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, A₂ and B₂, while some strains have elevation of esterase B₁ 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 B₁ esterase) and a Sri Lankan strain (PeIRR, with amplified B₂ 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 (PeISS) 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 B₁ esterase nucleotide sequences {MRES and the previously published TEM-R [Mouches, Pauplin, Agarwal, Lemieux, Herzog, Abadon, Beyssat-Arnaouty, Hyrien, De Saint Vincent, Georgiou 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 B₁ 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 carboxyl-esterases (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 A₂ and B₂, 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 B₁ has always initially been found amplified alone, and occurs in the U.S.A., Central America, the Caribbean and in Asia. Esterase B₂ is always elevated with esterase A₂ and has been found in more than 30 countries worldwide. The

amplification units on which the B₁ and B₂ 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 B₁ and B₂ esterase genes and their flanking regions suggests that they are alleles which have been independently amplified. With reference to the B₂ 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 A₂ and B₂ 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 B₁, B₂ and an unamplified esterase B from different strains of *C. quinquefasciatus*. The esterase B₁ was from an OP-resistant Cuban strain, MRES (Bisset et al., 1990). The esterase B₂ was from an OP-resistant Sri Lankan strain, namely PeIRR (Peiris and Hemingway 1990b) and the unamplified esterase B from a susceptible Sri Lankan strain, namely PeISS (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 (Cyglar et al., 1993). A number of residues are conserved in all these esterases, including the TEM-R B₁ 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 (PeIRR B₂ esterase), Z32695 (MRES B₁ 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 LD₅₀ 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 B₁ 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 LD₅₀ 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 N₂. 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 B₂ 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 *EcoRI* and separated by gel electrophoresis through 0.8% (w/v) agarose. The DNA was transferred to nylon membranes (Amersham) and hybridized with the ³²P-labelled probe (sp. radioactivity > 2 × 10⁶ 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 N₂. 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 B₁ 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 MgCl₂ 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' GACTCG-AGTCGACATCGA 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 μ l blunting reaction mixture contained 1 μ g of cDNA, 0.5 mM dNTPs and was buffered in T4 DNA polymerase repair buffer [18 mM $(\text{NH}_4)_2\text{SO}_4$ /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 *EcoRV*, 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 *EcoRI* RFLP and the B_2 esterase gene a 9 kb *EcoRI* 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 *EcoRI*-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 *EcoRI* 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-

[illegible]

PelRR B₂ 1610v 1620v
 CGTGAACGATCAGCTGTTTGA
 *****t*****
 TEM-R B₁ *****C*****
 MRES B₁ *****c*****
 pelss B₁ *****c*****

The full-length nucleotide sequence from PeRR esterase B₂ 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 PeSS 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 B₂), TEM-R [esterase B₁ (Mouches et al., 1990)], MRES (esterase B₁), PelSS (a non-amplified esterase B) and a previously published amplified B₂ esterase (Mouches et al., 1990)

The amino acid sequence for PelRR esterase B₂ 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 B₂ 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 (Cyglar 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 *EcoRI* and hybridized with a 1350 bp *Culex* PelRR B₂ 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 E₄, 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 E₄, an alignment was undertaken. Figure 4 shows the identity between the PelRR B₂ and aphid E₄ esterase amino acid sequences.

DISCUSSION

The Californian TEM-R resistant strain of *C. quinquefasciatus* has an amplified B₁ 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 B₂ and B₁ 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

		10v	20v	30v	40v	50v	60v	70v	
PelRR B ₂		MSLES	LTQTKY	GPVRG	KRSVSL	LGQEV	VSFQGI	PYARA	PEGELR
Aphid E ₄		MKNTCG	ILLNLF	FIGCFL	TCSAS	NTPK**	VHS*EIA*	GFEY	TYN*RKI
		10^	20^	30^	40^	50^	60^	70^	80^
		90v	100v	110v	120v	130v	140v	150v	160v
PelRR B ₂		GCEDSL	KINVF---	AKEIN	PSKPLP	VMLYI	YGGGFT	EGTSG	TELYGP
Aphid E ₄		*Q**C*	FL**Y	TPKLPQ	*NSAGD	LMN*IVH*	H**YYF	--EGI**	HY*LDNN
		110^	120^	130^	140^	150^	160^	170^	180^
		180v	190v	200v	210v	220v	230v	240v	250v
PelRR B ₂		AAFGGD	PKRVTL	VGHSA	GAASV	QYHLIS	DASKDL	FQRAIV	MSGSTY
Aphid E ₄		V*****	NS**IT	M****S	HN****	PM**G**	N**IQ**	AFCH**	TAENVA
		210^	220^	230^	240^	250^	260^	270^	280^
		280v	290v	300v	310v	320v	330v	340v	350v
PelRR B ₂		MQDDIF	TPFGPT	VEPYLT	EQCMIP	KEPFEM	ARTAWG	DKIDIM	IGGTSE
Aphid E ₄		FP-----	*****	VAGY*K	-FL*DI*	EKLVP	HD-----	PVL*SIA	QD***I
		310^	320^	330^	340^	350^	360^	370^	380^
		380v	390v	400v	410v	420v	430v	440v	450v
PelRR B ₂		YPDSSP	SMENNL	GYVHMS	DRVFWH	GLHRTI	LARAARS	RRFTFV	YRICL
Aphid E ₄		FG*KPI*	K*TKSN	LSK*I**	S*GY*TS	KAAQHI*	KNT*PV	YF*EFG	YSYSGN
		400^	410^	420^	430^	440^	450^	460^	470^
		480v	490v	500v	510v	520v	530v	540v	
PelRR B ₂		VDVFTA	VINGDP	NCMTAK	SGVVFEP	NAQTKP	TFKCLN	IANDG	VAFVDY
Aphid E ₄		*NIWAT*	IKS*V*	DENSE	WLP*SKN	L*DPFR	FT*ITQ	QTFEARE	QSTTG
		500^	510^	520^	530^	540^	550^		

Figure 4 Amino acid identity between PelRR B₂ esterase and the E₄ 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 (Cyglar 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 E₄ sequence, cysteine residues thought to be involved in disulphide-bridge formation are underlined (Cys⁸⁹–Cys¹⁰⁶ and Cys²⁶⁶–Cys²⁷⁷). There is a 31.0% identity over a 500-amino-acid region (from B₂⁸/E₄²⁹ to B₂⁵⁰⁷/E₄⁵²⁷).

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 B₁, PelRR B₂ 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 B₁ 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 B₁ and TEM-R B₁ 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 B₂ esterase is 97.4% with TEM-R B₁ and 98.0% with MRES B₁, whereas TEM-R B₁ and MRES B₁ are only 96.1% identical. In addition, the *Eco*RI RFLP for the amplified (when compared with PelSS) MRES B₁ 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 B₁ gene (Raymond et al., 1991). The differences between the two B₁ 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 B₂ esterase has from either of the two B₁ 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 B₁ 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 B₂ and partial B₂ (Mouches et al., 1990) esterase sequences. As for the two B₁ esterase genes, these differences could be due to a separate amplification event of the two B₂ 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 B₂ 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 B₁ 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 *PeIRR B₂* esterase gene has a typical amplified (when compared with *PeISS*) 9 kb *EcoRI* RFLP band (Figure 3; Raymond et al., 1991) and a fainter band equivalent to the *PeISS* 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 *PeISS* and *PeIRR* subcolonies, as the original parental strain contained several thousand field-collected insects. There are 15 amino acid differences between the *PeISS B* esterase and the amplified *PeIRR B₂* esterase. Thus, in *PeIRR*, resistance to OPs is conferred not only by an amplified *B₂* esterase, but also by a *B* esterase allele different from that of the susceptible *PeISS* strain. The differences at the amino acid level between the *B* esterases of *PeIRR* and *PeISS* must contribute to the ability of *PeIRR B₂* 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, *E₄*, 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 *E₄* esterase, as well as conferring OP resistance, also confers pyrethroid resistance. Although the *E₄* 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 *E₄* 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 *E₄* esterase.

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