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A New Esterase Gene Amplification Involved in OP Resistance in *Culex pipiens* Mosquitoes from China

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*Two overproduced esterases (A8 and B8) not previously described were found in southern China. They provide a low resistance level to organophosphate (OP) insecticides, and correspond to a coamplification of both esterase loci (*Est-2 *and* Est-3*) classically involved in OP resistance for this mosquito species. This coamplification is distinct from all other similar events thus far reported. The peculiar situation in southern China, where numerous OP resistance alleles at these two loci were found, is discussed in comparison with the Mediterranean situation, the only one with a similar diversity of overproduced esterases.*

KEY WORDS: insecticide resistance; adaptation; organophosphate; overproduction; mosquito.

INTRODUCTION

In the house mosquito *Culex pipiens,* increased detoxification by esterase overproduction is a frequent mechanism of resistance to organophosphorus (OP) insecticides. Two esterase loci are involved, *Est-2* and *Est-3,* and various overproduced allozymes have been described at these loci. These overproduced esterases have been named according to their esterase locus (A for *Est-3,* B for *Est-2*) and to their order of discovery or report in the literature: A1, A2, etc., and B1, B2, etc. In susceptible populations, these two esterase loci are highly polymorphic, and only nonoverproduced allozymes are present (Raymond *et al.,* 1996).

Esterase A1 was first described in southern France and is now found in the whole Mediterranean region (Pasteur *et al.,* 1981; Villani *et al.,* 1982, 1986; Severini *et al.,* 1993; Chevillon *et al.,* 1995). Esterase B1 was first discovered in

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California and has subsequently been found in North and South America and in China (Qiao and Raymond, 1995). Some overproduced esterases are always found in association, such as A2 and B2 (indicated as A2–B2), A4–B4, and A5–B5. The first pair is found on a worldwide basis (Africa, North and South America, Europe, Middle East, India, Southeast Asia), due to extensive recent migration facilitated by their advantage in insecticide-treated areas (Curtis and Pasteur, 1981; Villani *et al.,* 1986; Raymond *et al.,* 1987, 1991; Marchi and Addis, 1990; Rivet *et al.,* 1993). The other two pairs are located in the Mediterranean area, although substantial changes in their range and frequency are currently taking place (Poirié *et al.,* 1992; Ben Cheikh and Pasteur, 1993; Chevillon *et al.,* 1995; Severini *et al.,* 1997; Guillemaud *et al.,* 1998).

Esterase overproduction is the result of either gene regulation, as for A1 (Rooker *et al.,* 1996), or gene amplification, for all the others (Mouchès *et al.,* 1986; Raymond *et al.,* 1989; Poirié *et al.,* 1992; Vaughan and Hemingway, 1995; Guillemaud *et al.,* 1997). The consistent association of the paired esterases (A2–B2, A4–B4, and A5–B5) is the result of a coamplification of the *Est-2* and *Est-3* loci (Rooker *et al.,* 1996; Guillemaud *et al.,* 1997).

Few studies have been performed in China. A resistant strain from Beijing collected in 1992 showed that the B1 allele was present (Qiao and Raymond, 1995). A strain collected in 1992 from Foshan (near Guangzhou or Canton) contained B1, the pair A2–B2, and an additional overproduced esterase named B6 (Xu *et al.,* 1994). Another strain collected from Chengdu (Sichuan area) in 1992 displayed another overproduced esterase named B7 (Xu *et al.,* 1994). Here we describe a pair of overproduced esterases involved in OP resistance, isolated in a strain from Guangzhou. We show that they are coamplified, distinct from all previously described overproduced alleles, including the Chinese B6 and B7, and thus correspond to an independent amplification event. These new overproduced esterases are called A8–B8. The apparent high diversity of overproduced esterases in the *Culex pipiens* complex from China is discussed in the context of the current knowledge of their evolution.

MATERIALS AND METHODS

Mosquitoes

Three strains were used: S-Lab and Beijing [which are OP susceptible and lack high-activity esterases (Georghiou *et al.,* 1966; Dong Yan, 1990)] and MAO. The MAO strain was derived from a hypogeous natural population sampled in September 1994 in the suburbs of Guangzhou (southern China). After 1 year of mass rearing, the frequency of the new pair of overproduced esterase (later designated A8 and B8) was increased during four generations by allowing only females displaying A8–B8 to reproduce. Then single families were set up, and the

MAO strain was derived from two of these families in which both parents displayed A8–B8. The strain was not homozygous for the presence of A8–B8, however, 1 year later more than 87% ($N = 81$) displayed the overproduced A8–B8 esterases.

Insecticide Bioassays

Resistance characteristics of the MAO strain were compared by performing bioassays on fourth-instar larvae, following the method of Raymond and Marquine (1994). Five insecticides were used in ethanol solutions: chlorpyrifos (Dow Chemical, Midland, MI), temephos (American Cyanamid, Princeton, NJ), propoxur (Bayer, Leverkusen, Germany), dichlorvos (Qing Dao Insecticide Factory, China), and parathion (Qing Dao Insecticide Factory, China). The action of one synergist, DEF (*S,S,S*-tributyl phophorotrithioate; Interchim), an inhibitor of esterases and glutathione-*S*-transferases, was investigated by exposing larvae to a standard dose (0.08 mg/L) 4 hr before the addition of the insecticide solution. In each test, sets of 20 larvae were exposed to different insecticide doses for 24 hr. Results were analyzed using the probit analysis program of Ratsira *et al* (1993) based on Finney (1971). Resistance ratios (RR) were determined by dividing the LC_{50} of the resistant strain by the LC_{50} of the susceptible S-LAB reference strain. Synergism ratios (SR) were computed by dividing the LC_{50} for insecticide alone by the LC_{50} for insecticide plus synergist. To test whether a synergist was more efficient in the resistant than in the susceptible strain, the relative synergism ratios (RSR), described by Prabhaker *et al.* (1988), were compared. RSR is equal to the RR for insecticide alone divided by the RR for insecticide plus synergist. A RSR >1 indicates that there is a stronger effect of the synergist in the resistant than in the susceptible strain, i.e., that the detoxifying mechanism synergized is enhanced in the resistant strain; a RSR <1 shows that the two strains compared are not different as far as the synergist is concerned. When the hypothesis of parallelism between two dose–mortality lines could not be rejected, the confidence limits of RR and SR were computed (Ratsira *et al.*, 1993).

Detection of Highly Active Esterases

Esterases were identified in single individual homogenates analyzed by starch electrophoresis using TEM 7.4 buffer systems and revealed according to Pasteur *et al.* (1988). Overproduced esterases from reference strains were run as controls: A1 [strain Barriol (Chevillon *et al.*, 1995)], A2–B2 [strain Selax (Wirth *et al.*, 1990)], B1 [strain Edit (Qiao and Raymond, 1995)], A4–B4, and A5–B5 [strains Vim and Cyprus (Poirié *et al.*, 1992)].

DNA Analysis

Genomic DNA was extracted from pools of mosquitoes from different strains using the method of Raymond *et al.* (1989) and digested with one (or two) of the six restriction enzymes (*Bam*HI, *Bgl*2, *Eco*RI, *Eco*RV, *Hin*dIII, *Pst*I), in a total volume of 20 µl. Digested DNA was loaded onto 0.8% agarose gels: the fragments separated by electrophoresis were transferred onto Nylon membranes by Southern blotting using the method of Sambrook *et al.* (1989). The filters were prehybridized, hybridized at 65°C with the ^{32}P-labeled 1.8-kb A2 PCR product (Guillemaud *et al.*, 1996), and washed at a high stringency at 65°C according to Sambrook *et al.* (1989). Following autoradiography, filters were stripped of radioactive signal and reprobed with the ^{32}P-labeled 1.3-kb B1 (Mouchès *et al.*, 1990). A restriction map was built independently for each probe.

RESULTS

Esterase Pattern of the MAO Strain

Starch gel electrophoresis disclosed that all MAO individuals possess two esterases, designated A8 and B8, of high activity, each with a distinct migration from all other described overproduced esterases. A8 preferentially hydrolyzes α-naphthyl acetate; B8, β-naphthyl acetate (Fig. 1).

DNA Analysis

Restriction maps of the genomic region hybridizing with esterase A and esterase B probes were built for the strain MAO after digesting genomic DNA with restriction enzymes, and probing the membranes first with the 1.3-kb cDNA of esterase B and then with the DNA fragment of esterase A2 (1.8 kb). For all restriction enzymes cutting far outside the esterase A gene, DNA fragments were of the same size when hybridized with the esterase A or esterase B probe, 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. 2). Esterases A and B were found to be separated by a DNA fragment of 3.5 kb, which is within the range of sizes found in other strains displaying a coamplification (see Fig. 2 for examples). The two esterase genes were in an inverted position, with the two 5′ ends in close proximity, as always found in other strains (Fig. 2).

The intensity of hybridization of the restriction fragments in MAO mosquitoes was much stronger than in S-LAB (for the same quantity of DNA in each strain), indicating that they contain a larger number of esterase A and B gene copies than the susceptible insects. The copy number was not precisely estimated

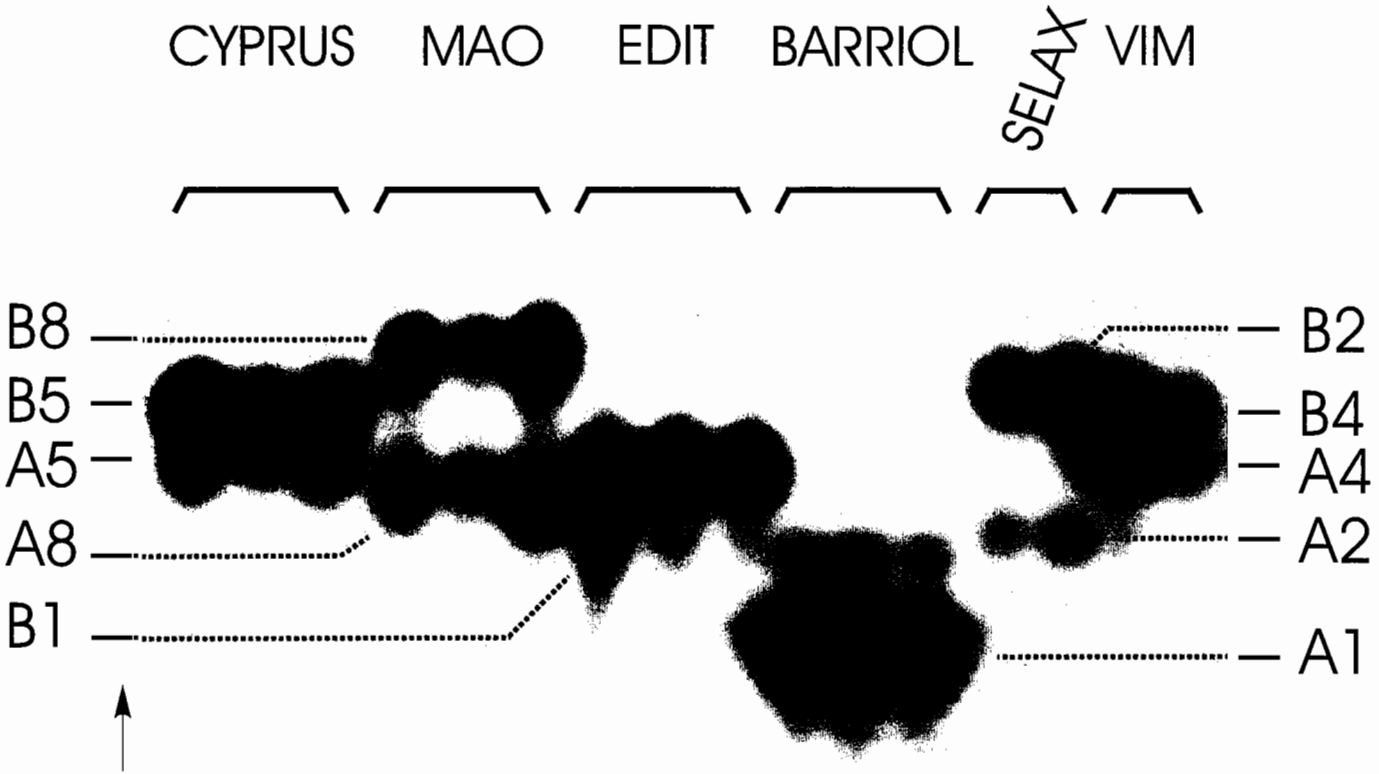

Fig. 1. High-activity esterases of single adults after starch gel electrophoresis. From left to right, strains Cyprus (esterases A5 and B5), MAO (esterases A8 and B8), Edit (esterase B1), Barriol (esterase A1), Selax (esterases A2 and B2), and Vim (esterases A4 and B4). Each esterase is labeled. The arrow indicates the electrophoretic migration.

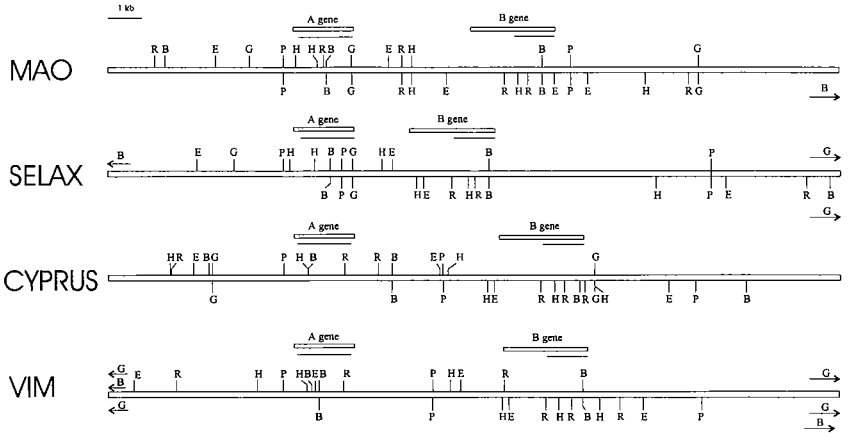

Fig. 2. Restriction maps of *A* and *B esterase* genes of the MAO strain, compared to the Selax, Cyprus, and Vim maps. In each case, the restriction sites detected by the *A esterase* probe are indicated above, and those detected by the *B esterase* probe are below. The position of A and B probes are indicated under the *A* and *B esterase* loci. B, *Bam*HI; G, *Bgl*II; E, *Eco*RI; R, *Eco*RV; H, *Hin*dIII; P, *Pst*I. The map of Selax (displaying esterases A2–B2) is adapted from Rooker *et al.* (1996), and the maps of Cyprus and Vim (displaying esterases A5–B5 and A4–B4, respectively) are adapted from Guillemaud *et al.* (1997).

but was obviously much lower than that of the amplification present in strains TEM-R (with amplified B1) or Cyprus (with amphified A5 and B5) (Guillemaud *et al.*, 1997).

Resistance Characteristics of the MAO Strain

Resistance of MAO mosquitoes toward OP insecticides was studied by bioassays, using the S-LAB (at the Montpellier laboratory) or Beijing (at the Chinese laboratory) strains as the susceptible controls (Table I). Dose–mortality curves were linear ($P > 0.05$) for the two strains with the insecticides studied, whether or not combined with DEF or PB synergists, indicating homogeneity of tolerance. The only exceptions were the mortality lines of MAO for chlorpyrifos or temephos plus PB, and S-LAB for chlorpyrifos plus PB, probably due to environmental variations.

MAO larvae displayed a small but significant ($P < 0.05$) resistance to all OP tested (resistance ratios RR = 4.65 for dichlorvos, RR = 2.08 for parathion, RR = 3.68 for temephos, and RR = 7.58 to chlorpyrifos). The addition of DEF to bioassays significantly decreased ($P < 0.05$) temephos and chlorpyrifos tolerance in MAO and S-LAB larvae. This decrease (or synergism ratio; SR) was higher in MAO (temephos, 8.68; chlorpyrifos, 5.74) than in S-LAB (temephos, 3.23; chlorpyrifos, 4.72), leading to relative synergism ratios RSR of 2.69 for temephos and 1.22 for chlorpyrifos. Thus, detoxification by esterase or GST is enhanced in

Table I. LC_{50} and Slope of the Mortality Lines Observed in Bioassays with Various Insecticides in MAO and S-LAB[a]

Insecticide	LC_{50} (range)	Slope (SE)	χ^2	df	H	RR (range)	SR
Dichlorvos							
Beijing	0.287 (0.255–0.325)	3.42 (0.37)	0.89	2	1	1	—
MAO	1.34 (1.21–1.48)	4.60 (0.59)	2.99	2	1	4.65 (3.68–5.89)*	—
Parathion							
Beijing	0.00089 (0.00079–0.00100)	2.45 (0.23)	2.54	3	1	1	—
MAO	0.00185 (0.00080–0.00457)	0.93 (0.18)	0.10	2	1	2.08 (1.62–2.69)*	—
Temephos							
S-LAB	0.00106 (0.00103–0.00110)	6.32 (0.53)	1.72	3	1	1	—
MAO	0.00391 (0.00371–0.00412)	4.22 (0.30)	3.78	4	1	3.68 (3.22–4.21)*	—
Temephos + DEF							
S-LAB	0.00033 (0.00031–0.00035)	5.43 (0.41)	2.0	1	1	1	3.23*
MAO	0.00045 (0.00042–0.00048)	5.89 (0.49)	1.23	2	1	1.37 (1.16–1.62)*	8.68*
Temephos + PB							
S-LAB	0.0120 (0.0114–0.0127)	9.37 (0.87)	0.35	2	1	1	0.09*
MAO	0.0268 (0.0208–0.0347)	2.24 (0.35)	27.0	5	5.4	2.24 (1.7–2.96)*	0.15*
Chlorpyrifos							
S-LAB	0.00076 (0.00074–0.00079)	11.4 (0.84)	1.67	2	1	1	—
MAO	0.00578 (0.00508–0.00657)	3.39 (0.33)	12.0	4	3.0	7.58 (6.15–9.34)*	—
Chlorpyrifos + DEF							
S-LAB	0.00016 (0.00015–0.00017)	6.21 (0.53)	0.66	1	1	1	4.72*
MAO	0.00101 (0.00092–0.00110)	4.61 (0.38)	0.35	2	1	6.24 (5.16–7.54)*	5.74*
Chlorpyrifos + PB							
S-LAB	0.00478 (0.00347–0.00659)	3.42 (0.96)	65	4	16	1	0.16*
MAO	0.0159 (0.0146–0.0173)	3.49 (0.20)	5.76	3		3.33 (2.21–5.02)*	0.36*

[a]LC_{50}, mg/L; H, heterogeneity factor. Ranges with 95% confidence.

*Different from 1 at the 5% confidence level.

MAO compared to S-LAB. However, the DEF synergism was not total in MAO, since its temephos or chlorpyrifos resistance ratio in the presence of the synergist was significantly ($P < 0.05$) different from 1 (RR = 1.37 and 6.24, respectively), indicating either differences in tolerance between the strains or the presence of another mechanism conferring low resistance. The addition of PB to bioassays significantly increased ($P < 0.05$) temephos and chlorpyrifos tolerance in MAO and S-LAB larvae. This increase in tolerance ratio was lower in MAO (temephos, SR = 0.15; chlorpyrifos, SR = 0.36) than in S-LAB (temephos, SR = 0.09; chlorpyrifos, SR = 0.16), leading to relative synergism ratios RSR of 1.66 for temephos and 2.25 for chlorpyrifos. It can be concluded that at least two OP resistance mechanisms are operating in MAO: esterase and oxidase detoxification.

DISCUSSION

Overproduction of new esterases, A8 and B8, is the result of a coamplification of *Est-2* and *Est-3* loci and is independent of all other similar coamplifications thus far described (A2–B2, A4–B4, and A5–B5). This is indicated by the restriction

map of the amplicon region encompassing both genes (indicating a coamplification) and by the distinct restriction map displayed by the MAO strain. As found in all other published cases (amplified or nonamplified strains), both esterase genes are in an inverted (head-to-head) position. Comparison with other coamplified esterase genes indicates that the intergenic distance between the two genes is variable. For the MAO strain, this distance (about 3.5 kb) is within the range currently observed (from about 2 up to 6 kb). The amplification level associated with the overproduction of A8–B8 in MAO has not been measured precisely, but it does not appear to be very high from the DNA data. This is consistent with the low level of resistance (<3-fold for the OP assayed) which is synergized by DEF. However, many OPs have been used in the field in southern China, and in the region of Guangzhou, where the MAO strain was collected, trichlorfon, malathion, temephos, phoxim, fenitrothion, dichlorvos, and fenthion have been used extensively against this mosquito (Xu *et al.,* 1994).

The number of overproduced esterases described in southern China is now quite large: B1, B6, B7, A2–B2, and A8–B8 (and all of them except B7 could be found near Guangzhou). This is reminiscent of the situation found in the Mediterranean. Both areas share the worldwide A2–B2, and both exhibit overproduced esterases not found elsewhere so far (A1, A4–B4, and A5–B5 for the Mediterranean and B6, B7, and A8–B8 for southern China). We know that the situation is evolving rapidly in the Mediterranean but we have relatively little information about the evolution in China.

The situation in southern China is probably the result of recent contact between relatively isolated OP treated areas. Within the near-future one or several of the existing alleles will probably be eliminated. However, A2–B2 is a good candidate as the "winning allele," as it has a universal geographic distribution (including southern China) and appears to show a particular ability to take over. However, it is not yet understood why this is so, and the observation of the southern China situation will be invaluable to follow the competition of A2–B2 against additional resistant alleles.

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