Esterase gene amplification in *Culex pipiens*

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Abstract
In the mosquito *Culex pipiens* one of the major resistance mechanisms to organophosphorous pesticides (OPs) is increased detoxification of insecticides. This resistance is the consequence of overproduction of two types of esterases, esterases A and B, coded at two loci, *Est-3 (A esterase)* and *Est-2 (B esterase)*. We have analysed the genomic structure of these genes in different strains resistant to OPs and have attempted to characterize the different types of mutations leading to the resistant phenotypes.

It is shown that, concerning the more frequent resistant phenotypes, mutations leading to resistance are of two main types. First, overproduction of one *A* esterase present in Southern France results from a regulatory mechanism. The second type of mutation is gene amplification which involves events that have initially generated the duplication of both the *A* and *B* esterase or only the *B* esterase locus. We report the point that the most frequent esterase overproductions are the results of eight different mutations and that, given the range of distribution of these genotypes, mutation leading to an efficient resistance gene is one of the most limiting factors for the evolution toward resistance in *Culex pipiens*.

Keywords: gene amplification, esterase, resistance.

Introduction
In insects two main resistance mechanisms to organophosphorous (OP) insecticides have been described. One corresponds to a modification of the target site, acetylcholinesterase (reviewed by Fournier & Mutero, 1994). A second mechanism is an increased detoxification of the insecticide (Oppenorth, 1985). In the house mosquito *Culex pipiens* such a mechanism has been widely described (Pasteur et al., 1981a, 1984), and is the consequence of overproduction of two types of carbamate esterases (E.C. 3.1.1.1), historically named A and B esterases, coded at two loci, *Est-2* (or *B* esterase) and *Est-3* (or *A* esterase). Three pairs of associated overproduced esterases have been observed in natural *Culex pipiens* populations: A2-B2 (Curtis & Pasteur, 1981; Raymond et al., 1987; Villani et al., 1983), A4-B4, and A5-B5 (Poirel et al., 1992). In some cases, overproduction is only found at one locus: B1 (Georgiou et al., 1990), B6, B7 (Xu et al., 1994) and possibly B8 (Taubhani et al., 1995) in the B esterase locus and A1 (Mutero et al., 1981a) at the A esterase locus.

The overproduction of all the B esterases investigated so far (B1, B2, B4, B5 and presumably B6, B7 and B8) is due to a tandem amplification of the structural gene (Mouchès et al., 1986; Raymond et al., 1989; Poirel et al., 1992; Xu et al., 1994; Vaughan et al., 1995). The recent cloning of an *A* esterase gene (Vaughan & Hemingway, 1995) has allowed the investigation of the molecular basis of *A* esterase overproduction. Restriction enzyme mapping showed that A2 esterase gene lies within 2.2 kb of the B2 esterase gene (Rooker et al., 1996). In mosquitoloines with overproduced esterases A2 and B2 the amplification level of *A* esterase was equal to that of *B* esterase, suggesting that the genes were co-amplified. Furthermore, Rooker et al. (1996) showed that in one strain with an overproduced *A* esterase (A1), gene amplification (2-fold at most) could not account for the increased protein level (70-fold (Mouchès et al., 1987)). This indicates that overproduction of *A* esterases may be the result of two different mechanisms: gene amplification and a regulatory mechanism (Rooker et al., 1996).
In this paper we characterize the enzymes coding for known overproduced esterases in the mosquito Culex pipiens and the different mechanisms by which esterase overproduction can be achieved.

Results

Estimation of amplification level

We estimated the amplification levels of genes coding for overproduced esterases A5-B5 in CYPRUS, A4-B4 in VIM, A3-B2 in SELAX, B1 in TEM-R and of genes coding for non-overproduced A esterases in TEM-R and non-overproduced A and B esterases in MSE. We used the susceptible reference S-LAB as control with no amplification at either the esterase A and B loci.

Hybridizations with the Ace probe revealed that, in a few cases, there was a non-linear relationship between the quantity of DNA blotted and the radioactive signal (data not shown). Consequently these results could not be used as a control for DNA concentration (see Rooker et al., 1996). As an alternative, we chose a method to estimate the amplification level that was not dependent on variation of hybridization conditions between experiments (see Experimental procedures). For all strains, linear regressions between the radioactive signal of A or B esterase gene hybridization and DNA dilution were significant ($P < 10^{-4}$).

Dot-blot assays were repeated twice (see one example in Fig. 1) and gave the estimates of amplification levels of A and B esterase loci indicated in Table 1. The amplification levels of A and B esterase loci were not different ($P > 0.05$) in each strain, with the exception of TEM-R ($P < 2 \times 10^{-5}$). Due to the low number of replicates, this result must be considered as conserva-

![Diagram](image1)

**Figure 1.** Example of autoradiographs of dot-blot of serially diluted genomic DNA of strains displaying CYPRUS, SELAX, TEM-R and VIM or not (S-LAB and MSE) overproduced esterases. The probes used for hybridization were indicated and their ratio (on the right) corresponds to the DNA dilution factors. Analysis was performed on the filmager measures and not on autoradiographs.

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Table 1. Amplification level of A and B esterase genes for strains displaying overproduced esterases and the reference strain S-LAB. P-value refers to a two-sided Student t-test of equality of A and B esterase gene amplification levels.

<table>
<thead>
<tr>
<th>Strains</th>
<th>A Esterase</th>
<th>B Esterase</th>
<th>P-value</th>
<th>A esterase</th>
</tr>
</thead>
<tbody>
<tr>
<td>TEM-R</td>
<td>20.4 ± 0.2</td>
<td>0.7 ± 0.1</td>
<td>&lt;2 x 10^-6</td>
<td>0.5</td>
</tr>
<tr>
<td>VM</td>
<td>7.5 ± 0.7</td>
<td>0.44 ± 0.6</td>
<td>0.24</td>
<td>4.3</td>
</tr>
<tr>
<td>CYPRUS</td>
<td>80.3 ± 3.3</td>
<td>43.3 ± 1.7</td>
<td>0.005</td>
<td>15</td>
</tr>
<tr>
<td>SELAX</td>
<td>32.4 ± 0.1</td>
<td>40.8 ± 7.4</td>
<td>0.51</td>
<td>19</td>
</tr>
</tbody>
</table>

Ase probe comparison

<table>
<thead>
<tr>
<th>Strains</th>
<th>A esterase</th>
</tr>
</thead>
<tbody>
<tr>
<td>S-LAB</td>
<td>1.12 ± 0.1</td>
</tr>
<tr>
<td>TEM-R</td>
<td>1.16 ± 0.1</td>
</tr>
<tr>
<td>VM</td>
<td>0.85</td>
</tr>
</tbody>
</table>

The highest amplification level was found in Cyprus where the A5 and B5 esterase genes were amplified 40-60-fold. In TEM-R the B1 gene was amplified 20-fold whereas the A gene was not amplified. In SELAX the A2 and B2 genes were amplified between 30- and 40-fold, which is not different from previous estimates; 21-40-fold for B2 (Raymond et al., 1989), and 30 (15-40) for A2 and 20 (10-40) for B2 (Rooker et al., 1999). Amplification levels of the A esterase locus obtained by the Southern blot method (Fig. 2) are also

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Figure 2. Autoradiography of southern blot of
DNA digested with EcoRI and hybridized with
the A2 probe of strains displaying (SELAX, VM
and CYPRUS) or not (TEM-R and S-LAB)
overproducing A esterase. The ratio indicates
the DNA dilution factors.
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Figure 3. Restriction maps of A and B esterase genes of strains displaying or not overproduced esterases. For each map, the restriction sites detected by the A esterase probe are indicated above and those detected by the B esterase probe are below. The positions of A and B probes are indicated under the A and B esterase lines. Homologous restriction sites between two or more maps are in bold and italic characters.

Abbreviations: A, Accl; C, BglI; D, BamHI; E, BstEII; F, EcoRI; H, HindIII; K, KpnI; P, PstI; S, Sall; X, XbaI; M, MspI; D, Dow.

The maps of BARRIOS and SELAX (displaying respectively A1 and A2-B2) are from Roome et al. (1999).

Esterase gene amplification in Culex pipiens

Second, the amplification level for A and B esterase genes are similar within each strain. This extends the results of Rooker et al. (1996) who found a similar situation for the strain SELAX (displaying A2 and B2). Differences in amplification levels were found depending on the method used (dot-blot or Southern blot) as already described by Rooker et al. (1996). In both methods, radioactivity levels were directly measured using a β-imager, so that a possible explanation is probably to be found during the extra step of DNA digestion, electrophoresis or transfer on membrane for the Southern method.

We now have evidence from three separate strains that when the A and B esterases are both overproduced, the corresponding structural genes are both amplified within the same amplicon. Hence, the amplification events leading to the overproduction of both A and B esterases must correspond to a unique event for each esterase pair.

In all strains except those with B1, the genomic region of A and B esterase loci have the same basic structure: in each strain, regardless of whether or not they were amplified, we found a cluster of esterase genes including the two loci in an inverted position, the two 5' ends being in close proximity. However, the length of the intergenic region was variable among strains. We observed two classes of length: 2.2 kb and 6.3 kb with errors due to the observation scale. These classes did not correspond to amplified versus non-amplified esterases. The A4 and B4, A5 and B5 esterases genes in VIM and CYP11RUS respectively, and non-amplified A and B esterases genes in MSE were found 6.3 kb apart, whereas A2 and B2 esterase genes in SELAX and non amplified A and B esterase genes in S-LAB were 2.2 kb apart. This implies that the mutational event that has generated the amplicon is not directly related to the difference of intergenic length.

The particular case of the B1 amplicon

An exception to the co-amplification of A and B esterase genes is found in strains displaying an overproduced B1 esterase. None of the four B1 strains originating from distinct geographical localities displayed an amplified A gene. It is concluded that the A esterase locus is not included in the amplicon in strains displaying overproduced B1 esterase.

Despite these findings, restriction mapping of these strains with the A2 probe indicated that they all display the same allele at the A locus. A previous comparison of the A gene between the B1 strains undertaken by Guillerna et al. (1990) showed that sequences of a highly variable intron of the A esterase locus were similar between the EDIT and TEM-R strains. Given
that the A esterase locus is highly variable in natural populations (Pasteur et al., 1981b; Guillermaud et al., 1998; Raymond et al., 1996; Parker, 1996), this observation indicates that the B1 amplicon and the A esterase allele detected in B1 strains are in strong linkage disequilibrium. From the analysis of the structure of other couples of overproduced esterases, we can hypothesize that this genetic linkage disequilibrium is due to a close physical proximity. The maps of A and B esterase areas obtained from TEM-R are not incompatible with the presence of one B gene at 20 kb apart from the A gene, the two maps sharing the two restriction enzymes BamHI and HpaI in their extremity (Fig. 3).

This schema could explain the presence of one of the two supernumerary bands μ and ν systematically found in hybridization of EcoRI digested DNA of strains or individual mosquitoes displaying B1 gene with the B1 probe (Qi, 1995). It could correspond to the digestion of the area where the B and A esterase genes are close together, whereas the published restriction map of B1 (Raymond et al., 1991) corresponds to the amplicon of B esterase genes without the A esterase structural gene. Fig. 4 summarizes the structure of amplification in strains where A and B genes are co-amplified and in strains where only the B esterase gene is amplified. The model clearly shows the functional amplicon, i.e. the DNA fragment originally duplicated, and the apparent amplicon, i.e. the DNA fragment surrounding the probe used. Thus the original breakpoint of amplification is not necessarily at the edge of the (apparent) amplicon established by RFLP studies. Further variation in amplification level could take place by, for example, unequal crossing-over at various places in the (apparent and functional) amplicons.

It is worthy of note that our estimate of the B1 amplification level in TEM-R is very much lower than that of Mouchel et al. (1988) or Raymond et al. (1991), who found respectively amplification levels of 250 and 150-fold. These differences could be the result of a change in mean copy number of B1 in the TEM-R strain or to the difference in the methods used. Although Raymond et al. (1993) did not find any statistically significant decrease in resistance in a strain displaying A2-B2 amplification (SELAX88) between May 1990 and October 1991 and raised without insecticide selection, it remains possible that a decrease in resistance took place during this 1-year period for a strain displaying B1 (TEM-938). Indeed, the resistance ratio at LCN (lethal concentration at which 50% of the mosquitoes die) for temephos decreased from 1280-fold to 64-fold for TEM-938 and from 32- to 19-fold for SELAX88 (Raymond et al., 1993) between 1990 and 1991, although this last change was marginally not statistically significant. Moreover, they showed that when a strain was not homozygous for esterase gene amplification, the resistance was rapidly lost probably.

Figure 4. Schematic structures of amplicons displaying A and B esterase genes (such as A2-B2, A4-B4 and A5-B5) or B esterase gene only, inferred from restriction maps of the study and results of Qi (1996). Model 1 occurs when the limits of the amplicon (here represented as vertical arrows) encompass both esterase genes. When one of the limits lies between both loci, only one gene is subsequently amplified and is represented by model 2. Circles, triangles, stars and squares represent variation in the flanking DNA regions of the two loci. The single copy B1 esterase region in model 2 is supposed to correspond to the initial cluster of esterase genes in B1 strain. This region, displaying a flanking area distinct from that of the B1 amplicon may be responsible for additional bands in B esterase hybridization such as μ and ν bands found in Qi et al. (1996). The amplicon constructed using probes for Esterase A and Esterase B genes (apparent amplicon) does not necessarily correspond to the functional amplicon. See text for explanations.

because of the fitness cost related to enzyme over-production. Given that homoyzogosity for amplification level has never been checked in any strains, this result may be more general, since differences in esterase gene copy number can produce differences in individual fitnesses. Therefore lower levels of amplification could be selected for in a strain homoyzogous for the presence of the amplification (but displaying variable amplification levels) in the absence of insecticide treatment. It is also known that using autoradiography to estimate amplification level, as was done by Mouchés et al. (1988) or Raymond et al. (1989), leads generally to an overestimation. If we consider the southern blot of Acc digested DNA (Fig. 2), optical density measures would have given amplification levels of about 64-fold for SELAX (versus 15-fold obtained from the β-imager), 128-fold for CVPRUS (versus 15-fold) and 8-fold for VIM (versus 2.5-fold). This reduction in amplification level possibly due to an improvement of the detection method may be compared with the sphid Myzus persicae, where the amplification level of esterase E4 in highly resistant clone was estimated by autoradiography around 64 X (Field et al., 1998), whereas only ten to twelve copies were present as revealed by phospho-imager and pulsed field gel electrophoresis analysis (Field et al., 1998).

Evolution of field resistance by enzyme overproduction in Culex pipiens.

From the work of Rooke et al. (1996), Vaughan et al. (1995), Xu et al. (1994) and the present study, it is now possible to have an estimate of the independent number of mutational events leading to OP resistance through esterase overproduction at detectable frequency in natural populations. The mutations are of two major classes, i.e. involving gene amplification or gene regulation. A1 esterase overproduction in mosquitoes from Southern France (Pastuer et al., 1981a) is the result of such an increased expression of A esterase gene lacking structural amplification (Rooker et al., 1996). Gene amplification in Culex mosquitoes involves events that have initially generated the dupli- cation of both the A and B esterase or only the B esterase locus. Each of the three couples of over-produced esterase, A2-B2, A4-B4 and A5-B5, must have resulted from the co-amplification of the two genes as a single event (Rooker et al., 1996; this study). In addition to B1, three different B esterase alleles (B6, B7 and possibly B8) are overproduced aore in mosquitoes (Xu et al., 1994; Vaughan et al., 1995), and at least for B1 and possibly for the others it arise from the single amplification of the B esterase gene. Therefore, since the 1980s, when OP treatments began against mosquitoes, only seven independent esterase gene amplifications and one mutation (sensu lato, i.e. not necessarily one nucleotide change) leading to A esterase overexpression have been reported in natural populations of Culex pipiens. Thus, given the geographical distributions and the frequencies of these resistance genes, it is obvious that the mutation leading to an efficient resistance gene corresponds to one of the most limiting factor in the evolution of resistance. It is likely that other overproduced esterases will be reported in the future, as some geographical areas are still not thoroughly studied. The actual figure of eight muta- tions events is mainly based on partial surveys of the Mediterranean area, Africa, North America, the Caribbbean and Asia. Moreover, infrequent descriptions of other overproduced esterases in single individuals from natural populations (e.g. Qi et al. (1995) or Callaghan (1989)) indicate that the range of mutations leading to overproduced esterases is larger than the number of mutations effectively selected in natural populations.

Experimental procedures

Mosquito strains

Mosquito strains of the Culex pipiens complex used were: MSE, a strain resistant to OP and carbamate insecticides and possessing an insensitive ACNE, collected in 1979 near Mon- tellier, Southern France (Raymond et al., 1986; Bourguet et al., 1986); S-LAB, a reference susceptible strain from California (Georgiou et al., 1986); TEM-R, an OP resistant strain from California (Georgiou & Pasteur, 1986), with amplified B7 esterase (Raymond et al., 1983); E/UJ/18, a strain collected in 1992 from Beijing, China, also with amplified B1 esterase (Giao & Raymond, 1989), E/D, a laboratory strain homoyzogous for B1 following single pair mating, derived from larvae collected in 1980 in California (Giao & Raymond, 1989); RICO, a natural population displaying B1 esterase, sampled in 1992 in San Juan (Puerto Rico) (Giao & Raymond, 1986); SELAX, the OP resistant reference strain with amplified A2 and B2, derived from a 1984 sample from California (Raymond et al., 1987); VIM, collected in 1964 in Montpelier, Southern France, with ampli- fied A4 and B4 esterases (Pons et al., 1992); BARRIO, a strain from Southern France without A1 esterase (Chevillon et al., 1995); and finally CYPRUS, derived from a strain incubated in Cyprus in 1967 with overproduced A5 and B5 esterases (Pons et al., 1992).

DNA analysis

Restriction mapping. Genomic DNA was extracted from a pool of 100 adults using the method of Raymond et al. (1989) and digested with one or two restriction enzymes (Accl, BssHII, BglII, EcoRI, EcoRV, HindIII, KpnI, PstI, SacI, SalI, XbaI, MphI and XhoI) in a total volume of 20 µl. Digested DNA was loaded onto 0.8% agarose gels, the fragments separated by electrophoresis and the whole transferred onto Nylon mem-
branes by Southern blotting using the method of Sambrook et al. (1989). The filters were prehybridized and hybridized at 65°C with the 32P-labeled 1.8 kb A2 PCR product (Guillenmaud et al., 1996), and washed at high stringency at 95°C (Sambrook et al., 1989). Following autoradiography, filters were stripped of radioactive signal and re-probed with the 1.3 kb B1 esterase cDNA probe of Mouchlis et al. (1996).

Estimation of esterase gene amplification levels. Genomic DNA was extracted according to the method of Sherwood et al. (1988) and diluted with water to a concentration of 10 ng/μl. The concentration of genomic DNA was assayed by using the Hoechst dye method.

To determine the presence of additional esterase copies, a PCR reaction was performed using the primers described by Mouchlis et al. (1996). After an initial denaturation step at 94°C for 2 min, the reaction mixture was subjected to 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min. The PCR products were visualized on a 1.5% agarose gel and stained with ethidium bromide. The amplified products were purified using the QIAquick gel extraction kit (Qiagen, Valencia, CA) and sequenced using the ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA). The sequences were aligned using the ClustalW program (Thompson et al., 1994) and analyzed for conserved motifs using the PROSITE database (Bairoch and Hochschild, 1994).

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References


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