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(54) Title: MALATHION CARBOXYLESTERASE

(57) **Abrégé/Abstract:**

The present invention provides an isolated DNA molecule encoding Malathion Carboxylesterase capable of hydrolysing at least one organophosphate selected from the group consisting of carboxylester organophosphates and dimethyloxon organophosphates. The DNA molecule comprises a nucleotide sequence having at least 60 %, preferably at least 80 % and more preferably at least 95 % homology with Lc α E₇, in which the protein encoded by the DNA molecule differs from E₃ at least in the substitution of Trip at position 251 with an amino acid selected from the group consisting of Leu, Ser, Ala, Ile, Val, Thr, Cys, Met and Gly. The preferred substituents are Leu and Ser.

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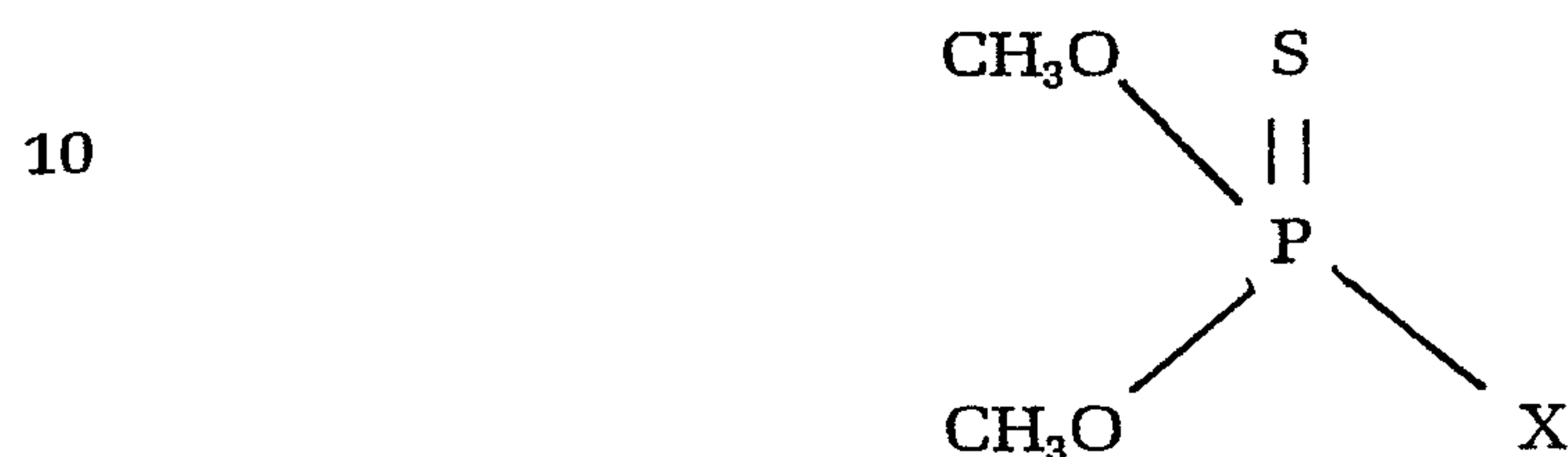
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(21) International Application Number: PCT/AU96/00746 (22) International Filing Date: 22 November 1996 (22.11.96) (30) Priority Data: PN 6751 23 November 1995 (23.11.95) AU (71) Applicant (for all designated States except US): COMMON-WEALTH SCIENTIFIC AND INDUSTRIAL RESEARCH ORGANISATION [AU/AU]; Limestone Avenue, Campbell, ACT 2601 (AU). (72) Inventors; and (75) Inventors/Applicants (for US only): RUSSELL, Robyn, Joyce [AU/AU]; 28 Sullivan Crescent, Wanniasa, ACT 2903 (AU). NEWCOMB, Richard, David [NZ/NZ]; 11/21 Ruarangi Road, Mt. Albert, Auckland (NZ). CAMPBELL, Peter, Malcolm [AU/AU]; 25 Wybalena Grove, Cook, ACT 2614 (AU). ROBIN, Geoffrey, Charles de Quetteville [AU/AU]; 10 Gundara Street, Aranda, ACT 2614 (AU). CLAUDIANOS, Charles [AU/AU]; 8/12 Babinda Place, Isabella Plains, ACT 2905 (AU). SMYTH, Kerrie-Ann [AU/US]; 137 Hughes Place, Syracuse, NY 13210 (US). BOYCE, Thomas, Mark [US/US]; 137 Hughes Place, Syracuse, NY 13210 (US). OAKESHOTT, John, Graham [AU/AU]; 28 Sullivan Crescent, Wanniasa, ACT 2903		(AU). BROWNLIE, Jeremy, Colin [AU/AU]; RMB 83 Burra Road, Via Queanbeyan, NSW 2620 (AU). (74) Agent: F.B. RICE & CO.; 28A Montague Street, Balmain, NSW 2041 (AU). (81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i>

(54) Title: MALATHION CARBOXYLESTERASE**(57) Abstract**

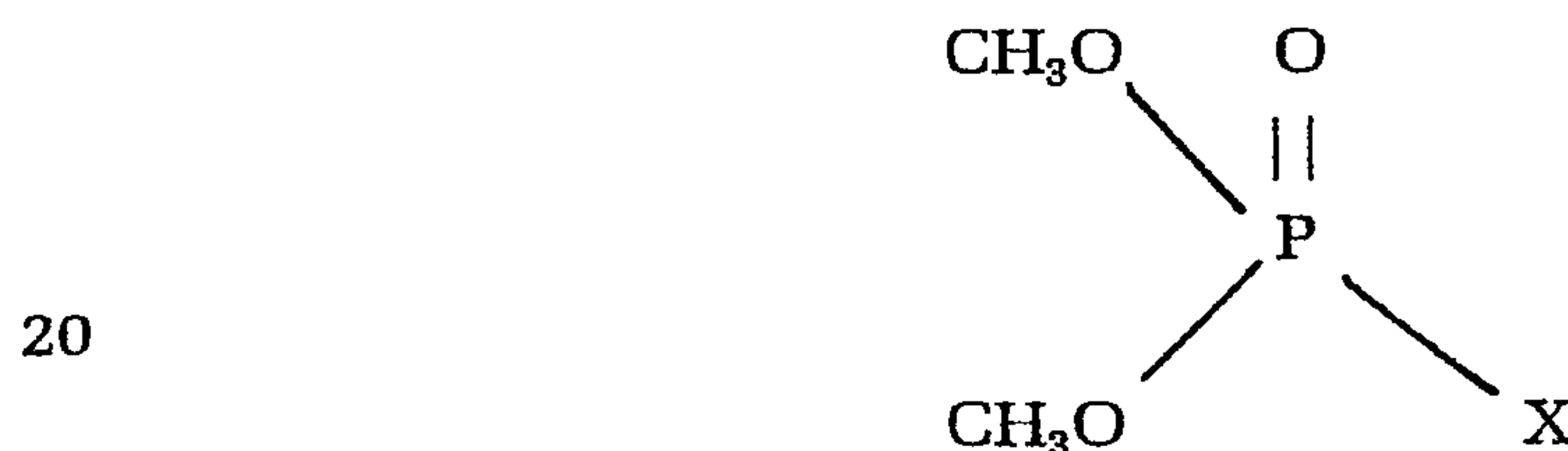
The present invention provides an isolated DNA molecule encoding Malathion Carboxylesterase capable of hydrolysing at least one organophosphate selected from the group consisting of carboxylester organophosphates and dimethyloxon organophosphates. The DNA molecule comprises a nucleotide sequence having at least 60 %, preferably at least 80 % and more preferably at least 95 % homology with Lc α E₇, in which the protein encoded by the DNA molecule differs from E₃ at least in the substitution of Trip at position 251 with an amino acid selected from the group consisting of Leu, Ser, Ala, Ile, Val, Thr, Cys, Met and Gly. The preferred substituents are Leu and Ser.

MALATHION CARBOXYLESTERASE

This invention relates to an enzyme (and the nucleic acid sequences
5 encoding this enzyme), termed malathion carboxylesterase (MCE) which is
able to efficiently hydrolyse a specific class of organophosphate (OP)
insecticides which have the general structures:



15 (eg. malathion, phenthoate)



(eg. malaoxon, phenthoate oxon)

where X contains one or more carboxylester groups for thion type
25 organophosphates but is unconstrained for oxon type organophosphates.

Residues of organophosphate insecticides are undesirable
contaminants of the environment and a range of commodities. Areas of
particular sensitivity include contamination of domestic water supplies and
soil, residues above permissible levels in various food and fibre exports and
30 contamination of domestic pets. Bioremediation strategies are therefore
required for eliminating or reducing these insecticide residues. One
proposed strategy involves the use of enzymes capable of immobilising or
degrading the insecticide residues. Such enzymes may be employed, for
example, in bioreactors through which contaminated water could be passed;
35 in production animal dips to reduce problems with contaminated pasture
and run off into water supplies; or in washing solutions after post harvest

disinfestation of fruit, vegetables or animal products to reduce residue levels and withholding times. Suitable enzymes for degrading pesticide residues include esterases. It is desirable that the esterases be relatively specific and hydrolyse the pesticide residues at a rapid rate.

5 The MCE enzyme has been purified from different malathion resistant strains of *L. cuprina*, RM and der-L (Whyard S., Russell R.J. and Walker V.K., Biochemical Genetics 32: 9, 1994; Whyard S. and Walker V.K., Pesticide Biochemistry and Physiology 50: 198,1994). It is a 60.5 kDa monomer with a K_m for malathion of $11.0 \pm 0.4 \mu M$ and a V_{max} of 775 ± 28 nmol malathion/min/mg. It also has a high turnover rate for malathion ($k_{cat} = 46 \text{ min}^{-1}$).

10 In order to enable the production of useful amounts of the MCE enzyme the present inventors sought to clone the putative MCE gene from a malathion resistant strain of *L. cuprina*(RM-8) using PCR and cloning techniques.

15 The MCE gene in *L. cuprina* has been mapped using classical genetic techniques to a position within 0.7 map units from the E3 gene on chromosome 4. The likely homologue of MCE in *Drosophila melanogaster*, *Mce*, has been mapped to the right arm of chromosome 3 in the vicinity of the genes encoding the major α -carboxylesterase, EST 9, and the orthologue of *L. cuprina* E3, EST23 (Spackman M.E., Oakeshott J.G., Smyth K-A., Medveczky K.M., and Russell R.J., Biochemical Genetics, 32: 39, 1994).

20 In order to clone the MCE gene from *L. cuprina*, it was decided to use the wealth of molecular genetic techniques available for *D. melanogaster* to clone the MCE homologue and use these clones as probes to isolate the *L. cuprina* genes themselves.

25 In summary, five esterase amplicons were isolated from *L. cuprina* genomic and cDNA. Four of the five *L. cuprina* amplicons obtained by PCR using cluster specific primers were designated Lc α E7, Lc α E8, Lc α E9 and Lc α E10 on the basis of homology to the corresponding *Drosophila* genes. The fifth, Lc#53, could not be assigned with any confidence on the basis of similarity to any of the *Drosophila* genes.

30 MCE specific activity is highest in the adult head, rather than the thorax or abdomen (Smyth,K-M., Walker,V.K., Russell,R.J. and Oakeshott,J.G. Pesticide Biochemistry and Physiology, 54:48, 1996). On this basis, Lc α E7, Lc α E8 and Lc α E10 were all MCE candidates. Previous

physiological studies of Parker, A.P., Russell, R.J., Delves, A.C. and Oakeshott, J.G. (Pesticide Biochemistry and Physiology 41:305, 1991) have shown that the E3 (Lc α E7) enzyme is present in the adult head. Moreover, the Lc α E8 and Lc α E10 genes are also expressed in the head since PCR using cluster-specific primers were able to amplify these genes from a head cDNA library. PCR failed to detect Lc α E9 and Lc#53 in either larval fat body or adult head cDNA and Northern analysis of the *D. melanogaster* α E9 homologue indicated that this gene was only expressed in embryos. Therefore both Lc α E9 and Lc#53 were discounted as candidates for the genes encoding E3 and MCE.

The Lc α E8 and Lc α E10 genes were initially chosen as prime MCE candidates on the basis of this distribution and due to the fact that it was known that Lc α E7 encodes the E3 enzyme involved in diazinon/parathion OP resistance in *L. cuprina* (PCT/AU 95/00016: "Enzyme based bioremediation") and it was thought that malathion resistance and diazinon/parathion resistance were encoded by separate genes.

The present inventors have made the surprising finding that it is a variant of Lc α E7 which encodes the MCE enzyme. This gene has been expressed *in vitro* and the product shown to have MCE activity. The expressed product can be formulated for use in degrading environmental carboxylester or dimethyl general OPs.

Accordingly, in a first aspect, the present invention consists in an isolated DNA molecule encoding an enzyme capable of hydrolysing at least one organophosphate selected from the group consisting of carboxylester organophosphates and dimethyl-oxon organophosphates, the DNA molecule comprising a nucleotide sequence having at least 60%, preferably at least 80% and more preferably at least 95% homology with Lc α E7, in which the protein encoded by the DNA molecule differs from E3 at least in the substitution of Trp at position 251 with an amino acid selected from the group consisting of Leu, Ser, Ala, Ile, Val, Thr, Cys, Met and Gly.

In a preferred embodiment the present invention the isolated DNA molecule has a sequence as shown in Figure 1 or a sequence which hybridises thereto with the proviso that the protein encoded by the DNA molecule differs from E3 at least in the substitution of Trp at position 251 with an amino acid selected from the group consisting of Leu, Ser, Ala, Ile, Val, Thr, Cys, Met and Gly.

In a preferred embodiment of the present invention the Trp at position 251 is substituted with Leu or Ser.

As is stated above the present invention includes nucleic acid molecules which hybridise to the sequence shown in Figure 1. Preferably
5 such hybridisation occurs at, or between, low and high stringency conditions. In general terms, low stringency conditions can be defined as 3xSSC at about ambient temperature to 65°C, and high stringency conditions as 0.1xSSC at about 65°C. SSC is the abbreviation of a buffer of 0.15M NaCl, 0.015M trisodium citrate. 3xSSC is three times as strong as SSC and so on.

10 In a second aspect the present invention consists in an isolated DNA molecule, the DNA molecule encoding a polypeptide having the amino acid sequence of RM-8Con shown in Figure 1 or the amino acid sequence of MdaE7 shown in Figure 3 in which Trp at position 251 is replaced with Ser.

Homologues of the MCE encoding sequence may also be present in
15 the genome of other insects, and particularly other species of Diptera. Thus, it is to be understood that the invention also extends to these homologues. An example of this is provided by the results set out hereunder regarding *Musca* MCE.

The isolated DNA molecules of the present invention may be cloned
20 into a suitable expression vector and subsequently transfected into a prokaryotic or eukaryotic host cell for expression of the enzyme. A particularly suitable system involves baculovirus vectors and an insect cell line.

In a third aspect the present invention consists in a method of
25 producing an enzyme capable of hydrolysing at least one organophosphate selected from the group consisting of carboxylester organophosphates and dimethyl-oxon organophosphates, or an enzymatically active portion thereof, the method comprising transforming a host cell with the DNA molecule of the first aspect of the present invention operatively linked to a control
30 sequence, culturing the transformed cell under conditions which allow expression of the DNA sequence and recovering the produced enzyme, or enzymatically active portion thereof.

It is also envisaged that as an alternative to using the enzyme *per se* as a bioremediation agent the bioremediation agent may be an organism
35 transformed with the DNA encoding the enzyme. In such an arrangement

the organism, transformed such that it expresses the enzyme, would be used as the bioremediation agent.

The invention further relates to methods for eliminating or reducing the concentration of carboxylester or dimethyl-oxon-type organophosphate insecticides
 5 residues in a contaminated sample or substance, involving the use of an esterase encoded by an isolated DNA molecule according to the present invention.

In order that the nature of the present invention may be more clearly understood preferred forms will now be described with reference to the following examples and Figures in which:

10 Figure 1 shows multiple nucleotide alignment of the three malathion-resistant clones (RM8A-C) and their consensus (RM8con) with the reference susceptible clone (Lc743) of Lc α E7 (E3). Dots indicate identity with the Lc743 susceptible clone. Below the ruler is the aligned nucleotide sequence and above is the inferred amino
 15 acid sequence of Lc743 with the one replacement found in Lc7RM8con indicated in bold text immediately below. Nucleotides are numbered from the predicted start of translation and amino acids from the predicted start methionine. Lc743 5' and Lc743 3' primer sequences are underlined.

Figure 2 shows amino acid alignment of the inferred Mdo α E7 protein from the Rutgers strain of *Musca domestica* compared to the Lc α E7 (E3) protein from the
 20 *Lucilia cuprina* Lc743 clone (WO 95/19440 "Enzyme Based Bioremediation"). Sequence comparison shows a 75% identity and 86% similarity between the same length, 570 residue proteins. Arrow indicates the conserved tryptophan residue at position 251 of the alignment.

Figure 3 shows the 1710bp nucleotide coding sequence of the Rutgers strain
 25 Mdo α E7 gene. Also shown is the inferred 570 protein sequence.

Figure 4 shows amino acid alignment of the PCR Ankara strain Mdo α E7 amplicon and the corresponding region of the RM-8 malathion resistant Lc α E7 protein. The structural mutations conferring malathion resistance (serine for Mdo α E7 and Leucine for Lc α E7) are indicated by arrow at residue position 251.

CLONING AND SEQUENCING OF THE MCE GENE FROM A MALATHION RESISTANT STRAIN OF LUCILIA CUPRINA

Two types of change in carboxylesterase activity have been
5 associated with resistance to OP insecticides in the higher Diptera. One type
of change results in resistance to OPs like diazinon and parathion, while the
other results in resistance to OPs like malathion, with one or more
carboxylester groups in addition to the phosphotriester moiety that defines it
as an OP (see above).

10 The two types of change were first described among OP resistant
strains of *Musca domestica*. In both types an increased degradation of OPs
was associated with reduced ali-esterase activity, where "ali-esterase" refers
to enzymes which are major contributors to the hydrolysis of the
carboxylester, methyl butyrate, or similar molecules (Oppenoorth, F.J.,
15 Entomology Experimental and Applied, 2: 304, 1959; Oppenoorth F.J. and
van Asperen, K., Entomology Experimental and Applied 4: 311, 1961). This
led to the formulation of the "mutant ali-esterase hypothesis", which
proposes that each type of resistance is due to a mutation in a specific
carboxylesterase that simultaneously enables it to hydrolyse the
20 phosphoester linkages common to the oxon form of all OPs and decreases its
activity toward certain carboxylester substrates (Oppenoorth, F.J. and van
Asperen, K., Science 132: 298, 1960).

Both types of change yielded resistance factors for diverse OPs
(except malathion) in the range of about 2-30 fold (Bell, J.D. and Busvine,
25 J.R., Entomology Experimental and Applied, 10: 263, 1967). However, the
malathion resistant *M. domestica* strains also showed exceptionally high
resistance to malathion (generally > 100 fold). This high resistance was
associated with cleavage of the carboxylester linkages in malathion (ie MCE
activity) in addition to the hydrolysis of the phosphoester linkage (OP
30 hydrolase activity). MCE activity accounted for the major breakdown
products *in vivo* and *in vitro* (Townsend, M.G. and Busvine, J.R., Entomology
Experimental and Applied 12: 243, 1969).

No recombination between the two types of resistance or between
them and ali-esterase activity was observed among the *M. domestica* strains
35 (Nguy, V.D. and Busvine, J.R., World Health Organisation 22: 531, 1960).
This suggests that while they are clearly distinct in respect of OP hydrolase

and MCE activities, the two types of resistance might nevertheless be allelic changes to the same carboxylesterase/ali-esterase gene/enzyme system (Oppenoorth, F. J. and Welling, W., in Insecticide Biochemistry and Pharmacology, Wilkinson, C.F. ed., Plenum Press, New York and London, pp. 507-551, 1976).

5 A malathion resistance phenotype has also been described in the blowfly, *Chrysomya putoria*, which parallels the malathion resistance phenotype of *M. domestica* in that it is associated with high MCE and low ali-esterase activities (Busvine, J.R., Bell, J.D. and Guneidy, A.M., Bulletin of Entomological Research 54: 589, 1963; Bell, J.D. and Busvine, J.R., Entomology Experimental and Applied 10: 263, 1967; Townsend, M.G. and Busvine, J.R., Entomology Experimental and Applied 12: 243, 1969). Further evidence for the similarity of the malathion resistance phenotypes in the two species is indicated by the spectrum of OP compounds which synergise malathion. Specifically, among a series of symmetrical trisubstituted phosphorus compounds, the best synergists (eg triphenylphosphate) were common to both species (Bell, J.D. and Busvine, J.R., Entomology Experimental and Applied 10: 263, 1967). However, little is known of diazinon/parathion type resistance in *C. putoria*.

20 The mutant ali-esterase hypothesis has also been invoked to explain diazinon/parathion resistance in *L. cuprina*, because these flies hydrolyse paraoxon more rapidly than susceptible flies (Hughes, P.B. and Devonshire, A.L., Pesticide Biochemistry and Physiology 18:289, 1982) and resistance is associated with reduced carboxylesterase activity. In this case the esterase isozyme E3 from resistant flies is not detected ("non-staining") after polyacrylamide gel electrophoresis (PAGE; Hughes, P.B. and Raftos, D.A., Bulletin of Entomological Research 75: 535, 1985). Evidence for a causal connection between the E3 change and resistance was obtained by EMS mutagenesis of an E3 staining, OP susceptible strain of *L. cuprina* and selection for OP resistant mutants; all resistant mutants recovered had the E3 non-staining PAGE phenotype (McKenzie, J.A., Parker, A.G. and Yen, J.L., Genetics 130: 613, 1992).

35 Like malathion resistant strains of *M. domestica*, strains of *L. cuprina* that are resistant to malathion exhibit very high resistance factors towards malathion and enhanced MCE activity. Also in common with *M. domestica*, malathion resistant *L. cuprina* generally do not exhibit

diazinon/parathion resistance, and *vice versa*. However, one difference from the situation in *M. domestica* is that the loci encoding the two resistance phenotypes appeared in some experiments to be genetically separable, albeit closely linked (Smyth, K-A., Russell, R.J. and Oakeshott, J.G., Biochemical Genetics 32: 437, 1994; 5 Smyth, K-A., Walker, V.K., Russell, R.J. and Oakeshott, J.G., Pesticide Biochemistry and Physiology 54: 48,1996).

An esterase gene cluster containing genes involved in OP resistance has been isolated from *L. cuprina* (Newcomb, R.D., East, P.D., Russell, R.J. and Oakeshott, J.G., Insect Molecular Biology 5: 211, 1996). One of these genes, Lc α E7, encodes 10 esterase E3 (Newcomb, R.D., Campbell, P.M., Russell, R.J. and Oakeshott, J.G. Insect Biochemistry and Molecular Biology, 22: 15, 1997), a structural mutation in the active site of which confers diazinon/parathion resistance on *L. cuprina*. These data are described in a previous patent application WO 95/19440, "Enzyme based bioremediation".

15 Below we describe the cloning and sequencing of the Lc α E7 gene from a malathion resistant strain of *L. cuprina*. We present molecular genetic evidence that this allele of esterase E3 is the MCE gene responsible for malathion resistance in *L. cuprina*.

20 a) Cloning the malathion resistant allele of Lc α E7

An RT-PCR (reverse transcriptase - PCR) approach was used to clone a cDNA allele of Lc α E7 from a malathion resistant strain of *L. cuprina* (RM-8) which is homozygous for the fourth chromosome.

25 Adults from the RM-8 strain were aged for three days before collection and stored at -70° C. RNA was prepared using a modified protocol of Chigwin et al. (Chigwin, J.M., Przybyla, A.E., MacDonald, R. J. & Rutter, W.J., 1979, Biochemistry 18, 5294). About 100 adults were thoroughly homogenised in 15ml of solution D (4M guanidinium thiocyanate, 25mM sodium citrate, pH 7.0, 0.5% sarkosyl, 0.1M 30 β -mercaptoethanol) using a Sorvall Omnimix blender. The resulting homogenate was filtered through glasswool and 6 ml layered on top of 5 ml of 4.8M CsCl, made up in 10mM Na- EDTA, pH 8, in an SW41 ultracentrifuge tube. These were spun at

35,000 rpm in an SW41 rotor for 16hr at 15° C. The supernatant was removed and the RNA pellet resuspended in 400 μ l of DEPC-treated H₂O. The RNA was precipitated by the addition of 800 μ l of ethanol and 10 μ l of 4M NaCl and stored under ethanol at -20°C. Before use the RNA pellet was washed in 75% ethanol and air dried before resuspension in DEPC-treated H₂O.

PolyA⁺ RNA was prepared from 500 μ g of total RNA using affinity chromatography on oligo-dT cellulose (Pharmacia; Sambrook, J., Fritsch, E.F., and Maniatis, T., 1989, Molecular Cloning: A Laboratory Manual, 2nd Edition, Cold Spring Harbor Laboratory Press, USA). The resulting mRNA (1-5 μ g) was again precipitated, washed and resuspended in 20 μ l of DEPC-treated H₂O. Oligo-dT primed cDNA was made from 1 μ g of mRNA using reverse transcriptase (Superscript II, BRL) as per the manufacturers instructions in a 20 μ l volume reaction. 200ng of cDNA was used as template in each of two PCR reactions using primers designed from the 5' (Lc743 5': 5' atgaatttcaacgtagtttgatgga 3') and complementary 3' (Lc743 3' : 5' ctaaaataaatctctatgttttcaaac 3') ends of the coding region of the Lc α E7 gene. Reactions used Taq DNA polymerase (BRL) and contained 100 pmoles of each primer, 0.2 mM of each dNTP, 10mM Tris-HCl, pH 8.3, 50mM KCl, 0.002% Tween 20 (v/v), 1.5 mM MgCl₂, and 200 ng of template. Two drops of mineral oil were layered over each 50 μ l reaction. Six units of Taq enzyme was added after a 5 minute "hot start" at 97°C and was followed by 40 cycles of 35 seconds at 97°C, 1 minute at 60°C and 2 minutes at 72°C. A final cycle of 72°C for 8 minutes was included. The 1.7 kb major product was gel purified and cloned into the EcoRV cleavage site of the pBSK⁻ (Stratagene) or pGEM-T (Promega) plasmid vectors using conventional cloning techniques (Sambrook, J., Fritsch, E.F., and Maniatis, T., 1989, Molecular Cloning: A Laboratory Manual, 2nd Edition, Cold Spring Harbor Laboratory Press, USA).

30 b) Sequencing the malathion resistant allele of Lc α E7

Methods:

Three clones were chosen for sequencing (RM8-A to -C), all of which were derived from independent PCR reactions. A set of twelve

21-mer sequencing primers (sequence shown below) were designed from the existing Lc α E7 sequence:

Table 1

5

primer seq (5' - 3')	primer name	5' position in Lc743 sequence (Figure 1)
ggatggtgtgcgtgattgttg	7F1	246
aaaaggatgtggtgttgatta	7F2	464
actaatgtcgggtaatgctat	7F3	723
cactatgatgggtaacacttc	7F4	1026
tgttacaggagaaacaccaac	7F5	1203
agaatcgcgtagaatacaaaac	7F6	1467
acggtataccctcaaaactgt	7R1	187
tcccaaacgatattgtatgtt	7R2	504
acatcatgtagtgggtagaag	7R3	685
ccgaggatgtttgggtaagac	7R4	990
tatcagctgttggtgtttctc	7R5	1231
acgcgattctttaggcatacg	7R6	1476

These were used in dye-terminator sequencing reactions (ABI) conducted following manufacturer's instructions in 25 μ l capillary tubes in a Corbett Research capillary thermal cycler, except that 50pmoles of primer was used per reaction, a "hot start" of 96°C for 3 minutes was included and 30 cycles were completed for each sequencing reaction. Dye primer reactions were also conducted on all clones using the ABI M13 forward and reverse primers as per ABI protocols. Sequencing reactions were resolved by electrophoresis on an ABI 370A automatic sequencing machine as per the manufacturer's instructions. This resulted in both strands being sequenced entirely.

Results:

20

Figure 1 shows a nucleotide and amino acid alignment of the three resistant clones (RM8 A-C) compared with the reference susceptible clone

(Lc743) of Lc α E7. A consensus sequence of the malathion-resistant Lc α E7 allele was determined (RM-8con). Differences between resistant clones were assumed to be errors incorporated by the Taq polymerase.

Comparison of the susceptible sequence (Lc743) with that of the malathion-resistant RM-8 consensus sequence (RM-8con) identified only one replacement site difference, a Trp to Leu substitution at amino acid position 251 (nucleotide position 752). The homologous amino acid was highlighted on a three-dimensional model of *T. californica* AChE, revealing that the Leu mutation was situated at the base of the active site gorge, 6.5 Angstroms from the active site Ser.

c) Sequencing the region surrounding nucleotide 752 from various Lc α E7 alleles

An esterase structural mutation conferring malathion resistance would be expected to occur in the active site region of the molecule. The Trp to Leu mutation at nucleotide position 752 in Lc α E7 is therefore an excellent candidate for the malathion resistance mutation.

The inventors have established a total of 14 strains of *L. cuprina* which are homozygous for chromosome IV and of known malathion resistance status. These lines fall into seven classes on the basis of an RFLP analysis of genomic DNA using the Lc α E7 gene as a probe. Nucleotide position 752 was therefore sampled over the entire range of classes.

Methods:

The complete cDNA sequence of the Lc α E7 alleles from strains representing several of the classes are available. For example, the sequence of Lc α E7 from RM-8 is shown in Figure 1. Moreover, the Lc α E7 cDNA sequences from strains LS2 and Llandillo 103, which represent two more classes, are described in patent application PCT/AU 95/00016 ("Enzyme based bioremediation"). The complete Lc α E7 cDNA sequence of the Gunning 107 strain, representing a fourth class, is described in J. Trott, B.Agr.Sc Thesis, 1995.

To obtain the sequence of Lc α E7 in the region of nucleotide 752 in strains LBB101, Llandillo 104 and Hampton Hill 6.2, representing the

remaining three classes, a PCR approach was taken. Genomic DNA was prepared from either eggs using the method of Davis, L.G., Dibner, M.D., and Batley, J.F., (1986. *Basis Methods in Molecular Biology*, Elsevier Science Publ. Co., New York, Section 5.3), or from adult flies using a C-TAB method (Crozier, Y.C., Koulouanos, S. & Crozier, R.H., 1991, *Experientia* 47, 9668-969). 1µg samples were then used as templates in PCR reactions using 100p moles of the primers 7F1 and 7R4. Also included in the reactions were 0.2mM of each dNTP, 10mM Tris-HCl, pH 8.3, 50mM KCl, 1.5mM MgCl₂. Two drops of mineral oil were layered over each 50µl reaction. 2.5 units of Taq polymerase was added after a 'hot start' of 97°C for 3 minutes while an annealing temperature of 55° was maintained. An initial extension at 72°C was held for 2 minutes. This was followed by 34 rounds of 97°C for 35 seconds, 55°C for 1 minute and 72°C for 1 minute. A final extension of 72°C for 9 minutes was included. A single product of about 1kb was produced. This was purified for sequencing using QIAquick™ spin columns (Qiagen), following manufacturer's instructions. 500 ng of template was used in dye-terminator sequencing reactions using the 7F7 (5': 5'tgctgcctctaccactacat 3') and 7R7 (3': 5' cctgtggcttggctttcataa 3') primers as described above.

Results:

Of the seven classes assayed, all five malathion-susceptible strains (LS2, LBB101, Llandillo 104, Gunning 107 and Llandillo 103) possess a G at nucleotide position 752, whereas both malathion-resistant strains (Hampton Hill 6.2 and RM-8) possess a T at this position, resulting in a Trp to Leu substitution at amino acid position 251 (Table 2). The presence of the same structural mutation in two malathion resistant strains with different fourth chromosomes strongly suggests that the mutation is responsible for resistance.

Table 2

Strain	Malathion resistance status	Class	Residue at amino acid position 251 ^a
LS2	Susceptible	A	Trp
LBB101	Susceptible	C	Trp
Llandillo 104	Susceptible	B	Trp
RM-8	Resistant	E	Leu
Hampton Hill 6.2	Resistant	F	Leu
Llandillo 103	Susceptible	D	Trp
Gunning 107	Susceptible	G	Trp

^aAmino acid at position 251 corresponds to nucleotide position 752 in Figure 1.

5

d) **Cloning and sequencing the orthologous α E7 gene from a malathion resistant strain of *Musca domestica***

As described above, the diazinon/parathion and malathion esterase-mediated OP resistance types exhibit many striking parallels between *L. cuprina*, *M. domestica* and *C. putoria*, and are probably caused by functionally equivalent mutations in orthologous genes. The orthologous gene was therefore cloned from the housefly, *M. domestica*, and the region surrounding nucleotide 752 examined for the presence of the malathion resistance mutation in a malathion resistant *Musca* strain.

15

PCR reactions:

Consensus generic α -esterase primers were designed to the conserved regions of the multiple amino acid alignments of *D. melanogaster* (Robin, C. Russell, R.J., Medveczky, K.M. and Oakeshott, R.J., Journal of Molecular Evolution 43: 241, 1996) and *L. cuprina* (Newcomb, R.D., Campbell, P.M., Russell, R.J. and Oakeshott, J.G. Insect Biochemistry and Molecular Biology, in press) α -esterase genes, and used in a PCR amplification experiment for the recovery of homologous α E7 gene sequence from *M. domestica*.

20

25

Genomic DNA was prepared using the Lifton method (Bender, W., Spierer, P. and Hogness, D.S., Journal of Molecular Biology 168: 17, 1989) from adult females of the Rutgers OP resistant housefly strain (Plapp, F.W. Jr., Tate, L.G. and Hodgson, E. 1976. Pestic. Biochem. Physiol. 6:175-182).

- 5 Rutgers strain genomic DNA was used as the template in a 50 μ l amplification reaction:

Table 3

	Final concentration/amount
Template DNA	100ng
primer Md1	50pmoles
primer Md2	50pmoles
Buffer	10mM Tris-HCl (pH8.3), 1.5mM MgCl ₂ , 50mM KCl
dNTP's	0.25mM (dATP,dCTP,dTTP,dGTP)
Taq polymerase	1 unit
Total volume	50 μ l

- 10 Primers:

Md1 (35mer)

5' TTCGAGGGIATICCTAYGCIMARCCICCIBTNGG 3'

corresponding to residues 58-69 in *L.cuprina* α E7

Md2 (32mer)

- 15 5' ACYTGRTCYTTIARICCGICRTTICCIIGNAC 3'

corresponding to residues 92-82 in *L. cuprina* α E7

Note: IUB codes used for mixed positions; I = inosine.

- 20 PCR conditions over 38 cycles:

95°C 3' 1 cycle

80°C Hold (addition of 1 unit Taq polymerase)

95°C 1' 50°C 1' 72°C 1' 1 cycle

95°C 1' 55°C 1' 72°C 1' 35 cycles

- 25 95°C 1' 55°C 1' 72°C 4' 1 cycle

Cloning and sequencing PCR amplicons:

The 540bp major product was eluted from an agarose gel, purified using QIAGEN QIAquick PCR purification kit and cloned into the pGEM-T plasmid vector (PROMEGA) using standard techniques (Sambrook, J., Fritsch, E.F. and Maniatis, T., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, USA, 1989). The ends of the cloned insert were sequenced using commercially available T7 and SP6 primers and TaqFS dye-terminator technology (ABI) on the Applied Biosystems Model 370A automated DNA sequencer. Translated amino acid sequences were aligned to predicted α -esterase protein sequences using PILEUP from the GCG computer package (Devereux, J., The GCG sequence analysis software package Version 6.0. Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, Wisconsin, USA, 1989); all proved to be homologous to the sequences of known α -esterase genes from *D. melanogaster* and *L. cuprina*.. The cloned 534bp amplicon showed 76% identity over the equivalent 135 amino acids of the *L. cuprina* α E7 predicted protein sequence.

Isolation of the complete α E7 gene from M. Domestica:

A λ DASH (Stratagene) genomic library of the Rutgers strain of *M. domestica* (Koener, J.F., Carino, F.A. and Feyereisen, R., Insect Biochemistry and Molecular Biology 23:439, 1993) was screened for a full-length genomic clone of α E7. Approximately 300,000 plaques were probed with the ^{32} P labelled 534bp amplicon described above. Library screening using conventional techniques (Sambrook, J., Fritsch, E.F. and Maniatis, T., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, USA, 1989) was performed at high stringency (50% formamide, 5X SSC, 3X Denhardt's, 0.5% SDS and 10 μ g/ml salmon sperm DNA at 45°C) and included a final high stringency wash (0.1% SSC, 0.1% SDS at 65°C). Restriction mapping indicated that a single λ DASH clone with a 17.5kb genomic insert contained the α E7 gene. A 4.5kb HindIII fragment was subcloned into the pBSK⁻ vector (Stratagene) and characterised using dye-terminator automatic sequencing technology, as described above. A set

a thirteen sequencing primers were designed and used to interpret the full length genomic sequence:

Table 4

primer name	5' - 3' primer sequence	size	5' position in Md α E7 coding sequence (Figure 3)
T7	end sequencing of 4.5kb pBSK ⁻ clone		polycloning site
T3	end sequencing of 4.5kb pBSK ⁻ clone		polycloning site
AE7.1	TTTGGTCCCGACTACTTTATGA	22mer	442
AE7.2	TGCCACTTATGAAATCTGTCTGTA	24mer	310
AE7.3	TACATGATGATAACCGAACAGACC	24mer	676
AE7.4	TCGATTATTTGGGTTTCATTTGT	23mer	107
AE7.5	ACAGACAGATTTTCATAAGTGG	21mer	288
AE7.6	TTTGCATTCTTTTCGGGTGTCA	21mer	913
AE7.7	ATTCGATACCCACATTGATAG	21mer	1016
AE7.8	GGCACTCCCATTATTTGTAT	21mer	1312
AE7.10	ATGACTTTTCTGAAGCAATTCAT	23mer	1
AE7.11	AAACAATTCCTTCTTTTATCGA	23mer	1710
AE7.12	GGCATGGAAAACCTCACCTGG	21mer	1558

5

The predicted coding sequence of 1710bp or 570 amino acids showed a very high 75% identity and 85% similarity to the equivalent full length 570 residues of the α E7 protein from *L. cuprina* (Figures 2 and 3). Southern hybridisation analysis as per standard methods (Sambrook, J., Fritsch, E.F. and Maniatis, T., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, USA, 1989) of genomic DNA from the Rutgers strain using the 534bp α E7 PCR amplicon as a probe and EcoRI, Hind III, Sal I digested DNA, showed a single 4.5kb hybridising band for the HindIII digest, a single 6.0kb SalI band and two EcoRI bands of 1.5kb and 0.8kb. The analysis confirms the restriction pattern interpreted from the sequencing and mapping data of the lambda genomic clone. No other aberrant hybridisation patterns occurred indicating a high probability that α E7 exists as a single copy gene.

10

15

Characterisation of a putative $\alpha E7$ malathion resistant allele of *M. domestic*:

Genomic DNA extracted (Bender, W., Spierer, P. and Hogness, D.S.,
5 Journal of Molecular Biology 168: 17, 1989) from single adult female flies of
the highly malathion resistant Ankara strain (Sisli, M.N., Bosgelmez, A.,
Kocak, O., and Porsuk, H. 1983. Mikrobiyol Bul. 17:49-46) was used for
sequence characterisation of a putative malathion resistance allele. A series
of PCR reactions were performed using single fly genomic DNA for the
10 characterisation of allelic variants of the $\alpha E7$ gene. PCR amplification using
conditions described above, with the specific housefly AE7.5 and AE7.6
primer pair, produced single amplicons of approximately 760bp. This
amplicon encompasses the highly conserved region involved in the catalytic
site of the enzyme, coding for residues 96-304 of the translated sequence,
15 including the site of the Trp to Leu mutation at amino acid residue 251
associated with malathion resistance in *L. cuprina* (Figure 4). Cloning and
sequencing of PCR amplicons (described earlier) from nine individual flies
showed that the Ankara strain segregates for two allelic variants of the $\alpha E7$
gene: one has a Trp residue at amino acid position 251, whereas the other
20 has a Ser at this same position. This replacement is synonymous with the
Trp to Leu substitution involved in malathion resistance in *L. cuprina*. Both
leucine and serine replace a bulky tryptophan residue within the active site
and we therefore propose that this change accounts for the observed changes
in the kinetic properties of the enzymes towards carboxylesters and OPs. In
25 a similar manner it is believed that the substitution of the bulky Trp residue
with other smaller residues such as Ala, Ile, Gly, Val, Thr, Cys and Met will
have a similar effect. The finding of these similar active site mutations in
malathion resistant strains of both *Lucilia* and *Musca* further supports our
conclusion that these mutations are responsible for malathion resistance in
30 these species.

**HYDROLYTIC ACTIVITY OF THE EXPRESSED PRODUCTS OF THE
SUSCEPTIBLE AND MALATHION RESISTANT ALLELES OF *Lc $\alpha E7$***

35 Below we describe the activities of the expressed products of the
susceptible and malathion resistant alleles of *Lc $\alpha E7$* for various

carboxylester and OP substrates. The results suggest a possible mechanism for malathion resistance in *L. cuprina* as a result of the mutation at nucleotide 752 in the LcαE7 gene.

5 a) **In vitro expression**

The *in vitro* expression of the OP susceptible allele of LcαE7 (clone Lc743) is described in patent application WO 95/19440 ("Enzyme based bioremediation").

10 The malathion resistant LcαE7 full-length cDNA was cloned into the baculovirus transfer vector, Bacpac 6 (Clontech) 3' of the polyhedrin promoter. Transfections were conducted using a lipofection method with DOTAP (Boehringer Mannheim) as per King and Possee (The Baculovirus Expression System: A Laboratory Guide, Chapman & Hall, London, 1992). One µg of DNA of each of the resulting constructs together with 200 ng of Bacpac 6 baculovirus DNA (Clontech),
15 linearised by digestion with the restriction enzyme BSU 36I (Promega), was incubated in a solution of HBS (hepes buffered saline) containing 15% DOTAP (Boehringer Mannheim) in a polystyrene container at room temperature for 10 minutes. The solution was then used to transfect a single well of a six well tissue culture plate preseeded 2 hrs previously with 10⁴ Sf9 (*Spodoptera frugiperda*) cells in
20 1.5 mls Grace's medium (King and Possee, The Baculovirus Expression System: A Laboratory Guide, Chapman & Hall, London, 1992). After 12 hours, the medium was replaced with 3 mls of Grace's medium containing 10% fetal calf serum. Construct plus DOTAP, linearised virus plus DOTAP and DOTAP only controls were conducted in parallel with transfections. The transfections were harvested 4-5 days
25 after infection and the cells isolated by centrifugation at 500g for 5 minutes. Aliquots of the resulting cell pellets were immediately stored on ice, resuspended in 10mM imidazole-HCl buffer, pH 7.0, containing 0.5% Triton™ X-100. Final protein concentrations in these cell extracts were between 5 and 40 mg/ml. Aliquots of the cell extracts were stored at -70°C prior to enzyme assays.

b) Malathion hydrolysis***Methods:***

5 MCE activity was assayed using the partition method of Ziegler, R., Whyard, S., Downe, A.E.R., Wyatt, G.R. and Walker, V.K., Pesticide Biochemistry and Physiology, 28:279 (1987) as modified by Whyard, S., Russell, R.J. and Walker V.K. Biochemical Genetics 32:9 (1994). Cell
10 extracts were diluted 300-fold in 10mM imidazole-HCl, pH 7.0, and 150 μ l aliquots were placed in triplicate microfuge tubes. Reactions were started by the addition of 1 μ l ethanol containing [^{14} C]-malathion [Amersham; 103 mCi/mmol, 280nCi, labelled at both the methylene carbons of the succinate moiety, adjusted to 15mM (or 375 μ M - 15mM for kinetic experiments) by the addition of unlabelled malathion (99%; Riedel-de-Haën Ag., Seelze,
15 Germany)]. The assay mixture was incubated at 25°C for 10 minutes, then 300 μ l of dilution buffer was added and the undegraded malathion extracted three times with 600 μ l of chloroform. The concentration of carboxylic acids of malathion in 300 μ l of aqueous phase was determined by liquid scintillation. Protein concentrations in the cell supernatants were
20 determined using the Biorad Protein Assay Kit by the method of Bradford, M., Analytical Biochemistry 72:248 (1976) with bovine serum albumin as the standard. Boiled enzyme controls were performed routinely. The specific MCE activity of an extract of cells infected with non-recombinant baculovirus was at least 700-fold lower than that of cells infected with
25 baculovirus encoding OP-susceptible or malathion-resistance alleles of *Lc α E7*. This slight MCE activity that was not due to alleles of *Lc α E7* was deemed a very minor source of error and subsequently ignored.

Results:

30

Using initial concentrations of malathion between 2.5 and 100 μ M, MCE encoded by malathion resistant and susceptible alleles of *Lc α E7* exhibited a good fit to Michaelis-Menten kinetics. K_m and V_{max} were calculated for both enzymes. K_{cat} was then calculated from the V_{max} and the
35 molarity of the *Lc α E7* products in their respective cell extracts. The molarity of susceptible *Lc α E7* product in the cell extract was determined by

titration with paraoxon as previously described in Newcomb, R. D., Campbell, P.M., Russell, R.J. and Oakeshott, J. G., *Insect Biochemistry and Molecular Biology* (22: 15, 1997). The molarity of the product of the malathion resistant allele of Lc α E7 was determined similarly, except that triphenylphosphate (TPP; 1 to 10×10^{-8} M) was used instead of paraoxon. In a control experiment TPP (8×10^{-8} M) was preincubated with cell extract in triplicate for 15, 30 or 45 minutes prior to addition of the substrate malathion. There was no significant difference between the residual MCE activity at each of the preincubation times, indicating firstly that the inhibition of MCE by TPP had gone to completion and secondly, that TPP was not being turned over by MCE.

The kinetic parameters for malathion hydrolysis for the products of the malathion resistant and susceptible alleles of Lc α E7 are:

Table 5

Expressed Lc α E7 Gene Product	K _m (μ M)	K _{cat} (min ⁻¹)
Malathion susceptible (strain LS2)	200 ± 30	70 ± 11
Malathion resistant (strain RM-8)	21 ± 1	43 ± 1

The K_m and K_{cat} for the malathion resistant product are in reasonable agreement with those determined for the MCE enzyme purified from malathion resistant flies ($11 \pm 0.4 \mu$ M and 46 ± 2 per min, respectively; Whyard, S. and Walker, V.K., *Pesticide Biochemistry and Physiology*, 50 198, 1994).

c) Sensitivity of MCE activity to TPP

High sensitivity to inhibition by TPP is a distinctive characteristic of the MCE activity associated with malathion resistance in *M. domestica*, *C. putoria* and *L. cuprina*, consistent with potent synergism of malathion by TPP in resistant strains of these species (Shono, T., *Applied and Entomological Zoology* 18: 407, 1983; Bell, J.D. and Busvine, J.R., *Entomology Experimental and Applied* 10: 263, 1967; Townsend. M.G. and Busvine, J.R., *Entomology Experimental and Applied* 12: 243, 1969; Hughes, P.B., Green, P.E. and Reichmann, K.G., *Journal of Economic Entomology* 77: 1400, 1984; Smyth, K-A., Walker, V.K., Russell, R.J. and Oakeshott, J.G.,

Pesticide Biochemistry and Physiology, 54: 48, 1996). MCE activity encoded by the malathion resistant allele of *LcαE7* is potently inhibited by TPP, as indicated by stoichiometric inhibition of the enzyme at concentrations below 10^{-7} M (see above).

5

d) *α*-Naphthyl acetate hydrolysis

Methods:

10 The initial rates of reactions between cell extracts containing the expressed products of the malathion resistant and susceptible *LcαE7* alleles and *α*-naphthyl acetate (*α*-NA) were determined at 25°C using a recording spectrophotometer and the method of Mastrapaolo and Yourno (Analytical Biochemistry 115: 188, 1981). 6-200μM *α*-NA dissolved in 10μl of
15 2-methoxyethanol was added to 0.1 M Tris-HCl pH 8.0 (980 μl) in a quartz cuvette. *α*-Naphthyl acetate is slowly hydrolysed in water so a background rate was recorded before starting the enzymic reaction by the addition of 10 μl of diluted cell extract. Control reactions were performed with extracts of both uninfected cells and Bacpac 6 infected cells. These controls
20 exhibited negligible enzymic hydrolysis.

Results:

25 Using initial concentrations of *α*-NA from 6 to 200 μM, the enzymes encoded by the malathion resistant and susceptible alleles of *LcαE7* exhibited a good fit to Michaelis-Menten kinetics. K_m and V_{max} were calculated for both enzymes. K_{cat} was then calculated from the V_{max} and the molarity of the *LcαE7* products in their respective cell extracts (determined above).

30

Table 6

Expressed <i>LcαE7</i> Gene Product	K_m (μM)	K_{cat} (min ⁻¹)
Malathion susceptible (strain LS2)	70±5	11,000±300
Malathion resistant (strain RM-8)	150±50	2270±30

The K_m and K_{cat} for the malathion resistant product are in reasonable agreement with those determined for the MCE enzyme purified from malathion resistant flies ($167 \pm 14 \mu M$ and 2063 per min; Whyard, S. and Walker, V.K., Pesticide Biochemistry and Physiology, 50: 198, 1994).

5

e) α -Naphthyl butyrate hydrolysis

Methods:

10 As described above for α -NA hydrolysis except that 6-200 μM α -naphthyl butyrate (α -NB) was used instead of α -NA.

Results:

15 Using initial concentrations of α -NB from 6 to 200 μM , the enzymes encoded by the malathion resistant and susceptible alleles of *Lc α E7* exhibited a good fit to Michaelis-Menten kinetics. K_m and V_{max} were calculated for both enzymes. K_{cat} was then calculated from the V_{max} and the molarity of the *Lc α E7* products in their respective cell extracts (determined
20 above).

Table 7

Expressed Lc α E7 Gene Product	K_m (μM)	K_{cat} (min^{-1})
Malathion susceptible (strain LS2)	20 ± 5	$18,000 \pm 2,000$
Malathion resistant (strain RM-8)	29 ± 4	$9,000 \pm 400$

25 The K_m and K_{cat} for the malathion resistant product are in reasonable agreement with those determined for the MCE enzyme purified from malathion resistant flies ($39 \pm 4 \mu M$ and 3700 per min; Whyard, S. and Walker, V.K., Pesticide Biochemistry and Physiology, 50: 198, 1994).

f) **General OP hydrolysis**

In *M. domestica* there is a pattern of cross-resistance among OPs (Bell, J.D. and Busvine, J.R., Entomology Experimental and Applied 10: 263, 1967) such that parathion/diazinon resistant flies generally exhibit greater resistance factors towards OPs with two ethoxy groups attached to the phosphorus atom ('diethyl OPs') rather than two methoxy groups ('dimethyl OPs'). The converse pattern (ie greater resistance to dimethyl OPs) was observed for malathion resistant strains of *M. domestica* and *C. putoria* (Bell, J.D. and Busvine, J.R., Entomology Experimental and Applied 10: 263, 1967; Townsend, M.G. and Busvine, J.R., Entomology Experimental and Applied 12: 243, 1969). This dimethyl OP preference applies both to malathion analogues (with carboxylester groups) and general OPs (without carboxylester groups). The implication of these studies is that there is a general OP hydrolase activity intimately associated with malathion type resistance and that this OP hydrolase exhibits a preference for dimethyl OPs.

There are insufficient published data to determine whether such a dimethyl/diethyl OP cross-resistance pattern occurs in *L. cuprina*. Here we determine firstly that there is such a cross resistance pattern and secondly that the enzyme encoded by the malathion resistance allele of *LcαE7* has hydrolytic activity against OPs which lack carboxylester groups.

Methods:

25 i) **Toxicology:**

The following organophosphorus compounds were used: diazinon (O,O-diethyl O-2-isopropyl-6-methylpyrimidin-4-yl phosphorothioate, 91%, Mallinckrodt), parathion-methyl (O,O-dimethyl O-4-nitrophenyl phosphorothioate, 97.0%, Bayer), parathion (O,O-diethyl O-4-nitrophenyl phosphorothioate, 99%, Pestanal grade, Riedel-de-Haën), fenthion (O,O-dimethyl O-[3-methyl-4-(methylthio)phenyl] phosphorothioate, 98.8%, Bayer), fenthion-ethyl (O,O-diethyl O-[3-methyl-4-(methylthio)phenyl] phosphorothioate, a gift from Dr. G. Levot), dichlorvos (2,2-dichlorovinyl dimethyl phosphate, 99%, Chem Service), diethyl-dichlorvos (2,2-dichlorovinyl diethyl phosphate, a gift from Dr. J. Desmarchelier), di-

isopropyl-dichlorvos (2,2-dichlorovinyl di-isopropyl phosphate, a gift from Dr. J. Desmarchelier), malathion (S-1,2-bis(ethoxycarbonyl)ethyl O,O-dimethyl phosphorodithioate, technical grade, Nufarm), isopropyl malathion (S-1,2-bis(ethoxycarbonyl)ethyl O,O-di-isopropyl phosphorodithioate, a gift
5 from Dr. J. Desmarchelier).

The toxicity of OPs in adult female *L. cuprina* was determined 3 or 4 days post-eclosion by application of OPs to the scutellum in 0.7 μ l dioctylphthalate (Busvine, J.R., Bell, J.D. and Guneidy, A.M., Bulletin of Entomological Research 54: 589, 1963;; Townsend, M.G. and Busvine, J.R.,
10 Entomology Experimental and Applied 12: 243, 1969). Each OP was applied to at least 20 flies at each of at least 5 different concentrations, spanning the dose causing 50% mortality (LD₅₀). Control groups were treated with solvent but no OP. Mortality was determined 24 hours later. Data were fitted to the probit curve using the Probit Or Logit (POLO-PC) computer
15 program (LeOra Software, 1987). This program corrects for natural mortality which was <5%. The statistic *g* ("index of significance for potency estimation") was always less than 0.5 for the 95% confidence limits of LD₅₀.

ii) Chlorfenvinphos hydrolysis assay:

20

Enzyme samples were diluted in 0.1 M imidazole-HCl buffer pH 7.0 ("imidazole buffer") to a final volume of 50 μ l. Reactions were started by the addition 0.5 μ l of (¹⁴C-ethyl)-chlorfenvinphos (CVP, 306.5 MBq/mmol, Internationale Isotope Munchen) from a 7.5mM stock solution in ethanol.
25 The reaction was incubated at 30°C and stopped by the addition of 300 μ l dichloromethane and 150 μ l of water followed by vigorous vortex mixing. The reactions were centrifuged to separate phases and 150 μ l of the upper, aqueous phase was taken for scintillation counting to determine the amount of ¹⁴C-diethylphosphate produced by hydrolysis of CVP. Incubations with
30 boiled enzyme were also performed to control for non-enzymic hydrolysis of CVP.

Results:**I) Toxicology:**

5 LD₅₀s of 10 OPs were determined for the Woodside 5.2 strain
(homozygous for a malathion resistance allele of *LcαE7*) and the Llandillo
103 strain (homozygous for a parathion/diazinon resistance allele of *LcαE7*).
LD₅₀s were also determined for the OP susceptible LS2 strain in order to
calculate resistance factors (Table 8). Woodside 5.2 flies exhibited about
10 two- to five-fold greater resistance factors towards the dimethyl OPs,
parathion-methyl, fenthion and dichlorvos than to their diethyl analogues,
parathion, fenthion-ethyl and ethyl dichlorvos. Conversely, Llandillo 103
flies exhibited about two-fold greater resistance factors towards the diethyl
15 OPs, parathion and ethyl dichlorvos, than to their dimethyl analogues,
parathion-methyl and dichlorvos. However, there was no significant
difference between the resistance factors of Llandillo 103 flies for fenthion
and fenthion-ethyl.

Among four diethyl OPs, Llandillo 103 flies have higher resistance
factors than Woodside 5.2 flies (except fenthion-ethyl with similar resistance
20 factors; Table 8). In contrast, Woodside 5.2 flies have higher resistance
factors than Llandillo 103 for each of four dimethyl OPs. Thus both strains
exhibit general OP resistance of similar potency, albeit with a bias towards
either dimethyl or diethyl OPs.

Neither resistant strain exhibited more than 3-fold resistance to the
25 di-isopropyl analogues of dichlorvos or malathion (Table 8).

Comparable data from *M. domestica* are available for seven of the
test compounds. In each case resistance factors are similar in strains of the
two species exhibiting parallel resistance types (Table 8).

Table 8 OP cross-resistance patterns in adult *L. cuprina* with comparisons to *M. domestica*.

OP Compounds ¹	LD ₅₀ ² LS2 (OP Susceptible)	LD ₅₀ Llandillo 103 (Dz/para Resistant)	LD ₅₀ Woodside 5.2 (Mal Resistant)	RF3 (Dz/para R)	RF (Mal R)
Diazinon (E)	57 (40-79), 3.5 ± 0.5	550 (490-630), 4.9 ± 0.7	270 (240-300), 5.1 ± 0.6	10 (20)	5 (2)
Parathion-methyl (M)	16 (11-20), 6.5 ± 0.8	185 (141-244), 3.4 ± 0.5	430 (390-490), 9.7 ± 1.5	12 (9)	27 (10)
Parathion (E)	52 (48-55), 9.2 ± 1.1	1050 (890-1280), 6.8 ± 1.2	290 (270-310), 13.3 ± 2.3	20 (35)	6 (3)
Fenthion (M)	61 (42-87), 5.2 ± 0.7	210 (180-240), 8.7 ± 1.5	320 (210-490), 3.9 ± 0.7	3 (3)	5 (7)
Fenthion-ethyl (E)	330 (290-370), 7.4 ± 1.1	690 (570-830), 8.7 ± 1.5	730 (660-870), 13.4 ± 2.6	2	2
Dichlorvos (M)	41 (35-51), 8.2 ± 1.1	150 (95-190), 5.1 ± 0.8	270 (210-340), 6.3 ± 1.0	4 (3)	7 (6)
Ethyl Dichlorvos (E)	360 (300-420), 5.0 ± 0.8	2370 (2320-2410), 54 ± 14	1100 (700-1500), 4.9 ± 1.2	7	3
Isopropyl Dichlorvos (P)	3500 (2200-4800), 4.1 ± 0.6	4600 (3400-5900), 4.1 ± 0.5	10200 (8800-12000), 8.1 ± 1.4	NS	3
Malathion (M)	550 (480-610), 6.4 ± 1.2	490 (360-600), 4.2 ± 0.9	4	NS (2)	>130 (157)
Isopropyl Malathion (P)	3600 (2700-4900), 6.3 ± 0.7	4900 (3700-6200), 8.5 ± 1.7	6400 (5900-7100), 10.1 ± 1.8	NS	1.8 (4)

1. Dimethyl (M), diethyl (E) or di-isopropyl (P) OPs.

2. LD₅₀ (ng/fly) with 95% confidence limits, slope and standard error of the probit regression line.3. Resistance Factors: ratio of the LD₅₀ of Llandillo 103 or Woodside 5.2 with the LD₅₀ of LS2. "NS" indicates an RF not significantly different from unity. Resistance factors of *M. domestica* of the appropriate resistance type are shown in parentheses (Bell, J.D. and Busvine, J.R., Entomology Experimental and Applied 10: 263, 1967; ; Townsend, M.G. and Busvine, J.R., Entomology Experimental and Applied 12: 243, 1969).

4. No mortality at this dose.

ii) Chlorfenvinphos hydrolytic activity of whole fly homogenates and expressed *LcαE7* gene products:

Whole fly homogenates of malathion resistant (strains RM-8, 60NE 1.1, 4.2, Beverly 6.2, Hampton Hill 6.1, Hampton Hill 6.2, Woodside 5.2, Rop Rmal 1, M22.2 6.3, M27.1 4.1), diazinon resistant (Gunning 107, Inverell 22, Q4, RM2.6, Llandillo 103, Sunbury 5.2) and susceptible (LBB 101, Llandillo 104, LS2) strains of *L. cuprina* were tested for esterase-mediated hydrolysis of CVP, a general OP (ie not a malathion-type OP). All 10 malathion resistant strains had greater CVP hydrolytic activities (1.5 -3.0 pmol/min/mg) than the 3 susceptible strains (0.5 -1.0 pmol/min/mg, but less activity than the 6 diazinon resistant strains (8.2 - 30.0 pmol/min/mg).

The expressed product of the malathion resistant *LcαE7* allele was tested for CVP hydrolytic activity. Turnover of 75μM CVP was about 1.2 hour⁻¹, which is approximately 50-fold less than that of the diazinon resistant (RM2-6) *LcαE7* gene product but much greater than that of the OP-susceptible (LS2) gene product, for which CVP activity was undetectable. [The CVP hydrolytic activity of the gene products of the RM2-6 and LS2 alleles of *LcαE7* are described in patent application PCT/AU 95/00016 : "Enzyme based bioremediation"].

g) Conclusions

1. We have discovered that dimethyl versus diethyl patterns of OP cross-resistance among strains of *L. cuprina* parallel those of OP resistant strains of *M. domestica* and *C. putoria* . The two OP resistance types are equally potent and general among most OPs (excluding malathion), albeit with a dimethyl or diethyl OP preference.

2. Diethyl OP hydrolytic activities encoded by the OP susceptible allele (nil), the malathion resistant allele (1.2 hour⁻¹) and the diazinon/parathion resistant alleles of *LcαE7* (~1 min⁻¹) parallel the diethyl OP hydrolytic activities in homogenates of OP susceptible (low), malathion resistant (intermediate) and diazinon/parathion resistant (high) *L. cuprina* strains.

3. Taking points 1 and 2 together we propose that the dimethyl versus diethyl pattern of general OP cross resistance reflects the substrate specificity of the general OP hydrolase activities encoded by the two alternative OP resistance alleles of the $\alpha E7$ gene. Thus we expect that products of malathion resistance alleles of $\alpha E7$ genes from *L. cuprina* and *M. domestica* will exhibit dimethyl OP hydrolysis with kinetics that are as favourable for bioremediation as the diazinon/parathion resistance $\alpha E7$ alleles are for diethyl OPs.
4. The enhanced MCE activity of the product of the malathion resistance alleles of $\alpha E7$ genes causes flies to survive more than 100-fold greater doses of malathion. The MCE activity is enhanced in two ways. Firstly, it has more favourable kinetics for malathion breakdown than that of the susceptible allele (K_{cat}/K_m is 6-fold greater). Secondly, it has acquired general OP hydrolase activity. The latter is important for both resistance and bioremediation because it enables the enzyme to recover its MCE activity after phosphorylation/inhibition by the 'activated' or 'P=O' form of OP insecticides. P=O OPs are encountered in an insect because they are generated by an insect's metabolism. For bioremediation the OP hydrolase is required for two reasons; firstly, to hydrolyse general OPs where these are the main contaminant, and secondly, to ensure that malathion hydrolysis by the enzyme will continue in the presence of minor contamination with 'P=O' OPs.

ALLELISM OF THE MALATHION AND DIAZINON/PARATHION RESISTANCE PHENOTYPES IN *L. CUPRINA*

It is clear from the above molecular genetic and biochemical data that malathion resistance in *L. cuprina* is conferred by a structural mutation in the active site of *Lc* $\alpha E7$ (esterase E3), the same gene that is involved in resistance to diazinon/parathion type OPs. However, two classical genetic studies have detected a small number of presumptive recombinants between the malathion and diazinon resistance phenotypes in *L. cuprina*, which would suggest that they are separate, albeit closely linked, genes (Raftos, D.A. and Hughes, P.B., Journal of Economic Entomology 77: 553, 1986;

Smyth, K-A., Russell, R.J. and Oakeshott, J.G., Biochemical Genetics 32: 437, 1994).

The availability of frozen extracts from three of the five presumptive recombinants generated by the study of Smyth *et al.* (1994) enabled the authors to test them directly for the predicted genotypes using PCR techniques. In this particular study, diazinon resistant males (strain Q4) were crossed to malathion resistant females (strain RM-8) and the F1 females backcrossed to Q4 males. Progeny were scored for the E3-non-staining, high MCE phenotypes as indicative of resistance to both diazinon and malathion. Five presumably recombinant individuals showing the MCE high/E3 non-staining phenotype were recovered from 692 backcross progeny; none were recovered with the reciprocal MCE low/E3 staining phenotype, which would presumably be susceptible to both malathion and diazinon.

15 **Methods:**

The regions of the *Lc σ E7* gene surrounding the diazinon resistance (Gly to Asp substitution at position 137) and malathion resistance (Trp to Leu at position 251) mutations were amplified from each of the three available extracts from the Smyth *et al.* (1994) study, using the 7F1/7R2 and 7F7/7R4 primer sets, respectively. The PCR conditions and primers (except 7F7) are as described above, except that an annealing temperature of 55°C and a buffer supplied by the manufacturer of Taq DNA polymerase (BRL; 0.2 mM of each dNTP, 20 mM Tris-HCl, pH8.4, 50 mM KCl, 1.5 mM MgCl₂) were used. The 7F7 primer has the sequence: 5' tgctgcctctaccactacat 3' and its 5' position in the Lc743 sequence is nucleotide 660 (see Figure 1).

The 330nt fragment of *Lc α E7* generated by the 7F7/7R4 primer set contains an RFLP polymorphism specific for each of the Q4 and RM-8 alleles: an *Nco*I cleavage site at nucleotide position 752 marks the Q4 allele (this polymorphism is at the site of the Trp to Leu mutation responsible for malathion resistance), while a *Bgl*I site at position 796 characterises the RM-8 allele. Therefore, in order to identify the Q4 and RM-8 alleles in each extract, PCR products were digested directly with each restriction enzyme and the products sized by agarose gel electrophoresis, using standard techniques (Sambrook, J., Fritsch, E.F. and Maniatis, T., Molecular Cloning:

A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, USA, 1989). Controls contained PCR products generated from Q4 and RM-8 genomic DNA.

No such convenient RFLP polymorphisms were contained in the
5 326nt fragment amplified by the 7F1/7R2 primer set (this fragment contains a 68nt intron at nucleotide position 360). However, three nucleotide polymorphisms distinguish the Q4 and RM-8 fragments: an A to T substitution at nucleotide position 303 , T to C at position 345 and G to A at position 410 in the Q4 sequence (the latter substitution is responsible for
10 diazinon/parathion resistance). PCR products were therefore cloned into the pGEM-T vector (Promega) using standard techniques (Sambrook, J., Fritsch, E.F. and Maniatis, T., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, USA, 1989) and individual clones sequenced using commercially available SP6 and T7
15 primers and dye terminator technology, as described above.

Results:

Digestion of the PCR products generated by the 7F7/7R4 primer set
20 with either *Nco1* or *Bgl1* revealed the presence of only the RM-8 chromosome in all three extracts (ie PCR products could be cleaved by *Bgl1* and not *Nco1*, whereas the products of the control Q4 genomic DNA were readily digested with *Nco1* ; data not shown). Curiously, the Q4 allele was not amplified from any of the extracts despite the fact that F1 progeny were
25 backcrossed to Q4 in the crossing regime.

Two out of three clones derived from the first extract, and two out of two clones derived from a second, contained polymorphisms characteristic of the Q4 allele at positions 303 and 345 and the polymorphism characteristic of RM-8 at position 410. On the other hand, the third clone
30 derived from the first extract and a single clone derived from the third extract contained all three polymorphisms characteristic of RM-8. Again, no fragments generated entirely from the Q4 chromosome were found among the cloned DNAs.

Conclusions:

1. The Q4 allele was not amplified from any of the extracts despite the fact that F1 progeny were backcrossed to Q4 and would therefore be expected to contain at least one copy of the Q4 fourth chromosome.
 2. Progeny homozygous for the Q4 allele would be expected if the malathion resistance mutation was located on a gene separate from *LcaE7*. No such progeny were found.
 3. No MCE low/E3 staining (presumably susceptible to both malathion and diazinon) progeny were recovered, which would be expected if E3 and MCE were separate genes.
 4. At least two of the extracts contained a fourth chromosome that was a Q4/RM-8 recombinant somewhere in the region of the *LcaE7* gene 5' to the Gly to Asp mutation at nucleotide position 410. The origin of flies carrying this fourth chromosome and the MCE activity / PAGE phenotype of the resultant chimeric protein are unknown.
 5. It is clear that none of the putative E3/MCE recombinants were the outcome of simple reciprocal recombination events during the crossing programs; they do not, therefore, constitute proof that the E3 and MCE genes are separate genes.
- It is clear from the present invention that malathion resistance in *L. cuprina* is the result of a structural mutation in the *LcaE7* (E3) gene, the same gene which mutates to give resistance to diazinon/parathion type OPs. In other words, the MCE and E3 genes are probably allelic and not separated by 0.7 map units as previous classical genetic studies had indicated. The allelism of the two resistance mutations explains the observation of Smyth, K-A., Russell, R.J. and Oakeshott, J.G. (Biochemical Genetics 32:437, 1994) that there is a negative association between malathion-type OP resistance and diazinon/parathion type OP resistance. The presence of malathion-type resistance alleles in a population would therefore suggest the use of diethyl

OPs for combating flystrike, while the presence of diazinon/parathion-type resistance alleles would suggest the use of dimethyl OPs.

5 The MCE enzyme produced by the method of the present invention may be used to develop a functional *in vitro* assay for the degradation of carboxylester and dimethyl type OPs in much the same way as the E3 enzyme (Patent: Enzyme Based Bioremediation) has been used to develop an *in vitro* assay for the general OP hydrolysis. Used together, such assays provide scope for screening for alternative OPs that might overcome resistance problems by E3-like enzymes.

10 It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

- (A) NAME: COMMONWEALTH SCIENTIFIC AND INDUSTRIAL
RESEARCH ORGANISATION
(B) STREET: Limestone Avenue
(C) CITY: Campbell
(D) COUNTRY: Australia
(E) POSTAL CODE: Act 2601

(ii) TITLE OF INVENTION: MALATHION CARBOXYLESTERASE

(iii) NUMBER OF SEQUENCES: 43

(iv) CORRESPONDENCE ADDRESS:

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SIM & MCBURNEY
330 UNIVERSITY AVENUE, 6TH FLOOR
TORONTO, CANADA M5G 1R7

(v) COMPUTER-READABLE FORM

- (A) COMPUTER: IBM PC COMPATIBLE
(B) OPERATING SYSTEM: PC-DOS/MS DOS
(C) SOFTWARE: PatentIn Release #1.0, Version#1.25 (EPO)

(vi) CURRENT APPLICATION DATA:

- (A) APPLICATION NUMBER: CA 2,236,793
(B) FILING DATE: November 22, 1996
(C) CLASSIFICATION: C12N-15/55

(vii) PRIOR APPLICATION DATA

- (A) APPLICATION NUMBER: PN 6751
(B) FILING DATE: November 23, 1995
(C) CLASSIFICATION: C12N-15/55

(viii) PATENT AGENT INFORMATION:

- (A) NAME: John H. Woodley
(B) REFERENCE NUMBER: JHW 2838-24

(2) INFORMATION FOR SEQUENCE ID NO.: 1

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1713 Nucleotides
(B) TYPE: DNA
(C) STRANDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULAR TYPE: *Lucilia cuprina*

(xi) SEQUENCE DESCRIPTION: SEQ ID NO. 1

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ccctgggatg gtgtgcgtga ttgttgcaat cataaagata agtcagtgca agttgatttt 300
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tcgatgtttg aaaaacatag agatttattt tag 1713

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(2) INFORMATION FOR SEQUENCE ID NO.: 2

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 570 Amino Acids
- (B) TYPE: PRT
- (C) STRANDNESS: single
- (D) TOPOLOGY: linear
- (E) ORIGINAL SOURCE: *Lucilia cuprina*

(Xi) SEQUENCE DESCRIPTION: SEQ ID NO. 2

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Cys Ile Glu Asn Lys Phe Leu Asn Tyr Arg Leu Thr Thr Asn Glu Thr
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Val Val Ala Glu Thr Glu Tyr Gly Lys Val Lys Gly Val Lys Arg Leu
      35              40              45

Thr Val Tyr Asp Asp Ser Tyr Tyr Ser Phe Glu Gly Ile Pro Tyr Ala
      50              55              60

Gln Pro Pro Val Gly Glu Leu Arg Phe Lys Ala Pro Gln Arg Pro Thr
      65              70              75              80

Pro Trp Asp Gly Val Arg Asp Cys Cys Asn His Lys Asp Lys Ser Val
      85              90              95

Gln Val Asp Phe Ile Thr Gly Lys Val Cys Gly Ser Glu Asp Cys Leu
      100             105             110

Tyr Leu Ser Val Tyr Thr Asn Asn Leu Asn Pro Glu Thr Lys Arg Pro
      115             120             125

Val Leu Val Tyr Ile His Gly Gly Gly Phe Ile Ile Gly Glu Asn His
      130             135             140

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Ser	Glu	Asp	Leu	Asn	Val	Pro	Gly	Asn	Ala	Gly	Leu	Lys	Asp	Gln	Val	180	185	190	
Met	Ala	Leu	Arg	Trp	Ile	Lys	Asn	Asn	Cys	Ala	Asn	Phe	Gly	Gly	Asn	195	200	205	
Pro	Asp	Asn	Ile	Thr	Val	Phe	Gly	Glu	Ser	Ala	Gly	Ala	Ala	Ser	Thr	210	215	220	
His	Tyr	Met	Met	Leu	Thr	Glu	Gln	Thr	Arg	Gly	Leu	Phe	His	Arg	Gly	225	230	235	240
Ile	Leu	Met	Ser	Gly	Asn	Ala	Ile	Cys	Pro	Leu	Ala	Asn	Thr	Gln	Cys	245	250	255	
Gln	His	Arg	Ala	Phe	Thr	Leu	Ala	Lys	Leu	Ala	Gly	Tyr	Lys	Gly	Glu	260	265	270	
Asp	Asn	Asp	Lys	Asp	Val	Leu	Glu	Phe	Leu	Met	Lys	Ala	Lys	Pro	Gln	275	280	285	
Asp	Leu	Ile	Lys	Leu	Glu	Glu	Lys	Val	Leu	Thr	Leu	Glu	Glu	Arg	Thr	290	295	300	
Asn	Lys	Val	Met	Phe	Pro	Phe	Gly	Pro	Thr	Val	Glu	Pro	Tyr	Gln	Thr	305	310	315	320
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Trp	Gly	Asn	Ser	Ile	Pro	Thr	Met	Met	Gly	Asn	Thr	Ser	Tyr	Glu	Gly	340	345	350	
Leu	Phe	Phe	Thr	Ser	Ile	Leu	Lys	Gln	Met	Pro	Met	Leu	Val	Lys	Glu	355	360	365	
Leu	Glu	Thr	Cys	Val	Asn	Phe	Val	Pro	Ser	Glu	Leu	Ala	Asp	Ala	Glu	370	375	380	
Arg	Thr	Ala	Pro	Glu	Thr	Leu	Glu	Met	Gly	Ala	Lys	Ile	Lys	Lys	Ala	385	390	395	400
His	Val	Thr	Gly	Glu	Thr	Pro	Thr	Ala	Asp	Asn	Phe	Met	Asp	Leu	Cys	405	410	415	
Ser	His	Ile	Tyr	Phe	Trp	Phe	Pro	Met	His	Arg	Leu	Leu	Gln	Leu	Arg	420	425	430	
Phe	Asn	His	Thr	Ser	Gly	Thr	Pro	Val	Tyr	Leu	Tyr	Arg	Phe	Asp	Phe	435	440	445	
Asp	Ser	Glu	Asp	Leu	Ile	Asn	Pro	Tyr	Arg	Ile	Met	Arg	Ser	Gly	Arg	450	455	460	

Gly Val Lys Gly Val Ser His Ala Asp Glu Leu Thr Tyr Phe Phe Trp
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 Asn Gln Leu Ala Lys Arg Met Pro Lys Glu Ser Arg Glu Tyr Lys Thr
 485 490 495
 Ile Glu Arg Met Thr Gly Ile Trp Ile Gln Phe Ala Thr Thr Gly Asn
 500 505 510
 Pro Tyr Ser Asn Glu Ile Glu Gly Met Glu Asn Val Ser Trp Asp Pro
 515 520 525
 Ile Lys Lys Ser Asp Glu Val Tyr Lys Cys Leu Asn Ile Ser Asp Glu
 530 535 540
 Leu Lys Met Ile Asp Val Pro Glu Met Asp Lys Ile Lys Gln Trp Glu
 545 550 555 560
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(2) INFORMATION FOR SEQUENCE ID NO.: 3

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1713 Nucleotides
 (B) TYPE: DNA
 (C) STRANDNESS: single
 (D) TOPOLOGY: linear
 (E) ORIGINAL SOURCE: *Lucilia cuprina*

(ii) MOLECULAR TYPE: other nucleic acid, primer

(Xi) SEQUENCE DESCRIPTION :

SEQ ID NO. 3

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 ccctgggatg gtgtgcgca ttgttgcaat cataaagata agtcagtgca agttgatttt 300
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 atggaaaatg tttcctggga tccaattaag aaatccgatg aagtatacaa gtgtttgaat 1620
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1713

(A) LENGTH: 570 Amino Acids
(B) TYPE: PRT
(C) STRANDNESS: single
(D) TOPOLOGY: linear
(E) ORIGINAL SOURCE: *Lucilia cuprina*

(Xi) SEQUENCE DESCRIPTION: SEQ ID NO. 4

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Thr	Val 50	Tyr	Asp	Asp	Ser	Tyr 55	Tyr	Ser	Phe	Glu	Gly 60	Ile	Pro	Tyr	Ala
Gln 65	Pro	Pro	Val	Gly	Glu 70	Leu	Arg	Phe	Lys	Ala 75	Pro	Gln	Arg	Pro	Thr 80
Pro	Trp	Asp	Gly	Val 85	Arg	Asp	Cys	Cys	Asn 90	His	Lys	Asp	Lys	Ser 95	Val
Gln	Val	Asp	Phe 100	Ile	Thr	Gly	Lys 105	Val	Cys	Gly	Ser	Glu	Asp 110	Cys	Leu
Tyr	Leu	Ser 115	Val	Tyr	Thr	Asn	Asn 120	Leu	Asn	Pro	Glu	Thr 125	Lys	Arg	Pro
Val 130	Leu	Val	Tyr	Ile	His	Gly 135	Gly	Gly	Phe	Ile	Ile 140	Gly	Glu	Asn	His
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Ile	Asn	Ile	Gln 165	Tyr	Arg	Leu	Gly	Ala	Leu 170	Gly	Phe	Leu	Ser	Leu 175	Asn
Ser	Glu	Asp	Leu 180	Asn	Val	Pro	Gly	Asn 185	Ala	Gly	Leu	Lys	Asp 190	Gln	Val
Met	Ala	Leu 195	Arg	Trp	Ile	Lys	Asn 200	Asn	Cys	Ala	Asn	Phe 205	Gly	Gly	Asn
Pro 210	Asp	Asn	Ile	Thr	Val	Phe 215	Gly	Glu	Ser	Ala	Gly 220	Ala	Ala	Ser	Thr
His 225	Tyr	Met	Met	Leu	Thr 230	Glu	Gln	Thr	Arg	Gly 235	Leu	Phe	His	Arg	Gly 240

Ile	Leu	Met	Ser	Gly	Asn	Ala	Ile	Cys	Pro	Leu	Ala	Asn	Thr	Gln	Cys	245	250	255
Gln	His	Arg	Ala	Phe	Thr	Leu	Ala	Lys	Leu	Ala	Gly	Tyr	Lys	Gly	Glu	260	265	270
Asp	Asn	Asp	Lys	Asp	Val	Leu	Glu	Phe	Leu	Met	Lys	Ala	Lys	Pro	Gln	275	280	285
Asp	Leu	Ile	Lys	Leu	Glu	Glu	Lys	Val	Leu	Thr	Leu	Glu	Glu	Arg	Thr	290	295	300
Asn	Lys	Val	Met	Phe	Pro	Phe	Gly	Pro	Thr	Val	Glu	Pro	Tyr	Gln	Thr	305	310	315
Ala	Asp	Cys	Val	Leu	Pro	Lys	His	Pro	Arg	Glu	Met	Val	Lys	Thr	Ala	325	330	335
Trp	Gly	Asn	Ser	Ile	Pro	Thr	Met	Met	Gly	Asn	Thr	Ser	Tyr	Glu	Gly	340	345	350
Leu	Phe	Phe	Thr	Ser	Ile	Leu	Lys	Gln	Met	Pro	Met	Leu	Val	Lys	Glu	355	360	365
Leu	Glu	Thr	Cys	Val	Asn	Phe	Val	Pro	Ser	Glu	Leu	Ala	Asp	Ala	Glu	370	375	380
Arg	Thr	Ala	Pro	Glu	Thr	Leu	Glu	Met	Gly	Ala	Lys	Ile	Lys	Lys	Ala	385	390	395
His	Val	Thr	Gly	Glu	Thr	Pro	Thr	Ala	Asp	Asn	Phe	Met	Asp	Leu	Cys	405	410	415
Ser	His	Ile	Tyr	Phe	Trp	Phe	Pro	Met	His	Arg	Leu	Leu	Gln	Leu	Arg	420	425	430
Phe	Asn	His	Thr	Ser	Gly	Thr	Pro	Val	Tyr	Leu	Tyr	Arg	Phe	Asp	Phe	435	440	445
Asp	Ser	Glu	Asp	Leu	Ile	Asn	Pro	Tyr	Arg	Ile	Met	Arg	Ser	Gly	Arg	450	455	460
Gly	Val	Lys	Gly	Val	Ser	His	Ala	Asp	Glu	Leu	Thr	Tyr	Phe	Phe	Trp	465	470	475
Asn	Gln	Leu	Ala	Lys	Arg	Met	Pro	Lys	Glu	Ser	Arg	Glu	Tyr	Lys	Thr	485	490	495
Ile	Glu	Arg	Met	Thr	Gly	Ile	Trp	Ile	Gln	Phe	Ala	Thr	Thr	Gly	Asn	500	505	510
Pro	Tyr	Ser	Asn	Glu	Ile	Glu	Gly	Met	Glu	Asn	Val	Ser	Trp	Asp	Pro	515	520	525
Ile	Lys	Lys	Ser	Asp	Glu	Val	Tyr	Lys	Cys	Leu	Asn	Ile	Ser	Asp	Glu	530	535	540
Leu	Lys	Met	Ile	Asp	Val	Pro	Glu	Met	Asp	Lys	Ile	Lys	Gln	Trp	Glu	545	550	555

Ser Met Phe Glu Lys His Arg Asp Leu Phe
565 570

(2) INFORMATION FOR SEQUENCE ID NO.: 5

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1713 Nucleotides
- (B) TYPE: DNA
- (C) STRANDNESS: single
- (D) TOPOLOGY: linear
- (E) ORIGINAL SOURCE: *Lucilia cuprina*

(ii) MOLECULAR TYPE: other nucleic acid, primer

(Xi) SEQUENCE DESCRIPTION: SEQ ID NO. 5

atgaatttca	acgttagttt	gatggagaaa	ttaaaatgga	agattaaatg	cattgaaaat	60
aagtttttta	actatcgttt	aactaccaat	gaaacgggtg	tagctgaaac	tgaatatggc	120
aaagtgaag	gcgttaaacg	tttaactgtg	tacgatgatt	cctactacag	ttttgagggg	180
ataccgtacg	cccaaccgcc	agtgggtgag	ctgagattta	aagcacccca	gcgaccaaca	240
ccctgggatg	gtgtgctga	ttgttgcaat	cataaagata	agtcagtgca	agttgatttt	300
ataacgggca	aagtgtgtgg	ctcagaggat	tgtctatacc	taagtgtcta	tacgaataat	360
ctaaatcccg	aaactaaacg	tcccgtttta	gtatacatat	atgggtgggtg	ttttattatc	420
ggtgaaaatc	atcgtgatat	gtatggctct	gattatttca	ttaaaaagga	tgtgggtgtg	480
attaacatac	aatatcgttt	gggagctcta	ggttttctaa	gtttaaattc	agaagacctt	540
aatgtgcccc	gtaatgccgg	ccttaaagat	caagtcattg	ccttgcatcg	gattaaaaat	600
aattgcgcca	actttgggtg	caatcccgat	aattattacag	tctttgggtg	aagtgccggg	660
gctgcctcta	cccactacat	gatgttaacc	gaacaaactc	gcggctcttt	ccatcgtggg	720
atactaattg	cgggtaattg	tattttgtcca	ttggctaata	cccaatgtca	acatcgtgcc	780
ttcaccttag	ccaaattggc	cggctataag	ggtgagaata	atgataagga	tgtttttgga	840
tttcttatga	aagccaagcc	acaggattta	gtaaaacttg	aggaaaaagt	tttaactcta	900
gaagagcgta	caaataaggt	catgtttcct	tttgggtcca	ctggttgagc	atatcagacc	960
gctgattgtg	tcttacccaa	acatcctcgg	gaaatgggta	aaactgcttg	gggtaattcg	1020
atacccacta	tgatgggtaa	cacttcatat	gagggtctat	ttttcacttc	aattcttaag	1080
caaatgccta	tgcttggtta	ggaattggaa	acttggtgca	atctttgtgc	aagtgaattg	1140
gctgatgctg	aacgcaccgc	cccagagacc	ttggaaatgg	gtgctaaaat	taaaaagggt	1200
catgttacag	gagaaacacc	aacagctgat	aatttttatg	atctttgtct	tcacatctat	1260
ttctggttcc	ccatgcacgc	tttggtgcaa	ttacgtttca	atcacacctc	cggtagaccc	1320
gtctacttgt	atcgcttcga	cttcgattcg	gaagatctta	tcaatcccta	tcgtattatg	1380
cgtagtggac	gtgggtgtta	gggtgttagt	catgctgatg	aattaacctt	tttcttctgg	1440
aatcaattgg	ccaaacgtat	gcctaaagaa	tcgcgtgaat	acaaaacaat	tgaacgtatg	1500
actggatat	ggatacaatt	tgccaccact	ggtaatcctt	atagcaatga	aattgaagggt	1560
atggaaaatg	tttcctggga	tccaattaag	aaatccgatg	aagtatacaa	gtgtttgaat	1620
attagtgatg	aattgaaaat	gattgatgtg	cctgaaatgg	ataagattaa	acaatggggag	1680
tcgatgtttg	aaaaacatag	agatttattt	tag			1713

(2) INFORMATION FOR SEQUENCE ID NO.: 6

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 570 Amino Acids
- (B) TYPE: PRT
- (C) STRANDNESS: single
- (D) TOPOLOGY: linear
- (E) ORIGINAL SOURCE: *Lucilia cuprina*

(ii) MOLECULAR TYPE: other nucleic acid, primer

(Xi) SEQUENCE DESCRIPTION: SEQ ID NO. 6

Met	Asn	Phe	Asn	Val	Ser	Leu	Met	Glu	Lys	Leu	Lys	Trp	Lys	Ile	Lys	1	5	10	15
Cys	Ile	Glu	Asn	Lys	Phe	Leu	Asn	Tyr	Arg	Leu	Thr	Thr	Asn	Glu	Thr	20	25	30	
Val	Val	Ala	Glu	Thr	Glu	Tyr	Gly	Lys	Val	Lys	Gly	Val	Lys	Arg	Leu	35	40	45	
Thr	Val	Tyr	Asp	Asp	Ser	Tyr	Tyr	Ser	Phe	Glu	Gly	Ile	Pro	Tyr	Ala	50	55	60	
Gln	Pro	Pro	Val	Gly	Glu	Leu	Arg	Phe	Lys	Ala	Pro	Gln	Arg	Pro	Thr	65	70	75	80
Pro	Trp	Asp	Gly	Val	Arg	Asp	Cys	Cys	Asn	His	Lys	Asp	Lys	Ser	Val	85	90	95	
Gln	Val	Asp	Phe	Ile	Thr	Gly	Lys	Val	Cys	Gly	Ser	Glu	Asp	Cys	Leu	100	105	110	
Tyr	Leu	Ser	Val	Tyr	Thr	Asn	Asn	Leu	Asn	Pro	Glu	Thr	Lys	Arg	Pro	115	120	125	
Val	Leu	Val	Tyr	Ile	His	Gly	Gly	Gly	Phe	Ile	Ile	Gly	Glu	Asn	His	130	135	140	
Arg	Asp	Met	Tyr	Gly	Pro	Asp	Tyr	Phe	Ile	Lys	Lys	Asp	Val	Val	Leu	145	150	155	160
Ile	Asn	Ile	Gln	Tyr	Arg	Leu	Gly	Ala	Leu	Gly	Phe	Leu	Ser	Leu	Asn	165	170	175	
Ser	Glu	Asp	Leu	Asn	Val	Pro	Gly	Asn	Ala	Gly	Leu	Lys	Asp	Gln	Val	180	185	190	
Met	Ala	Leu	Arg	Trp	Ile	Lys	Asn	Asn	Cys	Ala	Asn	Phe	Gly	Gly	Asn	195	200	205	
Pro	Asp	Asn	Ile	Thr	Val	Phe	Gly	Glu	Ser	Ala	Gly	Ala	Ala	Ser	Thr	210	215	220	
His	Tyr	Met	Met	Leu	Thr	Glu	Gln	Thr	Arg	Gly	Leu	Phe	His	Arg	Gly	225	230	235	240
Ile	Leu	Met	Ser	Gly	Asn	Ala	Ile	Cys	Pro	Leu	Ala	Asn	Thr	Gln	Cys	245	250	255	
Gln	His	Arg	Ala	Phe	Thr	Leu	Ala	Lys	Leu	Ala	Gly	Tyr	Lys	Gly	Glu	260	265	270	
Asp	Asn	Asp	Lys	Asp	Val	Leu	Glu	Phe	Leu	Met	Lys	Ala	Lys	Pro	Gln	275	280	285	
Asp	Leu	Ile	Lys	Leu	Glu	Glu	Lys	Val	Leu	Thr	Leu	Glu	Glu	Arg	Thr	290	295	300	
Asn	Lys	Val	Met	Phe	Pro	Phe	Gly	Pro	Thr	Val	Glu	Pro	Tyr	Gln	Thr	305	310	315	320

Ala	Asp	Cys	Val	Leu	Pro	Lys	His	Pro	Arg	Glu	Met	Val	Lys	Thr	Ala	325	330	335	
Trp	Gly	Asn	Ser	Ile	Pro	Thr	Met	Met	Gly	Asn	Thr	Ser	Tyr	Glu	Gly	340	345	350	
Leu	Phe	Phe	Thr	Ser	Ile	Leu	Lys	Gln	Met	Pro	Met	Leu	Val	Lys	Glu	355	360	365	
Leu	Glu	Thr	Cys	Val	Asn	Phe	Val	Pro	Ser	Glu	Leu	Ala	Asp	Ala	Glu	370	375	380	
Arg	Thr	Ala	Pro	Glu	Thr	Leu	Glu	Met	Gly	Ala	Lys	Ile	Lys	Lys	Ala	385	390	395	400
His	Val	Thr	Gly	Glu	Thr	Pro	Thr	Ala	Asp	Asn	Phe	Met	Asp	Leu	Cys	405	410	415	
Ser	His	Ile	Tyr	Phe	Trp	Phe	Pro	Met	His	Arg	Leu	Leu	Gln	Leu	Arg	420	425	430	
Phe	Asn	His	Thr	Ser	Gly	Thr	Pro	Val	Tyr	Leu	Tyr	Arg	Phe	Asp	Phe	435	440	445	
Asp	Ser	Glu	Asp	Leu	Ile	Asn	Pro	Tyr	Arg	Ile	Met	Arg	Ser	Gly	Arg	450	455	460	
Gly	Val	Lys	Gly	Val	Ser	His	Ala	Asp	Glu	Leu	Thr	Tyr	Phe	Phe	Trp	465	470	475	480
Asn	Gln	Leu	Ala	Lys	Arg	Met	Pro	Lys	Glu	Ser	Arg	Glu	Tyr	Lys	Thr	485	490	495	
Ile	Glu	Arg	Met	Thr	Gly	Ile	Trp	Ile	Gln	Phe	Ala	Thr	Thr	Gly	Asn	500	505	510	
Pro	Tyr	Ser	Asn	Glu	Ile	Glu	Gly	Met	Glu	Asn	Val	Ser	Trp	Asp	Pro	515	520	525	
Ile	Lys	Lys	Ser	Asp	Glu	Val	Tyr	Lys	Cys	Leu	Asn	Ile	Ser	Asp	Glu	530	535	540	
Leu	Lys	Met	Ile	Asp	Val	Pro	Glu	Met	Asp	Lys	Ile	Lys	Gln	Trp	Glu	545	550	555	560
Ser	Met	Phe	Glu	Lys	His	Arg	Asp	Leu	Phe							565	570		

(2) INFORMATION FOR SEQUENCE ID NO.: 7

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1713 Nucleotides
- (B) TYPE: DNA
- (C) STRANDNESS: single
- (D) TOPOLOGY: linear
- (E) ORIGINAL SOURCE: *Lucilia cuprina*

(ii) MOLECULAR TYPE: other nucleic acid, primer

(xi) SEQUENCE DESCRIPTION: SEQ ID NO. 7

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atgaattttca acgttagttt gatggagaaa ttaaaatgga agattaaatg cattgaaaat 60
aagttttttaa actatcgttt aactaccaat gaaacggtgg tagctgaaac tgaatatggc 120
aaagtgaaag gcgttaaacg tttaactgtg tacgatgatt cctactacag ttttgagggg 180
ataccgtacg cccaaccgcc agtgggtgag ctgagattta aagcacccca gcgaccaaca 240
ccctgggatg gtgtgctga ttgttgcaat cataaagata agtcagtgca agttgatttt 300
ataacgggca aagtgtgtgg ctcagaggat tgtctatacc taagtgtcta tacgaataat 360
ctaaatcccg aaactaaacg tcccgtttta gtatacatat atgggtgggtg ttttattatc 420
ggtgaaaatc atcgtgatat gtatggtcct gattatttca ttaaaaagga tgtgggtgttg 480
attaacatac aatatcgttt gggagctcta gggttttctaa gtttaaattc agaagacctt 540
aatgtgcccc gtaatgccgg ccttaaagat caagtcattg ccttgcggtg gattaaaaat 600
aattgcgcca actttgggtg caatcccgat aatattacag tctttgggtg aagtgccggg 660
gctgcctcta cccactacat gatgttaacc gaacaaactc gcggtctttt ccatcggtgg 720
ataactaatg cgggtaatgc tatttgcca tgggctaata ccaatgtca acatcggtgc 780
ttcaccttag ccaaattggc cggctataag ggtgaggata atgataagga tgttttggaa 840
tttcttatga aagccaagcc acaggattta ataaaacttg aggaaaaagt ttttaactcta 900
gaagagcgta caaataaggt catgtttcct tttgggtcca ctggtgagcc atatcagacc 960
gctgattgtg tcttacccaa acatcctcgg gaaatgggta aaactgcttg gggtaattcg 1020
atacccacta tgatgggtaa cacttcatat gaggggtctat ttttcacttc aattccttaag 1080
caaatgccta tgcttggtta ggaattggaa acttggtgca attttggtgc aagtgaattg 1140
gctgatgctg aacgcaccgc cccagagacc ttggaaatgg gtgctaaaat taaaaaggct 1200
catgttacag gagaaacacc aacagctgat aatttttatg atctttgctc tcacatctat 1260
ttctgggttc ccatgcatcg tttgttgcaa ttacgtttca atcacacctc cgttacaccc 1320
gtctacttgt atcgcttcga ctttgattcg gaagatctta ttaatcccta tcgtattatg 1380
cgtagtggac gtggtgttaa ggggtgttag catgctgatg aattaaccta tttcttctgg 1440
aatcaattgg ccaaacgtat gcctaaagaa tcgctggaat acaaaaacaat tgaacgtatg 1500
actggtatat ggatacaatt tgccaccact ggtaatcctt atagcaatga aattgaagg 1560
atggaaaatg tttcctggga tccaattaag aaatccgacg aagtatacaa gtgtttgaat 1620
attagtgacg aattgaaaat gattgatgtg cctgaaatgg ataagattaa acaatgggaa 1680
tcgatgtttg aaaaacatag agatttattt tag 1713

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(2) INFORMATION FOR SEQUENCE ID NO.: 8

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 570 Amino Acids
- (B) TYPE: PRT
- (C) STRANDNESS: single
- (D) TOPOLOGY: linear
- (E) ORIGINAL SOURCE: *Lucilia cuprina*

(ii) MOLECULAR TYPE: other nucleic acid, primer

(Xi) SEQUENCE DESCRIPTION: SEQ ID NO. 8

```

Met Asn Phe Asn Val Ser Leu Met Glu Lys Leu Lys Trp Lys Ile Lys
 1             5             10             15

Cys Ile Glu Asn Lys Phe Leu Asn Tyr Arg Leu Thr Thr Asn Glu Thr
      20             25             30

Val Val Ala Glu Thr Glu Tyr Gly Lys Val Lys Gly Val Lys Arg Leu
      35             40             45

Thr Val Tyr Asp Asp Ser Tyr Tyr Ser Phe Glu Gly Ile Pro Tyr Ala
      50             55             60

Gln Pro Pro Val Gly Glu Leu Arg Phe Lys Ala Pro Gln Arg Pro Thr
      65             70             75             80

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Pro	Trp	Asp	Gly	Val	Arg	Asp	Cys	Cys	Asn	His	Lys	Asp	Lys	Ser	Val	85	90	95	
Gln	Val	Asp	Phe	Ile	Thr	Gly	Lys	Val	Cys	Gly	Ser	Glu	Asp	Cys	Leu	100	105	110	
Tyr	Leu	Ser	Val	Tyr	Thr	Asn	Asn	Leu	Asn	Pro	Glu	Thr	Lys	Arg	Pro	115	120	125	
Val	Leu	Val	Tyr	Ile	His	Gly	Gly	Gly	Phe	Ile	Ile	Gly	Glu	Asn	His	130	135	140	
Arg	Asp	Met	Tyr	Gly	Pro	Asp	Tyr	Phe	Ile	Lys	Lys	Asp	Val	Val	Leu	145	150	155	160
Ile	Asn	Ile	Gln	Tyr	Arg	Leu	Gly	Ala	Leu	Gly	Phe	Leu	Ser	Leu	Asn	165	170	175	
Ser	Glu	Asp	Leu	Asn	Val	Pro	Gly	Asn	Ala	Gly	Leu	Lys	Asp	Gln	Val	180	185	190	
Met	Ala	Leu	Arg	Trp	Ile	Lys	Asn	Asn	Cys	Ala	Asn	Phe	Gly	Gly	Asn	195	200	205	
Pro	Asp	Asn	Ile	Thr	Val	Phe	Gly	Glu	Ser	Ala	Gly	Ala	Ala	Ser	Thr	210	215	220	
His	Tyr	Met	Met	Leu	Thr	Glu	Gln	Thr	Arg	Gly	Leu	Phe	His	Arg	Gly	225	230	235	240
Ile	Leu	Met	Ser	Gly	Asn	Ala	Ile	Cys	Pro	Trp	Ala	Asn	Thr	Gln	Cys	245	250	255	
Gln	His	Arg	Ala	Phe	Thr	Leu	Ala	Lys	Leu	Ala	Gly	Tyr	Lys	Gly	Glu	260	265	270	
Asp	Asn	Asp	Lys	Asp	Val	Leu	Glu	Phe	Leu	Met	Lys	Ala	Lys	Pro	Gln	275	280	285	
Asp	Leu	Ile	Lys	Leu	Glu	Glu	Lys	Val	Leu	Thr	Leu	Glu	Glu	Arg	Thr	290	295	300	
Asn	Lys	Val	Met	Phe	Pro	Phe	Gly	Pro	Thr	Val	Glu	Pro	Tyr	Gln	Thr	305	310	315	320
Ala	Asp	Cys	Val	Leu	Pro	Lys	His	Pro	Arg	Glu	Met	Val	Lys	Thr	Ala	325	330	335	
Trp	Gly	Asn	Ser	Ile	Pro	Thr	Met	Met	Gly	Asn	Thr	Ser	Tyr	Glu	Gly	340	345	350	
Leu	Phe	Phe	Thr	Ser	Ile	Leu	Lys	Gln	Met	Pro	Met	Leu	Val	Lys	Glu	355	360	365	
Leu	Glu	Thr	Cys	Val	Asn	Phe	Val	Pro	Ser	Glu	Leu	Ala	Asp	Ala	Glu	370	375	380	
Arg	Thr	Ala	Pro	Glu	Thr	Leu	Glu	Met	Gly	Ala	Lys	Ile	Lys	Lys	Ala	385	390	395	400

His Val Thr Gly Glu Thr Pro Thr Ala Asp Asn Phe Met Asp Leu Cys
 405 410 415
 Ser His Ile Tyr Phe Trp Phe Pro Met His Arg Leu Leu Gln Leu Arg
 420 425 430
 Phe Asn His Thr Ser Gly Thr Pro Val Tyr Leu Tyr Arg Phe Asp Phe
 435 440 445
 Asp Ser Glu Asp Leu Ile Asn Pro Tyr Arg Ile Met Arg Ser Gly Arg
 450 455 460
 Gly Val Lys Gly Val Ser His Ala Asp Glu Leu Thr Tyr Phe Phe Trp
 465 470 475 480
 Asn Gln Leu Ala Lys Arg Met Pro Lys Glu Ser Arg Glu Tyr Lys Thr
 485 490 495
 Ile Glu Arg Met Thr Gly Ile Trp Ile Gln Phe Ala Thr Thr Gly Asn
 500 505 510
 Pro Tyr Ser Asn Glu Ile Glu Gly Met Glu Asn Val Ser Trp Asp Pro
 515 520 525
 Ile Lys Lys Ser Asp Glu Val Tyr Lys Cys Leu Asn Ile Ser Asp Glu
 530 535 540
 Leu Lys Met Ile Asp Val Pro Glu Met Asp Lys Ile Lys Gln Trp Glu
 545 550 555 560
 Ser Met Phe Glu Lys His Arg Asp Leu Phe
 565 570

(2) INFORMATION FOR SEQUENCE ID NO.: 9

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1713 Nucleotides
- (B) TYPE: DNA
- (C) STRANDNESS: single
- (D) TOPOLOGY: linear
- (E) ORIGINAL SOURCE: *Lucilia cuprina*

(ii) MOLECULAR TYPE: other nucleic acid, primer

(Xi) SEQUENCE DESCRIPTION: SEQ ID NO. 9

atgaatttca acgtagttt gatggagaaa ttaaaatgga agattaaatg cattgaaaat 60
 aagtttttaa actatcgttt aactaccaat gaaacggtgg tagctgaaac tgaatatggc 120
 aaagtgaag gcgttaaacg tttaactgtg tacgatgatt cctactacag ttttgagggt 180
 ataccgtacg cccaaccgcc agtgggtgag ctgagattta aagcacccca gcgaccaaca 240
 ccctgggatg gtgtgcgtga ttgttgcaat cataaagata agtcagtga agttgatttt 300
 ataacgggca aagtgtgtgg ctgagaggat tgtctatacc taagtgtcta tacgaataat 360
 ctaaattccc aaactaaacg tcccgtttta gtatacatat atgggtgggtg ttttattatc 420
 ggtgaaaatc atcgtgatat gtatggtcct gattatttca ttaaaaagga tgtgggtgtg 480
 attaacatac aatatcgttt gggagctcta ggttttctaa gtttaaattc agaagacctt 540
 aatgtgcccg gtaatgccgg ccttaaagat caagtcattg ccttgcggtg gattaaaaat 600
 aattgcgcca actttgggtg caatcccgat aatattacag tctttgggtg aagtgccggg 660
 gctgcctcta cccactacat gatgttaacc gaacaaactc gcggtctttt ccatcgtggg 720
 atactaatgt cgggtaaatgc tattttgtcca ttggctaata cccaatgtca acatcgtgcc 780
 ttcaccttag ccaaattggc cggctataag ggtgaggata atgataagga tgttttggaa 840
 tttcttatga aagccaagcc acaggattta ataaaacttg aggaaaaagt tttaactcta 900
 gaagagcgta caaataaggt catgtttcct tttgggtcca ctggtgagcc atatcagacc 960

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gctgattgtg tcttaccctaa acatcctcgg gaaatgggta aaactgcttg gggtaattcg 1020
ataccacta tgatgggtaa cacttcatat gaggggtctat ttttcacttc aattcttaag 1080
caaatgccta tgcttggttaa ggaattggaa acttggtgtca attttgtgcc aagtgaattg 1140
gctgatgctg aacgcaccgc cccagagacc ttggaaatgg gtgctaaaat taaaaaggct 1200
catgttacag gagaaacacc aacagctgat aattttatgg atctttgctc tcacatctat 1260
ttctgggttc ccatgcatcg tttgttgcaa ttacgtttca atcacacctc cggtagaccc 1320
gtctacttgt atcgcttcga ctctgattcg gaagatctta tcaatcccta tcgtattatg 1380
cgtagtggac gtggtgttaa ggggtgttagt catgctgatg aattaaccta tttcttctgg 1440
aatcaattgg ccaaacgtat gcctaaagaa tcgcgtgaat acaaaacaat tgaacgtatg 1500
actggtatat ggatacaatt tgccaccact ggtaatcctt atagcaatga aattgaagg 1560
atggaaaatg tttcctggga tccaattaag aaatccgatg aagtatacaa gtgtttgaat 1620
attagtgatg aattgaaaat gattgatgtg cctgaaatgg ataagattaa acaatgggag 1680
tcgatgtttg aaaaacatag agatttattt tag 1713

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(2) INFORMATION FOR SEQUENCE ID NO.: 10

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 570 Amino Acids
- (B) TYPE: PRT
- (C) STRANDNESS: single
- (D) TOPOLOGY: linear
- (E) ORIGINAL SOURCE: *Lucilia cuprina*

(ii) MOLECULAR TYPE: other nucleic acid, primer

(Xi) SEQUENCE DESCRIPTION: SEQ ID NO. 10

```

Met Asn Phe Asn Val Ser Leu Met Glu Lys Leu Lys Trp Lys Ile Lys
 1             5             10             15

Cys Ile Glu Asn Lys Phe Leu Asn Tyr Arg Leu Thr Thr Asn Glu Thr
      20             25             30

Val Val Ala Glu Thr Glu Tyr Gly Lys Val Lys Gly Val Lys Arg Leu
      35             40             45

Thr Val Tyr Asp Asp Ser Tyr Tyr Ser Phe Glu Gly Ile Pro Tyr Ala
      50             55             60

Gln Pro Pro Val Gly Glu Leu Arg Phe Lys Ala Pro Gln Arg Pro Thr
      65             70             75             80

Pro Trp Asp Gly Val Arg Asp Cys Cys Asn His Lys Asp Lys Ser Val
      85             90             95

Gln Val Asp Phe Ile Thr Gly Lys Val Cys Gly Ser Glu Asp Cys Leu
      100            105            110

Tyr Leu Ser Val Tyr Thr Asn Asn Leu Asn Pro Glu Thr Lys Arg Pro
      115            120            125

Val Leu Val Tyr Ile His Gly Gly Gly Phe Ile Ile Gly Glu Asn His
      130            135            140

Arg Asp Met Tyr Gly Pro Asp Tyr Phe Ile Lys Lys Asp Val Val Leu
      145            150            155            160

Ile Asn Ile Gln Tyr Arg Leu Gly Ala Leu Gly Phe Leu Ser Leu Asn
      165            170            175

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Ser	Glu	Asp	Leu	Asn	Val	Pro	Gly	Asn	Ala	Gly	Leu	Lys	Asp	Gln	Val			
			180					185					190					
Met	Ala	Leu	Arg	Trp	Ile	Lys	Asn	Asn	Cys	Ala	Asn	Phe	Gly	Gly	Asn			
		195					200					205						
Pro	Asp	Asn	Ile	Thr	Val	Phe	Gly	Glu	Ser	Ala	Gly	Ala	Ala	Ser	Thr			
	210					215					220							
His	Tyr	Met	Met	Leu	Thr	Glu	Gln	Thr	Arg	Gly	Leu	Phe	His	Arg	Gly			
225					230					235					240			
Ile	Leu	Met	Ser	Gly	Asn	Ala	Ile	Cys	Pro	Leu	Ala	Asn	Thr	Gln	Cys			
				245					250					255				
Gln	His	Arg	Ala	Phe	Thr	Leu	Ala	Lys	Leu	Ala	Gly	Tyr	Lys	Gly	Glu			
			260					265					270					
Asp	Asn	Asp	Lys	Asp	Val	Leu	Glu	Phe	Leu	Met	Lys	Ala	Lys	Pro	Gln			
		275					280					285						
Asp	Leu	Ile	Lys	Leu	Glu	Glu	Lys	Val	Leu	Thr	Leu	Glu	Glu	Arg	Thr			
	290					295					300							
Asn	Lys	Val	Met	Phe	Pro	Phe	Gly	Pro	Thr	Val	Glu	Pro	Tyr	Gln	Thr			
305					310					315					320			
Ala	Asp	Cys	Val	Leu	Pro	Lys	His	Pro	Arg	Glu	Met	Val	Lys	Thr	Ala			
				325				330						335				
Trp	Gly	Asn	Ser	Ile	Pro	Thr	Met	Met	Gly	Asn	Thr	Ser	Tyr	Glu	Gly			
			340					345					350					
Leu	Phe	Phe	Thr	Ser	Ile	Leu	Lys	Gln	Met	Pro	Met	Leu	Val	Lys	Glu			
		355					360					365						
Leu	Glu	Thr	Cys	Val	Asn	Phe	Val	Pro	Ser	Glu	Leu	Ala	Asp	Ala	Glu			
	370					375					380							
Arg	Thr	Ala	Pro	Glu	Thr	Leu	Glu	Met	Gly	Ala	Lys	Ile	Lys	Lys	Ala			
385					390					395					400			
His	Val	Thr	Gly	Glu	Thr	Pro	Thr	Ala	Asp	Asn	Phe	Met	Asp	Leu	Cys			
				405					410					415				
Ser	His	Ile	Tyr	Phe	Trp	Phe	Pro	Met	His	Arg	Leu	Leu	Gln	Leu	Arg			
			420					425					430					
Phe	Asn	His	Thr	Ser	Gly	Thr	Pro	Val	Tyr	Leu	Tyr	Arg	Phe	Asp	Phe			
		435					440					445						
Asp	Ser	Glu	Asp	Leu	Ile	Asn	Pro	Tyr	Arg	Ile	Met	Arg	Ser	Gly	Arg			
	450					455					460							
Gly	Val	Lys	Gly	Val	Ser	His	Ala	Asp	Glu	Leu	Thr	Tyr	Phe	Phe	Trp			
465					470					475					480			
Asn	Gln	Leu	Ala	Lys	Arg	Met	Pro	Lys	Glu	Ser	Arg	Glu	Tyr	Lys	Thr			
				485					490					495				
Ile	Glu	Arg	Met	Thr	Gly	Ile	Trp	Ile	Gln	Phe	Ala	Thr	Thr	Gly	Asn			
			500					505					510					

Ser Met Phe Glu Lys His Arg Asp Leu Phe
565 570

Cys Met Val Asn Lys Tyr Thr Asn Tyr Arg Leu Ser Thr Asn Glu Thr
20 25 30

Gln	Ile	Ile	Asp	Thr	Glu	Tyr	Gly	Gln	Ile	Lys	Gly	Val	Lys	Arg	Met
		35					40					45			
Thr	Val	Tyr	Asp	Asp	Ser	Tyr	Tyr	Ser	Phe	Glu	Ser	Ile	Pro	Tyr	Ala
	50					55					60				
Lys	Pro	Pro	Val	Gly	Glu	Leu	Arg	Phe	Lys	Ala	Pro	Gln	Arg	Pro	Val
65					70					75					80
Pro	Trp	Glu	Gly	Val	Arg	Asp	Cys	Cys	Gly	Pro	Ala	Asn	Arg	Ser	Val
				85					90					95	
Gln	Thr	Asp	Phe	Ile	Ser	Gly	Lys	Pro	Thr	Gly	Ser	Glu	Asp	Cys	Leu
			100					105					110		
Tyr	Leu	Asn	Val	Tyr	Thr	Asn	Asp	Leu	Asn	Pro	Asp	Lys	Arg	Arg	Pro
		115					120					125			
Val	Met	Val	Phe	Ile	His	Gly	Gly	Asp	Phe	Ile	Phe	Gly	Glu	Ala	Asn
	130					135					140				
Arg	Asn	Trp	Phe	Gly	Pro	Asp	Tyr	Phe	Met	Lys	Lys	Pro	Val	Val	Leu
145					150					155					160
Val	Thr	Val	Gln	Tyr	Arg	Leu	Gly	Val	Leu	Gly	Phe	Leu	Ser	Leu	Lys
				165				170						175	
Ser	Glu	Asn	Leu	Asn	Val	Pro	Gly	Asn	Ala	Gly	Leu	Lys	Asp	Gln	Val
			180					185					190		
Met	Ala	Leu	Arg	Trp	Val	Lys	Ser	Asn	Ile	Ala	Ile	Phe	Gly	Gly	Asp
		195				200						205			
Val	Asp	Asn	Ile	Thr	Val	Phe	Gly	Glu	Ser	Ala	Gly	Gly	Ala	Ser	Thr
	210					215					220				
His	Tyr	Met	Met	Ile	Thr	Glu	Gln	Thr	Arg	Gly	Leu	Phe	His	Arg	Gly
225					230					235					240
Ile	Met	Met	Ser	Gly	Asn	Ser	Met	Cys	Ser	Trp	Ala	Ser	Thr	Glu	Cys
				245				250						255	
Gln	Ser	Arg	Ala	Leu	Thr	Met	Ala	Lys	Arg	Val	Gly	Tyr	Lys	Gly	Glu
			260					265					270		
Asp	Asn	Glu	Lys	Asp	Ile	Leu	Glu	Phe	Leu	Met	Lys	Ala	Asn	Pro	Tyr
		275					280					285			
Asp	Leu	Ile	Lys	Glu	Glu	Pro	Gln	Val	Leu	Thr	Pro	Glu	Arg	Met	Gln
	290					295					300				
Asn	Lys	Val	Met	Phe	Pro	Phe	Gly	Pro	Thr	Val	Glu	Pro	Tyr	Gln	Thr
305					310					315					320
Ala	Asp	Cys	Val	Val	Pro	Lys	Pro	Ile	Arg	Glu	Met	Val	Lys	Ser	Ala
				325				330						335	
Trp	Gly	Asn	Ser	Ile	Pro	Thr	Leu	Ile	Gly	Asn	Thr	Ser	Tyr	Glu	Gly
			340					345					350		

Leu Leu Ser Lys Ser Val Ala Lys Gln Tyr Pro Glu Val Val Lys Glu
 355 360 365
 Leu Glu Ser Cys Val Asn Tyr Val Pro Trp Glu Leu Ala Asp Ser Glu
 370 375 380
 Arg Ser Ala Pro Glu Thr Leu Glu Arg Ala Ala Ile Val Lys Lys Ala
 385 390 395 400
 His Val Asp Gly Glu Thr Pro Thr Leu Asp Asn Phe Met Glu Leu Cys
 405 410 415
 Ser Tyr Phe Tyr Phe Leu Phe Pro Met His Arg Phe Leu Gln Leu Arg
 420 425 430
 Phe Asn His Thr Ala Gly Thr Pro Ile Tyr Leu Tyr Arg Phe Asp Phe
 435 440 445
 Asp Ser Glu Glu Ile Ile Asn Pro Tyr Arg Ile Met Arg Phe Gly Arg
 450 455 460
 Gly Val Lys Gly Val Ser His Ala Asp Glu Leu Thr Tyr Leu Phe Trp
 465 470 475 480
 Asn Ile Leu Ser Lys Arg Leu Pro Lys Glu Ser Arg Glu Tyr Lys Thr
 485 490 495
 Ile Glu Arg Met Val Gly Ile Trp Thr Glu Phe Ala Thr Thr Gly Lys
 500 505 510
 Pro Tyr Ser Asn Asp Ile Ala Gly Met Glu Asn Leu Thr Trp Asp Pro
 515 520 525
 Ile Lys Lys Ser Asp Asp Val Tyr Lys Cys Leu Asn Ile Gly Asp Glu
 530 535 540
 Leu Lys Val Met Asp Leu Pro Glu Met Asp Lys Ile Lys Gln Gly Ala
 545 550 555 560
 Ser Ile Phe Asp Lys Lys Lys Glu Leu Phe
 565 570

2) INFORMATION FOR SEQUENCE ID NO.: 14

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1710 Nucleotides
- (B) TYPE: DNA
- (C) STRANDNESS: single
- (D) TOPOLOGY: linear
- (E) ORIGINAL SOURCE: *Musca domestica*

(ii) MOLECULAR TYPE: other nucleic acid, primer

(Xi) SEQUENCE DESCRIPTION: SEQ ID NO. 14

atgacttttc tgaagcaatt catatttcgc ctgaaactat gctttaaatg catggtcaat 60
 aaatacacia actaccgtct gagtacaaat gaaacccaaa taatcgatac tgaatatgga 120
 caaattaagg gtgttaagcg aatgaccgtc tacgatgatt cttactacag tttcgagagt 180
 ataccctatg ctaagcctcc agtgggtgag ttgagattca aggcaccca gcggcctgta 240
 ccatgggagg gtgtacgtga ttgctgtggg ccagccaaca gatcggtaca gacagatttc 300
 ataagtggca aaccacacagg ttcggaggat tgtctatacc tgaatgtgta taccaatgac 360
 ttgaaccag acaaaaggcg tcctgttatg gttttcatcc atggcggaga ttttattttc 420

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ggcgaagcaa atcgtaactg gtttgggtccc gactacttta tgaagaaacc cgtgggtcttg 480
gtaaccgtgc aatatcgttt ggggtgtgttg ggtttcctta gcctgaaatc ggaaaatctc 540
aatgtccccg gcaacgctgg cctcaaggat caagtaatgg ccttgagatg ggtcaagagt 600
aatattgcca ttttcggtgg cgatgtagac aatattaccg tcttcggcga aagtgtctggt 660
ggggcctcaa cccattacat gatgataacc gaacagaccc gtgggtttatt ccatcgtggg 720
atcatgatgt ccggtaatc catgtgctca tgggcctcta cagaatgcca aagtcgtgcg 780
ctcaccatgg ccaaacgtgt tggctataag ggagaggaca atgaaaaaga tatcctggaa 840
ttcctaataa aagccaatcc ctatgatttg atcaaagagg agccacaagt tttgacaccc 900
gaaagaatgc aaaataaggt catgtttcct tttggacca ctgtagaacc ataccagaca 960
gccgactgtg tggtaaccaa accaatcaga gaaatgggtg agagcgcctg gggaaattcg 1020
ataccacat tgataggcaa tacctcctac gaaggtttgc tttccaaatc aattgccaaa 1080
caatatccgg aggttgtaaa agagttggaa tcctgtgtga attatgtgcc ttgggagttg 1140
gctgacagtg aacgcagtgc cccggaaccc ctggagaggg ctgccattgt gaaaaaggcc 1200
catgtggatg gggaaacacc tactctggat aattttatgg agctttgctc ctatttctat 1260
ttcctcttcc ccatgcatcg cttcctacaa ttgcgcttca accacacagc tggcactccc 1320
atattattgt atcgtttcga tttcgattcc gaagaaatta ttaacccta tcgtattatg 1380
cgttttggcc gtggcgtaa aggtgtaagc catgccgatg agctaacct tctcttctgg 1440
aacattttgt cgaaacgcct gccaaaggaa agccgcgaat acaaaacat tgaacgcatg 1500
gttggcattt ggacggaatt cgccaccacc ggcaaacat acagcaatga tatagccggc 1560
atggaaaacc tcacctggga tcccataaaa aaatccgatg atgtctataa atgtttaaat 1620
atcggcgatg aattgaaagt tatggatttg ccagaaatgg ataaaattaa acaatgggca 1680
agtatattcg ataaaaagaa ggaattgttt 1710

```

2) INFORMATION FOR SEQUENCE ID NO.: 15

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 207 Amino Acids
- (B) TYPE: PRT
- (C) STRANDNESS: single
- (D) TOPOLOGY: linear
- (E) ORIGINAL SOURCE: *Musca domestica*

(ii) MOLECULAR TYPE: other nucleic acid, primer

(Xi) SEQUENCE DESCRIPTION: SEQ ID NO. 15

```

Gln Thr Asp Phe Ile Ser Gly Lys Pro Thr Gly Ser Glu Asp Cys Leu
 1             5             10             15
Tyr Leu Asn Val Tyr Thr Asn Asp Leu Asn Pro Asp Lys Lys Arg Pro
      20             25             30
Val Met Val Phe Ile His Gly Gly Gly Phe Ile Phe Gly Glu Ala Asn
      35             40             45
Arg Asn Trp Tyr Gly Pro Asp Tyr Phe Met Lys Lys Pro Val Val Leu
      50             55             60
Val Thr Val Gln Tyr Arg Leu Gly Val Leu Gly Phe Leu Ser Leu Lys
      65             70             75             80
Ser Glu Asn Leu Asn Val Pro Gly Asn Ala Gly Leu Lys Asp Gln Val
      85             90             95
Met Ala Leu Arg Trp Phe Lys Ser Asn Ile Ala Ile Phe Gly Gly Asp
      100            105            110
Val Asp Asn Ile Thr Val Phe Gly Glu Ser Ala Gly Gly Ala Ser Thr
      115            120            125

```

His Tyr Met Met Ile Thr Glu Gln Thr Arg Gly Leu Phe His Arg Gly
 130 135 140

Ile Met Met Ser Gly Asn Ser Met Cys Ser Ser Ala Ser Thr Glu Cys
 145 150 155 160

Gln Ser Arg Ala Leu Thr Met Ala Lys Arg Val Gly Tyr Lys Gly Glu
 165 170 175

Glu Asn Glu Lys Asp Ile Leu Glu Phe Leu Met Lys Ala Asn Pro Tyr
 180 185 190

Asp Leu Ile Lys Glu Glu Pro Gln Val Leu Thr Pro Glu Arg Met
 195 200 205

2) INFORMATION FOR SEQUENCE ID NO.: 16

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 Nucleotides
- (B) TYPE: DNA
- (C) STRANDNESS: single
- (D) TOPOLOGY: linear
- (E) ORIGINAL SOURCE: Musca domestica

(ii) MOLECULAR TYPE: other nucleic acid, primer

(Xi) SEQUENCE DESCRIPTION: SEQ ID NO. 16
 ggatggtgtg cgtgattgtt g

21

2) INFORMATION FOR SEQUENCE ID NO.: 17

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 Nucleotides
- (B) TYPE: DNA
- (C) STRANDNESS: single
- (D) TOPOLOGY: linear
- (E) ORIGINAL SOURCE: Musca domestica

(ii) MOLECULAR TYPE: other nucleic acid, primer

(Xi) SEQUENCE DESCRIPTION: SEQ ID NO. 17
 aaaaggatgt ggtgttgatt a

21

2) INFORMATION FOR SEQUENCE ID NO.: 18

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 Nucleotides
- (B) TYPE: DNA
- (C) STRANDNESS: single
- (D) TOPOLOGY: linear
- (E) ORIGINAL SOURCE: Lucilia cuprina

(ii) MOLECULAR TYPE: other nucleic acid, primer

(Xi) SEQUENCE DESCRIPTION: SEQ ID NO. 18
 actaatgtcg ggtaatgcta t

21

2) INFORMATION FOR SEQUENCE ID NO.: 19

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 Nucleotides
- (B) TYPE: DNA
- (C) STRANDNESS: single
- (D) TOPOLOGY: linear
- (E) ORIGINAL SOURCE: *Lucilia cuprina*

(ii) MOLECULAR TYPE: other nucleic acid, primer

(Xi) SEQUENCE DESCRIPTION: SEQ ID NO. 19
cactatgatg ggtaacactt c

21

2) INFORMATION FOR SEQUENCE ID NO.: 20

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 Nucleotides
- (B) TYPE: DNA
- (C) STRANDNESS: single
- (D) TOPOLOGY: linear
- (E) ORIGINAL SOURCE: *Lucilia cuprina*

(ii) MOLECULAR TYPE: other nucleic acid, primer

(Xi) SEQUENCE DESCRIPTION: SEQ ID NO. 20
tggttacagga gaaacaccaa c

21

2) INFORMATION FOR SEQUENCE ID NO.: 21

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 Nucleotides
- (B) TYPE: DNA
- (C) STRANDNESS: single
- (D) TOPOLOGY: linear
- (E) ORIGINAL SOURCE: *Lucilia cuprina*

(ii) MOLECULAR TYPE: other nucleic acid, primer

(Xi) SEQUENCE DESCRIPTION: SEQ ID NO. 21
agaatcgcgt gaatacaaaa c

21

2) INFORMATION FOR SEQUENCE ID NO.: 22

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 Nucleotides
- (B) TYPE: DNA
- (C) STRANDNESS: single
- (D) TOPOLOGY: linear
- (E) ORIGINAL SOURCE: *Lucilia cuprina*

(ii) MOLECULAR TYPE: other nucleic acid, primer

(Xi) SEQUENCE DESCRIPTION: SEQ ID NO. 22
acggtatacc ctcaaaactg t

21

2) INFORMATION FOR SEQUENCE ID NO.: 23

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 Nucleotides
- (B) TYPE: DNA
- (C) STRANDNESS: single

(D) TOPOLOGY: linear
 (E) ORIGINAL SOURCE: *Lucilia cuprina*

(ii) MOLECULAR TYPE: other nucleic acid, primer

(Xi) SEQUENCE DESCRIPTION: SEQ ID NO. 23
 tcccaaacga tattgtatgt t 21

2) INFORMATION FOR SEQUENCE ID NO.: 24

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 21 Nucleotides
 (B) TYPE: DNA
 (C) STRANDNESS: single
 (D) TOPOLOGY: linear
 (E) ORIGINAL SOURCE: *Lucilia cuprina*

(ii) MOLECULAR TYPE: other nucleic acid, primer

(Xi) SEQUENCE DESCRIPTION: SEQ ID NO. 24
 acatcatgta gtgggtagaa g 21

2) INFORMATION FOR SEQUENCE ID NO.: 25

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 21 Nucleotides
 (B) TYPE: DNA
 (C) STRANDNESS: single
 (D) TOPOLOGY: linear
 (E) ORIGINAL SOURCE: *Lucilia cuprina*

(ii) MOLECULAR TYPE: other nucleic acid, primer

(Xi) SEQUENCE DESCRIPTION: SEQ ID NO. 25
 ccgaggatgt ttgggtaaga c 21

2) INFORMATION FOR SEQUENCE ID NO.: 26

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 21 Nucleotides
 (B) TYPE: DNA
 (C) STRANDNESS: single
 (D) TOPOLOGY: linear
 (E) ORIGINAL SOURCE: *Lucilia cuprina*

(ii) MOLECULAR TYPE: other nucleic acid, primer

(Xi) SEQUENCE DESCRIPTION: SEQ ID NO. 26
 tatcagctgt tgggtgttct c 21

2) INFORMATION FOR SEQUENCE ID NO.: 27

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 21 Nucleotides
 (B) TYPE: DNA
 (C) STRANDNESS: single
 (D) TOPOLOGY: linear
 (E) ORIGINAL SOURCE: *Lucilia cuprina*

(ii) MOLECULAR TYPE: other nucleic acid, primer

(Xi) SEQUENCE DESCRIPTION: SEQ ID NO. 27
acgcgattct ttaggcatac g

21

2) INFORMATION FOR SEQUENCE ID NO.: 28

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 Nucleotides
- (B) TYPE: DNA
- (C) STRANDNESS: single
- (D) TOPOLOGY: linear
- (E) ORIGINAL SOURCE: *Lucilia cuprina*

(ii) MOLECULAR TYPE: other nucleic acid, primer

(Xi) SEQUENCE DESCRIPTION: SEQ ID NO. 28
tgctgcctct acccactaca t

21

2) INFORMATION FOR SEQUENCE ID NO.: 29

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 Nucleotides
- (B) TYPE: DNA
- (C) STRANDNESS: single
- (D) TOPOLOGY: linear
- (E) ORIGINAL SOURCE: *Lucilia cuprina*

(ii) MOLECULAR TYPE: other nucleic acid, primer

(Xi) SEQUENCE DESCRIPTION: SEQ ID NO. 29
cctgtggctt ggctttcata a

21

2) INFORMATION FOR SEQUENCE ID NO.: 30

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 35 Nucleotides
- (B) TYPE: DNA
- (C) STRANDNESS: single
- (D) TOPOLOGY: linear
- (E) ORIGINAL SOURCE: Artificial Sequence

(ii) MOLECULAR TYPE: other nucleic acid, primer

FEATURE:

OTHER INFORMATION: Degenerate Primer

(Xi) SEQUENCE DESCRIPTION: SEQ ID NO. 30
ttcgaggga tncctaygc nmarccnccn btngg

35

2) INFORMATION FOR SEQUENCE ID NO.: 31

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 32 Nucleotides
- (B) TYPE: DNA
- (C) STRANDNESS: single
- (D) TOPOLOGY: linear
- (E) ORIGINAL SOURCE: Artificial Sequence

(ii) MOLECULAR TYPE: other nucleic acid, primer

FEATURE:

OTHER INFORMATION: Degenerate Primer

(Xi) SEQUENCE DESCRIPTION: SEQ ID NO. 31
acytgrtcyt tnarnccngc rttncnggn ac

32

2) INFORMATION FOR SEQUENCE ID NO.: 32

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 22 Nucleotides
- (B) TYPE: DNA
- (C) STRANDNESS: single
- (D) TOPOLOGY: linear
- (E) ORIGINAL SOURCE: Musca domestica

(ii) MOLECULAR TYPE: other nucleic acid, primer

(Xi) SEQUENCE DESCRIPTION: SEQ ID NO. 32
tttggtcccg actactttat ga

22

2) INFORMATION FOR SEQUENCE ID NO.: 33

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 Nucleotides
- (B) TYPE: DNA
- (C) STRANDNESS: single
- (D) TOPOLOGY: linear
- (E) ORIGINAL SOURCE: Musca domestica

(ii) MOLECULAR TYPE: other nucleic acid, primer

(Xi) SEQUENCE DESCRIPTION: SEQ ID NO. 33
tgccacttat gaaatctgtc tgta

24

2) INFORMATION FOR SEQUENCE ID NO.: 34

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 Nucleotides
- (B) TYPE: DNA
- (C) STRANDNESS: single
- (D) TOPOLOGY: linear
- (E) ORIGINAL SOURCE: Musca domestica

(ii) MOLECULAR TYPE: other nucleic acid, primer

(Xi) SEQUENCE DESCRIPTION: SEQ ID NO. 34
tacatgatga taaccgaaca gacc

24

2) INFORMATION FOR SEQUENCE ID NO.: 35

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 23 Nucleotides
- (B) TYPE: DNA
- (C) STRANDNESS: single
- (D) TOPOLOGY: linear
- (E) ORIGINAL SOURCE: Musca domestica

(ii) MOLECULAR TYPE: other nucleic acid, primer

(Xi) SEQUENCE DESCRIPTION: SEQ ID NO. 35
tcgattattt gggtttcatt tgt

23

2) INFORMATION FOR SEQUENCE ID NO.: 36

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 Nucleotides
- (B) TYPE: DNA
- (C) STRANDNESS: single
- (D) TOPOLOGY: linear
- (E) ORIGINAL SOURCE: Musca domestica

(ii) MOLECULAR TYPE: other nucleic acid, primer

(Xi) SEQUENCE DESCRIPTION: SEQ ID NO. 36
acagacagat ttcataagtg g

21

2) INFORMATION FOR SEQUENCE ID NO.: 37

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 Nucleotides
- (B) TYPE: DNA
- (C) STRANDNESS: single
- (D) TOPOLOGY: linear
- (E) ORIGINAL SOURCE: Musca domestica

(ii) MOLECULAR TYPE: other nucleic acid, primer

(Xi) SEQUENCE DESCRIPTION: SEQ ID NO. 37
tttgcattct ttcgggtgtc a

21

2) INFORMATION FOR SEQUENCE ID NO.: 38

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 Nucleotides
- (B) TYPE: DNA
- (C) STRANDNESS: single
- (D) TOPOLOGY: linear
- (E) ORIGINAL SOURCE: Musca domestica

(ii) MOLECULAR TYPE: other nucleic acid, primer

(Xi) SEQUENCE DESCRIPTION: SEQ ID NO. 38
attcgatacc cacattgata g

21

2) INFORMATION FOR SEQUENCE ID NO.: 39

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 Nucleotides
- (B) TYPE: DNA
- (C) STRANDNESS: single
- (D) TOPOLOGY: linear
- (E) ORIGINAL SOURCE: Musca domestica

(ii) MOLECULAR TYPE: other nucleic acid, primer

(Xi) SEQUENCE DESCRIPTION: SEQ ID NO. 39
ggcactccca tttatttgta t

21

2) INFORMATION FOR SEQUENCE ID NO.: 40

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 23 Nucleotides
- (B) TYPE: DNA
- (C) STRANDNESS: single

(D) TOPOLOGY: linear

(E) ORIGINAL SOURCE: Musca domestica

(ii) MOLECULAR TYPE: other nucleic acid, primer

(Xi) SEQUENCE DESCRIPTION: SEQ ID NO. 40

atgacttttc tgaagcaatt cat

23

2) INFORMATION FOR SEQUENCE ID NO.: 41

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 23 Nucleotides

(B) TYPE: DNA

(C) STRANDNESS: single

(D) TOPOLOGY: linear

(E) ORIGINAL SOURCE: Musca domestica

(ii) MOLECULAR TYPE: other nucleic acid, primer

(Xi) SEQUENCE DESCRIPTION: SEQ ID NO. 41

aaacaattcc ttctttttat cga

23

2) INFORMATION FOR SEQUENCE ID NO.: 42

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 Nucleotides

(B) TYPE: DNA

(C) STRANDNESS: single

(D) TOPOLOGY: linear

(E) ORIGINAL SOURCE: Musca domestica

(ii) MOLECULAR TYPE: other nucleic acid, primer

(Xi) SEQUENCE DESCRIPTION: SEQ ID NO. 42

ggcatggaaa acctcacctg g

21

2) INFORMATION FOR SEQUENCE ID NO.: 43

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 207 Amino Acids

(B) TYPE: PRT

(C) STRANDNESS: single

(D) TOPOLOGY: linear

(E) ORIGINAL SOURCE: Lucilia cuprina

(ii) MOLECULAR TYPE: other nucleic acid, primer

(Xi) SEQUENCE DESCRIPTION: SEQ ID NO. 43

Gln	Val	Asp	Phe	Ile	Thr	Gly	Lys	Val	Cys	Gly	Ser	Glu	Asp	Cys	Leu
1				5				10						15	

Tyr	Leu	Ser	Val	Tyr	Thr	Asn	Asn	Leu	Asn	Pro	Glu	Thr	Lys	Arg	Pro
			20					25					30		

Val	Leu	Val	Tyr	Ile	His	Gly	Gly	Gly	Phe	Ile	Ile	Gly	Glu	Asn	His
			35					40				45			

Arg	Asp	Met	Tyr	Gly	Pro	Asp	Tyr	Phe	Ile	Lys	Lys	Asp	Val	Val	Leu
			50				55					60			

Ile	Asn	Ile	Gln	Tyr	Arg	Leu	Gly	Ala	Leu	Gly	Phe	Leu	Ser	Leu	Asn
65					70					75					80
Ser	Glu	Asp	Leu	Asn	Val	Pro	Gly	Asn	Ala	Gly	Leu	Lys	Asp	Gln	Val
				85					90					95	
Met	Ala	Leu	Arg	Trp	Ile	Lys	Asn	Asn	Cys	Ala	Asn	Phe	Gly	Gly	Asn
			100					105					110		
Pro	Asp	Asn	Ile	Thr	Val	Phe	Gly	Glu	Ser	Ala	Gly	Ala	Ala	Ser	Thr
		115					120					125			
His	Tyr	Met	Met	Leu	Thr	Glu	Gln	Thr	Arg	Gly	Leu	Phe	His	Arg	Gly
	130					135					140				
Ile	Leu	Met	Ser	Gly	Asn	Ala	Ile	Cys	Pro	Leu	Ala	Asn	Thr	Gln	Cys
145					150					155					160
Gln	His	Arg	Ala	Phe	Thr	Leu	Ala	Lys	Leu	Ala	Gly	Tyr	Lys	Gly	Glu
				165					170					175	
Asp	Asn	Asp	Lys	Asp	Val	Leu	Glu	Phe	Leu	Met	Lys	Ala	Lys	Pro	Gln
			180					185					190		
Asp	Leu	Ile	Lys	Leu	Glu	Glu	Lys	Val	Leu	Thr	Leu	Glu	Glu	Arg	
		195					200					205			

THE EMBODIMENTS OF THE INVENTION IN WHICH AN EXCLUSIVE PROPERTY OR PRIVILEGE IS CLAIMED ARE DEFINED AS FOLLOWS:

1. An isolated DNA molecule encoding an enzyme capable of hydrolysing at least one organophosphate selected from the group consisting of carboxylester organophosphates and dimethyl-oxon organophosphates, the DNA molecule comprising a nucleotide sequence having at least 75% identity with SEQ ID NO:7, in which the enzyme encoded by the DNA molecule differs from SEQ ID NO:8 at least in the substitution of Trp at position 251 with an amino acid selected from the group consisting of Leu, Ser, Ala, Ile, Val, Thr, Cys, Met and Gly.
2. An isolated DNA molecule as claimed in claim 1 in which the DNA molecule has at least 80% identity with SEQ ID NO:7.
3. An isolated DNA molecule as claimed in claim 1 or claim 2 in which the DNA molecule has at least 95% identity with SEQ ID NO:7.
4. An isolated DNA molecule as claimed in any one of claims 1 to 3 in which the Trp at position 251 is substituted with Leu or Ser.
5. An isolated DNA molecule, the DNA molecule encoding an enzyme having the amino acid sequence of SEQ ID NO:13 in which Trp at position 251 is replaced with Ser, or the amino acid sequence of SEQ ID NO:10.
6. A transformed cell which expresses an enzyme capable of hydrolysing at least one organophosphate selected from the group consisting of carboxylester organophosphates and dimethyl-oxon organophosphates, in which the cell is transformed with the DNA molecule as claimed in any one of claims 1 to 5,
7. A transformed cell as claimed in claim 6 in which the cell is a prokaryotic cell or an insect cell.

8. An enzyme capable of hydrolysing at least one organophosphate selected from the group consisting of carboxylester organophosphates and dimethyl-oxon organophosphates, the enzyme being produced by the transformed cell as claimed in claim 6 or claim 7.

5

9. An isolated enzyme capable of hydrolysing at least one organophosphate selected from the group consisting of carboxylester organophosphates and dimethyl-oxon organophosphates, the enzyme comprising an amino acid sequence having at least 75% identity with SEQ ID NO:8, and differs from SEQ ID NQ:8 at least in the substitution of Trp at position 251 with an amino acid selected from the group consisting of Leu, Ser, Ala, Ile, Val, Thr, Cys, Met and Gly.

10

10. An isolated enzyme as claimed in claim 9 in which the enzyme comprises an amino acid sequence having at least 80% identity with SEQ ID NO:8.

15

11. An isolated enzyme as claimed in claim 9 or claim 10 in which the enzyme comprises an amino acid sequence having at least 95% identity with SEQ ID NO:8.

12. An isolated enzyme as claimed in any one of claims 9 to 11 in which the Trp at position 251 is substituted with Leu or Ser.

20

13. A method of eliminating or reducing the concentration of organophosphate pesticide residues in a contaminated sample or substance in which the organophosphate is selected from the group consisting of carboxylester organophosphates and dimethyl-oxon organophosphates, the method comprising contacting the sample or substance with the enzyme capable of hydrolysing at least one organophosphate selected from the group consisting of carboxylester organophosphates and dimethyl-oxon organophosphates encoded by the DNA molecule as claimed in any one of claims 1 to 5.

25

30

14. A method of eliminating or reducing the concentration of organophosphate pesticide residues in a contaminated sample or substance in which the

organophosphate is selected from the group consisting of carboxylester organophosphates and dimethyl-oxon organophosphates, the method comprising contacting the sample or substance with the transformed cell as claimed in claim 6 or claim 7.

5

15. A method of eliminating or reducing the concentration of organophosphate pesticide residues in a contaminated sample or substance in which the organophosphate is selected from the group consisting of carboxylester organophosphates and dimethyl-oxon organophosphates, the method comprising
10 contacting the sample or substance with the enzyme according to any one of claims 8 to 12.

16. A method of producing an enzyme capable of hydrolysing at least one organophosphate selected from the group consisting of carboxylester
15 organophosphates and dimethyl-oxon organophosphates, the method comprising transforming a host cell with the DNA molecule as claimed in any one of claims 1 to 5 operatively linked to a control sequence, culturing the transformed cell under conditions which allow expression of the DNA sequence and recovering the produced enzyme, or enzymatically active portion thereof.

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Lc743	M N F N V S L M E K L K W K I K C I E N	20
RM8con		
1	-----+-----+-----+-----+-----+	60
Lc743	<u>ATGAATTTCAACGTTAGTTTGATGGAGAAATTAAAATGGAAGATTAAATGCATTGAAAAT</u>	
RM8A	Lc743/5'	
RM8B		
RM8C		
RM8con		
Lc743	K F L N Y R L T T N E T V V A E T E Y G	40
RM8con		
61	-----+-----+-----+-----+-----+	120
Lc743	AAGTTTTTAAACTATCGTTTAACTACCAATGAAACGGTGGTAGCTGAAACTGAATATGGC	
RM8A		
RM8B		
RM8C		
RM8con		
Lc743	K V K G V K R L T V Y D D S Y Y S F E G	60
RM8con		
121	-----+-----+-----+-----+-----+	180
Lc743	AAAGTGAAAGGCGTTAAACGTTTAACTGTGTACGATGATTCCTACTACAGTTTTGAGGGT	
RM8A		
RM8B		
RM8C		
RM8con		
Lc743	I P Y A Q P P V G E L R F K A P Q R P T	80
RM8con		
181	-----+-----+-----+-----+-----+	240
Lc743	ATACCGTACGCCCAACCGCCAGTGGGTGAGCTGAGATTTAAAGCACCCCAGCGACCAACA	
RM8A		
RM8B		
RM8C		
RM8con		
Lc743	P W D G V R D C C N H K D K S V Q V D F	100
RM8con		
241	-----+-----+-----+-----+-----+	300
Lc743	CCCTGGGATGGTGTGCGTGATTGTTGCAATCATAAAGATAAGTCAGTGCAAGTTGATTTT	
RM8A		
RM8B	C.....	
RM8C		
RM8con		

FIGURE 1

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Lc743	I T G K V C G S E D C L Y L S V Y T N N	120
RM8con		
301	- - - + - - - + - - - + - - - + - - - + - - - +	360
Lc743	ATAACGGGCAAAGTGTGTGGCTCAGAGGATTGTCTATACCTAAGTGTCTATACGAATAAT	
RM8A	.	
RM8B	.	
RM8C	.	
RM8con	.	
Lc743	L N P E T K R P V L V Y I H G G G F I I	140
RM8con		
361	- - - + - - - + - - - + - - - + - - - + - - - +	420
Lc743	CTAAATCCCGAAACTAAACGTCCCGTTTTAGTATACATCATGGTGGTGGTTTATTATC	
RM8A	.	
RM8B	.	
RM8C	.	
RM8con	.	
Lc743	G E N H R D M Y G P D Y F I K K D V V L	160
RM8con		
421	- - - + - - - + - - - + - - - + - - - + - - - +	480
Lc743	GGTGAAAATCATCGTGATATGTATGGTCCTGATTATTTTCATTAAAAAGGATGTGGTGT TG	
RM8A	.	
RM8B	.	
RM8C	.	
RM8con	.	
Lc743	I N I Q Y R L G A L G F L S L N S E D L	180
RM8con		
481	- - - + - - - + - - - + - - - + - - - + - - - +	540
Lc743	ATTAACATACAATATCGTTTGGGAGCTCTAGGTTTTCTAAGTTTAAATT CAGAAGACCTT	
RM8A	.	
RM8B	.	
RM8C	.	
RM8con	.	
Lc743	N V P G N A G L K D Q V M A L R W I K N	200
RM8con		
541	- - - + - - - + - - - + - - - + - - - + - - - +	600
Lc743	AATGTGCCCGGTAATGCCGGCCTTAAAGATCAAGTCATGGCCTTGCGTTGGATTAAAAAT	
RM8A	.	
RM8B	.	
RM8C	.	
RM8con	.	

Figure 1 continued

Lc743	N C A N F G G N P D N I T V F G E S A G	220
RM8con		
601	-----+-----+-----+-----+-----+-----+	660
Lc743	AATTGCGCCAACTTTGGTGGCAATCCCGATAATATTACAGTCTTTGGTGAAAGTGCCGGT	
RM8A	.	
RM8B	.	
RM8C	.	
RM8con	.	
Lc743	A A S T H Y M M L T E Q T R G L F H R G	240
RM8con		
661	-----+-----+-----+-----+-----+-----+	720
Lc743	GCTGCCTCTACCCACTACATGATGTTAACCGAACAACTCGCGGTCTTTTCATCGTGGT	
RM8A	.	
RM8B	.	
RM8C	.	
RM8con	.	
Lc743	I L M S G N A I C P W A N T Q C Q H R A	260
RM8con	 L	
721	-----+-----+-----+-----+-----+-----+	780
Lc743	ATACTAATGTCGGGTAATGCTATTTGTCCATGGGCTAATACCCAATGTCAACATCGTGCC	
RM8A	T.	
RM8B	T.	
RM8C	T.	
RM8con	T.	
Lc743	F T L A K L A G Y K G E D N D K D V L E	280
RM8con		
781	-----+-----+-----+-----+-----+-----+	840
Lc743	TTCACCTTAGCCAAATTGGCCGGCTATAAGGGTGAGGATAATGATAAGGATGTTTTGGAA	
RM8A	.	
RM8B	.	
RM8C	A.	
RM8con	.	
Lc743	F L M K A K P Q D L I K L E E K V L T L	300
RM8con		
841	-----+-----+-----+-----+-----+-----+	900
Lc743	TTTCTTATGAAAGCCAAGCCACAGGATTTAATAAAAAGTTTAACTCTA	
RM8A	.	
RM8B	.	
RM8C	G.	
RM8con	.	

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Lc743	E	E	R	T	N	K	V	M	F	P	F	G	P	T	V	E	P	Y	Q	T	320
RM8con	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
901	-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+																				960
Lc743	GAAGAGCGTACAAATAAGGTCATGTTTCCTTTTGGTCCCAGTGTGAGCCATATCAGACC																				
RM8A																					
RM8B																					
RM8C																					
RM8con																					

[illegible]

Lc743	I	P	T	M	M	G	N	T	S	Y	E	G	L	F	F	T	S	I	L	K	360
RM8con	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
1021	-----+-----+-----+-----+-----+-----+-----+-----+1080																				
Lc743	ATACCCACTATGATGGGTAACACTTCATATGAGGGTCTATTTTTCATTCAATTCTTAAG																				
RM8A																					
RM8B																					
RM8C																					
RM8con																					

Lc743		Q	M	P	M	L	V	K	E	L	E	T	C	V	N	F	V	P	S	E	L	380
RM8con		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	1081	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+1140
Lc743		CAAATGCCTATGCTTGTTAAGGAATTGGAAACTTGTGTCAATTTTGTGCCAAGTGAATTG																				
RM8A																						
RM8B																						
RM8C																						
RM8con																						

Lc743	A D A E R T A P E T L E M G A K I K K A	400
RM8con		
1141	- - - + - - - + - - - + - - - + - - - + - - - + - - - +	1200
Lc743	GCTGATGCTGAACGCACCGCCCCAGAGACCTTGCAAATGGGTGCTAAAATTAAAAAGGCT	
RM8A	T.	
RM8B		
RM8C		
RM8con		

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Lc743 H V T G E T P T A D N F M D L C S H I Y 420
RM8con
1201 -----+-----+-----+-----+-----+-----+1260
Lc743 CATGTTACAGGAGAAACACCAACAGCTGATAATTTTATGGATCTTTGCTCTCACATCTAT
RM8A
RM8B
RM8C
RM8con

Lc743 F W F P M H R L L Q L R F N H T S G T P 440
RM8con
1261 -----+-----+-----+-----+-----+-----+1320
Lc743 TTCTGGTTCCCCATGCATCGTTTGTTGCAATTACGTTTCAATCACACCTCCGGTACACCC
RM8A
RM8B
RM8C
RM8con

Lc743 V Y L Y R F D F D S E D L I N P Y R I M 460
RM8con
1321 -----+-----+-----+-----+-----+-----+1380
Lc743 GTCTACTTGTATCGCTTCGACTTTGATTGCGGAAGATCTTATTAATCCCTATCGTATTATG
RM8A C.....C.....
RM8B C.....C.....
RM8C C.....C.....
RM8con C.....C.....

Lc743 R S G R G V K G V S H A D E L T Y F F W 480
RM8con
1381 -----+-----+-----+-----+-----+-----+1440
Lc743 CGTAGTGGACGTGGTGTAAAGGGTGTAGTCATGCTGATGAATTAACCTATTTCTTCTG
RM8A
RM8B
RM8C
RM8con

Lc743 N Q L A K R M P K E S R E Y K T I E R M 500
RM8con
1441 -----+-----+-----+-----+-----+-----+1500
Lc743 AATCAATTGGCCAAACGTATGCCTAAAGAATCGCGTGAATACAAAACAATTGAACGTATG
RM8A
RM8B
RM8C
RM8con

Figure 1 continued

Lc743	T	G	I	W	I	Q	F	A	T	T	G	N	P	Y	S	N	E	I	E	G	520
RM8con	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
1501	-----+-----+-----+-----+-----+-----+1560																				
Lc743	ACTGGTATATGGATACAATTTGCCACCACTGGTAATCCTTATAGCAATGAAATTGAAGGT																				
RM8A																					
RM8B																					
RM8C																					
RM8con																					

[illegible]

Lc743	I	S	D	E	L	K	M	I	D	V	P	E	M	D	K	I	K	Q	W	E	560
RM8con	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
1621	-----+-----+-----+-----+-----+-----+-----+-----+1680																				
Lc743	ATTAGTGACGAATTGAAAATGATTGATGTGCCTGAAATGGATAAGATTAAACAATGGGAA																				
RM8A	T.....G																				
RM8B	T.....G																				
RM8C	T.....G																				
RM8con	T.....G																				

Lc743	S	M	F	E	K	H	R	D	L	F	*	570
RM8con	.	.	.	.	.	.	.	.	.	.	.	
1681	-----+-----+-----+---										1713	
Lc743	<u>TCGATGTTTGAAAAACATAGAGATTTATTTTAG</u>											
RM8A	Lc743/3'.....											
RM8B												
RM8C												
RM8con												

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Figure 2.

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Figure 3.

ATGACTTTTCTGAAGCAATTCATATTTTCGCCTGAAACTATGCTTTAAATGCATGGTCAAT
1 -----+-----+-----+-----+-----+-----+ 60
TACTGAAAAGACTTCGTTAAGTATAAAGCGGACTTTGATACGAAATTTACGTACCAGTTA
M T F L K Q F I F R L K L C F K C M V N -
AAATACACAAACTACCGTCTGAGTACAAATGAAACCCAAATAATCGATACTGAATATGGA
61 -----+-----+-----+-----+-----+-----+ 120
TTTATGTGTTTGATGGCAGACTCATGTTTACTTTGGGTTTATTAGCTATGACTTATACCT
K Y T N Y R L S T N E T Q I I D T E Y G -
CAAATTAAGGGTGTTAAGCGAATGACCGTCTACGATGATTCTTACTACAGTTTCGAGAGT
121 -----+-----+-----+-----+-----+-----+ 180
GTTTAATTCCCACAATTTCGCTTACTGGCAGATGCTACTAAGAATGATGTCAAAGCTCTCA
Q I K G V K R M T V Y D D S Y Y S F E S -
ATACCCTATGCTAAGCCTCCAGTGGGTGAGTTGAGATTCAAGGCACCCCAGCGGCCTGTA
181 -----+-----+-----+-----+-----+-----+ 240
TATGGGATACGATTTCGGAGGTCACCCACTCAACTCTAAGTTCCGTGGGGTCGCCGGACAT
I P Y A K P P V G E L R F K A P Q R P V -
CCATGGGAGGGTGTACGTGATTGCTGTGGGCCAGCCAACAGATCGGTACAGACAGATTTC
241 -----+-----+-----+-----+-----+-----+ 300
GGTACCCTCCCACATGCACTAACGACACCCGGTCGGTTGTCTAGCCATGTCTGTCTAAAG
P W E G V R D C C G P A N R S V Q T D F -
ATAAGTGGCAAACCCACAGGTTCCGGAGGATTGTCTATACCTGAATGTGTATACCAATGAC
301 -----+-----+-----+-----+-----+-----+ 360
TATTCACCGTTTGGGTGTCCAAGCCTCCTAACAGATATGGACTTACACATATGGTTACTG
I S G K P T G S E D C L Y L N V Y T N D -
TTGAACCCAGACAAAAGGCGTCCTGTTATGGTTTTTCATCCATGGCGGAGATTTTATTTTC
361 -----+-----+-----+-----+-----+-----+ 420
AACTTGGGTCTGTTTTCCGCAGGACAATACCAAAAGTAGGTACCGCCTCTAAAATAAAAG
L N P D K R R P V M V F I H G G D F I F -
GGCGAAGCAAATCGTAACTGGTTTGGTCCCGACTACTTTATGAAGAAACCCGTGGTCTTG
421 -----+-----+-----+-----+-----+-----+ 480
CCGCTTCGTTTAGCATTGACCAAACCAGGGCTGATGAAATACTTCTTTGGGCACCAGAAC
G E A N R N W F G P D Y F M K K P V V L -

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GTAACCGTGCAATATCGTTTGGGTGTGTTGGGTTTCCTTAGCCTGAAATCGGAAAATCTC
481 -----+-----+-----+-----+-----+-----+ 540
CATTGGCACGTTATAGCAAACCCACACAACCCAAAGGAATCGGACTTTAGCCTTTTAGAG
V T V Q Y R L G V L G F L S L K S E N L -
AATGTCCCCGGCAACGCTGGCCTCAAGGATCAAGTAATGGCCTTGAGATGGGTCAAGAGT
541 -----+-----+-----+-----+-----+-----+ 600
TTACAGGGGCGGTTGCGACCGGAGTTCCTAGTTCATTACCGGAACTCTACCCAGTTCTCA
N V P G N A G L K D Q V M A L R W V K S -
AATATTGCCATTTTCGGTGGCGATGTAGACAATATTACCGTCTTCGGCGAAAGTGCTGGT
601 -----+-----+-----+-----+-----+-----+ 660
TTATAACGGTAAAAGCCACCGCTACATCTGTTATAATGGCAGAAGCCGCTTTCACGACCA
N I A I F G G D V D N I T V F G E S A G -
GGGGCCTCAACCCATTACATGATGATAACCGAACAGACCCGTGGTTTATTCCATCGTGGT
661 -----+-----+-----+-----+-----+-----+ 720
CCCCGGAGTTGGGTAATGTACTACTATTGGCTTGTCTGGGCACCAAATAAGGTAGCACCA
G A S T H Y M M I T E Q T R G L F H R G -
ATCATGATGTCCGGTAATTCCATGTGCTCATGGGCCTCTACAGAATGCCAAAGTCGTGCG
721 -----+-----+-----+-----+-----+-----+ 780
TAGTACTACAGGCCATTAAGGTACACGAGTACCCGGAGATGTCTTACGGTTTCAGCACGC
I M M S G N S M C S W A S T E C Q S R A -
CTCACCATGGCCAAACGTGTTGGCTATAAGGGAGAGGACAATGAAAAAGATATCCTGGAA
781 -----+-----+-----+-----+-----+-----+ 840
GAGTGGTACCGGTTTGCACAACCGATATTCCCTCTCCTGTTACTTTTTCTATAGGACCTT
L T M A K R V G Y K G E D N E K D I L E -
TTCCTAATGAAAGCCAATCCCTATGATTTGATCAAAGAGGAGCCACAAGTTTTGACACCC
841 -----+-----+-----+-----+-----+-----+ 900
AAGGATTACTTTCGGTTAGGGATACTAACTAGTTTCTCCTCGGTGTTCAAACTGTGGG
F L M K A N P Y D L I K E E P Q V L T P -
GAAAGAATGCAAAATAAGGTCATGTTTCCTTTTGGACCCACTGTAGAACCATACCAGACA
901 -----+-----+-----+-----+-----+-----+ 960
CTTCTTACGTTTTATTCCAGTACAAAGGAAAACCTGGGTGACATCTTGGTATGGTCTGT
E R M Q N K V M F P F G P T V E P Y Q T -
GCCGACTGTGTGGTACCCAAACCAATCAGAGAAATGGTGAAGAGCGCCTGGGGAAATTCG
961 -----+-----+-----+-----+-----+-----+ 1020
CGGCTGACACACCATGGGTTTGGTTAGTCTCTTTACCACTTCTCGCGGACCCCTTTAAGC
A D C V V P K P I R E M V K S A W G N S -

Figure 3 continued

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ATACCCACATTGATAGGCAATACCTCCTACGAAGGTTTGCTTTCCAAATCAATTGCCAAA
1021 -----+-----+-----+-----+-----+-----+ 1080
TATGGGTGTAACCTATCCGTTATGGAGGATGCTTCCAAACGAAAGGTTTAGTTAACGGTTT

I P T L I G N T S Y E G L L S K S I A K -

CAATATCCGGAGGTTGTAAAAGAGTTGGAATCCTGTGTGAATTATGTGCCTTGGGAGTTG
1081 -----+-----+-----+-----+-----+-----+ 1140
GTTATAGGCCTCCAACATTTTCTCAACCTTAGGACACACTTAATACACGGAACCCTCAAC

Q Y P E V V K E L E S C V N Y V P W E L -

GCTGACAGTGAACGCAGTGCCCCGGAACCCCTGGAGAGGGCTGCCATTGTGAAAAAGGCC
1141 -----+-----+-----+-----+-----+-----+ 1200
CGACTGTCACCTTGCCTCACGGGGCCTTTGGGACCTCTCCCGACGGTAACACTTTTTCGG

A D S E R S A P E T L E R A A I V K K A -

CATGTGGATGGGGAAACACCTACTCTGGATAATTTTATGGAGCTTTGCTCCTATTTCTAT
1201 -----+-----+-----+-----+-----+-----+ 1260
GTACACCTACCCCTTTGTGGATGAGACCTATTAAAATACCTCGAAACGAGGATAAAGATA

H V D G E T P T L D N F M E L C S Y F Y -

TTCCTCTTCCCATGCATCGCTTCCTACAATTGCGCTTCAACCACACAGCTGGCACTCCC
1261 -----+-----+-----+-----+-----+-----+ 1320
AAGGAGAAGGGGTACGTAGCGAAGGATGTTAACGCGAAGTTGGTGTGTGACCGTGAGGG

F L F P M H R F L Q L R F N H T A G T P -

ATTTATTTGTATCGTTTCGATTTTCGATTCCGAAGAAATTATTAACCCCTATCGTATTATG
1321 -----+-----+-----+-----+-----+-----+ 1380
TAAATAAACATAGCAAAGCTAAAGCTAAGGCTTCTTTAATAATTGGGGATAGCATAATAC

I Y L Y R F D F D S E E I I N P Y R I M -

CGTTTTGGCCGTGGCGTTAAAGGTGTAAGCCATGCCGATGAGCTAACCTATCTCTTCTGG
1381 -----+-----+-----+-----+-----+-----+ 1440
GCAAAACCGGCACCGCAATTTCCACATTCCGGTACGGCTACTCGATTGGATAGAGAAGACC

R F G R G V K G V S H A D E L T Y L F W -

AACATTTTGTGCGAAACGCCTGCCAAAGGAAAGCCGCGAATACAAAACCATTTGAACGCATG
1441 -----+-----+-----+-----+-----+-----+ 1500
TTGTAAAACAGCTTTGCGGACGGTTTCCTTTCCGGCGCTTATGTTTTGGTAACTTGCGTAC

N I L S K R L P K E S R E Y K T I E R M -

GTTGGCATTGACGGAATTCGCCACCACCGGCAAACCATACAGCAATGATATAGCCGGC
1501 -----+-----+-----+-----+-----+-----+ 1560
CAACCGTAAACCTGCCTTAAGCGGTGGTGGCCGTTTGGTATGTCGTTACTATATCGGCCG

V G I W T E F A T T G K P Y S N D I A G -

Figure 3 continued

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ATGGAACCTCACCTGGGATCCCATAAAAAATCCGATGATGTCTATAAATGTTTAAAT
1561 -----+-----+-----+-----+-----+-----+ 1620
TACCTTTTGGAGTGGACCCTAGGGTATTTTTTTAGGCTACTACAGATATTTACAAATTTA

M E N L T W D P I K K S D D V Y K C L N -

ATCGGCGATGAATTGAAAGTTATGGATTGCCCAGAAATGGATAAAATTAAACAATGGGCA
1621 -----+-----+-----+-----+-----+-----+ 1680
TAGCCGCTACTTAACTTTCAATACCTAAACGGTCTTTACCTATTTTAATTTGTTACCCGT

I G D E L K V M D L P E M D K I K Q W A -

AGTATATTCGATAAAAAGAAGGAATTGTTT
1681 -----+-----+-----+ 1710
TCATATAAGCTATTTTTCTTCCTTAACAAA

S I F D K K K E L F
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Figure 3 continued

Figure 4..

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