African Journal of Biotechnology Vol. 9(28), pp. 4309-4313, 12 July, 2010
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2010 Academic Journals

Full Length Research Paper

Partial cloning and estimation of the *Culex pipiens* (SELAX strain) B2 amplicon

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Accepted 27 October, 2009

The mechanism of resistance towards organophosphate insecticides in *Culex* complex mosquitoes has been shown to be associated with amplification of the structural esterase gene. The cloned portion of the SELAX strain A2-B2 amplicon was compared to that of the B1 amplicon which had been partially characterized. Fragments of known sizes from clones were used as probes to estimate the length of the B2 amplicon in the SELAX strain of mosquitoes. The two types of clones that were isolated and designated as N4 and N6, differed from each other by their size of inserts. Both clones hybridized with the 1.8 kb A2 probe and the 1.3 kb B1 probe; revealing that both N4 and N6 clones contained A2 and B2 esterase genes. Restriction enzyme digests of the clones suggested that the cloned portion of the B2 amplicon was 16 kb.

Key words: B2 gene, A2B2 esterase, insecticide resistance.

INTRODUCTION

Organophosphate resistance in *Culex pipiens* complex mosquitoes has been shown to be correlated with the presence of highly active non-specific A or B carboxylesterases (Yasutomi, 1970, Curtis and Pasteur, 1981, Villani et al., 1983). Both A and B esterases have been classified as carboxylesterases. In *Culex quinquefasciatus*, the highly active enzymes, ‘B’ esterases preferentially hydrolyses βNA; whereas in *Culex pipiens* the active enzyme ‘A’ esterase is observed only in the presence of EDTA and preferentialy hydrolyses αNA. These esterases can be distinguished by either appearing red or blue with hydrolysis to α- or β- naphthyl acetate substrates.

The SELAX strain of *C. quinquefasciatus* has been documented as being resistant towards organophosphate insecticides with the overproduced A2-B2 esterases for resistance. Field collections of the SELAX strain were carried out around the South East LAX airport in Los Angeles California hence the name, SELAX. This strain is thus considered to be the reference strain for A2-B2 amplification and has been reared in various laboratories.

In order to study and characterize the SELAX strain of the *C. pipiens* mosquito, a portion of its genome had to be cloned, which may however, contain part or the whole of its amplification unit; the amplicon or the B2 gene. A substantial amount of the work on esterase gene amplification and gene structure has focused on the B1 esterase gene for the Tem-R strain (Mouchés et al., 1986).The esterase B1 from the resistant Tem-R strain of *C. quinquefasciatus*, was first purified to homogeneity and was used to prepare antibodies that specifically immunoprecipitated the B esterase enzyme (Fourniér et al., 1987). Immunoassays using these antibodies have shown that the highly resistant *C. quinquefasciatus* contains at least 500-fold higher levels of the B1 esterase as compared to the susceptible strains (Mouchés et al., 1987). RNA from larvae was used to prepare cDNA which was inserted into the *EcoR* I site of the expression phage λ gt II and screened with a B1 antiserum. The recombinant phage that was immunoreactive with the B1 antiserum contained a 700 bp cDNA insert that was shown to be able to select by hybridization an mRNA that coded for esterase B1 (Mouchés et al., 1986). The studies identified a 2.1 kb fragment that strongly hybridized with the labeled B1 probe that was assumed to contain a portion of the B1 esterase gene. The probe

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however hybridized slightly with a 2.8 kb fragment of the susceptible S-Lab DNA.

Using the B1 clone as a probe, a 0.7 kb fragment of the B2 gene was cloned and sequenced (Raymond et al., 1991). The two genes showed a 96% nucleotide sequence homology as well as 97% homology in the amino acid sequence. Beyond this, there has been no closer study of the esterase amplifications especially in terms of the B2 esterase gene, its surrounding structures or the size of its amplification unit or amplicon.

The study aimed at cloning a portion of the B2 amplicon from the SELAX strain and to determine the size of the B2 repeat. From the cloned B2 DNA, probes from the 3' and 5' end of the B gene were generated in order to extend the B2 restriction map to give an estimate of the B2 amplicon size.

MATERIALS AND METHODS

Extraction and partial digests of genomic DNA

Genomic DNA was extracted from whole adult mosquitoes using the method described by Raymond et al. (1989). The concentration of DNA was quantified by measuring the absorbance at 260 nm using a spectrophotometer.

Partial digests of the mosquito genomic DNA of the SELAX strain were carried out using *Mun* I restriction enzyme; which was chosen because its sticky ends are compatible with the sticky ends of the *EcoR* I vector arms. Partial digestion of the genomic DNA was necessary in order to minimize the size range of the genomic fragments that had to be cloned. The digest which comprised of 25.0 µl of the insert DNA, 12.5 µl of 10 x reaction (at 1 x working strength), 87.0 µl sterile H_2O and 0.5 µl *Mun* I restriction enzyme, was set up in a total volume of 125 µl. At 5 min intervals, 31 µl was removed and the digest stopped by the addition of 5 µl of 0.5M EDTA. Ten microlitres of the products were run on 0.8% agarose gel, Southern blotted and hybridized to the cDNA 1.3 kb B1 probe.

The remaining digests were dephosphorylated to prevent self-ligation of fragments using Calf Intestine Alkaline Phosphate (CIAP) which was obtained from Sigma®. In a total volume of 50 µl, 5 µl of 10x CIAP buffer, 1 µl of CIAP enzyme and 44 µl of sterile water were added to the *Mun* I partially digested genomic DNA. The reaction mixture was incubated at 37℃ for 1 h after which the CIAP enzyme was inactivated by heating at 75℃ for 10 min after the addition of 2 µl of 5 mM EDTA. The reaction mixture was extracted once with phenol: chloroform and then twice with chloroform. The DNA was precipitated with 0.5 volume of 5 M ammonium acetate and 2 volumes of ethanol and kept at - 20℃ for 20 min after which it was centrifuged and the pellet resuspended in 25 µl of sterile water.

Preparation of Lambda dash II vector DNA

The Lambda Dash II Bacteriophage vector was obtained pre-digested as *EcoR* I arms, from Stratagene®. In this case the stuffer fragment had been removed by digest with *EcoR* I restriction enzyme and the arms were ready for direct cloning of the insert; the *Mun* I partially digested genomic DNA. However direct cloning did not require any desphophorylation of the vector arms.

Ligation, packaging and library amplification

Ligation of the Vector DNA to the insert DNA was carried out using the following mixture; 1 µl of λ DNA, 1.5 µl insert DNA, 0.5 µl 10x ligation buffer, 0.5 µl 10 mM rATP, 1.0 µl T4 ligase 4 U/µl and 0.5 µl sterile water. The mixture was left overnight at 4℃. The ligated material of the vector DNA and the partial genomic digests were packaged *in vitro* using the Gigapack®II Gold Packaging Extract from Stratagene. The general procedure for the library amplification was based on the Stratagene protocol. Aliquots of the packaged material were mixed with 600 µl of host cells OD_{600} = 0.5 in Falcon tubes. The tubes were incubated for 15 min at 37℃. Molten LB (Luria-Bertoni) top agarose, (6.5 ml) was then added to the above tubes and plated out on 150 mm plates that contained LB bottom agar. The dilutions carried out were in the ratio of 50: 600 (that is, 50 µl of the packaged material to 600 µl of the host cells). When the plaques were observed, the plates were overlaid with 10 ml SM (Phage Dilution Buffer) buffer to elute the DNA. The suspension was recovered from each of the plates, pooled into tubes, vortexed and centrifuged. The supernatant was decanted and 3% chloroform and 7% DMSO [Dimethyl Sulfoxide (Sigma)] in 1 ml volumes were added and stored.

Screening for positive clones

In order to isolate the positive recombinant clones that may contain the esterase B gene in the cloned DNA, series of screening and identification of positive clones by hybridizing with a labeled complementary probe was carried out. The 1 ml volumes of the suspension recovered from the plates were placed on 150 mm LB plates with 6.5 ml LB top agarose. The plates were incubated overnight at 30℃. Plates with well isolated plaques were transferred onto Biotrace NT nitrocellulose discs for plaque hybridization. Plaque transfer was carried out for 2 min with ink markings made on the agar for orientation. The filter membranes were subjected to denaturing and neutralization and dried and baked in an oven at 80℃ for 1 - 2 h. These filters were later prehybridised and hybridized with a cDNA 1.3 kb B1 probe (Mouches et al., 1986) at 65℃ overnight and thereafter washed and autoradiographed. Positive plaques that were identified on the radiography films by orientation to the master plates were removed and the λ DNA was extracted for the production of probes.

Production of probes from the cloned DNA

By choosing restriction enzyme sites upstream of the A2 gene and downstream of the B1 gene for appropriate sizes, double digests on 5 µg DNA of clones in a total volume of 40 µl were carried out to produce probes. The digest mix was incubated at 37℃ for 2 h after which 5 µl of Endo R stop was added and the tube spun briefly. The digest samples were run on a 1.5% agarose gel for 5 h at 42V; with a 1kb molecular DNA marker alongside. After a good separation of DNA bands, the required size bands were excised from the gel using a blunt scalpel and purified using siliconised glass wool. The DNA was quantified, radiolabelled and then used as probes to construct restriction maps of the clones.

RESULTS AND DISCUSSION

Library screening using the B1 probe produced 670 plaques. More than 100 were found to be positive after autoradiography and 30 positive clones were analysed. The clones were of different types with their differences being attributed to the different fragment sizes obtained for each of the clones when cut with certain restriction enzymes and hybridized to either a B1 or an A2 probe.

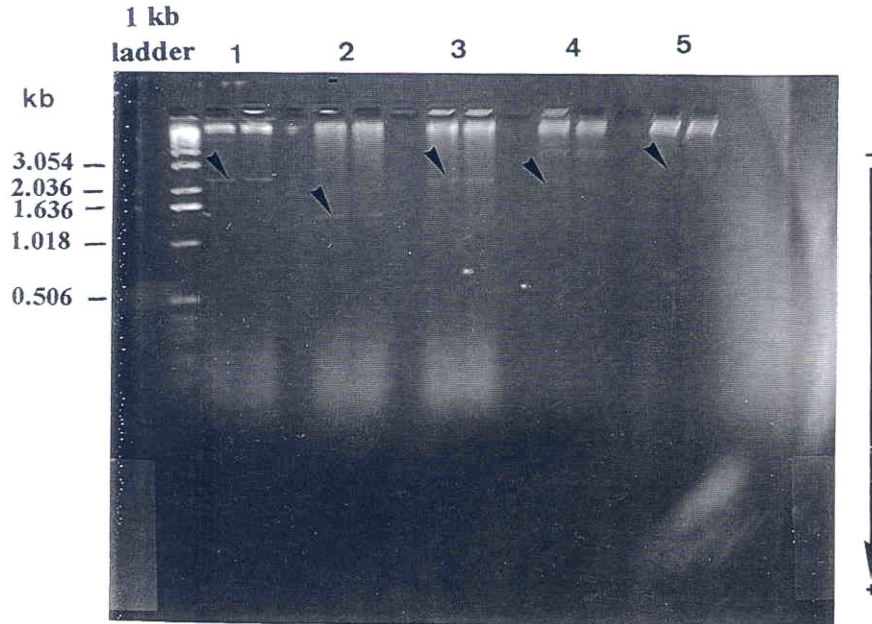

Figure 1A. 1.5% Agarose gel showing the separation of probes after digests of clones, N4 and N6 respectively. Numbers 1 - 5 represent probes generated by double digests 1: BH1 (Xho I/Sal I), 2: BH3 (Xho I/Sac I), 3: BH2 (Xho/Xba I) 4: AC1 (Bgl II/Sac I) and 5: AC2 (Sac I/Sal I). The arrows indicate the 2.2 and 1.4 kb fragment bands that were excised.

However, only two types of clones were isolated and were described as N4 and N6. The two clones differed from each other by the size of inserts and they both hybridized with the 1.8 kb A2 probe and the 1.3 kb B1 probe; revealing that both N4 and N6 clones contained A2 and B2 esterase genes.

Sections of the amplicon clones were excised using restriction enzymes in order to produce DNA probes of the flanking DNA. Several probes were generated from double digests with certain restriction enzymes. From the 3'-end of the B2 gene on the N4 clone, a 2.2 kb probe was generated and designated as BH1. This resulted from a double digest of clone N4 and N6 with the restrict-tion enzymes *Xho* I and *Xba* I. Another probe from the 3'-end of the same N4 and N6 clones that was 1.4 kb, was generated and named BH2. This was from a double digest of *Xho* I and *Sac* I (Figures 1A and B). Some of the generated probes using restriction enzymes on both N4 and N6 clones were BH3 (*Xho* I/*Sac* I), AC1 (*Bgl* II/*Sac* I), AC2 (*Sac* I/*Sal* I), J1 (*BamH* I/*Sac* I), J2 (*Acc* I/*Hind* III) and BJ1 (*Pst* I/*Xho* I).

Single and double digests of the clones and hybridization using the B1 and the A2 probe resulted in the construction of restriction maps which also showed the position of the *Mun* I sites within the clones (Figure 2). Hybridisation of the clones using these probes positioned the A2 esterase gene as being located upstream of the B2 gene. For clone N4, there were 2 *Mun* I sites whereas N6 had 3 *Mun I* sites. The fragment size for N4 clone was estimated to be 11.0 kb and that for N6 clone was 16.0 kb. The N6 restriction map extends further upstream of the A2 gene. The region around the two genes starting from the *Sal* I site upstream of the A2 gene to the *Xba* I site further downstream of the B2 gene were found to be the same.

Partial digests of SELAX DNA with the enzyme *Mun* I which had *EcoR* I compatible cohesive ends, produced amplicon fragments of 10 - 15 kb which were successfully cloned into the pre-digested *EcoR* I λ DASH vector arms. The large percentage of positive clones obtained after hybridization with the B1 probe in the screening indicated that the B2 gene was over-represented in the genomic library. Although a number of clones with different insert sizes were found, only the designated N4 and N6 clones that were analysed were found to be the largest. The N4 type clones had insert sizes of 11 kb and the N6 type clones had insert sizes of 16 kb. This suggested that the B2 amplicon may be identical at least up to 16 kb flanking the B2 gene. This reflects the findings of Mouchés et al. (1990), who calculated that the B1 genes were carried as single copies on a highly conserved 25 kb sequence. The sequence however represented the core of each amplification unit which in most cases was much longer, from 30 - 50 kb.

Hybridisation of the A2 PCR product to the clones demonstrated that both A2 and B2 were present in the clones. The A2 and the B1 probes hybridized to some of the same fragments, where the DNA fragment encom-

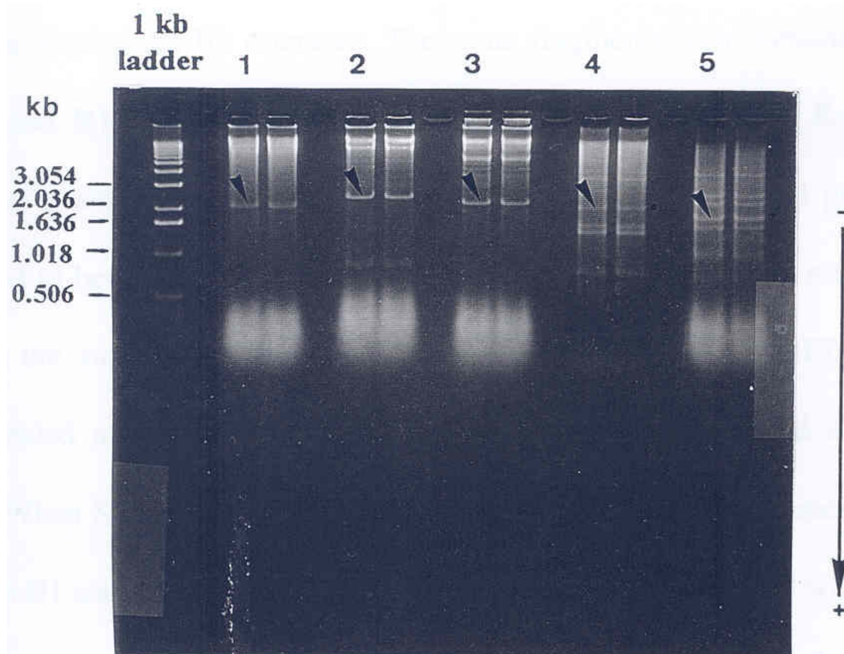

Figure 1B. 1.5% Agarose gel showing the separation of probes after digests of clones, N4 and N6 respectively. Numbers 1-5 represent probes generated by double digests 1: BH2 (Xho/Xba I) 2: AC2 (Sac I/Sal I) 3: J1 (BamH I/Sac I), 4: J2 (Acc I/Hind III) and BJ1 (Pst/Xho I). The arrows indicate the 2.2 kb and 1.4 kb fragment bands that were excised.

passed both genes, but for the most part picked up different fragment sizes for many of the enzymes. This proved that the A esterase gene did not pick up the B esterase gene sequences. This however was expected since the two esterases were known to be immunological and biochemically distinct (Mouchés et al., 1987; Fournier et al., 1987; Callaghan et al., 1991). Some previous study had also demonstrated that the B1 probe did not hybridise to the A esterase genes (Raymond et al., 1989).

The flanking DNA probes BH1 and AC1 were used, along with the A2 probe to extend the published amplicon restriction map using the *Culex* complex genomic DNA (Raymond et al., 1991). The map was extended to cover a total of 35 kb, which was 10-15 kb longer than had been estimated. The extent to which this represented a constant 'core' or a variable region outside of the 16 kb core sequence could not be estimated. It was conceivable that the B2 amplicon was less than 35 kb in size. Field et al., (1996) analyzed the esterase gene amplification in insecticide resistant *Myzus persicae* using pulsed-field gel electrophoresis. This revealed that the E4 genes were amplified 12-fold in a tandem array of direct repeats of about 24 kb. It is probable that some of the B1 amplicons are about the same size as the *Myzus* amplicons at only 30 kb in size (Mouchés et al., 1990, Callaghan, 1993) Therefore the 35 kb section mapped around the A2-B2 genes may have contained the B2 amplicon repeat.

The A2B2 amplification in SELAX should be at least 35 kb with a 16 - 32-fold amplification, that is, 600 - 2100 kb per chromosome in length. This compares to an estimated 30-50 kb and 250-fold amplification (7500 - 125000 kb per chromosome) of the B1 amplicon sequence in TermR (Mouchés et al., 1986, 1990, Raymond et al., 1989). The TemR B1 amplicon 'core' carries a single copy of the esterase gene as well as other flanking sequences and Juan elements (Mouchés et al., 1990). A study using PFGE (Pulse field Gel Electrophoresis) has shown that DNA fragments containing the B1 amplicon were up to 250 kb in size following complete digestion with restriction enzymes (Callaghan and Laias, 1995). It was thus concluded that the similarity of all the clones suggests that the B2 amplicon has a conserved core of at least 16 kb.

The presence of the Juan elements in B1 has raised speculation that they may be involved in the amplification process. The B2 clone covered the region of DNA containing the Juan element in the B1 allele. If then the Juan element was in the homologous region of the B2 amplicon, the BH1 fragment that was excised and used a probe should contain part of the Juan sequence.

Recent results from the cloning and sequencing of a 12 kb section of genomic DNA containing two gene loci coding for carboxylesterase alleles A5 and B5; revealed that there was the presence of putative Juan and trans-

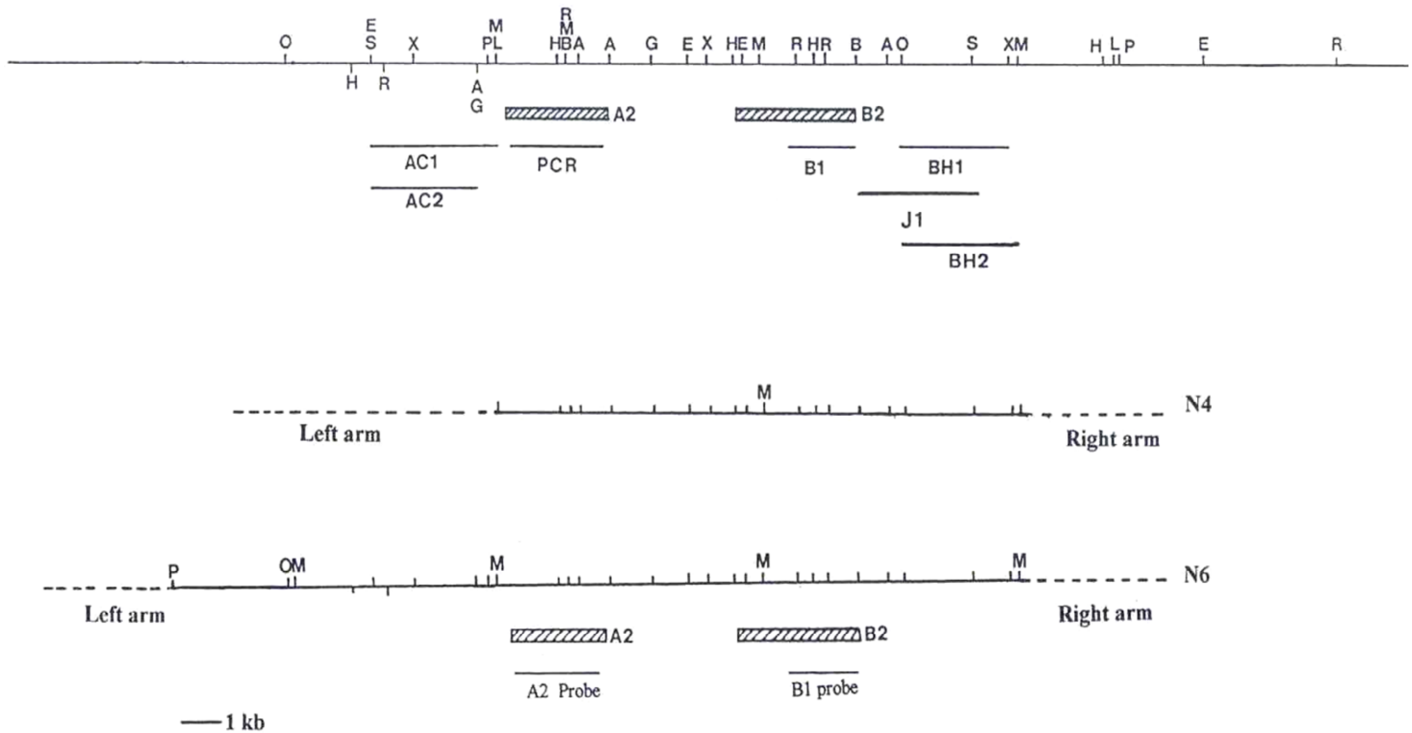

Figure 2. Map showing SELAX restriction genomic map above, in relation to the position of probes on the clone maps N4 and N6, below. M represents the *Mun* I sites on the clone maps.

posable elements upstream of B5 (Buss and Callaghan, 2004). A5-B5 is however restricted to the Mediterranean and is responsible for resistance towards the kill of insecticides. It can thus be concluded that the presence of these structures in the amplification core of the alleles B1, B2 and B5 can be speculated as also playing a role in the amplification of the resistant gene.

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