Heredity **77** (1996) 555–561 *Received 19 January 1996*

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

SERENA ROOKER‡, THOMAS GUILLEMAUD, JEAN BERGÉ†, NICOLE PASTEUR, MICHEL RAYMOND*

Institut des Sciences de l'Evolution (UMR CNRS 5554), Laboratoire Génétique et Environnement, Université de Montpellier II (C.C. 065), F-34095 Montpellier cedex 05 and †Institut National de la Recherche Agronomique, Boulevard Meilland, B.P. 2078, F-06606 Antibes, France

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 (Oppenoorth, 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 (Georghiou *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 B*i* and A*i*, respectively, *i* representing an identifying number (Pasteur *et al.*, 1981b, 1984; Raymond *et al.*, 1989; Georghiou, 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,

*Correspondence.
‡Present address: Department of Molecular Genetics, National Heart and Lung Institute, Heart Science Centre, Harefield Hospital, Middlesex, U.K.

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-MESH), 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′-GCAACGGGGGTCGATTACTAC-3′ and 5′-ACTTCATTCGTTCCTGCTCCG-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 of each dNTP, 1.5 mM $MgCl_2$ 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, Pleasenton, 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 70°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 PelRR 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 (*Acc*I, *Bcl*I, *Bam*HI, *Bgl*II, *Eco*RI, *Eco*RV, *Hin*dIII, *Kpn*I, *Pst*I, *Sac*I, *Sal*I, *Xba*I and *Xho*I). 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 ^{32}P-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 Mouchès *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: *Acc*I 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 autoradiographies 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 MSE), 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 *Acc*I 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

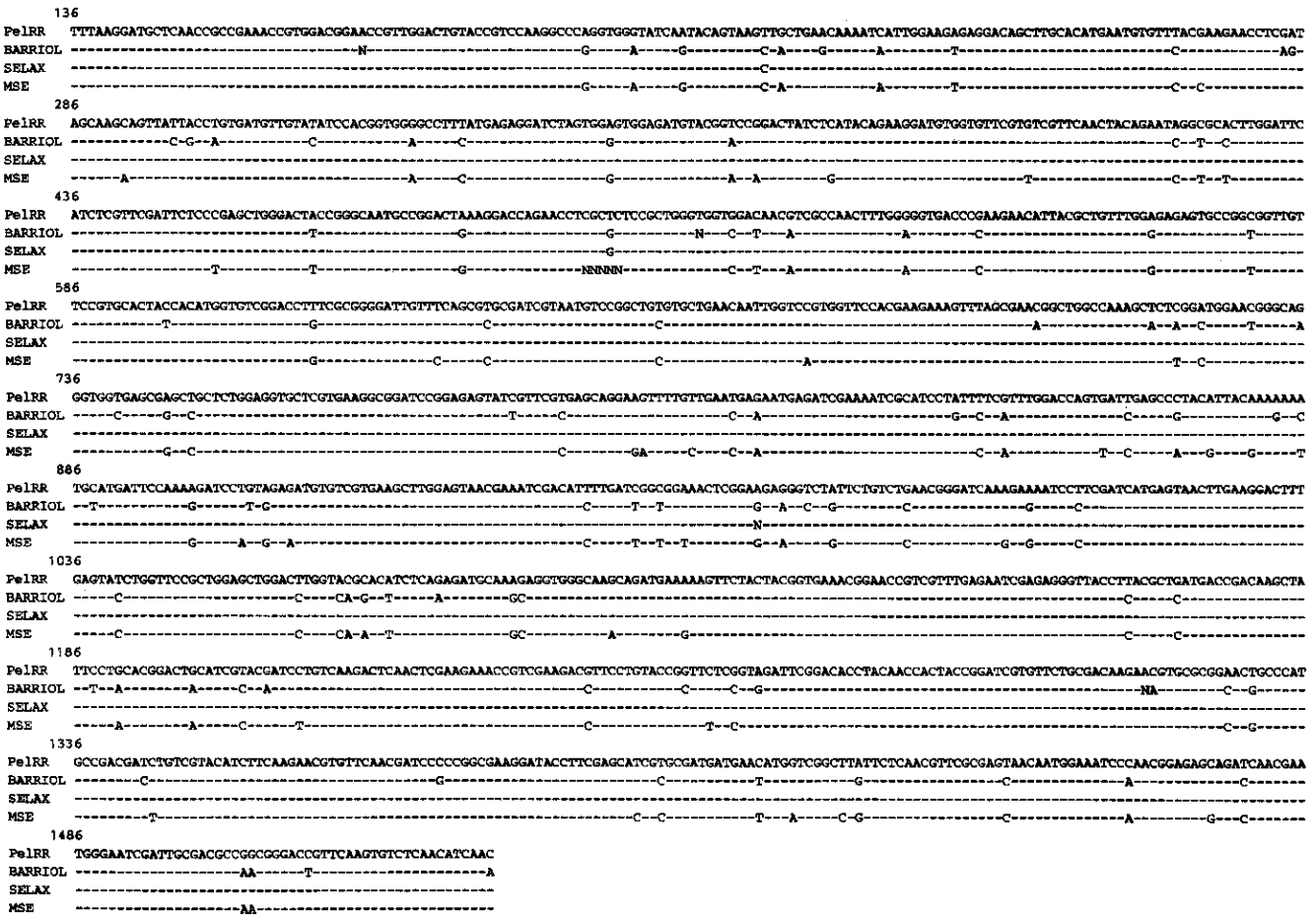

Fig. 1 Nucleic acid sequence alignment of esterase A genes from *Culex pipiens* mosquitoes of strains PelRR (Vaughan & Hemingway, 1995), SELAX, BARRIOL and MSE (accession numbers U43544, U43545, U43546 in Genbank database). The numbering corresponds to that of the PelRR A2 esterase sequence of Vaughan & Hemingway (1995).

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 (Mouchès *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 coamplified 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 disequilibirum 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.

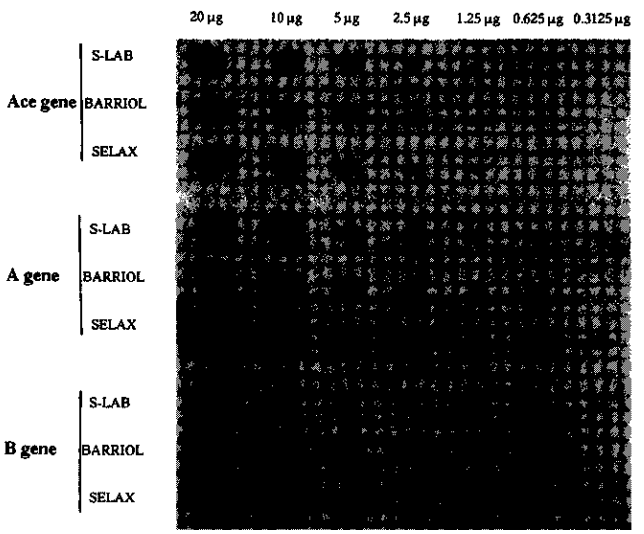

Fig. 2 Example of dot-blots of serial dilutions of genomic DNA hybridized with the 0.7 kb acetylcholinesterase probe (Ace gene), the 1.52 kb esterase A2 PCR product (A gene) and the 1.3 kb esterase B1 cDNA probe (B gene) (see text for details) showing differences in level of gene amplification between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

Relation between amplification and overproduction

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

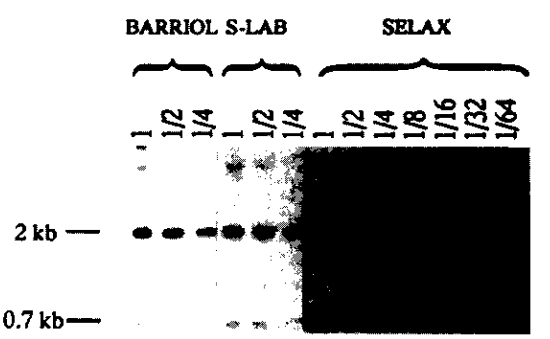

Fig. 3 Southern blot of serially diluted *Acc*I-digested genomic DNA from BARRIOL, SELAX and S-LAB *Culex pipiens* strains hybridized with the 1.52 kb A2 PCR product.

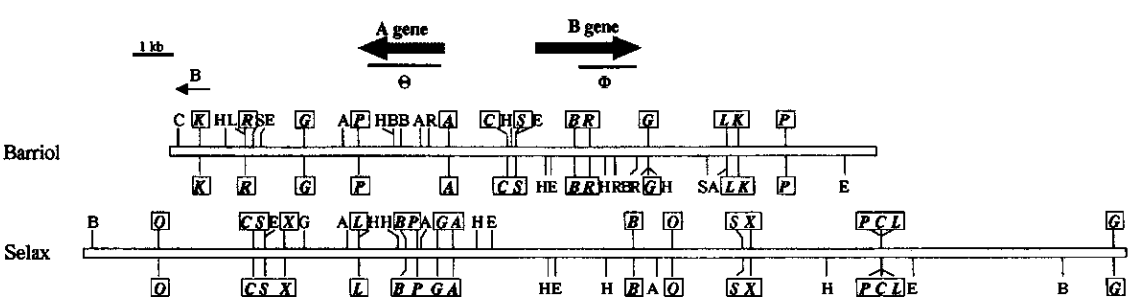

Fig. 4 Restriction enzyme maps showing the position of the esterase A gene in relation to esterase B in SELAX and BARRIOL *Culex pipiens* strains. Θ refers to the 1520 bp PCR product of esterase A2 from SELAX and Φ refers to the 1.3 kb cDNA of esterase B1. The lengths of esterases A and B and of probes Θ and Φ take into account the length of introns (details not shown). For each map, the restriction sites detected by A esterase are indicated above and those detected by B esterase are below. For each enzyme (A: *Acc*I, C: *Bcl*I, B: *Bam*HI, G: *Bgl*II, E: *Eco*RI, R: *Eco*RV, H: *Hin*dIII, K: *Kpn*I, P: *Pst*I, S: *Sac*I, L: *Sal*I, X: *Xba*I and O: *Xho*I), 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.

Poirié *et al.*, 1992) are correlated with overproduction of the enzymes. In the case of A esterases, large increases in activity have been reported (Curtis & Pasteur, 1981; Pasteur *et al.*, 1981b; Poirié *et al.*, 1992) and overproduction was shown to occur but few precise quantitative studies have been undertaken (Mouchès *et al.*, 1987). Here we show that two strains displaying overproduced A esterases possess variable levels of amplified A esterase gene relative to the susceptible S-LAB strain. The A2 gene is amplified 20–30 fold and the A1 gene twofold at most. This emphasizes the need to investigate the part of amplification and regulation that can be attributed to the overproduction in different cases where increased esterase A activity has been reported (e.g. A4, A5 and A6). Alternatively, increased esterase activity could result from (at least in part) different kinetics of the esterases involved.

Acknowledgments

We thank C. Bernard and G. Pistre for technical assistance, and P. Fort, D. Bourguet and J. Lagnel for helpful comments and discussions. This work was financed in part by a GDR 1105 du programme Environnement, Vie & Sociétés du CNRS and a CEE grant (no. ERBCHRXCT930172). S.R. benefitted from a CEE fellowship and T.G. from a MESR fellowship (no. 94137). This is contribution ISEM 96.114 of the Institut des Sciences de l'Evolution (UMR CNRS 5554).

References

BOURGUET, D., CAPELA, R. AND RAYMOND, M. 1996. An insensitive acetylcholinesterase in *Culex pipiens* L. mosquitoes from Portugal. *J. Econ. Entomol.* (in press).

CHEVILLON, C., PASTEUR, N., MARQUINE, M., HEYSE, D. AND RAYMOND, M. 1995. Population structure and dynamics of selected genes in the mosquito *Culex pipiens*. *Evolution*, **49**, 997–1007.

CURTIS, C. F. AND PASTEUR, N. 1981. Organophosphate resistance in vector populations of *Culex pipiens* L. (Diptera: Culicidae). *Bull. ent. Res.*, **71**, 153–161.

FIELD, L. M., DEVONSHIRE, A. L. AND FORDE, B. G. 1988. Molecular evidence that insecticide resistance in peach–potato aphids (*Myzus persicae* Sulz.) results from amplification of an esterase gene. *Biochem. J.*, **251**, 309–312.

GEORGHIOU, G. P. 1992. World distribution of esterases involved in organophosphate insecticide resistance in *Culex* mosquitoes, and methods for detection. In: Otto, D. and Weber, B. (eds) *Insecticides: Mechanism of Action and Resistance*, pp. 407–408. Intercept, Andover.

GEORGHIOU, G. P., METCALF, R. L. AND GIDDEN, F. E. 1966. Carbamate resistance in mosquitoes: selection of *Culex pipiens fatigans* Wied for resistance to Baygon. *Bull. W. H. O.*, **35**, 691–708.

GEORGHIOU, G. P., PASTEUR, N. AND HAWLEY, M. K. 1980. Linkage relationships between organophosphate resistance and a highly active esterase-B in *Culex quinquefasciatus* from California. *J. Econ. Entomol.*, **73**, 301–305.

MOUCHÈS, C., MAGNIN, M., BERGÉ, J.-B., DE SILVESTRI, M., BEYSSAT, V., PASTEUR, N. AND GEORGHIOU, G. P. 1987. Overproduction of detoxifying esterases in organophosphate-resistant *Culex* mosquitoes and their presence in other insects. *Proc. Natl. Acad. Sci. U.S.A.*, **84**, 2113–2116.

MOUCHÈS, C., PASTEUR, N., BERGÉ, J.-B., HYRIEN, O., RAYMOND, M., ROBERT DE SAINT VINCENT, B. *ET AL.* 1986. Amplification of an esterase gene is responsible for insecticide resistance in a California *Culex* mosquito. *Science*, **233**, 778–780.

MOUCHÈS, C., PAUPLIN, Y., AGARWAL, M., LEMIEUX, L., HERZOG, M., ABANDON, M. *ET AL.* 1990. Characterization of amplification core and esterase B1 gene responsible for insecticide resistance in *Culex*. *Proc. Natl. Acad. Sci. U.S.A.*, **87**, 2574–2578.

OPPENOORTH, F. J. 1985. Biochemistry and genetics of insecticide resistance. In: Kerkut, G. A. and Gilbert, L. I. (eds) *Comprehensive Insect Physiology, Biochemistry and Pharmacology*, pp. 731–773. Pergamon Press, Oxford.

PASTEUR, N., ISEKI, A. AND GEORGHIOU, G. P. 1981a. Genetic and biochemical studies of the highly active esterases A′ and B associated with organophosphate resistance in mosquitoes of the *Culex pipiens* complex. *Biochem. Genet.*, **19**, 909–919.

PASTEUR, N., SINÈGRE, G. AND GABINAUD, A. 1981b. *Est-2* and *Est-3* polymorphism in *Culex pipiens* L. from southern France in relation to organophosphate resistance. *Biochem. Genet.*, **19**, 499–508.

PASTEUR, N., GEORGHIOU, G. P. AND ISEKI, A. 1984. Variation in organophosphate resistance and esterase activity in *Culex quinquefasciatus* Say from California. *Génét. Sél. Évol.*, **16**, 271–284.

POIRIÉ, M., RAYMOND, M. AND PASTEUR, N. 1992. Identification of two distinct amplifications of the esterase B locus in *Culex pipiens* L. mosquitoes from Mediterranean countries. *Biochem. Genet.*, **30**, 13–26.

QIAO, C.-L. AND RAYMOND, M. 1995. The same esterase B1 haplotype is amplified in insecticide-resistant mosquitoes of the *Culex pipiens* complex from the Americas and China. *Heredity*, **74**, 339–345.

RAYMOND, M. AND MARQUINE, M. 1994. Evolution of insecticide resistance in *Culex pipiens* populations: the Corsican paradox. *J. Evol. Biol.*, **7**, 315–337.

RAYMOND, M. AND PASTEUR, N. 1995. Evolution of insecticide resistance in the mosquito *Culex pipiens*: the migration hypothesis of amplified esterase genes. In: Brown, T. M. (ed.) *Molecular Genetics and Ecology of Pesticide Resistance*. American Chemical Society, Washington,

DC (in press).

RAYMOND, M., FOURNIER, D., BRIDE, J.-M., CUANY, A., BERGÉ, J., MAGNIN, M. AND PASTEUR, N. 1986. Identification of resistance mechanisms in *Culex pipiens* (Diptera: Culicidae) from southern France: insensitive acetylcholinesterase and detoxifying oxidases. *J. Econ. Entomol.*, **79**, 1452–1458.

RAYMOND, M., PASTEUR, N., GEORGHIOU, G. P., MELLON, R. B., WIRTH, M. C. AND HAWLEY, M. K. 1987. Detoxification esterases new to California, USA, in organophosphate-resistant *Culex quinquefasciatus* (Diptera: Culicidae). *J. Med. Entomol.*, **24**, 24–27.

RAYMOND, M., BEYSSAT-ARNAOUTY, V., SIVASUBRAMANIAN, N., MOUCHÈS, C., GEORGHIOU, G. P. AND PASTEUR, N. 1989. Amplification of various esterase B's responsible for organophosphate resistance in *Culex* mosquitoes. *Biochem. Genet.*, **27**, 417–423.

RAYMOND, M., CALLAGHAN, A., FORT, P. AND PASTEUR, N. 1991. Worldwide migration of amplified insecticide resistance genes in mosquitoes. *Nature*, **350**, 151–153.

RAYMOND, M., MARQUINE, M. AND PASTEUR, N. 1992. Role of mutation and migration in the evolution of insecticide resistance in the mosquito *Culex pipiens*. In: Denholm, I., Devonshire, A. L. and Hollomon, D. W. (eds) *Resistance '91: Achievements and Developments in Combating Pesticide Resistance,* pp. 19–27. Elsevier Applied Science, London.

RAYMOND, M., QIAO, C. L. AND CALLAGHAN, A. 1996. Esterase polymorphism in insecticide susceptible populations of the mosquito *Culex pipiens. Genet. Res.*, **67**, 19–26.

ROOKER, S. 1994. *Analysis of Insect Serine Esterase Genes from Medically and Agriculturally Important Pest Species.* Ph. D. Thesis, University of London.

ROUSSET, F., BOUCHON, D., PINTUREAU, B., JUCHAULT, P. AND SOLIGNAC, M. 1992. *Wolbachia* endosymbionts responsible for various alterations of sexuality in arthropods. *Proc. R. Soc. B*, **250**, 91–98.

SAMBROOK, J., FRITSCH, E. F. AND MANIATIS, T. 1989. *Molecular Cloning: a Laboratory Manual.* Cold Spring Harbor Laboratory Press, New York.

VAUGHAN, A. AND HEMINGWAY, J. 1995. Mosquito carboxylesterase Est$\alpha 2_1$ (A_2). *J. Biol. Chem.*, **270**, 17044–17049.

VAUGHAN, A., RODRIGUEZ, M. AND HEMINGWAY, J. 1995. The independent gene amplification of electrophoretically indistinguishable B esterases from the insecticide-resistant mosquito *Culex quinquefasciatus. Biochem. J.*, **305**, 651–658.

XU, J., QU, F. AND LIU, W. 1994. Diversity of amplified esterase B genes responsible for organophosphate resistance in *Culex quinquefasciatus* from China. *J. Med. Coll. PLA*, **9**, 20–23.