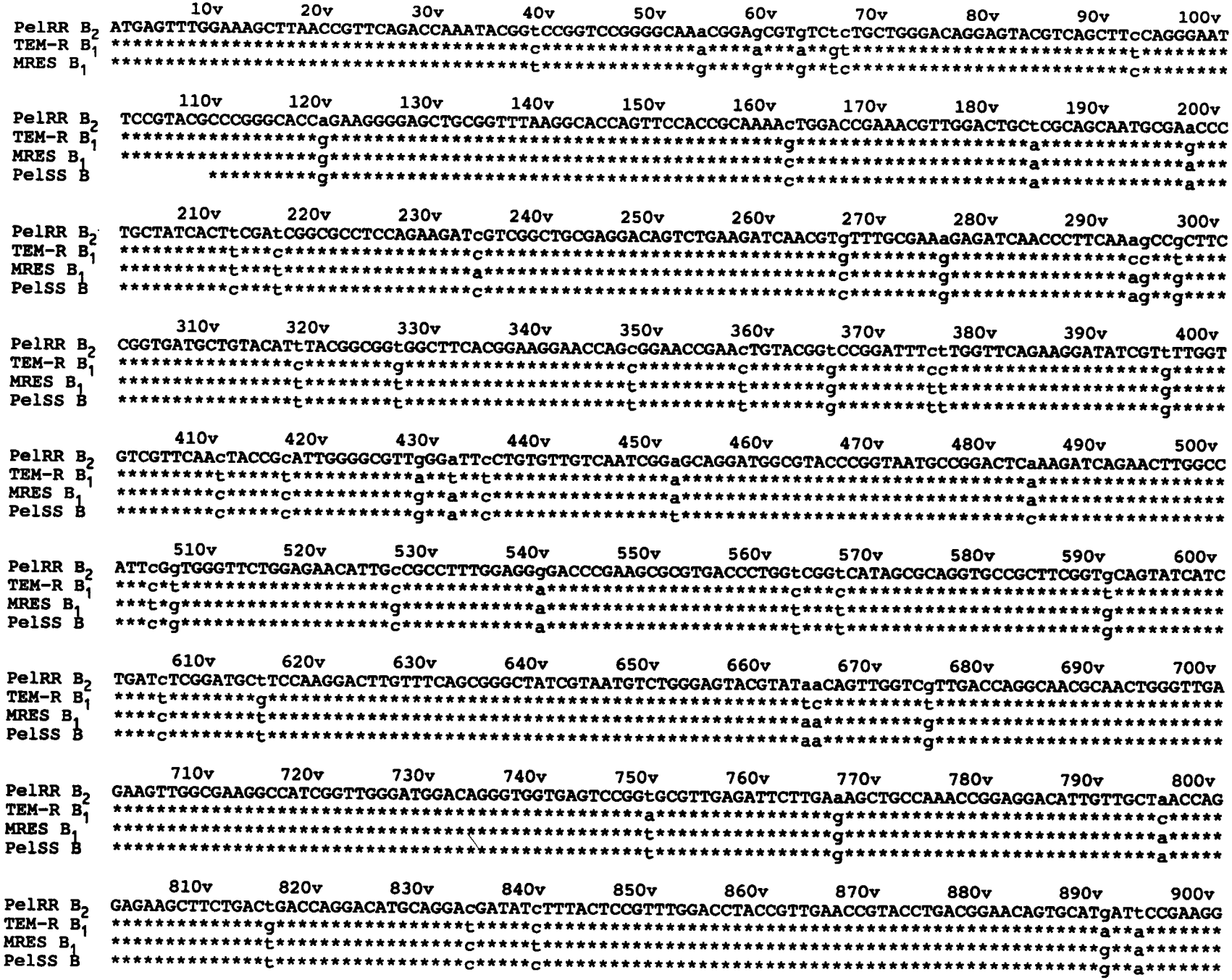
10v 20v 30v 40v 50v 60v 70v 80v 90v 100v
PelRR B2 ATGAGTTTGGAAAGCTTAACCGTTCAGACCAAATACGGtCCGGTCCGGGGCAAaCGGAgCGTgTCtcTGCTGGGACAGGAGTACGTCAGCTTcCAGGGAAT
TEM-R B1 **********c**********a****a***a**gt**********t********
MRES B1 **********t**********g****g***g**tc**********c********
110v 120v 130v 140v 150v 160v 170v 180v 190v 200v
PelRR B2 TCCGTACGCCCGGGCACCaGAAGGGGAGCTGCGGTTTAAGGCACCAGTTCCACCGCAAAAcTGGACCGAAACGTTGGACTGCtCGCAGCAATGCGAaCCC
TEM-R B1 **********g**********g**********a**********g***
MRES B1 **********g**********c**********a**********a***
PelSS B **********g**********c**********a**********a***
210v 220v 230v 240v 250v 260v 270v 280v 290v 300v
PelRR B2 TGCTATCACTtCGAtCGGCGCCTCCAGAAGATcGTCGGCTGCGAGGACAGTCTGAAGATCAACGTgTTTGCGAAaGAGATCAACCCTTCAAagCCgCTTC
TEM-R B1 **********t***c**********c**********g*******g**********cc**t****
MRES B1 **********t***t**********a**********c*******g**********ag**g****
PelSS B **********c***t**********c**********c*******g**********ag**g****
310v 320v 330v 340v 350v 360v 370v 380v 390v 400v
PelRR B2 CGGTGATGCTGTACATtTACGGCGGtGGCTTCACGGAAGGAACCAGcGGAACCGAAcTGTACGGtCCGGATTTctTGGTTCAGAAGGATATCGTtTTGGT
TEM-R B1 **********c*******g**********c*******g*******cc**********g*****
MRES B1 **********t*******t**********t*******t*******g*******tt**********g*****
PelSS B **********t*******t**********t*******t*******g*******tt**********g*****
410v 420v 430v 440v 450v 460v 470v 480v 490v 500v
PelRR B2 GTCGTTCAAcTACCGcATTGGGGCGTTgGGaTTcCTGTGTTGTCAATCGGaGCAGGATGGCGTACCCGGTAATGCCGGACTCaAAGATCAGAACTTGGCC
TEM-R B1 **********t*****t**********a**t**t**********a**********a**********
MRES B1 **********c*****c**********g**a**c**********a**********a**********
PelSS B **********c*****c**********g**a**c**********t**********c**********
510v 520v 530v 540v 550v 560v 570v 580v 590v 600v
PelRR B2 ATTcGgTGGGTTCTGGAGAACATTGcCGCCTTTGGAGGgGACCCGAAGCGCGTGACCCTGGtCGGtCATAGCGCAGGTGCCGCTTCGGTgCAGTATCATC
TEM-R B1 ***c*t**********c**********a**********c***c**********t**********
MRES B1 ***t*g**********g**********a**********t***t**********g**********
PelSS B ***c*g**********c**********a**********t***t**********g**********
610v 620v 630v 640v 650v 660v 670v 680v 690v 700v
PelRR B2 TGATcTCGGATGCttCCAAGGACTTGTTTCAGCGGGCTATCGTAATGTCTGGGAGTACGTATaaCAGTTGGTCgTTGACCAGGCAACGCAACTGGGTTGA
TEM-R B1 ****t*******g**********tc*********t**********
MRES B1 ****c*******t**********aa*********g**********
PelSS B ****c*******t**********aa*********g**********
710v 720v 730v 740v 750v 760v 770v 780v 790v 800v
PelRR B2 GAAGTTGGCGAAGGCCATCGGTTGGGATGGACAGGGTGGTGAGTCCGGtGCGTTGAGATTCTTGAaAGCTGCCAAACCGGAGGACATTGTTGCTaACCAG
TEM-R B1 **********a**********g**********c*****
MRES B1 **********t**********g**********a*****
PelSS B **********t**********g**********a*****
810v 820v 830v 840v 850v 860v 870v 880v 890v 900v
PelRR B2 GAGAAGCTTCTGACtGACCAGGACATGCAGGAcGATATcTTTACTCCGTTTGGACCTACCGTTGAACCGTACCTGACGGAACAGTGCATgATtCCGAAGG
TEM-R B1 **********g**********t*****c**********a**a*******
MRES B1 **********t**********c*****t**********g**a*******
PelSS B **********t**********c*****c**********g**a*******

Figure 1 Nucleotide identities between the coding regions of B esterases from four *Culex* strains: PelRR (esterase B_2), TEM-R (esterase B_1), MRES (esterase B_1) and PelSS (a partial-length non-amplified esterase B)

The full-length nucleotide sequence from PelRR esterase B_2 is shown in the top row. Below are the other three sequences. An asterisk denotes the same nucleotide. Lower-case letters are used when any one sequence differs from another. The partial-length PelSS B esterase sequence starts at nucleotide position 110.

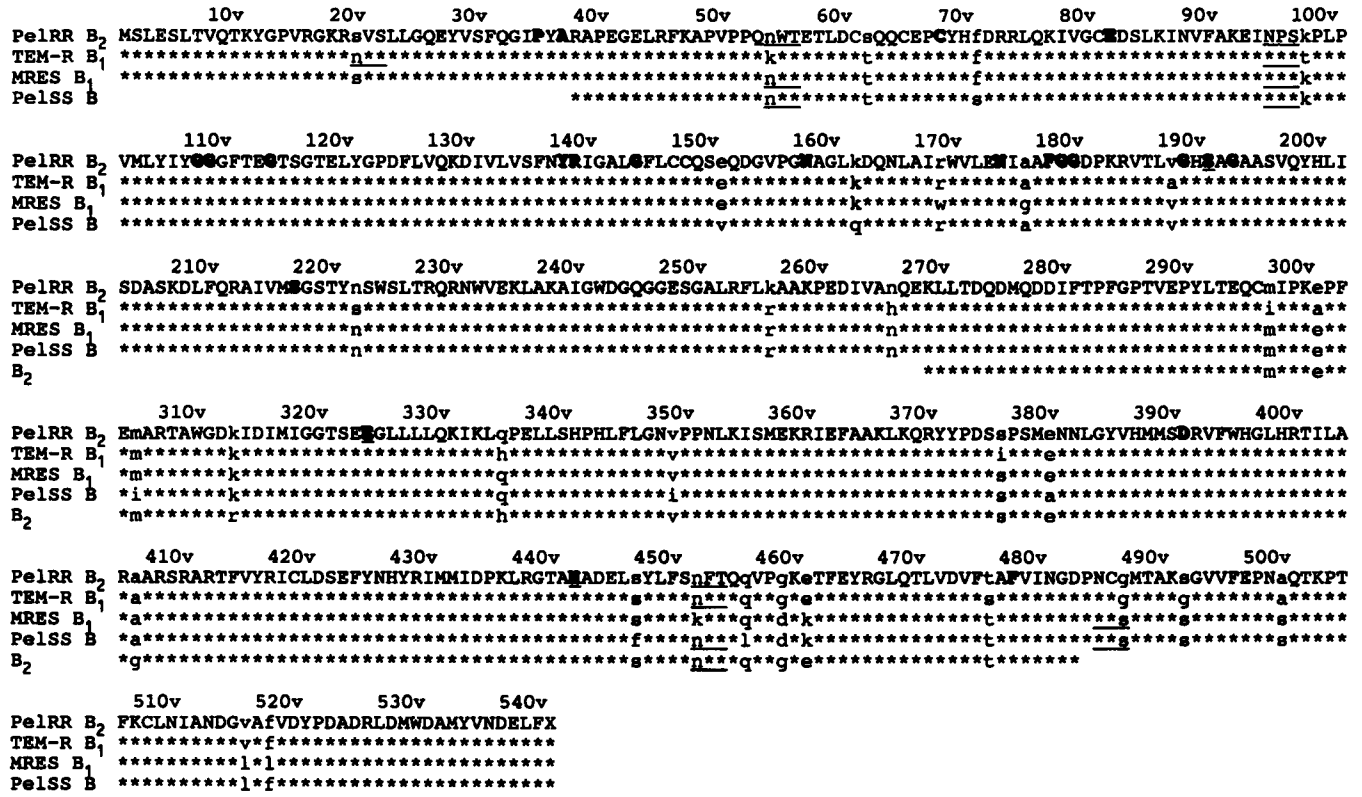

Figure 2 Identities between the inferred amino acid sequences of B esterases from five *Culex* strains: PelRR (esterase B_2), TEM-R [esterase B_1(Mouches et al., 1990)], MRES (esterase B_1), PelSS (a non-amplified esterase B) and a previously published amplified B_2 esterase (Mouches et al., 1990)

The amino acid sequence for PelRR esterase B_2 is shown in the top row. The same amino acid in the other esterases is shown by an asterisk. The PelSS B esterase sequence starts at position 38, and the previously published esterase B_2 sequence starts at position 269 and ends at position 483. Lower-case letters are used when one sequence differs from any other sequence. Amino acids preserved amongst a large family of 25 esterases (Cygler et al., 1993) are in **bold** type. Possible N-linked glycosylation sequences are underlined. The three amino acids (Ser-His-Glu) which are thought to make up the active-site triad are in **bold** type and underlined.

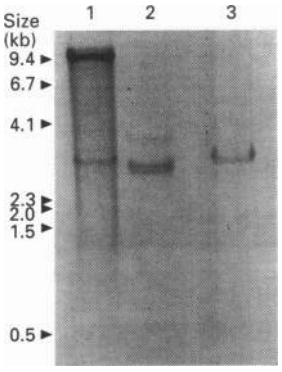

Figure 3 Genomic DNA digested with *Eco*RI and hybridized with a 1350 bp *Culex* PelRR B_2 esterase cDNA probe

Lanes: 1, PelRR; 2, MRES; 3, PelSS. Positions of size markers are shown on the left.

sylation (this can occur at Asn-Xaa-Ser or at Asn-Xaa-Thr in an amino acid sequence) in the B esterases, and these are shown in Figure 2.

Resistance to OPs in the peach-potato aphid, *Myzus persicae*, as in *Culex*, is due to the overproduction of a non-specific esterase. A full-length cDNA coding for the amplified esterase from the aphid, esterase E_4, has been sequenced and its amino acid sequence determined (Field et al., 1993). To determine the similarity between the functionally comparable *Culex* B esterases and esterase E_4, an alignment was undertaken. Figure 4 shows the identity between the PelRR B_2 and aphid E_4 esterase amino acid sequences.

DISCUSSION

The Californian TEM-R resistant strain of *C. quinquefasciatus* has an amplified B_1 esterase which confers OP resistance (Mouches et al., 1986; Raymond et al., 1989). The genomic sequence and its inferred amino acid sequence is known (Mouches et al., 1990). In the present study a further three B esterase cDNAs have been isolated from one susceptible (PelSS) and two OP-resistant *C. quinquefasciatus* (MRES and PelRR) strains. Resistance in both the MRES and PelRR strains has been shown to be esterase-based and linked to the amplification of B_2 and B_1 esterase respectively (Peiris and Hemingway, 1990a,1993; Bisset et al., 1991). The use of PCR to amplify the resistance-linked B esterase cDNA could also amplify the susceptible variants. Presence of unamplified susceptibility-linked alleles is shown by the RFLP analysis of PelRR and MRES B esterase genes (Figure 3). A weak non-amplified band is present at 3.3 kb in PelRR and a 3.8 kb band is present in MRES, but they are much less abundant than the amplified sequence. Thus the amplified

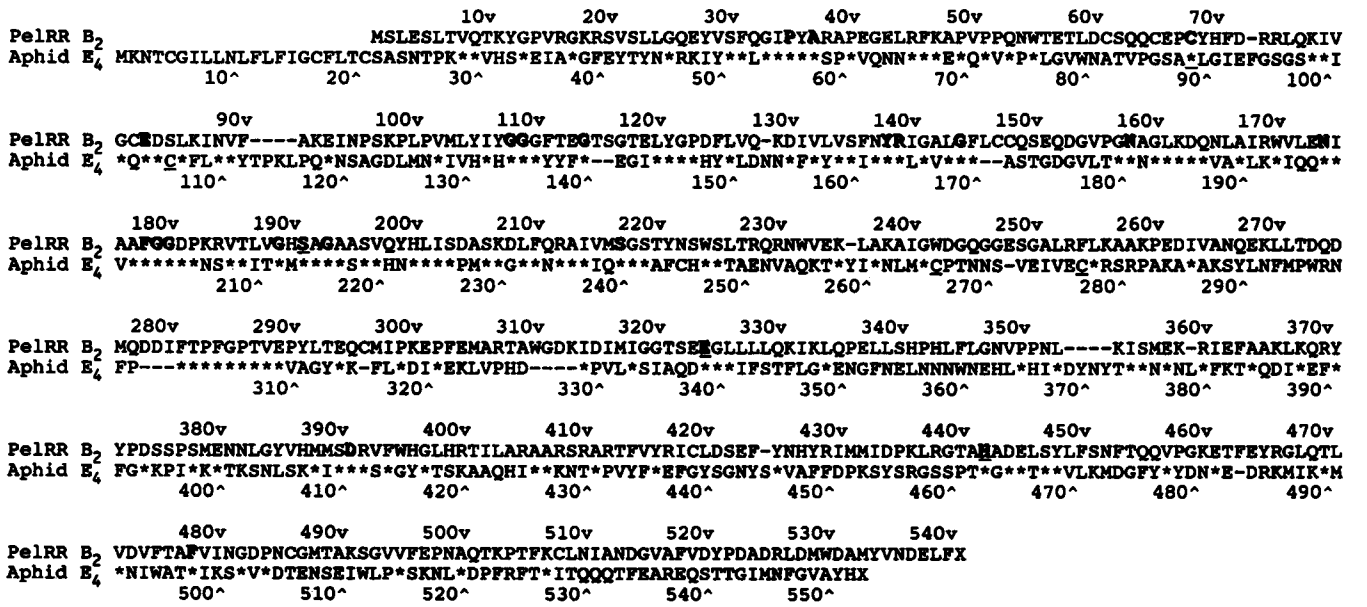

Figure 4 Amino acid identity between PelRR B_2 esterase and the E_4 esterase responsible for insecticide resistance in the peach-potato aphid, *Myzus persicae* (Sulzer) (Field et al., 1993)

The same amino acid is indicated by an asterisk. Amino acids preserved among a large family of 25 serine esterases (Cygler et al., 1993) are in **bold** type. The three amino acids (Ser-His-Glu) thought to make up the active site triad are in **bold** type and underlined. In the E_4 sequence, cysteine residues thought to be involved in disulphide-bridge formation are underlined (Cys^{89}–Cys^{106} and Cys^{266}–Cys^{277}). There is a 31.0% identity over a 500-amino-acid region (from B_2^{8}/E_4^{29} to B_2^{507}/E_4^{527}).

resistance-linked B esterase cDNA (and the related PCR product) make up the vast majority of the total B esterase cDNAs present. Multiple overlapping PCR products for both the MRES B_1, PelRR B_2 and PelSS B esterase cDNAs were sequenced, and interstrain products were found to be identical. The PelRR sequence was also significantly different (31 nucleotides) from that of PelSS, although both were selected from the same parental colony and contain the weak 3.3 kb RFLP band. Two variants of the MRES B_1 were found, although these differed only in the 5′ non-coding region. We are confident, therefore, that the resistance-linked B esterase cDNAs have been isolated.

The high identities at the molecular level (> 95.2% for nucleotide and amino acid comparisons) suggest that the esterase B genes are allelic. The majority (70.5%, 55) of the nucleotide differences between the MRES B_1 and TEM-R B_1 esterase cDNAs do not lead to a change in their amino acid sequences. There are, however, 21 amino acid differences between the two proteins. Furthermore, the percentage identity of the amino acid sequence from PelRR B_2 esterase is 97.4% with TEM-R B_1 and 98.0% with MRES B_1, whereas TEM-R B_1 and MRES B_1 are only 96.1% identical. In addition, the *Eco*RI RFLP for the amplified (when compared with PelSS) MRES B_1 esterase gene, which has two bands of equal intensity of 3.2 kb and 3.0 kb, is different from the 2.1 kb band of the TEM-R esterase B_1 gene (Raymond et al., 1991). The differences between the two B_1 esterase genes suggest that they arose from different amplification events. Alternatively, if a single amplification event occurred, the genes have since diverged to a greater extent than the electrophoretically distinct PelRR B_2 esterase has from either of the two B_1 esterases. The implication of this, coupled with the high percentage of silent to non-silent nucleotide changes, is that, if a single amplification event of these B_1 esterases has occurred, it is not within the time span over which OPs have been used.

There are three amino-acid-residue differences between the PelRR B_2 and partial B_2 (Mouches et al., 1990) esterase sequences. As for the two B_1 esterase genes, these differences could be due to a separate amplification event of the two B_2 esterase genes in the two strains or a recent divergence following a single amplification. Either of these hypotheses would explain the kinetic differences of insecticide interaction seen between purified B_2 esterases from a number of resistant strains (Ketterman et al., 1993).

The changes that have occurred between the B esterase alleles are due to single nucleotide substitutions, which are scattered throughout the coding sequence. Hence differences must be due to point mutations that have accumulated over time. The present work shows there are different alleles for electrophoretically identical B esterases, as judged by nucleotide and inferred amino acid sequence. On the basis of these data and previous RFLP patterns, there are at least five B alleles which have probably been independently amplified (Poirie et al., 1992). It is likely that the sequencing of more B esterase genes from strains which have already been shown to have kinetically distinct enzymes, will reveal further variants.

The kinetic differences between the B esterases from the susceptible and resistant strains are evidence for a positive selection pressure exerted by the OPs for those esterases from the resistant strains, as these esterases bind the oxon analogues of the OPs much more rapidly than the esterase from the susceptible strain (Karunaratne et al., 1994). RFLP analysis of the B locus and its flanking regions in susceptible *Culex* (which has a non-amplified B esterase gene), using B_1 esterase gene probes, has shown a high degree of polymorphism (Raymond et al., 1991). The PelRR and PelSS strains were selected from a single parental strain, namely Pel. On the basis of the *Eco*RI RFLP analysis for the PelSS B esterase (a faint single 3.3 kb band; Figure 3), the PelSS strain (which was selected by combining single families with low esterase activity) probably has a single B esterase allele.

The PelRR B_2 esterase gene has a typical amplified (when compared with PelSS) 9 kb *Eco*RI RFLP band (Figure 3; Raymond et al., 1991) and a fainter band equivalent to the PelSS band at 3.3 kb. It is curious, given the variability reported in the susceptible strains by Raymond et al. (1991), that the same 'susceptible' RFLP pattern is present in both PelSS and PelRR subcolonies, as the original parental strain contained several thousand field-collected insects. There are 15 amino acid differences between the PelSS B esterase and the amplified PelRR B_2 esterase. Thus, in PelRR, resistance to OPs is conferred not only by an amplified B_2 esterase, but also by a B esterase allele different from that of the susceptible PelSS strain. The differences at the amino acid level between the B esterases of PelRR and PelSS must contribute to the ability of PelRR B_2 esterase to bind oxon analogues of the OPs more rapidly than the susceptible esterase. It is possible that selection has favoured amplifications of B alleles from *Culex* which interact strongly with insecticides, and amplifications which contain less favourable alleles have been lost through negative selection pressure.

The amplified non-specific esterase, E_4, from *M. persicae* is the only other non-specific amplified esterase causing OP resistance for which a gene sequence is known (Field et al., 1993). Unlike the amplified *Culex* B esterases, the E_4 esterase, as well as conferring OP resistance, also confers pyrethroid resistance. Although the E_4 esterase and the amplified B esterases confer OP resistance in the same way, namely by interacting with the insecticides to prevent them reaching their target site, acetylcholinesterase, their amino acid sequences share only a 31.0% identity over 500 residues. The E_4 esterase and the *Culex* B esterases share residues that are conserved in 25 serine esterases (Cygler et al., 1993). Two disulphide bridges are conserved in all the esterases, apart from the *Culex* B esterases and *Heliothis* (Tobacco budworm) juvenile-hormone esterase. These bridges are also seen in the E_4 esterase.

A.V. was supported by Zeneca Public Health, and J.H. by The Royal Society. We thank G. Small and S. H. P. P. Karunaratne for technical assistance.

Received 4 May 1994/14 September 1994; accepted 21 September 1994

REFERENCES

Amin, A. M. and Peiris, H. T. R. (1990) Med. Vet. Entomol. **4**, 269–273
Bisset, J. A., Rodriguez, M. M., Diaz, C., Ortiz, E., Marquetti, M. C. and Hemingway, J. (1990) Bull. Entomol. Res. **80**, 245–250
Bisset, J. A., Rodriguez, M. M., Hemingway, J., Diaz, C., Small, G. J. and Ortiz, E. (1991) Med. Vet. Entomol. **5**, 223–228
Cygler, M., Schrag, J. D., Sussman, J. L., Harel, M., Silman, I., Gentry, M. K. and Doctor, B. P. (1993) Protein Sci. **2**, 366–382
Field, L. M., Williamson, M. S., Moores, G. D. and Devonshire, A. L. (1993) Biochem. J. **294**, 569–574
Karunaratne, S. H. P. P., Jayawardena, K. G. I., Hemingway, J. and Ketterman, A. J. (1993) Biochem. J. **294**, 575–579
Karunaratne, S. H. P. P., Jayawardena, K .G. I., Hemingway, J. and Ketterman, A. J. (1994) Insect Biochem, Mol. Biol., in the press
Ketterman, A. J., Jayawardena, K. G. I. and Hemingway, J. (1992) Biochem. J. **287**, 355–360
Ketterman, A. J., Karunaratne, S. H. P. P., Jayawardena, K. G. I. and Hemingway, J. (1993) Pestic. Biochem. Physiol. **47**, 142–148
Marchuk, D., Drumm, M., Saulino, A. and Collins, F. S. (1991) Nucleic Acids Res. **19**, 1154
Miller, S. A., Dykes, D. D. and Polesky, H. F. (1988) Nucleic Acids Res. **16**, 1215
Mouches, C., Pasteur, N., Berge, J. B., Hyrien, O., Raymond, M., De Saint Vincent, B. R., De Silvestri, M. and Georghiou, G. P. (1986) Science **233**, 778–780
Mouches, C., Pauplin, Y., Agarwal, M., Lemieux, L., Herzog, M., Abadon, M., Beyssat-Arnaouty, V., Hyrien, O., De Saint Vincent, B. R., Georghiou, G. P. and Pasteur, N. (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 2574–2578
Peiris, H. T. R. and Hemingway, J. (1990a) Bull. Entomol. Res. **80**, 453–457
Peiris, H. T. R. and Hemingway, J. (1990b) Bull. Entomol. Res. **80**, 49–55
Peiris, H. T. R. and Hemingway, J. (1993) Bull. Entomol. Res. **83**, 127–132
Poirie, M., Raymond, M. and Pasteur, N. (1992) Biochem. Genet. **30**, 13–26
Raymond, M., Beyssat-Arnaouty, V., Sivasubramanian, N., Mouches, C., Georghiou, G. P. and Pasteur, N. (1989) Biochem. Genet. **27**, 417–423
Raymond, M., Callaghan, A., Fort, P. and Pasteur, N. (1991) Nature (London) **350**, 151–153
Raymond, M., Pasteur, N. and Georghiou, G. P. (1987) Heredity **58**, 351–356
Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) Molecular Cloning: a Laboratory Manual, Cold Spring Harbour Laboratory Press, Cold Spring Harbor, NY
Tessier, D. C., Brousseau, R. and Vernet, T. (1986) Anal. Biochem. **158**, 171–178
Troutt, A. B., McHeyzer–Williams, M. G., Pulendran, B. and Nossal, G. J. V. (1992) Proc. Natl. Acad. Sci. U.S.A. **89**, 9823–9825
Villani, F., White, G. B., Curtis, C. F. and Miles, S. J. (1983) Bull. Entomol. Res. **73**, 153–170