Biochemical Genetics, Vol. 34, Nos. 9/10, 1996

Duplication of the *Ace.1* Locus in *Culex pipiens* Mosquitoes from the Caribbean

Denis Bourguet,[1,5] Michel Raymond,[1] Juan Bisset,[2] Nicole Pasteur,[1] and Martine Arpagaus[3,4]

Received 25 Apr. 1996—Final 4 June 1996

In Culex pipiens *mosquitoes, AChE1 encoded by the locus* Ace.1 *is the target of organophosphorus and carbamate insecticides. In several resistant strains homozygous for* Ace.1^{RR}, *insensitive AChE1 is exclusively found. An unusual situation occurs in two Caribbean resistant strains where each mosquito, at each generation, displays a mixture of sensitive and insensitive AChE1. These mosquitoes are not heterozygotes,* Ace.1^{RS}, *as preimaginal mortalities cannot account for the lethality of both homozygous classes. This situation is best explained by the existence of two* Ace.1 *loci, coding, respectively, a sensitive and an insensitive AChE1. Thus, we suggest that in the Caribbean a duplication of the* Ace.1 *locus occurred before the appearance of insecticide resistance at one of the two copies.*

KEY WORDS: acetylcholinesterase; gene duplication; *Culex pipiens;* insecticide resistance.

INTRODUCTION

Acetylcholinesterase (AChE; EC 3.1.1.7) is a key enzyme of cholinergic synapses, where it hydrolyzes the neurotransmitter. This enzyme is the target of organophosphorus (OP) and carbamate (CX) insecticides (Smissaert, 1964), and intensive pest controls have selected resistance due to less

[1] Institut des Sciences de l'Evolution, Laboratoire Génétique et Environnement (CC 065), UMR 5554, Université Montpellier II, F-34095 Montpellier, Cedex 05, France.
[2] Instituto de Medicina Tropical Pedro Kouri, Havana City, Cuba.
[3] INRA, Différenciation cellulaire et croissance, 2 Place Viala, 34060 Montpellier Cedex 01, France.
[4] INRA, Laboratoire de biologie des invertébrés, Boulevard Francis Meilland, 06606 Antibes Cedex, France.
[5] To whom correspondence should be addressed.

0006-2928/96/1000-0351$09.50/0

sensitive AChEs in more than 25 insect species (Fournier and Mutéro, 1994). In *Drosophila,* decreased sensitivity of AChE to insecticides results from a single or a combination of point mutations in the *Ace* gene (Mutéro *et al.,* 1994). A similar mechanism occurs in other insect species (Fournier, personal communication) and may be the cause of all resistance due to less sensitive AChEs.

In the mosquito *Culex pipiens,* an unusual situation occurs since two AChEs, AChE1 and AChE2, differing in substrate specificity and inhibitor sensitivity, have recently been characterized (Bourguet *et al.,* 1996a). These two enzymes are probably encoded by two genes, *Ace.1* and *Ace.2,* although different tissue-specific posttranscriptional modifications or alternative splicing of a single *Ace* gene cannot presently be ruled out. Only AChE1 is involved in the hydrolysis of acetylcholine at synapses, whereas the physiological function of AChE2 is not yet known. Insecticide resistance is due to a modification of AChE1, which becomes less sensitive to inhibitors, and not of AChE2 (Bourguet *et al.,* 1996a). Recently, a test has been described to determine the *Ace.1* genotypes (i.e., $Ace.1^{RR}$, $Ace.1^{RS}$, $Ace.1^{SS}$) in a single mosquito (Bourguet *et al.,* 1996b).

Mosquitoes from homozygous resistant strains are all $Ace.1^{RR}$ and therefore contain exclusively insensitive AChE1 enzyme. In this paper, we describe an unusual situation where mosquitoes from two homozygous resistant strains from Caribbean islands possess a mixture of sensitive and insensitive AChE1 protein. We show that this situation is best explained by a duplication of the *Ace.1* locus.

MATERIALS AND METHODS

Mosquito Strains

Four strains of mosquitoes were used: S-LAB, a susceptible reference strain isolated by Georghiou *et al.* (1966), and three strains resistant to OP and CX insecticides: (1) MSE, collected in Southern France in 1979 (Raymond *et al.,* 1986; Bourguet *et al.,* 1996c), (2) MRES, collected in Cuba in 1987 (Bisset *et al.,* 1990), and (3) MARTINIQUE, derived from a natural population collected in Martinique in 1994, which was mass-selected during 15 generations by exposing fourth-instar larvae to propoxur (a carbamate insecticide) at concentrations that induced 90% mortality.

AChE Extraction and Ultracentrifugation

About 100 fourth-instar larvae were mass-homogenized in 500 μl of a low-salt Triton (LST) buffer containing 10^{-2} *M* Tris–HCl, pH 7.0, 0.1 mg/ml

bacitracin (Sigma), 25 U/ml aprotinin (Sigma), 5×10^{-3} *M* EDTA, and 0.5% Triton X-100 (Sigma). Homogenates were centrifuged twice for 15 min at 12,000*g* and supernatants were used for ultracentrifugation. Ultracentrifugations were performed in 5–20% sucrose gradients in low-salt buffer (as above) containing 150 m*M* NaCl and 0.5% Triton X-100 (LST gradients). Three hundred microliters of supernatant was loaded on each gradient and centrifuged at 200,000*g* for 18 hr at 4°C in a SW41 rotor. Forty fractions were collected from the bottom of each gradient and assayed for AChE activity. Fractions with the highest AChE activity were recovered, used for electrophoresis, and assayed for AChE activities under different conditions.

Nondenaturing Electrophoresis

Nondenaturing electrophoreses were performed in the LKB Multiphor II horizontal electrophoresis unit using 7.5% polyacrylamide gels (2 mm thick) as described previously (Arpagaus and Toutant, 1985). Gels and running buffers contained 50 m*M* Tris–glycine (pH 8.9) and 0.5% Triton X-100. Gels were preelectrophoresed for 1 hr before loading 40 μl of recovered fractions of each strain. At the end of the migration (3 to 4 hr at 10 V/cm at 20°C), the gels were rinsed extensively in distilled water and the AChE activity was revealed following the method of Karnovsky and Roots (1964) in the absence or in the presence of 5×10^{-4} *M* propoxur.

AChE1 Purification

To analyze the kinetic properties of AChE1, AChE was purified from heads of adult mosquitoes, which contain less than 5% AChE2 (Bourguet *et al.*, 1996a). For each strain, ≈1000 heads were mass-homogenized in 20 ml extraction buffer (20 m*M* Tris, pH 7.0, containing 0.1% Triton X-100) with a glass pestle. Homogenates were centrifuged at 10,000*g* for 5 min. Supernatants were loaded on affinity chromatography columns containing procainamide (Sigma), a specific ligand of the AChE active site (Pasteur *et al.*, 1996). Columns were washed with 20 m*M* Tris–HCl, pH 9.5, 0.1% Triton X-100, and the bound enzyme was eluted with 50 m*M* Tris–HCl, pH 9.5, 0.4% Chaps (Sigma), 2.5 m*M* decamethonium (Sigma). The eluted fractions were dialyzed three times during 2 hr against the extraction buffer. The resulting purified AChE was used for kinetic analyses of AChE1.

Inhibitor Sensitivity

Inhibitor sensitivity was estimated by calculating the bimolecular velocity constant (k_i) following the dilution method of Aldridge (1950). Briefly,

AChE was incubated with the inhibitor for various times before tipping these inhibited mixtures into a substrate–reagent solution [final concentrations: 1.7 m*M* DTNB (Sigma) and 5 m*M* acetylthiocholine (Sigma)]. The plot of the ln of residual activity (A_i/A_o) against time for a given inhibitor concentration is a linear function when only one type of AChE is present in the reaction. The slope of the line divided by the inhibitor concentration gives the k_i. For each strain, the k_i was estimated for five inhibitors: two OPs in their oxon forms (malaoxon and paraoxon) and three carbamates (eserine, propoxur, and aldicarb). For each compound, the k_i was estimated at three insecticide concentrations.

AChE Activity and Sensitivity in Single Adult Mosquitoes

In single individuals, the relative activities of sensitive AChE1, insensitive AChE1, and AChE2 can be estimated by testing two discriminating concentrations of propoxur (Bourguet *et al.,* 1996b). Thus, (1) at 10^{-4} *M* propoxur, sensitive AChE1 is fully inhibited, whereas insensitive AChE1 and AChE2 are not affected; and (2) at 10^{-2} *M* propoxur, AChE2 is inhibited, whereas insensitive AChE1 is still not affected. Single adult mosquitoes were homogenized in 0.25 *M* phosphate buffer containing 1% Triton X-100 and centrifuged for 5 min at 10,000*g*. Supernatants were used to estimate AChE activities (according to Ellman *et al.,* 1961) both without insecticide (A_0) and in the presence of 10^{-4} *M* (A_1) or 10^{-2} *M* (A_2) propoxur. For each individual, the relative proportion of sensitive AChE1 activity was given by $1 - (A_1/A_0)$, of AChE2 activity by $(A_1 - A_2)/A_0$, and of insensitive AChE1 activity by A_2/A_0. For each strain, the relative proportion of each AChE activity in a single mosquito was estimated from 60 individuals for three generations.

Mortality During Preimaginal Development

Mortality of MRES and MARTINIQUE mosquitoes was recorded at different developmental stages. Hatching (HM), larval (LM), emergence (EM), and developmental (DM) mortalities were measured by the following ratios: $HM = 1 - (N_L/N_E)$, $LM = 1 - (N_P/N_L)$, $EM = 1 - (N_A/N_P)$, and $DM = 1 - (N_A/N_E)$, where N_E corresponds to the number of eggs, N_L to the number of larvae hatching from these eggs, N_P to the number of pupae issued from these larvae, and N_A to the number of adults emerging from these pupae. N_E was, respectively, 2833 and 1745 for the MRES and MARTINIQUE strains.

RESULTS

Electrophoretic Characterization of AChEs

AChE activity was recovered as a single peak, sedimenting at 7S, after separation of LST extracts of the strains S-LAB, MSE, MARTINIQUE, and MRES on sucrose gradients. Fractions corresponding to the top of this peak were then submitted to nondenaturing polyacrylamide electrophoresis. As observed previously in the absence of insecticide three bands, labeled 1, 2, and 4, with AChE activity were revealed in each strain (Fig. 1A): band 1 corresponds to AChE1, and bands 2 and 4 to AChE2 (Bourguet *et al.*, 1996a). Bands 1 and 2 are glycolipid-anchored amphiphilic dimers and band 4 is the hydrophilic dimer that is the lytic counterpart of band 2 (Bourguet *et al.*, 1996a). It should be noted that, in the present experiments, band 3, the lytic counterpart of band 1, was not detected. In the presence of 5×10^{-4} *M* propoxur, AChE2 bands (2 and 4) are unaffected in all strains (Fig. 1B). AChE1 (band 1) is totally inhibited at this concentration in S-LAB and unaffected in MSE mosquitoes, which agrees with the fact that these two strains are, respectively, susceptible and resistant homozygous strains (Raymond *et al.*, 1986; Bourguet *et al.*, 1996c). In MARTINIQUE and MRES, AChE1 is only partially inhibited at 5×10^{-4} *M* propoxur (Fig. 1B).

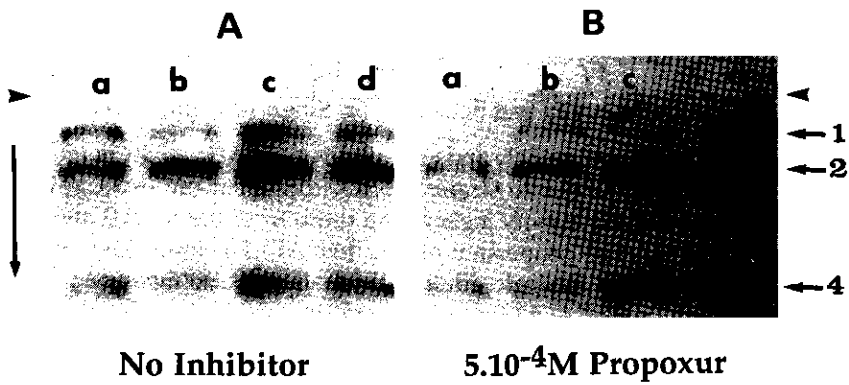

Fig. 1. Nondenaturing electrophoresis of susceptible and resistant strains. Two portions of the gel were stained with acetylthiocholine as substrate in the absence of inhibitor (A) or in the presence of 5.10^{-4} *M* propoxur (B). Lanes a, b, c, and d: peak fractions of the preparative gradients of S-LAB, MSE, MRES, and MARTINIQUE, respectively. Arrowheads: origin of migration. Band 1 corresponds to the amphiphilic dimer of AChE1. Bands 2 and 4 correspond, respectively, to amphiphilic and hydrophilic dimers of AChE2. AChE1 is totally inhibited in S-LAB, partially inhibited in MARTINIQUE and MRES, and not inhibited in MSE.

Inhibitor Sensitivity of AChE1s

The intermediate sensitivity to propoxur of AChE1 in the two Caribbean strains could be due to the presence of a different, less sensitive AChE1 allele. To test this hypothesis, the sensitivity of AChE1 to inhibitors was compared in the different strains. For each strain, the k_i of AChE1 was measured for three CXs (eserine, propoxur, and aldicarb) and two OPs in their oxon forms (malaoxon and paraoxon; Table I). The inhibition constant k_i was lower in the resistant strain MSE than in the susceptible strain S-LAB, indicating that insecticides bind less efficiently to the insensitive AChE1. Under our hypothesis, the k_i of the insensitive AChE1s of MARTINIQUE and MRES strains was expected to be different from that of MSE. For example, the partial inhibition observed with 5×10^{-4} M propoxur was expected to correspond to a higher k_i.

Plots of the *ln* of residual AChE1 activity against time for a given propoxur concentration were not linear (Fig. 2). The inhibition curves show two components, corresponding to the mixture of two AChE1 with different k_i values (i.e., of different propoxur sensitivity). This was confirmed when other inhibitors were tested (details not shown). One AChE1 possesses a k_i similar to that of the AChE1 of S-LAB, and the other AChE1 has a k_i similar to that of AChE1 of MSE (Table I).

This suggests that the two Caribbean strains possess a mixture of two AChE1 enzymes: one sensitive and similar to that found in S-LAB, the other insensitive and similar to that of MSE. This ruled out the possibility that Caribbean strains bear a different insensitive AChE1 allele.

AChE Activity and Sensitivity in Single Mosquitoes

We then analyzed qualitatively and quantitatively AChE activity in individuals of the different mosquito strains. Mosquitoes from the two Caribbean strains have an AChE activity higher than those from the S-LAB and MSE strains (Table II). The proportion of sensitive AChE1, insensitive AChE1, and AChE2 activities in single adult individuals was also measured (Table II). The proportion of AChE2 was low and similar in all strains (less than 10%). At each of the three generations considered, each of the 60 individuals tested from the MARTINIQUE and MRES strains contained approximately 60% sensitive AChE1 and 30% insensitive AChE1. The presence of the two AChE1 forms in each insect may be explained if, at each successive generation, all tested mosquitoes were heterozygotes. This implies that homozygotes of both types (i.e., for sensitive and insensitive AChE1s) did not reach the adult stage. To determine whether this could be the case, mortality rates were recorded during the whole developmental process (from egg to adult).

Table I. Inhibition Constant (k_i) of Sensitive (sAChE1) and Insensitive (iAChE1) AChE1s (±SE) Present in the Mosquito Strains Studied (Units: $mM^{-1} \cdot min^{-1}$)

			MRES		MARTINIQUE	
	S-LAB, sAChE1	MSE, iAChE1	sAChE1	iAChE1	sAChE1	iAChE1
Eserine	15,000 ± 426	61 ± 5	13,500 ± 1500	52 ± 3	18,000 ± 845	57 ± 9
Propoxur	145 ± 5.3	0.0005 ± 6.10^{-5}	132 ± 8.3	0.0007 ± 9.10^{-5}	151 ± 16	0.0007 ± 6.10^{-5}
Malaoxon	1.2 ± 0.03	0.18 ± 0.03	1.7 ± 0.5	0.16 ± 0.06	1.6 ± 0.09	0.18 ± 0.05
Paraoxon	159 ± 3	0.37 ± 0.08	148 ± 23	0.33 ± 0.15	136 ± 12	0.28 ± 0.08
Aldicarb	6.04 ± 0.13	0.58 ± 0.09	6.2 ± 0.12	0.78 ± 0.11	6.4 ± 0.23	0.59 ± 0.13

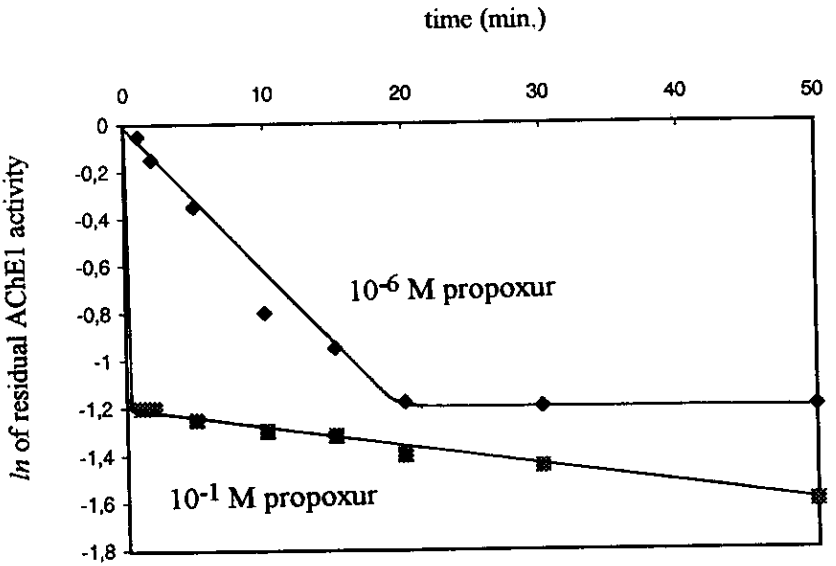

Fig. 2. Plots of the remaining AChE activity against the inhibition period from MRES at two propoxur concentrations.

Preimaginal Mortality

The mortality was measured during one generation from egg to adult (Table III). Mortality in MARTINIQUE and MRES did not exceed 15% at each preimaginal stage. Developmental mortality (from the egg to the adult) was not different between the two strains (Fisher exact test, $P = 0.37$) and below 25% (23% for MRES and 24% for MARTINIQUE). This is significantly lower than the expected 50% mortality necessary to explain the absence of both homozygote classes ($\chi^2 = 812$, df = 1, $P < 0.0001$, for MRES and $\chi^2 = 457$, df = 1, $P < 0.0001$, for MARTINIQUE).

Table II. Single Mosquito AChE Activity (±SE) and Relative Proportion of Sensitive AChE1 (sAChE1), Insensitive AChE1 (iAChE1), and AChE2 Activity in Adults of the Different Mosquito Strains

	AChE activity (mDo/min/adult)	AChE activity proportion		
		sAChE1	iAChE1	AChE2
S-LAB	249.8 ± 9.7	0.962 ± 0.002	—	0.038 ± 0.002
MSE	122.9 ± 2.6	—	0.909 ± 0.004	0.085 ± 0.016
MRES	318.5 ± 14.7	0.632 ± 0.014	0.291 ± 0.012	0.077 ± 0.005
MARTINIQUE	287.9 ± 15.2	0.616 ± 0.021	0.313 ± 0.018	0.071 ± 0.004

Table III. Mortality Rate at Different Stages During the Preimaginal Developmental Process in MRES and MARTINIQUE[a]

	MRES	MARTINIQUE
Hatching mortality (N_E)	0.07 (2833)	0.14 (1745)
Larval mortality (N_L)	0.02 (2627)	0.04 (1500)
Emergence mortality (N_P)	0.15 (2565)	0.08 (1434)
Developmental mortality (N_A)	0.23 (2175)	0.24 (1319)

[a] N_E, N_L, N_P, and N_A correspond, respectively, to the number of eggs, larvae, pupae, and adults used for mortality rate estimations.

DISCUSSION

As shown previously, sensitive and insensitive AChE1 in the two homozygous strains S-LAB and MSE are alleles of the same gene (Bourguet *et al.*, 1996a). All MSE mosquitoes are *Ace.1*^{RR} and display only insensitive AChE1, whereas S-LAB mosquitoes are *Ace.1*^{SS} and possess only sensitive AChE1 (Table II).

In contrast, each Caribbean mosquito from Martinique and Cuba has a sensitive and an insensitive AChE1 (Table II). We showed that the absence of mosquitoes similar to S-LAB and MSE cannot be explained by the lethality of homozygotes since the developmental mortality in MARTINIQUE and MRES (from the egg to the adult) is significantly lower than the required (50%) level (Table III). Furthermore, the level of preimaginal mortality observed in the MARTINIQUE and MRES strains is not higher than usually found for other *C. pipiens* strains. For example, the hatching mortality in other *Culex pipiens* strains is about 10% (Irving-Bell, 1983; Magnin *et al.*, 1987; Guillemaud, personal communication), which is close to the hatching mortalities found in the two Caribbean strains. Finally, the preimaginal mortalities found in MARTINIQUE and MRES are similar to those of other *Culex* species reared under the same conditions (Mottram *et al.*, 1994).

Thus, the AChE1 phenotype of Caribbean mosquitoes cannot be explained by a heterozygosity at the *Ace.1* locus (*Ace.1*^{RS}). We suggest that MRES and MARTINIQUE strains carry a duplication of the *Ace.1* locus and that only one copy encodes an insensitive AChE1. This hypothesis is supported by the following points. First, both insensitive and sensitive AChE1s have the same molecular forms, electrophoretic mobilities, and sedimentation coefficients (Fig. 1). Second, the overall AChE activity observed in the Caribbean mosquitoes is significantly higher than that in either the S-LAB or the MSE strain (Table II). A similar conclusion was drawn from a study of a resistant strain of the leafhopper *Nephotettix cincticeps,*

where all individuals displayed both sensitive and insensitive AChE (Hama, 1980; Oppenoorth, 1985). As Hama found no evidence for zygote mortality, he suggested that the strain carried a duplication of the *Ace* gene, one of the two copies being modified and thus insensitive.

Because insensitive AChE1s of MRES and MARTINIQUE have the same catalytic properties (Table I), they are probably identically modified. In *Drosophila,* AChE insensitivity is conferred by point mutations (Mutéro *et al.,* 1994) and each mutation or combination of them confers specific kinetic properties. An identical feature occurs in other insect species (Fournier, personal communication) and possibly also in *Culex pipiens.* Thus, the insensitive AChE1s of MRES and MARTINIQUE probably share the same mutation or combination of mutations and would correspond to the same allele.

These biochemical data allow some hypotheses on the historical events which occurred in Caribbean mosquito populations. It is likely that the duplication of the *Ace.1* locus took place before the modification(s) leading to an insensitive AChE: if not, insensitive alleles would have been carried by the two copies. Furthermore, pest management (using OPs) against *Culex pipiens* in Martinique and Cuba began, respectively, 4 and 14 years ago (Bisset *et al.,* 1990; Yébakima *et al.,* 1995). Thus, selection for AChE insensitivity is a very recent feature, and as a consequence, modifications are likely to have been selected after the duplication event.

Finally, the question arises whether duplication and modification of *Ace.1* in the two Caribbean islands correspond to a unique event that has been spread from island to island. A unique origin for mutation(s) ensuring insecticide resistance and subsequently spread by migration has already been described (e.g., Raymond and Marquine, 1994; ffrench-Constant *et al.,* 1993; Qiao and Raymond, 1995; Guillemaud *et al.,* 1996). All these studies are based on DNA sequence or restriction profile comparisons. As *Ace.1* probes are not yet available, this question cannot be investigated at the moment.

ACKNOWLEDGMENTS

We are grateful to C. Bernard, M. Marquine, and G. Pistre for technical assistance, to A. Roig for the electrophoresis illustration, to A. Yébakima for providing Martinique Mosquitoes, and to J.-P. Toutant for helpful comments and discussions. This work was financed in part by the GDR 1105 du Programme Environnement Vie et Société du CNRS, by the Institut National de la Recherche Agronomique, by ENIGMA (CEE grant No. ERBCHRXCT930172), by Acc-SV3 (No. 9503037), and by a MESR fellowship to D.B. (No. 93082). This is contribution 96-054 of the Institut des Sciences de l'Evolution (UMR 5554).

REFERENCES

Aldridge, W. N. (1950). Some properties of specific cholinesterase with particular reference to the mechanism of inhibition by diethyl *p*-nitrophenyl thiphosphate (E605) and analogues. *Biochem. J.* **46**:451.

Arpagaus, M., and Toutant, J.-P. (1985). Polymorphism of acetylcholinesterase in adult *Pieris brassicae* heads. Evidence for detergent-insensitive and Triton X-100-interacting forms. *Neurochem. Int.* **7**:793.

Bisset, J., Rodriguez, M., Diaz, C., Ortiz, E., Marquetti, M. C., and Hemingway, J. (1990). The mechanisms of organophosphate and carbamate resistance in *Culex quinquefasciatus* (Diptera: Culicidae) from Cuba. *Bull. Entomol. Res.* **80**:245.

Bourguet, D., Raymond, M., Fournier, D., Malcolm, C. A., Toutant, J.-P. and Arpagaus, M. (1996a). Existence of two acetylcholinesterases in the mosquito *Culex pipiens* (Diptera: Culicidae). *J. Neurochem.* **67**:2115.

Bourguet, D., Pasteur, N., Bisset, J., and Raymond, M. (1996b). Determination of *Ace.1* genotypes in single mosquitoes: toward an ecumenical biochemical test *Pest. Biochem. Physiol.* **55** (in press).

Bourguet, D., Capela, R., and Raymond, M. (1996c). An insensitive acetylcholinesterase in *Culex pipiens* L. mosquitoes from Portugal. *J. Econ. Entomol.* **89** (in press).

Ellman, G. L., Courtney, K. D., Andres, V., and Featherstone, R. M. (1961). A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem. Pharmacol.* **7**:88.

ffrench-Constant, R. H., Rocheleau, T. A., Steichen, J. C., and Chalmers, A. E. (1993). A point mutation in a *Drosophila* GABA receptor confers insecticide resistance. *Nature* **363**:449.

Fournier, D., and Mutéro, A. (1994). Modification of acetylcholinesterase as a mechanism of resistance to insecticides. *Comp. Biochem. Physiol.* **108**:19.

Georghiou, G. P., Metcalf, R. L., and Gidden, F. E. (1966). Carbamate resistance in mosquitoes: selection of *Culex fatigans* Wied. for resistance to Baygon. *Bull. WHO* **35**:691.

Guillemaud, T., Rooker, S., Pasteur, N., and Raymond, M. (1996). Testing the unique amplification and the worldwide migration hypothesis of insecticide resistance genes with sequence data. *Heredity* (in press).

Hama, H. (1980). Mechanism of insecticide resistance in green rice leafhopper and small brown planthopper. *Rev. Plant Prot. Res.* **13**:54.

Irving-Bell, R. J. (1983). Cytoplasmic incompatibility within and between *Culex molestus* and *Cx. quinquefasciatus* (Diptera: Culicidae). *J. Med. Entomol.* **20**:44.

Karnovsky, M. J., and Roots, L. J. (1964). A "direct-coloring" thiocholine method for cholinesterases. *J. Histochem. Cytochem.* **12**:219.

Magnin, M., Pasteur, N., and Raymond, M. (1987). Multiple incompatibilities within populations of *Culex pipiens* L. in southern France. *Genetica* **74**:125.

Mutéro, A., Pralavorio, M., Bride, J.-M., and Fournier, D. (1994). Resistance-associated point mutations in insecticide-insensitive acetylcholinesterase. *Proc. Natl. Acad. Sci. USA* **91**: 5922.

Mottram, P., Kay, B. H., and Fanning, I. D. (1994). Development and survival of *Culex sitiens* Wiedemann (Diptera: Culicidae) in relation to temperature and salinity. *J. Aust. Entomol. Soc.* **33**:81.

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, Vol. 12,* Pergamon, New York, p. 731.

Pasteur, N., Raymond, M., Rousset, F., Bergé, J.-B., Amichot, M., Pauron, D., Cuany, A., and Fournier, D. (1996). Cloning and detection of insecticide resistance genes. In Crampton, J. M., Beard, C. B., and Louis, K. (eds.), *The Molecular Biology of Insect Disease Vectors: Method Manual,* World Health Organization, Chapman. London pp. 399–419.

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.

Raymond, M., and Marquine, M. (1994). Evolution of insecticide resistance in *Culex pipiens* populations: The Corsican paradox. *J. Evol. Biol.* **7**:315.