ORIGINAL ARTICLE

The evolution of new enzyme function: lessons from xenobiotic metabolizing bacteria versus insecticide-resistant insects

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Abstract

Here, we compare the evolutionary routes by which bacteria and insects have evolved enzymatic processes for the degradation of four classes of synthetic chemical insecticide. For insects, the selective advantage of such degradative activities is survival on exposure to the insecticide, whereas for the bacteria the advantage is simply a matter of access to additional sources of nutrients. Nevertheless, bacteria have evolved highly efficient enzymes from a wide variety of enzyme families, whereas insects have relied upon generalist esterase-, cytochrome P450- and glutathione-S-transferase-dependent detoxification systems. Moreover, the mutant insect enzymes are less efficient kinetically and less diverged in sequence from their putative ancestors than their bacterial counterparts. This presumably reflects several advantages that bacteria have over insects in the acquisition of new enzymatic functions, such as a broad biochemical repertoire from which new functions can be evolved, large population sizes, high effective mutation rates, very short generation times and access to genetic diversity through horizontal gene transfer. Both the insect and bacterial systems support recent theory proposing that new biochemical functions often evolve from 'promiscuous' activities in existing enzymes, with subsequent mutations then enhancing those activities. Study of the insect enzymes will help in resistance management, while the bacterial enzymes are potential bioremediants of insecticide residues in a range of contaminated environments.

Introduction

Living organisms have been coping with xenobiotics since life began and, as life has evolved, the nature of the xenobiotic challenge has changed. For example, as photosynthesis evolved, the toxic xenobiotic oxygen was produced for the first time; organisms not only evolved strategies to cope with this new xenobiotic, but also learnt to use it to provide energy (Storz and Imlay 1999; Imlay 2003; Falkowski and Godfrey 2008). Since the industrial revolution, anthropogenic xenobiotics have entered the biosphere in copious quantity and variety. While it is true that many man-made chemicals have very close structural relatives in nature (Wackett 2009), specific classes of chemical compounds that were not there before are also now abundant in the environment. Indeed, like oxygen in earlier times, many of these anthropogenic compounds are toxic and, in the case of pesticides and herbicides, they are toxic by design.

Many evolutionary outcomes associated with the introduction of anthropogenic xenobiotics have been documented, although few have been as well documented as those associated with the introduction of modern insecticides. The consequences of synthetic insecticide use have been extremely well described in both the eukaryotic target organisms (insects) and in incidentally exposed prokaryotes (bacteria in particular), often to the molecular level. In both cases, the exposed organisms have

evolved altered physiologies because of the presence of insecticides, although clearly in response to different selection pressures.

Bacteria incidentally exposed to these insecticides have evolved mechanisms to degrade them so that they can use them as sources of nutrients to increase their rates of reproduction and proliferation. Within a few decades, entirely new catabolic pathways dedicated to insecticide mineralization have emerged. For example, at least three distinct metabolic routes have developed specifically for the catabolism of the insecticidal carbamate carbofuran (e.g. Behki and Topp 1993; Chaudhry et al. 2002; Yan et al. 2007). Many bacterial enzymes and pathways have been extensively characterized at the molecular level (Ellis et al. 2001; Scott et al. 2008; Gao et al. 2010), although it is very likely that we are just beginning to understand the extent of this catabolic diversity.

Conversely, insects have not adapted to the introduction of insecticides for opportunistic reasons, as is the case with bacteria, but rather as a requirement for survival. They have exercised this by the development of resistances based on either mutant target sites that are no longer sensitive to the insecticides (e.g. Cassanelli et al. 2006; Liu et al. 2006, 2009; Ramphul et al. 2009) or by mutations in xenobiotic detoxification pathways to adapt them for the detoxification of the insecticides (detailed below). Several hundred cases of insecticide resistance have now been reported (Whalon et al. 2008), although molecular analyses suggest that many cases across a wide range of species involve quite precisely orthologous mechanisms (Claudianos et al. 2006; Hartley et al. 2006; Liu et al. 2006; Li et al. 2007).

Here, we review the metabolic adaptations of the insects targeted by insecticides and the bacteria that are exposed to these xenobiotics in the environment, with a focus on the enzymes that are altered to achieve these degradative functions. Our goal is to abstract insights into evolutionary processes and in particular the mechanisms by which organisms acquire new functions. We will consider four classes of insecticide chemistry: the organophosphates (OPs), organochlorines (OCs), carbamates and synthetic pyrethroids (SPs). The enzymes and metabolic pathways that have evolved in response to these four classes of insecticide are the best characterized, with at least some biochemical and molecular data available on the mechanisms of degradation in both bacteria and insects.

Organophosphate metabolizing enzymes

Organophosphate triesters, such as parathion and diazinon, are potent acetylcholinesterase (AChE) inhibitors and have been widely used as insecticides around the world. Most OPs have three phosphoester linkages, two of which (R1 and R2 in Fig. 1) are generally short aliphatic groups (most commonly o-methyl or o-ethyl). The leaving group at the third position (R3) can be one of a large number of phosphoesters or phosphothioesters (O'Brien 1960; Tomlin 2006).

The OPs are substrates for AChE, although they are hydrolysed far more slowly by AChE than is its natural substrate, acetylcholine. Acetylcholine is hydrolysed by AChE in a two-step reaction: nucleophic attack on the carboxylester by the AChE active-site serine, forming a planar acylated intermediate (acylation; Fig. 2), followed by regeneration of the active-site serine via an activated water molecule (deacylation). When the substrate is a phosphotriester, a phosphorylated serine intermediate is formed by the same mechanism. However, the phosphoryl group of the phosphorylated serine is tetrahedral, rather than planar (as is the acetylated serine), and it is sterically prevented from undergoing dephosphorylation via an activated water molecule as per the deacylation of the acylated intermediate. Indeed, solvent water is used to complete the reaction, which is far slower. Furthermore, a second process (aging) can also occur in which the dialkylphophoserine intermediate is dealkylated. The monoalkylphophoserine product of aging cannot then be regenerated to a serine.

Phosphotriesters that contain a thiophosphate group, rather than a phosphate, are not turned over by AChE (or other carboxylesterases) and are therefore not toxic themselves but act as protoxins that are activated by cytochromes P450 via oxidation of the thiophosphate to a phosphate. As this is a much faster process in insects than mammals, the result is that thiophosphates have a greater selectivity for insects than do their phosphate counterparts. For this reason, commercially available OP insecticides are predominantly thiophosphates. The OP triester chemistry is new to nature and, by definition, enzymes that can degrade these compounds have recently evolved catalytic functions.

Bacterial enzymes

Three classes of bacterial enzymes are known to degrade phosphotriesters, namely phosphotriesterases (PTEs), methyl parathion hydrolases (MPHs) and organophosphorus acid anhydrolases (OPAA), and all operate via the hydrolysis of the R3 phosphoester bond (Fig. 1 and Table 1). Although all three of these enzymes are hydrolases (see below), there is some evidence for an as yet uncharacterized oxidative route to the degradation of some OPs as well (Munnecke and Hsieh 1976). The three classes of hydrolases liberate a phosphodiester and an alcohol as the first step in the mineralization of the OP compound.

Class	General structure	Formulations	Isomers	Commercially available insecticidal isomers
OP				
OC		HCH Lindane	4 (major) 1	γ-HCH
		DDT		
Carbamate				
Type I SP		Permethrin	4	(1R)-cis (1R)-trans
		Bifenthrin	2	(Z)-(1R)-cis
Type II SP		Cypermethrin Alpha-cypermethrin Beta-cypermethrin Theta-cypermethrin Zeta-cypermethrin	8 2 4 2 4	(1R)-cis-α(S) (1R)-trans-α(S)
		Deltamethrin	1	(1R)-cis-α(S)
		Cyhalothrin Lambda-cyhalothrin Gamma-cyhalothrin	4 2 1	(Z)-(1R)-cis-α(S)
		Fenvalerate Esfenvalerate	4 1	(2S)-α(S)

Figure 1 Structures of insecticides considered in this work. The structures of type 1 and type 2 synthetic pyrethroids (SP), organophosphate insecticides (OP), organochlorides (OC), and insecticidal carbamates are shown. Isomeric centres are indicated with an asterisk, the numbers of common isomers are indicated, and the structures of the commercially available insecticidal isomers are shown. Arrows indicate the bonds cleaved during enzymatic detoxification.

Further degradation then occurs; the phosphodiester provides a source of phosphorous for the organism, and the alcohol can provide carbon or energy. For example, the alcohol produced from parathion hydrolysis (*p*-nitrophenol) is further degraded by well-characterized catabolic pathways (Singh et al. 2002; Paul et al. 2004, 2008; Scott et al. 2008; Chauhan et al. 2010).

The best characterized of the three families of hydrolases that degrade OPs are the PTEs, which were initially isolated from a *Flavobacterium* sp. and *Pseudomonas diminuta* in the 1980s (PTE$_{Pd}$; Brown 1980; Dumas et al.

1989). Subsequently, a PTE with 90% amino acid sequence identity to this enzyme was also isolated from *Agrobacterium radiobacter* (PTE$_{Ar}$; Horne et al. 2002). The crystal structures of both enzymes have been determined, and both possess a TIM(β/α)$_8$-barrel fold, contain two metal atoms (Fe^{2+} and Zn^{2+}) in the active site and belong to the amidohydrolase superfamily of proteins (Benning et al. 2001; Yang et al. 2003; Jackson et al. 2005). PTEs are highly efficient enzymes; they act specifically on phosphotriesters with diffusion-limited kinetics, resulting in a rate enhancement over the noncatalysed reaction (i.e.
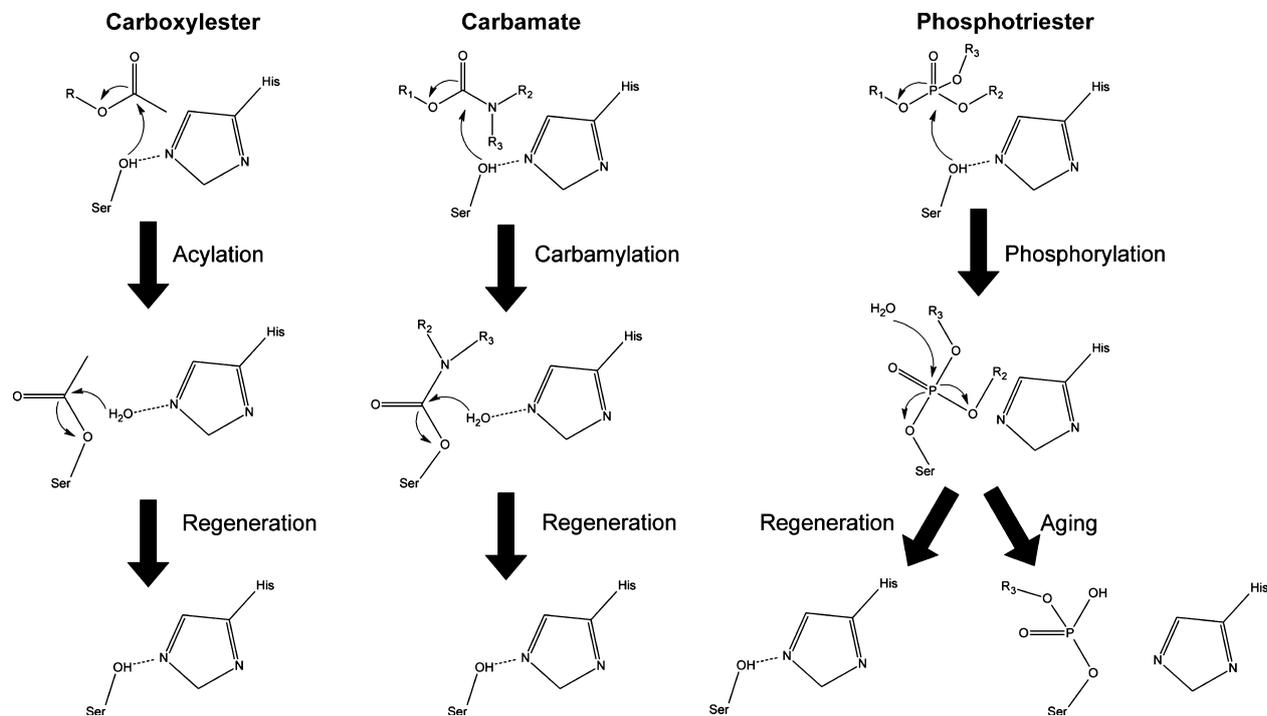
Figure 2 Reactions of insecticidal organophosphates (OPs) and carbamates with acetylcholine esterase (AChE). The physiological reaction of AChE with a carboxyl ester is shown (left), whereby the active-site serine is first acylated as a consequence of its nucleophilc attack of the substrates ester bond. Deacylation (regeneration) is catalysed by histidine-activated water. In the reaction with an insecticidal carbamate (middle), nucleophilc hydrolysis of the substrate results in a carbamylated serine intermediate, which is then regenerated by histidine-activated water. Phosphorylation of the active-site serine by OP (right) results in an intermediate that is sterically hindered from regeneration by a histidine-activated water. Instead, a solvent water acts as a nucleophile either regenerating the active-site serine or removing an alkyl side chain from the phosphorous moiety and resulting in a stable phosphodiester of serine (aging).

spontaneous decomposition) of $\sim 10^{11}$-fold (see Table 1). Hydrolysis of phosphotriesters is achieved via a nucleophilic substitution ($S_N 2$) using metal co-ordinated activated water (Holden and Raushel 2002; Jackson et al. 2005). The two different PTE enzymes vary somewhat in their substrate preferences: the *Agrobacteriun* PTE prefers dimethyl alkoxy groups in the R1 and R2 positions (see Fig. 1), whereas the *Flavobacterium*/*Pseudomonas* enzyme prefers diethyl groups at these positions (Brown 1980; Dumas et al. 1989; Horne et al. 2002). The substrate preference and catalytic rate for OPs are constrained by both the structure (Di Sioudi et al. 1999; Cho et al. 2004, 2006; Jackson et al. 2009a) and pK_a (Hong and Raushel 1996; Jackson et al. 2009b) of the R3 group. Leaving groups with a pK_a of ~ 8 or less are hydrolysed extremely efficiently, while leaving groups with higher pK_a values are generally turned over at a rate related to their pK_a value, although other factors such as hydrophobicity and stereochemical interactions can also affect the rate (Jackson et al. 2005, 2009b).

No close homologues of the PTEs have been identified, so their evolutionary origins are unknown. The genes

(*oph*) encoding PTE in the *Flavobacterium* sp. and *P. diminuta* are identical in sequence and located on large, albeit different plasmids, within a highly conserved region of ~ 5.1 kb (Mulbry et al. 1987), suggesting a recent genetic exchange between the plasmids. The *opdA* gene encoding PTE$_{Ar}$, while not plasmid-borne, is associated with a transposase gene (Horne et al. 2003), so it is likely that lateral gene transfer has also played a role in PTE evolution.

Recently, Afriat et al. (2006) discovered that PTE$_{Pd}$ has considerable promiscuous lactonase activity ($k_{cat}/K_m = 160 - 6.5 \times 10^5$ s^{-1} M^{-1} for different lactones and thiolactones). They therefore tested both the PTE and lactonase activities of three PTE-like lactonases ($\sim 30\%$ sequence identity to PTE) and found that they all proficiently hydrolyse lactones ($k_{cat}/K_m = 6 \times 10^4 - 1.7 \times 10^6$ s^{-1} M^{-1}) and have measurable paraoxonase activity (10^2- to 10^6-fold lower than the lactonase activity, and 10^4- to 10^8-fold lower than the activity of PTE for paraoxon). The authors suggested that the physiological role for these PTE-like lactonases might be in processing quorum-sensing lactones, in which case they probably

Table 1. Bacterial enzymes with activity against man-made insecticidal xenobiotics.

Insecticide class	Bacterial enzyme	Enzyme activity	Comments	Reference
Organophosphates (OPs)	Phosphotriesterase (PTE) (amidohydrolase fold) from *Flavobacterium* sp., *Pseudomonas diminuta*, and *Agrobacterium radiobacter* P230	For the *Pseudomonas/Flavobacterium* enzyme, using paraoxon as substrate: $k_{cat} = 4 \times 10^3$ s^{-1}, $K_m = 1 \times 10^{-4}$ m; $k_{cat}/K_m > 4 \times 10^7$ s^{-1} m^{-1}	Diffusion-limited kinetics; rate enhancement of 2.8×10^{11} over the uncatalysed reaction. The *Pseudomonas* and *Flavobacterium* enzymes are identical, while the *Agrobacterium* enzyme shares 90% amino acid identity. Very broad substrate range	Brown (1980), Dumas et al. (1989), Horne et al. (2002) and Horvat and Wolfenden (2005)
	Methyl parathion hydrolases (MPH) (β-lactamase fold) from *Plesiomonas* sp. strain M6, *Pseudomonas* sp. WBC-3	For methyl parathion as substrate: $K_m = 3.7 \times 10^{-5}$ m; $k_{cat} = 37$ s^{-1}	Limited substrate range, with parathion the major substrate. Enzyme is most closely related to glyoxylase II. Bimetal centre is nearly identical to that found in PTE	Cui et al. (2001) and Dong et al. (2005)
	Organophosphorus acid anhydrolases (OPAA) (Pita bread-fold, prolidase/proline dipeptidase) from *Alteromonas* sp. strain JD6.5	For paraoxon as substrate: $K_m = 1.4 \times 10^{-2}$ m; $k_{cat} = 2.3 \times 10^2$ s^{-1}; For diisopropyl fluorophosphate: $k_{cat} = 583$ s^{-1}	OPAA appears to be a proline dipeptidase with a high level of promiscuous activity against OPs. Other prolidases (e.g. PepQ from *E. coli*) also have OP hydrolase activity Mn^{2+}-dependent; broad substrate range	Defrank and Cheng (1991) and Park et al. (2004)
Organochlorines	LinA (unique dehydrochlorinase) from various species, predominantly *Sphingomonads*	For the LinAγ1-7 enzyme at 1.7 μm γ-HCH (lindane), reaction rate is 197 s^{-1}; for 1.7 μm α-HCH: 50 s^{-1}; for 1.7 μm δ-HCH: 5 s^{-1}	Very modest kinetics for the preferred isomers; rate enhancement of $>10^{10}$ over the uncatalysed reaction ($k_{non} \sim 160$ years)	Cortes and Hites (2000), Lal et al. (2010) and Pandey et al. (unpublished manuscript)
	LinB (hydrolytic dechlorinase) from various species, predominantly *Sphingomonads*	For the LinB SS04-5 enzyme at 1.7 μm β-HCH, reaction rate is: 3 s^{-1}; for 1.7 μm δ-HCH: 0.1 s^{-1}	A very broad substrate preference for halogenated compounds up to a chain length of eight carbon atoms	Lal et al. (2010) and Pandey et al. (unpublished manuscript)
Carbamates	MCD (β-lactamase fold) from *Achromobacter* WM111	For carbaryl as substrate: $k_{cat} = 1.7$ s^{-1}; $K_m = 3.1 \times 10^{-5}$ m; $k_{cat}/K_m = 5.4 \times 10^4$ s^{-1} m^{-1}	Also has a high level of carboxylesterase activity against 1-naphthyl acetate, and low PTE activity	Wolfe et al. (1978), Derbyshire et al. (1987) and Naqvi et al. (2009)
	CahA (amidase, Pnc1p fold) from *Arthrobacter* sp. RC100	For carbaryl as substrate: $k_{cat} = 0.2$ s^{-1}; $K_m = 2.3 \times 10^{-5}$ m; $k_{cat}/K_m = 8.7 \times 10^3$ s^{-1} m^{-1}	Retains the lysine/serine catalytic dyad of other known amidases; active against a range of carbamates	Hayatsu et al. (2001) and Hashimoto et al. (2006)
	CehA (structure unknown) from *Rhizobium* sp AC100	For carbaryl as substrate: $K_m = 6.2 \times 10^{-5}$ m	Associated with transposable elements, unknown mechanism, no known relatives; active against a wide range of *N*-methyl carbamates	Hashimoto et al. (2002)

Table 1. (Continued)

Insecticide class	Bacterial enzyme	Enzyme activity	Comments	Reference
Synthetic pyrethroids	PytH (α/β-hydrolase fold) from *Sphingobium* sp. strain JZ-1	For permethrin (*cis*- and *trans*-) as substrate: $K_m = 6 \times 10^{-8}$ m; $k_{cat} = 3$ s^{-1} cypermethrin (*cis*- and *trans*-): $K_m = 1 \times 10^{-7}$ m; $k_{cat} = 2.5$ s^{-1} cyhalothrin: $K_m = 3 \times 10^{-7}$ m; $k_{cat} = 1.3$ s^{-1} bifenthirin: 1.59×10^{-6} m; $k_{cat} = 0.4$ s^{-1} deltamethrin: $K_m = 0.79 \times 10^{-7}$ m; $k_{cat} = 0.8$ s^{-1} fenvalerate: $K_m = 5.9 \times 10^{-7}$ m; $k_{cat} = 0.9$ s^{-1}	No isomer specificity	Wang et al. (2009)
	Permethrinase (structure unknown) from *Bacillus cereus* SM3	Up to 70% degradation of *trans*-permethrin after 8 h	Demonstrates isomer specificity on permethrin; activity towards a wide range of α-cyanopyrethroids	Maloney et al. (1993)
	EspA (carboxylesterase) from *Klebsiella* ZD 112	For permethrin (*cis*- and *trans*-) as substrate: $K_m = 1.6 \times 10^{-7}$ m; $k_{cat} = 1$ s^{-1} cypermethrin $K_m = 2.1 \times 10^{-7}$ m; $k_{cat} = 0.29$ s^{-1} fenvalerate $K_m = 9.1 \times 10^{-7}$ m; $k_{cat} = 0.1$ s^{-1} deltamethrin $K_m = 1.2 \times 10^{-6}$ m; $k_{cat} = 1.6 \times 10^{-2}$ s^{-1}	No isomer specificity, very broad substrate range; also active against the carboxylester bonds of the OP insecticide, malathion	Wu et al. (2006)

evolved many millions of years ago. They further suggested that PTEs may have evolved from an unknown PTE-like lactonase, using its low level of promiscuous paraoxonase activity as an essential starting point. The paraoxonase activity would previously have had no physiological relevance, so it could have been selectively neutral, remaining latent in these enzymes. Empirical structures have also now been determined for three additional lactonases with promiscuous PTE activity (Del Vecchio et al. 2009; Hawwa et al. 2009; Xiang et al. 2009). These lactonases share both the fold and active site architecture with PTE, supporting the speculation of Afriat and co-workers.

Phosphotriesters are also hydrolysed by members of the MPH and OPAA families, albeit with considerably lower catalytic rates (k_{cat}) than those for the PTEs. The activity of MPH is effectively restricted to methyl parathion, with a k_{cat} value of 37 s^{-1} and a K_m of 37 μM (Table 1). OPAA has a k_{cat} for paraoxon of 230 s^{-1}, which is an order of magnitude slower than the PTEs. In contrast to MPH, OPAA has a relatively broad substrate range, including diisopropyl fluorophosphate (DFP), cyclohexyl methyl fluorophosphate, paraoxon and the G-series nerve gasses (sarin, soman and tabun), which are turned over more rapidly than the 'model' substrate methyl parathion (k_{cat} of 583 s^{-1} for DFP; Defrank and Cheng 1991).

Methyl parathion hydrolase has a β-lactamase-like structure and contains a bimetallic catalytic centre that is analogous in structure and presumably in mechanism to that of the PTEs (Dong et al. 2005). Therefore, it appears that a virtually identical catalytic mechanism for OP hydrolysis has evolved independently from two structurally distinct, but mechanistically similar ancestors, suggesting that it was mechanistic rather than structural considerations that predisposed the ancestors of both the PTEs and MPH to evolve OP hydrolase activity.

Organophosphorus acid anhydrolases is phylogenetically distinct from both the MPH and PTEs and is an Mn^{2+}-dependent hydrolase, rather than an Fe/Zn- or Zn/Zn-dependent enzyme like the PTEs and MPH. OPAA is therefore likely to be mechanistically dissimilar from the other PTE families, although its catalytic mechanism is yet to be elucidated. The evolutionary origin of the OPAA class of PTEs is much clearer than that for either the PTEs or MPH. OPAA has a high degree of amino acid identity ($\sim$50%) to the prolidase family of dipeptidases (e.g. PepQ from *Escherichia coli*; Cheng et al. 1996). Indeed, OPAA from *Alteromonas haloplanktis* maintains high levels of proline dipeptidase activity (650 μmol min^{-1} mg^{-1} for the ALA-PRO dipeptide; Cheng et al. 1996). Moreover, prolidases from other organisms, including *E. coli* aminopeptidase P, have levels of PTE activity that closely match that of OPAA (Jao et al. 2004).

This suggests that the OP hydrolase activity of the OPAA family is simply a promiscuous activity, albeit a moderately efficient one, and that the major cellular role of these enzymes is as a prolidase.

Insect enzymes

More than 150 separate cases of OP resistance have been described in insects where at least something is known about the biochemical mechanism (for reviews, see Feyereisen 2005; Oakeshott et al. 2005a,b; Li et al. 2007; and Farnsworth et al. 2010). Known enzyme-dependent mechanisms involved in resistance fall into just four classes (Table 2), only one of which (certain carboxylesterases) involves mutations in the active site to produce a new enzyme function (OP hydrolysis, which reduces the toxicity of the OP by several orders of magnitude; Singh and Walker 2006). The others confer resistance via gene amplification and/or over-expression of general detoxifying systems [glutathione-*S*-transferases (GSTs), cytochromes P450 or other carboxylesterases], or by mutations in AChE that decrease its sensitivity to the insecticide (Table 2).

So far there have been nine cases characterized in detail at the molecular level in which amino acid substitutions have conferred OP hydrolase activity on insect carboxylesterases. All of these fall into the same, orthologous clade of esterases, the best characterized of which is the αE7 enzyme of the sheep blowfly, *Lucilia cuprina*. Carboxylesterases, like AChE, can carry out the first step of the two-step reaction required to hydrolyse OPs at physiologically relevant rates (Fig. 2). In the nine cases of mutant carboxylesterases conferring OP resistance, a mutation in the active site greatly enhances the second step (regeneration by dephosphorylation). Remarkably, these nine cases essentially involve just two independent amino acid substitutions (Table 2). The first involves substitution of a tryptophan in the acyl pocket of the active site with a smaller residue, generally a leucine but sometimes a serine or a glycine, while the second involves substitution of a glycine in the oxyanion hole of the active site with an aspartic acid residue (Newcomb et al. 1997a; Campbell et al. 1998; Claudianos et al. 1999; Zhu et al. 1999; Heidari et al. 2004; Oakeshott et al. 2005a; de Carvalho et al. 2006, 2009, 2010). The two mutations do not occur together, each conferring resistance and OP hydrolase activity. Because these two very specific changes in orthologous esterase enzymes are the only ways yet known for an insect to evolve an enzyme with OP hydrolase activity, it appears that the options for doing so are very limited. Even the more active of the two *Lc*αE7 mutants (Gly137Asp) is many fold less active in its OP hydrolytic activity than any of the bacterial OP hydrolases (Tables 1 and 2). It is interesting to note that the activity of the

Table 2. Insect enzymes with activity against man-made insecticidal xenobiotics.*

Insecticide class	Insect enzyme	Enzyme activity	Comments	Reference
Organophosphates (OPs)	*LcαE7*G137D (E3 carboxylesterase) from *Lucilia cuprina* and orthologs in *Lucilia sericata*, *Musca domestica*, *Cochliomyia hominivorax*	*LcαE7*G137D: using diethyl 7-hydroxycoumaryl phosphate (dECP) as a substrate, $k_{cat} = 8 \times 10^{-4}$ s^{-1} for baculovirus expressed enzyme; dimethyl 4-methylumbelliferyl phosphate (dMUP): $k_{cat} = 9 \times 10^{-4}$ s^{-1}, chlorfenvinphos (CVP): specific activity = 143 ± 0.2 pmol min^{-1} mg^{-1} baculovirus cell extracts. *MdαE7*G137D (*M. domestica* orthologue): CVP: specific activity = 146 ± 0.5 pmol min^{-1} mg^{-1} *E. coli* cell extracts	Resistance to OPs resulting from mutations in *LcαE7*, the gene encoding carboxylesterase E3, that enhance the enzyme's ability to hydrolyse insecticides. The G137D mutation enhances hydrolysis of diethyl and dimethyl OPs by 55- and 33-fold, respectively, although general carboxylesterase activity is diminished	Newcomb et al. (1997a), Campbell et al. (1998), Claudianos et al. (1999), Devonshire et al. (2003), Heidari et al. (2004), Oakeshott et al. (2005a) and de Carvalho et al. (2006, 2009, 2010)
	LcαE7W251L/S/G (E3 carboxylesterase) from *L. cuprina* and orthologs in *M. domestica*, *L. sericata*, *Anisopteromalus calandrae*, *C. hominivorax*	LcαE7W251L: dECP: $k_{cat} = 1.5 \times 10^{-4}$ s^{-1} for baculovirus expressed enzyme; dMUP: $k_{cat} = 1 \times 10^{-3}$ s^{-1}, malathion: $k_{cat} = 3.7$ s^{-1}	The W251L mutation increases hydrolysis of diethyl and dimethyl OPs by 10- and 33-fold, respectively. Unlike the G137D mutation above, it retains the ability to hydrolyse carboxylesters in the leaving group of malathion, conferring strong resistance to this compound	Campbell et al. (1998), Claudianos et al. (1999), Zhu et al. (1999), Devonshire et al. (2003), Heidari et al. (2004), Oakeshott et al. (2005a) and de Carvalho et al. (2006, 2009, 2010)
	E4 and FE4 (carboxylesterases from paralogous genes) from *Myzus persicae*	E4: Paraoxon: $k_{cat} = 9 \times 10^{-5}$ s^{-1} (or 0.33 h^{-1})	Resistance resulting from the over-production of E4 or FE4 via gene amplification; results in sequestration and enhanced degradation of a wide range of OPs, carbamates and synthetic pyrethroids (SPs); *E4*-amplified genes are methylated leading to their over-expression	Devonshire and Moores (1982), Field and Devonshire (1997, 1998), Field (2000) and Field and Blackman (2003)
	Cytochromes P450 (mainly members of the CYP4 and CYP6 families) from various species	CYP6A1 from the Rutgers strain of *M. domestica*: diazinon: turnover = 32.2 pmol min^{-1} mg^{-1} *E. coli* expressed CYP6A1	Cytochrome P450-mediated resistance is attributable to over-expression via up-regulation of *CYP* genes. In the Rutgers strain of *M. domestica*, over-expression of *CYP6A1* is at least in part because of loss-of-function mutations in *trans*- regulatory loci	Carino et al. (1994), Feyereisen (2005) and Li et al. (2007)

Table 2. (Continued)

Insecticide class	Insect enzyme	Enzyme activity	Comments	Reference
	Glutathione-S-transferases (GSTs) from various species	PxGST3 from the MPA methyl parathion-resistant strain of Plutella xylostella: specific activity towards parathion, methyl parathion and paraoxon 13- to 40-fold higher than that of other GST	GST-mediated resistance is attributable to gene amplification and/or over-expression of GSTs, e.g. amplified MdGSTD3 and MdGST-6A are responsible for OP resistance in the Cornell strain of M. domestica, although several GSTs are amplified (but have no activity); over-expressed PxGSTE1 (encoding PxGST3) is responsible for OP resistance in P. xylostella	Chiang and Sun (1993), Wei et al. (2001) and Li et al. (2007)
	Acetylcholinesterases (AChEs) from various species	(nonover-expressed) isozymes in the same strain	Resistance is attributable to mutations in AChE (target site for OPs) that decrease sensitivity to the insecticide	Russell et al. (2004) and Oakeshott et al. (2005b)
Organochlorines	Cytochromes P450 (mainly members of the CYP4 and CYP6 families) from various species	Activity of Anopheles gambiae CYP6Z1 for DDT = 5.7 pmol min^{-1} mg^{-1} of baculovirus expressed protein; no activity was detected for expressed CYP6Z2	Cytochrome P450-mediated resistance is generally attributable to over-expression via up-regulation of CYP genes. In the DDT- and malathion-resistant 91-R and MHIII-D23 strains of Drosophila melanogaster, over-expression of CYP6A2 and CYP6A8 is at least in part because of loss-of-function mutations in trans-regulatory loci. DDT resistance in D. melanogaster is largely because of a series of multiple adaptive steps occurring in rapid succession at the CYP6G1 locus and involves three different transposable element insertions and a CYP6G1 gene duplication event. Both CYP6Z1 and CYP6Z2 are over-expressed in DDT-resistant A. gambiae, but only CYP6Z1 is capable of metabolizing DDT	Maitra et al. (2000), Catania et al. (2004), Feyereisen (2005), Li et al. (2007), Chiu et al. (2008) and Schmidt et al. (2010)
	CYP6A2 (cytochrome P450) of the RaleighDDT strain of Drosophila melanogaster	DDT metabolism rates were up to 20 times higher than those of the wild-type enzyme	Three point mutations in CYP6A2 in the RaleighDDT strain confer >1000-fold R to DDT; CYP6A2 is also over-expressed in this strain of D. melanogaster	Amichot et al. 2004 and Feyereisen (2005)
	GSTs from various species	DDT dehydrochlorinase (DDTase) activity of: AgGSTE2: 0.023 pmol min^{-1} mg^{-1}; DmGSTD1: >0.0072 pmol min^{-1} mg^{-1}; AaGSTE2: 0.0345 pmol min^{-1} mg^{-1}	GST-mediated resistance is attributable to over-expression of GSTs (e.g. AgGSTE2 in DDT-resistant Anopheles gambiae, DmGSTD1 in DDT-resistant D. melanogaster, and AaGSTD1 and AaGSTE2 in DDT/SP-resistant Aedes aegypti). Some insect GSTs detoxify DDT by dehydrochlorination, using reduced glutathione as a cofactor	Tang and Tu (1994), Ortelli et al. (2003), Lumjuan et al. (2005) and Li et al. (2007)

Table 2. (*Continued*)

Insecticide class	Insect enzyme	Enzyme activity	Comments	Reference
Carbamates	Carboxylesterase A1 from *Heliothis virescens*		Thiodicarb and cypermethrin resistance linked to an over-expressed carboxylesterase A1 in *H. virescens* that is related to the E4 esterase from *M. persicae*	Goh et al. (1995)
	E4 and FE4 (carboxylesterases from paralogous genes) from *M. persicae*	E4: pirimicarb: k_{cat} = 2.6 × 10⁻⁵ s⁻¹ (or 0.096 h⁻¹); propoxur: k_{cat} = 2.6 × 10⁻⁵ s⁻¹ (0.093 h⁻¹)	Resistance resulting from the over-production of E4 or FE4 via gene amplification (see above)	Devonshire and Moores (1982)
	Cytochromes P450 from a limited number of insect species		Only a small number of reports in the literature of P450-mediated resistance to carbamate insecticides (i.e. over-expressed *CYP6A1* in the Rutgers strain of *M. domestica*, *CYP6G1* in the WC2 strain of *D. melanogaster* and *CYP9A1* in the Macon Ridge strain of *H. virescens*)	Sabourault et al. (2001) and Feyereisen (2005)
Synthetic pyrethroids	Carboxylesterases from various species		Higher intensities of specific zones of esterase isozymes associated with high levels of resistance to SPs in various species; resistance mechanisms have not yet been elucidated at the molecular level	Oakeshott et al. (2010) and Farnsworth et al. (2010)
	LcαE7 wild-type (carboxylesterase) from *L. cuprina*	Wild-type αE7 has relatively high SP hydrolytic activity (specificity constants of up to 90 000 m⁻¹ s⁻¹), although its efficiency is much reduced for the more insecticidal isomers of the more recent type 2 SPs	Strong preferences for type 1 over type 2 SPs, dichloro- over dibromo-, and *trans-* over *cis*-isomers across the cyclopropane ring of the SP. Wild-type *L. cuprina* is presumably sensitive to OPs	Heidari et al. (2005), Devonshire et al. (2007) and Oakeshott et al. (2010)
	E4 and FE4 (carboxylesterases from paralogous genes) from *M. persicae*	E4 hydrolysed (1S)trans-permethrin rapidly (i.e. 2500 times faster than for paraoxon above), but hydrolysis of the other three isomers could not be detected	Resistance resulting from the over-production of E4 or FE4 via gene amplification (see above)	Devonshire and Moores (1982)
	Cytochrome P450s (mainly members of the CYP4 and CYP6 families) from various species	*MdCYP6D1* from LPR strain of *M. domestica*: rates of SP metabolism are extremely low e.g. deltamethrin – <0.88 pmol min⁻¹ mg⁻¹; cypermethrin – 8.3 pmol min⁻¹ mg⁻¹; *AgCYP6P3* from permethrin-resistant *Anopheles gambiae*: trans-permethrin isomers – 0.59 ± 0.04 min⁻¹; cis-permethrin isomers – 0.37 ± 0.02 min⁻¹; deltamethrin – 0.86 ± 0.03 min⁻¹	Cytochrome P450-mediated SP resistance is generally attributable to over-expression via up-regulation of CYP genes e.g. CYP4G8 and CYP6B7 in SP-resistant *H. virescens*; CYP6X1 in SP-resistant *Lygus lineolaris*; CYP6D1 from LPR strain of *M. domestica* (also contains other resistance genes, such as pen and kdr). Several P450s (e.g. CYP6P3, CYP6M2 and CYP6AK1) are over-expressed in field-caught permethrin-resistant *A. gambiae*, but so far only CYP6P3 has been expressed in *E. coli* and shown to have activity for both type 1 and type 2 SPs	Wheelock and Scott (1992), Zhang and Scott (1996), Feyereisen (2005), Li et al. (2007) and Muller et al. (2008)

Table 2. (Continued)

Insecticide class	Insect enzyme	Enzyme activity	Comments	Reference
	GSTs from various species		GST-mediated SP resistance is attributable to gene amplification and/or over-expression of GSTs (e.g. amplification of *NlGSTD1* in SP-resistant *Nilaparvata lugens*; over-expression of *AaGSTD1* and *AaGSTE2* in DDT/SP-resistant *A. aegypti*). GSTs are involved in sequestration of SPs and/or detoxification of lipid peroxidation products induced by SPs, rather than direct metabolism of SPs	Li et al. (2007)

*Where possible k_{cat} and K_m values are given. In other cases, specific activities are given in pmol min^{-1} mg^{-1} for easy comparison and may have been converted from their originally published units of measure. If these values were not available, other measures of activity are shown.

wild-type *Lcα*E7 enzyme for OPs with either of the most common alkoxy group substituents is not zero, so again the start point for the evolution of the 'new' function has been an enzyme that already has low promiscuous activity for that function.

The Gly to Asp mutation greatly reduces the native carboxylesterase activity and, in *L. cuprina* at least, this impairs development, producing asymmetric bristle and wing vein patterns and measurable loss of fitness (McKenzie 1994, 1997; Batterham et al. 1996; Newcomb et al. 1997a,b). The Trp to Leu mutation in *Lcα*E7 reduces the stability of the enzyme *in vitro* (C. J. Jackson, J-W. Liu and J. G. Oakeshott, unpublished data), although its effects on fitness, if any, are unknown.

Hartley et al. (2006) traced the evolutionary history of the two *Lcα*E7 mutants by sequencing pinned Australasian specimens collected before OPs were used commercially. The G137D mutation was not recovered from any of the 16 pre-1950 blowflies sampled for this allele; however, four of 21 flies sampled for the W241 allele carried a resistance mutation (either W251L or W251T), indicating that it was in the population prior to the introduction of OPs. On the other hand, the G137D allele now dominates post-OP Australasian *L. cuprina* populations, substantively replacing the susceptible wild-type and the poorer W251L-resistant mutant (Oakeshott et al. 2005a). This is in part because the fitness cost associated with the G137D mutation has been ameliorated in two ways. First, a modifier mutation arose at a second locus, which corrects the developmental instability problem (McKenzie 1994). Second, there has been a rise in the frequency of duplication events that put both a susceptible and a resistant alleles onto the one chromosome (Smyth et al. 2000; Newcomb et al. 2005), so that the fly can be simultaneously homozygous for both the original function and the new one. However, the selective sweep of the G137D mutation to high frequencies has greatly reduced the level of polymorphism at other positions in the *Lcα*E7 gene, thereby diminishing significantly the genetic diversity on which to select for further improvements in OP hydrolytic activity (Hartley et al. 2006).

Organochlorine metabolizing enzymes

Relatively few OC-based insecticides are now used, as a result of the concerns about their mammalian toxicity and bioaccumulation. The environmental fate and development of resistance to OC insecticides are best documented for DDT (1,1,1-trichloro-2,2-di(4-chlorophenyl)ethane) and lindane (the gamma isomer of hexachlorocyclohexane; HCH). Enzymes have been identified that degrade other OC insecticides, such as the bacterial monooxygenases that degrade endosulfan and endosulfate (Sutherland et al.

2002; Weir et al. 2006). However, we have not discussed such cases here as they lack the level of molecular detail available for the HCH- and DDT-degrading enzymes in either bacteria or insects.

DDT is a potent neurotoxin that binds the voltage-gated axonic sodium channels, causing uncontrolled and persistent excitation of the neurones. This mode of action is shared by the SPs, and mutations that cause target-site insensitivity for DDT often also reduce sensitivity for the SPs (Brengues et al. 2003).

Lindane acts by binding and blocking the gamma-aminobutyric acid (GABA) receptors of neuronal GABA-gated chloride channels. GABA is an antagonist of these channels, so lindane effectively blocks this antagonistic effect, resulting in continuous stimulation of the associated neurones (Joy 1982). Technical grade HCH comprises four major isomers that differ in the spatial orientation of the chlorines around the cyclohexane ring (Fig. 1), although only γ-HCH is insecticidal. The production of 1 tonne of lindane therefore generates 8–12 tonnes of an unwanted mixture of variously stable HCH isomers (predominantly α-, β- and δ-HCH), 4–6 million tonnes of which have been dumped worldwide (Lal et al. 2010).

Bacterial enzymes

Numerous bacteria have been discovered that can degrade DDT (Stenerse 1965; Nadeau et al. 1994; Bidlan and Manonmani 2002; Ahuja and Kumar 2003; Kamanavalli

and Ninnekar 2004; Blasco et al. 2008; Wang et al. 2010). However, the enzymes responsible for the early steps in this degradation have yet to be identified.

There have been over 45 reports of HCH-degrading bacteria, mostly from a single family, the *Sphingomonadaceae*, with most of the others also from relatively closely related Gram-negative bacteria (for review, see Lal et al. 2010). All the strains tested at a biochemical/molecular level appear to have very similar pathways for HCH mineralization, comprising a 10-enzyme pathway (encoded by the *linA – linJ* genes; Lal et al. 2010). The ten-enzyme pathway accommodates the enormous isomeric complexity of HCH. The two key enzymes at the head of the pathway are LinA, which is a unique HCH dehydrochlorinase, and LinB, which is a haloalkane dehalogenase in the α/β hydrolase superfamily of enzymes (Ollis et al. 1992; Nagata et al. 1993, 1997; Kmunicek et al. 2005; Brittain et al. 2011; Lal et al. 2010; Okai et al. 2010). LinA degrades α-, δ- and γ-HCH, performing either one or two dehydrochlorination reactions before LinB enters the pathway with its hydrolytic dechlorination reaction (Fig. 3). LinB also degrades of β-and δ-HCH without these isomers first being dehydrochlorinated by LinA (Ito et al. 2007; Lal et al. 2010). Each chloride removal reduces the toxicity of HCH by about 10-fold, and the removal of 3–4 chlorides is necessary before LinC and subsequent enzymes mineralize the compound (Kumari et al. 2002; Suar et al. 2004).

There is considerable evidence that the rapid assembly of the HCH catalabolic pathway has involved lateral gene
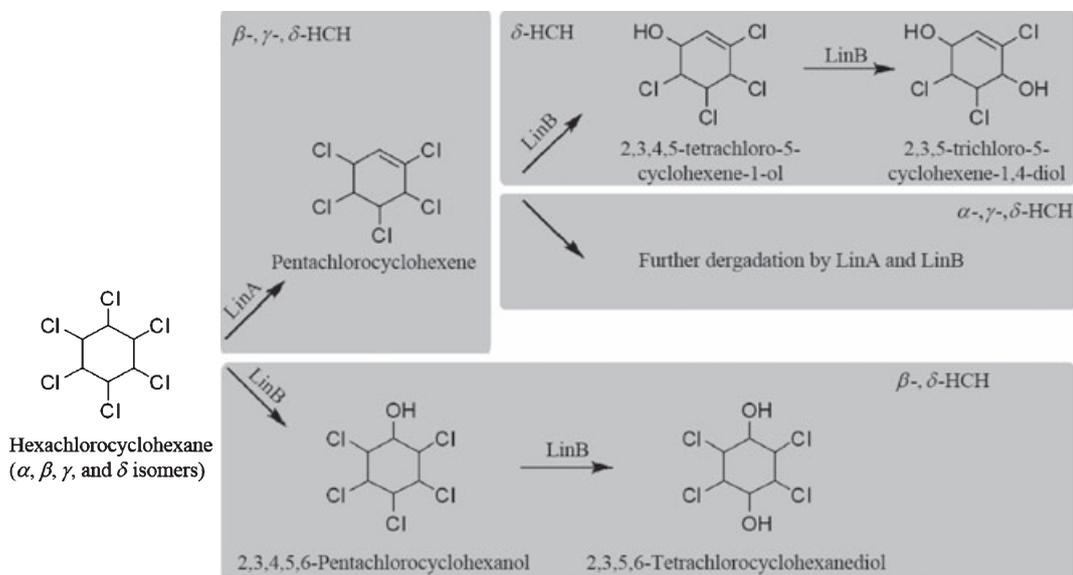
Figure 3 Simplified scheme of hexachlorocyclohexane (HCH) degradation pathway by LinA and LinB. Four different HCH isomers, α, β, γ and δ are degraded by the actions of LinA and LinB. The LinA (dehydrochlorinase)-initiated pathway occurs for β-, γ- and δ-HCH, and the respective pentachlorocyclohexanes (PCCH) are formed, which are further degraded by LinA and LinB. The haloalkane dehalogenase-, LinB-initiated pathway occurs for β- and δ-HCH, which are degraded to 2,3,5,6-tetrachlorocyclohexanediol via 2,3,4,5,6-pentachlorocyclohexanol. For details, see Lal et al. (2010).

transfer across species and insertion sequence-mediated transposition within species. For example, there is strong sequence similarity in the genes across both Gram-positive and Gram-negative species, and the gene organization amongst species is variable and bears no relationship to phylogeny (Singh et al. 2007; Manickam et al. 2008). Moreover, many *lin* genes within the various species are located on plasmids, and most are associated with the IS*6100* transposable element (Lal et al. 2010). As the *lin* pathway is most commonly found in a single genus, the Sphingomonads, it is possible that much of the downstream pathway and also some of the progenitors existed in sphingomonads for other reasons. Interestingly, *linA* has a very different codon usage from all the other genes of the pathway, the others being typical for sphingomonads, suggesting that *linA* at least originated elsewhere (Nagata et al. 1999; Dogra et al. 2004; Lal et al. 2010). There is also a high level of amino acid sequence variation between LinA and LinB variants from different species; at least some of this variation affects the isomer and enantiomer preferences of the encoded enzymes (Lal et al. 2010). Indeed, LinA and LinB between them can apparently degrade all of the >20 still-toxic compounds generated during the first three or four chloride removals from the various HCH isomers, which is a remarkable feat.

Insect enzymes

Only two classes of enzymes, the cytochromes P450 and GSTs, have so far been implicated in metabolic resistance to DDT in insects (Table 2). Cytochromes P450 typically catalyse an oxidation or hydroxylation that renders these highly hydrophobic insecticides more susceptible to further modification by increasing their hydrophilicity and capacity to cross lipid membranes. GSTs are then involved in conjugating the activated OC metabolites with large hydrophilic compounds such as glutathione or glucuronic acid, which can then be excreted (Josephy and Mannervik 2006). Most cases of DDT resistance appear to result from over-expression of these enzymes via gene amplification and/or up-regulation, by mutations in either *trans*-acting regulatory loci, or *cis*-acting elements within the promoters of P450 and GST genes (Feyereisen 2005; Li et al. 2007). It is assumed that the over-expression allows for more effective sequestration (in the case of the GSTs) or an enhancement of otherwise slow rates of oxidation/hydroxylation (in the case of the P450s).

One case of *cis*-inherited changes in a P450 gene leading to DDT resistance has been elucidated at a molecular level. In this case, involving the *CYP6G1* locus of *Drosophila melanogaster*, a series of adaptive steps was found to have occurred in rapid succession (Schmidt et al. 2010). A fragment of the *Accord* transposable element was originally inserted into the promoter region of the *CYP6G1* gene, causing an increase in transcription, and this was followed by a *CYP6G1* gene duplication and two additional transposable element insertions (a *P* and an *HMS-Beagle*), all of which increased DDT resistance.

Notwithstanding this instance of *cis*-inherited changes at an individual P450 locus, many studies have now reported the up-regulation of multiple P450 and GST genes in DDT-resistant insects (Feyereisen 2005; Oakeshott et al. 2005a). Whether or not all these genes are causally involved, the pattern suggests that the resistance mutation involves a master regulator gene with *trans*-acting effects on the expression of these general detoxifying genes. There is certainly the scope for many P450 and GST genes to be involved. Most insect genomes sequenced to date have more than 80 P450 and more than 20 cytosolyic GST genes, with notable exceptions in some specialized insects (honey bee, louse; Weinstock et al. 2006; Kirkness et al. 2010). Many of the P450 and GST genes are clustered within the genome (Oakeshott et al. 2010), and many are also known to be inducible by various chemical stresses (Plapp 1984; Maitra et al. 2000). Therefore, there is a huge repertoire of inducible P450 and GST genes within an insect species that could be associated with responses to chemical stresses such as insecticides (Feyereisen 2005).

However, the work of Chiu et al. (2008) on DDT resistance in the mosquito *Anopheles gambiae* shows that care should be taken in assuming that the increased expression of all the P450 and GST genes associated with resistance to these (and possibly other) insecticides reflects a causal connection. Previous microarray studies had identified five P450 loci (*CYP4C27*, *CYP4H15*, *CYP6Z1*, *CYP6Z2* and *CYP12F1*) that showed significantly higher expression in a DDT-resistant strain (David et al. 2005). Chiu and co-workers expressed both *CYP6Z1* and *CYP6Z2* in a heterologous expression system (Sf9 cells) and found that only CYP6Z1 was capable of metabolizing DDT. Thus, not all P450s that are up-regulated in resistant strains actually contribute causally to the resistance. Presumably, the others are up-regulated because they are involved in the same *trans*-acting regulatory networks.

While most cases of P450- and GST-based metabolic resistance to DDT characterized at the molecular level show enhanced expression of various P450/GST genes, there has been one case identified in which three amino acid substitutions in *CYP6A2* of a DDT-resistant strain of *D. melanogaster* resulted in an enzyme with up to 20 times higher DDT metabolism rates than the wild-type enzyme (Amichot et al. 2004). None of these mutations was located in the active site or near the substrate binding site, so the mechanism by which they confer an increase in activity is unclear. Interestingly, CYP6A2 was also over-expressed in this strain of *D. melanogaster*.

Insecticidal carbamate metabolizing enzymes

Like the OPs, the insecticidal carbamates are potent AChE inhibitors. However, unlike the phosphotriester chemistry, which is not synthesized naturally, the insecticidal carbamates are derived from carbamic acid, a chemistry used in a wide range of common cellular metabolites (carbamoyl phosphate and urea, for example). Again, like the OPs, carbamates are usually applied as chemically modified protoxins that are more readily activated in insects than mammals, such as the N-sulfonyl carbamates (Kinoshita and Fukuto 1980; Fahmy and Fukuto 1981).

The nitrogen of the carbamate structures is generally either a secondary (N-methyl) or a tertiary (N-dimethyl) amine, whilst the esterified group tends to be a bulky aromatic or an oxime group (Fig. 1). The mode of action for the carbamates is assumed to be analogous to that of the OPs (Casida 1963; Sogorb and Vilanova 2002). A carbamylated form of the AChE active-site serine is formed upon hydrolysis of the carbamate carboxyl ester, forming a relatively stable intermediate that completely inhibits its reaction with acetylcholine. In fact, this intermediate more closely resembles the covalent intermediate formed during the physiologically relevant reaction with acetylcholine than does the phosphorylated form produced during OP intoxication (Fig. 2). Because the carbamylated intermediate is planar, like the acylated form, the active-site serine can be regenerated by the 'normal' mechanism (i.e. an AChE activated water), albeit much more slowly than is the case when acetylcholine is the substrate, and the 'aging' reaction is thought to not occur (Fig. 2).

Bacterial enzymes

Although a large number of bacterial strains degrading insecticidal carbamates have been described in the literature, only three of the enzymes responsible for the first step in the degradation pathways have been identified and characterized: MCD, CehA and CahA (Derbyshire et al. 1987; Hayatsu et al. 2001; Hashimoto et al. 2002; Cheesman et al. 2007). Once the carbamate is hydrolysed by these enzymes, the reaction products are then catabolized by other cellular pathways as carbon sources. Both MCD from *Achromobacter* WM111 (Derbyshire et al. 1987) and CehA from *Rhizobium* sp AC100 (Hashimoto et al. 2002) are esterases capable of hydrolysing the carboxylester bond of 1-naphthyl acetate. Indeed, MCD hydrolyses 1-naphthyl acetate 30 times faster than it does carbaryl, with a k_{cat} value of 52 s^{-1} for 1-naphthyl acetate (Naqvi et al. 2009). MCD is a Mn^{2+}-dependent enzyme, as demonstrated by chelator and reconstitution studies (Karns and Tomasek 1991; Naqvi et al. 2009). The catalytic machinery of CehA remains a mystery, as it is inhibited

by neither serine hydrolase inhibitors nor chelators, and contains no recognizable catalytic motif (Bornscheuer 2002). CahA has no activity for 1-naphthyl actetate and is likely to be an amidase, based on the conservation of a catalytic serine/lysine dyad (Labahn et al. 2002) and the fact that it shares the same structural fold (the Pnc1p fold) as pyrazinamidase from *Mycobacterium tuberculosis* (Hu et al. 2007).

All three carbamate-degrading enzymes are able to degrade carbaryl, with k_{cat} values of approximately 2 and 0.2 s^{-1} for MCD and CahA (k_{cat} for CehA is not available), respectively, and K_m values for MCD, CehA and CahA in the range of 2.3–6.2 μM. MCD, CehA and CahA have all been described as possessing broad (although not identical) substrate ranges (Table 1). Interestingly, MCD has been demonstrated to have low (promiscuous) levels of PTE activity, with a k_{cat}/K_m for dimethyl umbelliferyl phosphate of 3×10^{-3}, 10^7 times lower than its k_{cat}/K_m for carbaryl (Naqvi et al. 2009). MCD shares the β-lactamase structural fold with the PTE MPH (see above), and it is possible that promiscuous PTE activity is common within this fold, because of the underlying catalytic mechanism.

Insect enzymes

Field resistance towards the carbamate chemistries were detected a mere 5 years after their introduction in the 1950s, whereas it often took a decade or more for many of the OPs (Brattsten 1990), suggesting that enzymes capable of degrading carbamates may have been present already, at least at low frequencies, in some insect populations. Despite the high levels of 'natural' and acquired carbamate resistance and insensitivity that are present in some insect populations, there are relatively few data concerning the molecular basis for resistance to carbamate insecticides in insects (Table 2). Nevertheless, there have been a few reports of over-expressed carboxylesterases and P450s contributing to carbamate resistance in various strains of *Heliothis virescens*, *Musca domestica* and *D. melanogaster* (Goh et al. 1995; Feyereisen 2005; Farnsworth et al. 2010) and synergist studies have implicated the involvement of an esterase-based mechanism in some *Culex* species (Tanga and Wood 1986; Bisseta et al. 1990). The lack of molecular detail concerning the mechanisms of carbamate resistance mechanisms clearly affords fertile ground for future research effort.

Synthetic pyrethroid-metabolizing enzymes

The SPs are neurotoxins that target the voltage-gated axonic sodium channels in a similar manner to DDT, causing uncontrolled and persistent excitation of the neurones (Soderlund and Bloomquist 1990). The chemistry for

this class of insecticide has been inspired by naturally occurring insecticides, the pyrthrums, produced by the genus *Chrysanthemum* (Silcox and Roberts 1994). So, like the carbamates, this chemistry has been present in nature for a considerable period of time.

Bacterial enzymes

To date, three pyrethroid-degrading enzymes of bacterial origin have been characterized: PytH from *Sphingobium* sp. strain JZ-1 (Wang et al. 2009), permethrinase from *Bacillus cereus* SM3 (Maloney et al. 1993) and EspA from *Klebsiella* ZD 112 (Wu et al. 2006). These enzymes are the first steps in catabolic pathways, and the hydrolysis products of these enzymes are likely further catabolized, as each of these organisms mineralize the SPs. The identities of these pathways and the subsequent enzymes in them have not yet been elucidated.

PytH has greatest homology with members of the α/β-hydrolase fold superfamily of proteins and is likely to be a serine hydrolase in that it is inhibited by diethylpyrocarbonate (DEPC) and phenylmethylsulfonyl fluoride (PMSF) and contains a conserved serine/aspartate/histidine motif that forms the catalytic triad of other serine hydrolases. Although the sequence of the *Bacillus* permethrinase is not known, inhibitor studies have revealed that it is likely to be a serine hydrolase as well. On the other hand, EspA has no close homologues of known function in the sequence databases, and the lack of conserved serine hydrolase motifs in its primary sequence suggests that it may operate by a different mechanism from that of PytH and the *Bacillus* permethrinase.

EspA and PytH are kinetically similar, with extremely low K_m values (in the range of 10 nM to 1 μM) for a wide range of pyrethroid substrates (Table 1). PytH has somewhat higher k_{cat} values than EspA, 0.4–3 s^{-1} for PytH compared with 0.01–1 s^{-1} for EspA, albeit both these values are low compared with the k_{cat} values for the OP-degrading enzymes. EspA has also been shown to be active against a broad range of ester-containing substrates (*p*-nitrophenyl esters with side chains of varying length), although with K_m values for these substrates typically in the order of 1000- to 10 000-fold greater than for the SPs. This observation may suggest that EspA may have evolved specifically towards a pyrethroid hydrolase activity, consistent with the inferred presence of a pathway for mineralization of SPs (Wu et al. 2006). The apparent steady state kinetics for the *Bacillus* permethrinase is unavailable but, like EspA and PytH, its rates appear to be consistently low across a range of substrates. However, unlike EspA and PytH, which appear to have no isomer specificity, permethrinase has a strong preference for the *trans*-isomer of permethrin (Maloney et al. 1993).

Insect enzymes

Carboxylesterases, P450s and GSTs have all been implicated in metabolic resistance to SPs in insects (Table 2). While increased intensities of specific zones of esterase isozymes (intense bands of carboxylesterase activity in nondenaturing polyacrylaminde gels) have been associated with resistance in various insect species, there is still no case where the resistance mechanism has been elucidated at the molecular level (Farnsworth et al. 2010). It is interesting that the *Lc*αE7 carboxylesterase from wild-type *L. cuprina* has relatively high SP hydrolytic activity (up to 90 000 M^{-1} s^{-1}; see Table 2) for some SPs. However, the blowfly is presumably susceptible, probably because the efficiency of the enzyme is much reduced for the more insecticidal isomers of the more recent type 2 SPs (Heidari et al. 2005; Devonshire et al. 2007).

Over-expression of CYP6D1 (at least in part because of loss-of-function mutations in repressor loci on another chromosome) has been implicated in SP resistance in the LPR strain of *M. domestica*, albeit other resistance genes are also present in this strain and their relative importance in the resistance phenotype is unknown (Wheelock and Scott 1992; Zhang and Scott 1996). There are point mutations in the LPR *CYP6D1* gene, and their contributions to the rate of SP metabolism by the encoded enzyme are unknown (Feyereisen 2005).

Microarray and RT-PCR studies found that several P450s were significantly over-expressed in field-caught, permethrin-resistant *Anopheles gambiae* mosquitoes (Muller et al. 2008). One of these enzymes, CYP6P3, was expressed in *E. coli* and shown to have activity for both permethrin and deltamethrin, although turnover numbers were modest, at less than one per minute. Again, a *kdr* target-site resistance allele was also segregating in this mosquito population, and the mechanism of P450 up-regulation is unknown.

Glutathione-*S*-transferase-mediated SP resistance is attributable to over-expression of GSTs, either as a result of gene amplification or up-regulation of *GST* genes, resulting in either sequestration of SPs and/or detoxification of lipid peroxidation products induced by SPs (Li et al. 2007; Table 2). However, the molecular detail for this mechanism is not yet understood.

Conclusions

Some learnings about evolutionary processes

Both target species of pest insects and incidentally exposed microorganisms have evolved enzymatic routes for the degradation of many insecticides. Both do so in response to selection, albeit very different forms of selection. For insects, it is a case of survival, and for bacteria

it is a matter of utilizing a new source of nutrients. Nevertheless, the two forms both translate to a need to generate new, or enhance existing, xenobiotic detoxification mechanisms, so that they can degrade the insecticides efficiently at generally low but physiologically significant concentrations.

Comparisons across the four classes of chemistry we have reviewed show that the bacteria have evolved far better catabolic systems than the insects, even though the insects would seem to have more incentive to do so. Considerably higher catalytic rates have been found for the primary detoxification enzymes in the bacteria, with k_{cat} values typically in the range of several to several thousand turnovers per second (Table 1), compared with one turnover per thousand seconds or slower for the insect enzymes (where kinetic data are available; Table 2). Additionally, the range of enzyme architectures and mechanisms employed by bacteria in degrading the four chemistries studied is much greater than those so far described for the insects, which essentially involve just three enzyme classes: cytochromes P450, GSTs and carboxyl/choline esterases. The bacterial insecticide-degrading enzymes also appear to have diverged much further from their nearest relatives than their insect counterparts, which generally only differ from their wild-type ancestors by a small number of mutational events. In many cases, these latter mutations simply act to increase expression of enzymes that are still relatively poor catalysts of the detoxification reaction required. The mutant carboxylesterases that have acquired OP hydrolase activity are a rare case where a substantively new enzymatic function has evolved to confer insecticide resistance in insects.

We suggest that the differences in the evolutionary outcomes for insects and bacteria stem from the differences in the biochemical and genetic systems under selection. Bacteria have a broader biochemical repertoire than insects from which new functions can develop, larger population sizes than insects, relatively high effective rates of mutation, very short generation times and, of course, greater access than insects to genetic diversity in other species via horizontal gene transfer.

The extensive biochemical repertoire of bacterial physiology is as varied as the environments in which they live; they can assimilate sulphur from a large variety of inorganic and organic sources, respire a wide variety of both inorganic (including sulphur and nitrogen compounds and metal oxides) and organic compounds (e.g. dimethyl sulfoxide, trimethylamine and organohalides), hydrolyse an enormous range of carbon/nitrogen, carbon/sulphur, carbon/halide and carbon/oxygen bonds, and produce a diverse array of secondary metabolites. By contrast, eukaryotes are conservative in regard to their primary metabolic pathways, using essentially the same pathways for energy transduction, anabolism and catabolism.

The size of insect populations can be enormous by eukaryotic standards, and their generation times are amongst the shortest for animals. It is unsurprising then that resistance mechanisms have emerged for most of the insecticide chemistries introduced over the last 50 years or so. However, as impressive as insect population sizes and generation times are in the context of other complex eukaryotes, they are considerably smaller and slower, respectively, than those of bacteria. Bacterial generation times can be in the order of 3 h^{-1}, and a 1-mL solution of laboratory-grown bacteria will often contain more than 10^9 individuals.

The 'normal' rate of mutation is very similar between eukaryotes and prokaryotes; it is estimated that both *D. melanogaster* and *E. coli* have mutation rates of $3–5 \times 10^{-10}$ per nucleotide per replication (Drake et al. 1998; Nishant et al. 2009). However, the effective rate of mutation for bacteria in the environment is likely to be considerably greater because of stress-induced adaptive evolution (induced modulation of the fidelity of DNA replication; Ponder et al. 2005; Foster 2007; Galhardo et al. 2007; Roth 2010) and the occurrence of mutator strains (strains with mutations that affect the fidelity of DNA replication; Tenaillon et al. 1999). There is no estimate for the prevalence of mutator genotypes in the environment; however, even at low frequency within a population, bacteria with these characteristics would contribute enormously to the generation of new functionality.

Bacterial species also have access to an immense 'inter-species gene pool' on which selection can act. Bacterial genomes are extremely plastic and are continually changed by gene duplication and loss and by lateral gene transfer (the acquisition of novel DNA segments from unrelated sources). Lateral gene transfer is common in bacteria and mediated by bacterial viruses (transduction), self-transmissible (mobilizable) plasmids or conjugative transposons (conjugation), and the direct uptake of DNA (transformation) (Achtman and Wagner 2008). Several of the bacterial enzymes described herein are located on plasmids or adjacent to transposable elements, and the data for the *lin* system strongly support a major and ongoing role for horizontal gene transfer in the assembly and dissemination of the pathway. In this respect, there are close parallels with the spread of antibiotic resistance since the 1960s; for example, *Staphylococcus aureus* has acquired 28- to 67-kb stretches of DNA encoding various antibiotic resistance genes on at least 20 separate occasions (Grundmann et al. 2006). Dagan and Martin (2007) studied >57 000 gene families distributed across 190 sequenced bacterial genomes and found that at least two-thirds (and probably all) have been affected by lateral

gene transfer at least at some time in their evolutionary past. In fact, there is such extensive genomic diversity even amongst strains of a single species that the concept of the 'bacterial species' is being called into question (Buckley and Roberts 2006). In a sense, all of the genomes in the bacterial biosphere are available to each species, and this number is huge.

By contrast, not only is the gene pool available to insects limited by their inability to access other species' genes via horizontal gene transfer, but their own gene pools are also significantly compromised by insecticide selection. The sheep blowfly example described previously shows how the selective sweeps accompanying the rapid rise in frequencies of resistance alleles can drastically reduce the amount of polymorphism in the chromosomal regions containing those resistance alleles. Furthermore, it is becoming apparent from a series of insect genome sequencing projects now that the P450, esterase and GST genes involved in xenobiotic resistance often co-occur in clusters of closely related, closely linked genes (Claudianos et al. 2006; Oakeshott et al. 2010). Thus, a selective sweep for a resistance allele in one gene involved in xenobiotic detoxification may well eliminate much of the polymorphism in other genes involved in xenobiotic detoxification as well. This in turn could mean that the insect is then less able to respond rapidly to selection for resistance for another insecticide class.

Much effort has been expended in understanding the mechanisms that underpin the evolution of new enzyme functions in both prokarya and eukarya. In the 1960s and 1970s, Ohno (1967) and Nei (1969) suggested that an early step in the process involved the duplication of an 'old' gene to generate a spare template on which to build the new function, leaving the other copy free to retain the original function. It is clear from the genomic data now available that gene duplication has indeed accompanied the evolution of proteins with new functions in both prokaryotes and eukaryotes. However, Ohno and Nei's model does not address the problem of how to assemble the multiple mutations still likely to be needed to produce an effective version of the new function/phenotype, nor the apparent lack of selective advantage associated with gene duplication *per se*. More recently, Tawfik and co-workers (Khersonsky et al. 2006; Amitai et al. 2007; Peisajovich and Tawfik 2007; Tokuriki and Tawfik 2009) have proposed that a rudimentary form of the new phenotype could be a pre-existing, previously neutral, 'side' activity of an enzyme with promiscuity of function. Enhancement of the rudimentary new phenotype would then occur because of the steady divergence of the new template as it acquires new mutations, suggesting that the process depends, at least for a while, on a pre-existing pool of previously neutral mutations. Because of the

relatively recent prevalence of insecticides, the new metabolic pathways of bacteria and the few examples found thus far of truly new biochemical functionality associated with insecticide resistance in insects provide unique opportunities to characterize this evolutionary process as it unfolds in real time.

Some practical implications

Arguably, the two greatest problems in the management of chemical insecticides are the evolution of resistance in the target pests and the off-target toxicities caused by residues of the pesticides that have dispersed away from their point of application. Obviously, the evolutionary responses we have described in insects have implications for the resistance problem. Not so obvious is that the evolutionary responses we have summarized from bacteria have implications for residue management.

The relevance of the bacterial responses to the residue problem lies in the potential to exploit the microbes, or the enzymes that they have developed, for the bioremediation of the pesticides (Alcalde et al. 2006; Whiteley and Lee 2006; Scott et al. 2008). Successful field trials have been reported for the use of *Sphingobium* species expressing the *lin* genes in the microbial bioremediation of HCH-contaminated soil (Raina et al. 2008). The OpdA enzyme is already being used commercially for free-enzyme bioremediation of OP residues in various contaminated liquids like spent animal and horticultural dip liquors (Sutherland et al. 2004; Scott et al. 2008). There are even the prospects of using the free-enzyme approach in the clean up of surface localized residues on commodities (Sutherland et al. 2004) and treating OP insecticide poisoning in humans (Bird et al. 2008; Gresham et al. 2010; Jackson et al. 2010). Given the facility with which microbes develop effective detoxifying enzymes for a range of pesticides, it is likely that enzymes will evolve that target most, if not all, insecticide chemistries. In some cases, these enzymes may fall short of the requirements for bioremediation (Scott et al. 2008), but there is considerable potential to evolve and engineer the enzymes further *in vitro* to make them fit for purpose (Ang et al. 2005; Jackson et al. 2009b; Bottcher and Bornscheuer 2010). There would therefore seem to be considerable opportunities to expand the scope of these approaches considerably, both across different types of pesticides and across different types of contamination problem.

The potential benefits in understanding the genetics and biochemistry of the mutant detoxifying enzymes that have evolved in insecticide-resistant insects lie both in improved management of existing chemistries and in the possibility of designing new or modified chemistries to which it may be more difficult for pests to evolve resistance. The

potential for improving insecticide management comes about because of the insights now possible through the use of 'next gen' genome sequencing technologies (Morozova and Marra 2008). With these technologies, it will very soon be possible to gain genome-wide scans of target species for the polymorphism available in all their e.g. cytochrome P450, esterase and GST genes that might potentially play a role in resistance to relevant chemistries. Such data will afford us with unprecedented predictive power in planning best use of available insecticide options. Functional genomics will also afford researchers the ability to seek new targets for insecticides with greater selectivity than ever (Ishaaya et al. 2007). There is also potential for designing new or modified chemistries through the use of the growing body of empirical and modelled structural data on the mutant enzymes (Bohm and Stahl 2000; Doucet-Personeni et al. 2001; Walter 2002; Baudry et al. 2003; Wang et al. 2008; Yan et al. 2009) recurrently associated with resistances to help in the design of new or modified chemistries most likely to be recalcitrant to resistance development by these mechanisms. More detailed consideration of biochemical, physiological and genomic factors relating to modern insecticide design is given in comprehensive reviews by Appelt et al. (1991), Walter (2002), Tietjen et al. (2005) and Ishaaya et al. (2007).

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