Coamplification of esterase A and B genes as a single unit in *Culex pipiens* mosquitoes

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In *Culex pipiens* mosquitoes, resistance to organophosphorous insecticides often results from increased detoxification by two types of esterases, A and B, which are closely linked. Overproduction of all esterases B so far investigated (B1, B2, B4, B5 and B6) is from gene amplification. An esterase A gene (esterase A2) has recently been cloned from mosquitoes with the overproduced esterases A2 and B2, and amplification of this gene has also been reported. We describe the cDNA sequences of three additional esterase genes from insecticide-resistant strains of *Culex pipiens* originating from France and California which show at least 93 per cent homology with the esterase A2 gene sequence. Restriction enzyme mapping shows that the esterase A gene lies within 2.2 kb of the esterase B gene. In mosquitoes with overproduced esterases A2 and B2, the amplification level of esterase A is equal to that of esterase B suggesting that the genes are coamplified. Furthermore, we show that in one strain with an overproduced A esterase (A1), gene amplification cannot account for the increased protein level. This indicates that overproduction of esterases A can be achieved through two different mechanisms: gene amplification and a regulatory mechanism – the nature of which remains to be identified.

Keywords: evolution, gene amplification, resistance, sequences.

**Introduction**

In insects, increased detoxification is a common mechanism of resistance to pesticides (Oppenorth, 1985). In the house mosquito *Culex pipiens*, such a mechanism is often involved in resistance to organophosphorous (OP) insecticides (Pasteur *et al.*, 1981a, 1984). Increased detoxification results from the overproduction of two types of esterases (A and B), coded at two loci, *Est-2* (or esterase *B*) and *Est-3* (or esterase *A*), located on the same chromosome at less than 1 per cent recombination. In some cases, overproduction concerns only one type of esterase (esterase A1 (Pasteur *et al.*, 1981b) and esterase B1 (Georgiou *et al.*, 1980; Vaughan *et al.*, 1995)), whereas in others both types are overproduced simultaneously. Five distinct overproduced alleles have been described so far at the esterase B locus and three at the esterase A locus, and they are labelled B1 and A1, respectively, i representing an identifying number (Pasteur *et al.*, 1981b; Raymond *et al.*, 1989; Georgiou, 1992; Poirié *et al.*, 1992; Xu *et al.*, 1994; Raymond & Pasteur, 1995; Vaughan *et al.*, 1995). Four pairs of linked overproduced esterases have been observed in natural populations: A2-B2 (Curtis & Pasteur, 1981; Raymond *et al.*, 1989), A4-B4, A5-B5 (Poirié *et al.*, 1992) and A6-B6 (Xu *et al.*, 1994), based on their electrophoretic properties and on the RFLP pattern of the genomic sequences hybridizing with esterase B1 cDNA (Mouchès *et al.*, 1990). The molecular basis of esterase B overproduction has been shown to be gene amplification (Mouchès *et al.*, 1986; Raymond *et al.*, 1989; Poirié *et al.*, 1992; Xu *et al.*, 1994; Vaughan *et al.*, 1995). Recently, Vaughan & Hemingway (1995) have shown that the gene coding for esterase A2 is also amplified. The molecular mechanisms underlying such amplifications are still unknown. Focusing on DNA polymorphism of the esterase B region, Raymond and his collaborators (Raymond *et al.*, 1991, 1996; Qiao & Raymond, 1996).
1995) have proposed a scenario involving a single initial amplification event for each esterase B allele and a wide dispersal of the amplified amplicon by migration.

Here we have focused our studies on overproduced A esterases in an attempt to answer two main questions: (i) is overproduction of all esterases A the result of gene amplification as is the case for all the overproduced esterases B identified so far and for esterase A2; and (ii) have the overproduced esterases A2 and B2 which are always associated been amplified simultaneously or independently?

Materials and methods

Mosquito strains

One susceptible (S-LAB) and three OP-resistant strains (SELAX, BARRIOL, MSE) of mosquitoes from the Culex pipiens complex were used: (i) S-LAB, a susceptible reference strain from California (Georghiou et al., 1966) containing no overproduced esterases; (ii) SELAX, a strain from California possessing overproduced esterases A2 and B2 (Raymond et al., 1987); (iii) BARRIOL, a strain from southern France possessing overproduced esterase A1 (N. Pasteur & M. Raymond, unpublished data); and (iv) MSE, a strain from southern France possessing an insensitive acetylcholinesterase but no overproduced esterases (Raymond et al., 1986; Bourguet et al., 1996).

Isolation and sequencing of esterase

cDNA synthesis Total RNA was extracted from 100 fourth-instar larvae by homogenization in 1 ml of guanidium hydrochloride buffer (8 M guanidium HCl, 20 mM MES, 20 mM EDTA and 50 mM β-MESII), centrifugation at 11 600 g for 20 min, followed by a first extraction of the supernatant with acid phenol/chloroform (pH 4.3) and then with water-saturated ether. RNA was precipitated by adding 600 μl isopropanol, incubated on ice for 15 min and centrifuged at 11 000 g for 15 min. The RNA pellet was washed with 70 per cent and 100 per cent ethanol, air dried and resuspended in 200 μl of sterile distilled water. Poly (A)* RNA was isolated from 250 μg of total larval RNA using the Oligotex mRNA kit (QIAGEN, Chatsworth, CA, USA) which involved hybridization of the mRNA to an oligo dT-labelled resin, washing to remove rRNA and elution of the mRNA into 20 μl of sterile water. Oligo dT-primed cDNA was synthesized from 1 μl of mRNA solution using Clontech’s first strand cDNA synthesis kit (Clontech Laboratories, Palo Alto, CA, USA), following the manufacturer’s instructions.

PCR and sequencing PCR primers were designed from the cDNA sequence of esterase A2 (Vaughan & Hemingway, 1995), to amplify specifically esterase A genes in preference to esterase B. The primer sets 5'-GACAAGGGTTGCATTACCT-3' and 5'-ACTTCATTCTCTTTGCGTCC-3' were used to amplify a 1520 bp DNA fragment (88 per cent of the coding gene). The 50 μl PCR reaction contained 5 ng of first strand cDNA, 2 μM of each primer, 1.25 mM dNTP, 1.5 mM MgCl₂ in 1× enzyme buffer (Goldstar Polymerase, Eurogentec, Louvain-la-Neuve, Belgium). The reaction mixture was overlaid with mineral oil and heated in a Crocodile thermocycler (Appligene, Pleasanton, CA, USA) at 95°C for 3 min before adding 2.5 units of Taq polymerase (Goldstar polymerase). The amplification programme was continued with a further 3 min incubation at 95°C, followed by 30 cycles of 95°C for 1 min, 60°C for 1.5 min and 72°C for 2 min. A final extension step was included at 72°C for 10 min. A 5 μl aliquot was removed for agarose gel electrophoresis and the PCR product was visualized by ethidium bromide staining. PCRs were repeated at least twice for each studied mosquito strain. Both DNA strands of PCR products were sequenced following a direct method (Rousset et al., 1992), using individual PCR primers and oligonucleotides designed from the obtained sequence to direct DNA synthesis. The sequences obtained were aligned with the PeIRR A2 esterase sequence of Vaughan & Hemingway (1995).

RFLP analysis Genomic DNA was extracted from a pool of 100 adults according to Raymond et al. (1989) and digested with one or several restriction enzymes (AccI, BclI, BamHI, BglII, EcoRI, EcoRV, HindIII, KpnI, PstI, SacI, SalI, XbaI and XhoI). Aliquots of digested DNA, equivalent to an individual mosquito, were loaded onto 0.8 per cent agarose gels, and transferred onto Nylon membranes according to Sambrook et al. (1989). The filters were prehybridized and hybridized at 65°C with a 32P-labelled probe derived from the 1520 bp A2 PCR product, and washed at high stringency at 65°C (Sambrook et al., 1989). After an autoradiography, filters were stripped of radioactive label and re-probed with the 1.3 kb esterase B1 cDNA probe of Mouches et al. (1990).
Estimation of esterase gene amplification levels. Genomic DNA was extracted as above and treated by RNase. DNA was quantified using a GenQuant RNA/DNA Calculator (Pharmacia, Cambridge, UK). Two methods were used: (i) a dot-blot method, described by Field et al. (1988), using increasing dilutions of DNA from different mosquito strains fixed onto a Nylon membrane, was employed to estimate the quantities of genomic DNA hybridizing with radioactive probes (i.e. the SELAX 1520 bp PCR product for esterase A and the esterase B1 1.3 kb cDNA for esterase B); and (ii) a Southern blot method: AccI digestions of serially diluted DNAs from different strains were electrophoresed on agarose gels and blotted as described above to estimate the amplification level of the esterase A gene. For the first method, hybridization with an acetylcholinesterase gene probe (Rooker, 1994) has been carried out. This gene is known to be present in Culex pipiens in a single copy (D. Bourguet, personal communication) and thus hybridization with this probe allows an estimate of the number of haploid genomic sets in each blot. The quantity of genomic DNA hybridizing with radioactive probes was estimated from autoradiographs and from a β-imager (Phosphoimager 445 SI, Molecular Dynamics, Sunnivale, CA, USA) analysis. The latter method gives a direct estimate of the quantity of β-ray produced by each hybridization without artefacts of saturation so that this estimate is linearly correlated to the quantity of DNA hybridized.

Results

Sequence of A esterases

RT-PCR products of 1520 bp were obtained for mosquitoes of the three resistant strains tested (SELAX, BARRIOL and MSF), using PCR primers designed to amplify specifically the esterase A gene (details not shown). These cDNA products were of the predicted size based on the sequence of the esterase A2 gene (Vaughan & Hemingway, 1995). A nucleic sequence alignment for all three esterases A is shown in Fig. 1. Similarity among pairs of esterase A genes is at least 93 per cent at the nucleotide level, and 97 per cent at the predicted amino acid level, confirming that esterase A genes were sequenced.

Amplification level

Two estimations of A esterase gene amplification levels for BARRIOL and SELAX were obtained, using S-LAB as reference. First, five independent dot-blot replicates with the three strains hybridized on the same membranes indicated that the esterase A gene displays a twofold amplification in BARRIOL and a 30-fold amplification in SELAX (Fig. 2). For the B esterase gene, dot-blots gave estimates of twofold in BARRIOL and 20-fold in SELAX. The variability between different replicas of dot-blots hybridized with the same probe indicated that the ‘true’ amplification level is between half and twice each value. Southern blots of AccI digests of serial DNA dilutions which produced one fragment containing most of the A esterase gene in each strain (Fig. 4) provided estimates of esterase A amplification levels of one to twofold for BARRIOL and 20-fold for SELAX (Fig. 3). It was not possible to use this last method for esterase B gene quantification because hybridization with the B probe gave different patterns for SELAX, BARRIOL and S-LAB.

Coamplification of A and B esterases

Restriction maps of the genomic region hybridizing with esterase A and of the esterase B probes were built for BARRIOL and SELAX mosquitoes after digesting genomic DNA with restriction enzymes and probing the membranes first with the cDNA fragment of esterase A2 (1520 bp) and then with the 1.3 kb cDNA of esterase B. For all restriction enzymes cutting far outside the esterase A gene, DNA fragments were of the same size when hybridized with esterase A or esterase B probes, indicating that the two genes lay within the same DNA region. This was confirmed by the complete overlapping of restriction maps built independently with the two probes (Fig. 4). Esterases A and B were found to be separated by a DNA fragment of 2.2 kb, and in the two strains, esterase A and B genes were in an inverted position with the two 5′ ends in close proximity (Fig. 4). The amplified regions in the SELAX and BARRIOl strains were estimated to be at least 22 kb.

Discussion

A single amplicon containing A and B esterase genes

RFLP analyses indicate that the esterase A and B genes are located within the same DNA region for both strains (SELAX and BARRIOL) studied. The distance between the two genes is about 2.2 kb. This observation, associated with the similar amplification
level of A and B esterase genes in the SELAX strain displaying both overproduced esterases A2 and B2, suggests that both genes are within the same amplicon in strains displaying an amplification. This is also consistent with the fact that the amplicon is far larger than only one esterase gene (Mouches et al., 1990). These results have three important implications. First, the amplifications of esterase A and esterase B in the resistant strain SELAX do not correspond to independent events: most likely, the same event has amplified the two closely linked esterase genes. It further strengthens the hypothesis that the simultaneous occurrence of overproduced A2-B2 in distinct geographical locations is the result of migration and not multiple mutational events (Raymond et al., 1991, 1992, 1996; Qiao & Raymond, 1995). Secondly, the strong linkage disequilibrium observed between overproduced A2 and B2 esterases in natural populations is now clearly explained: it is unlikely that random recombination could occur precisely between A and B esterase genes within each amplicon. The linkage disequilibrium is initially created by the molecular event generating the coamplification of A and B esterase genes, and further increased by selection in treated areas because of the advantage of the resulting esterase overproduction. Thirdly, it is unlikely that classical genetic analysis could be performed to study the relative involvement in resistance of both esterases in such situations: A2 and B2 cannot be easily separated by recombination. Only recombinant molecular genetics or biochemical studies with purified esterase proteins could solve this problem.

The particular situation of A1

There are generally two overproduced esterases found together in resistant mosquitoes displaying an
amplification, one A and one B. Two noteworthy exceptions exist: esterase A1 from southern France (Pasteur et al., 1981a) and esterase B1 from the Americas and China (Mouchès et al., 1986; Qiao & Raymond, 1995), which are found alone (Raymond et al., 1989). In the BARRIOL strain, the overproduction level of esterase A1 is about 70-fold (Mouchès et al., 1987), and we showed that the esterase A and B genes had an amplification level of no more than twofold by dot-blot analyses. This suggests that the large overproduction of A1 is the result of a regulatory process which does not affect the nearby esterase B gene. The exact mechanism (e.g. increased transcription or translation, higher stability of mRNA) of this regulation is still unknown. It is worthy of note that despite the absence of apparent coamplification (twofold at most), there is a widespread linkage disequilibrium between the A1 gene and an allele at the esterase B locus (Pasteur et al., 1981b; Raymond & Marquine, 1994; Chevillon et al., 1995). This indicates that the occurrence of the regulatory change generating A1 overproduction was probably a unique event.

Relation between amplification and overproduction

Previous studies have shown that amplification levels of B esterase genes (Mouchès et al., 1986, 1987;

![Diagram](image)

Fig. 2 Example of dot-blots of serial dilutions of genomic DNA hybridized with the 0.7 kb acetylcholinesterase probe (Ace gene), the 1.52 kb esterase A2 PCR product (A gene) and the 1.3 kb esterase B1 cDNA probe (B gene) (see text for details) showing differences in level of gene amplification between insecticide-resistant (BARRIOL and SELAX) and susceptible (S-LAB) Culex pipiens strains.

![Diagram](image)

Fig. 3 Southern blot of serially diluted AccI-digested genomic DNA from BARRIOL, SELAX and S-LAB Culex pipiens strains hybridized with the 1.52 kb A2 PCR product.

![Diagram](image)

Fig. 4 Restriction enzyme maps showing the position of the esterase A gene in relation to esterase B in SELAX and BARRIOL Culex pipiens strains. θ refers to the 1520 bp PCR product of esterase A2 from SELAX and φ refers to the 1.3 kb cDNA of esterase B1. The lengths of esterases A and B and of probes θ and φ take into account the length of introns (details not shown). For each map, the restriction sites detected by θ esterase are indicated above and those detected by B esterase are below. For each enzyme (A: AccI, C: BclI, B: BamHI, G: BglII, E: EcoRI, R: EcoRV, H: HindIII, K: KpnI, P: PsiI, S: SacI, L: SalI, X: XbaI and O: XhoI), only the first two restriction sites outside the probe location could be detected. For each strain, the restriction sites which are shared by the maps built with θ and φ probes are framed and in italic characters.


DC (in press).


