Molecular Biology and Evolution of Resistance to Toxicants

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To the prevailing biochemical/physiological classification of mechanisms of organismal resistance to toxicants, an additional molecular dimension is proposed. Predictions are developed regarding the relative prevalence of different classes of mutations and are found to compare favorably with reports from the literature. In particular, point mutations in target loci were the dominant form of resistance for both lab and field selection. Amplifications of target loci were less common than structural mutations, and more common for lab selected than for field-selected strains. Amplification was the most common mechanism of up-regulation of metabolizing enzymes. In comparison, only one mutation involving cis-regulation and several involving trans-acting regulation were found. Mutations involving gene disruption and down-regulation were uncommon, but were found in appropriate cases, i.e., when toxicants stimulated rather than inhibited target function and when metabolizing enzymes converted toxicants into more toxic metabolites. Additional phenomena of likely but uncertain importance are genetic “succession,” recombinational limitation, and negative cross-resistance. More work on these phenomena and on quantification of fitness costs of resistance is recommended.

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Introduction

The last 50 years have witnessed a massive transition in medicine and agriculture toward almost total dependence on toxic chemicals designed to control unwanted organisms. This arsenal of toxicants has been directed at numerous taxa in every kingdom: bacteria, protozoa, weeds, fungi, insects, and rodents. The use of such chemicals in everyday life is now widespread. Who has not heard of antibiotics, pesticides, or chemotherapeutic agents, and which large chemical company is not involved in one way or another in the production of such compounds? Evolution of resistance of target organisms to toxicants has been observed in every specialized field.

Key words: toxicant resistance, molecular biology, mutation, evolution, population genetics.

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and there is an extensive literature on antibiotic, drug, and pesticide resistance. Even viruses and cancer cells have become resistant to drugs used to control them. This literature addresses mostly detection, mechanisms, and operational problems of resistance. Only a small proportion of studies focus on resistance primarily as an evolutionary problem.

Reviews and books on resistance to one or another class of toxicants have rarely crossed disciplinary boundaries. The diagnosis of resistance, the characterization of resistant phenotypes, and the genetic and sometimes molecular identification of resistance is usually juxtaposed with some theoretical modeling of resistance evolution, with primary relevance to a goal of management. Rarely is progress in one field seen to influence the other. This disciplinary fragmentation of research on different taxa has led to parallel developments and idiosyncratic vocabularies. In this review we will refer to the diverse array of xenobiotics such as antibiotics, drugs, herbicides, insecticides, and acaricides collectively as toxicants. The population biologies of different organisms are another source of diversity. The spread of genes in populations of sexually reproducing organisms, such as DDT-resistant mosquitoes, obeys different rules than that in clonal organisms, such as AZT-resistant or protease-inhibitor resistant HIV virus. Our aim in this review is to attempt to integrate knowledge of resistance mutations in a variety of organisms and to a variety of toxicants so that common themes may emerge and contribute to a better understanding of resistance.

First, we propose a molecular classification of resistance that is meant to improve upon rather than replace the classical biochemical or physiological classifications of resistance phenotypes. Second, we discuss how the initial population frequency of resistance genes (i.e., prior to application of toxicant) is expected to relate to the molecular basis of resistance and review how the available literature matches these predictions. We conclude by offering some suggestions for future research.

Molecular Mechanisms of Resistance

There are many possible adaptations that permit an organism to survive lethal doses of a toxicant and can be classified as either mechanisms of decreased response to the toxicant or mechanisms of decreased exposure. These two major categories are also sometimes referred to as pharmacodynamically derived resistance and pharmacokinetically derived resistance. Under pharmacodynamically derived resistance are the various mechanisms of target insensitivity to toxicant, and under pharmacokinetically derived resistance are included behavioral avoidance, reduced uptake, increased detoxification, elimination, or sequestration. A third and less commonly discussed mechanism is circumvention, a mechanism by which the organism can bypass inhibited processes with alternate metabolic pathways. This classification of resistance mechanisms is also very useful from an operational point of view and it has been discussed in numerous reviews (e.g., for insecticides, Brattsten et al. 1986; for herbicides, Powles and Holtum 1994; for antibiotics, Davies 1994).

We propose to add a molecular dimension that classifies the types of mutations leading to resistance to the existing biochemical/physiological classification of resistance mechanisms. Mutations, whether in receptors, transporters, or enzymes, may be classified into those that alter binding/catalysis by structural changes, up-regulation (including gene amplification), or down-regulation (including gene disruption or silencing). Regulation can be altered either by cis- or trans-acting control of expression, by duplication or amplification, or by post-translational modification of, for example, the level of glycosylation, phosphorylation, or cellular localization. This molecular biological dimension in the classification of resistance mechanisms is best expressed as a two-dimensional table which we believe is an innovation in our understanding of the biology of resistance.

Little is known about posttranslational modification as a resistance mechanism. If such modifications have any heritable basis they are expected to appear as trans-acting regulatory factors. Similarly, the genetic control of avoidance behavior is not as well understood as biochemical mechanisms, although information is accumulating (Sparks et al. 1989; Riesgoescovar et al. 1992). Hence, for simplicity, these mechanisms have been omitted from our scheme.

Prior to selection, the size and composition of the pool of potential resistance mutants, comprising the set of loci and the set of alleles at each locus that could confer resistance, depends crucially on the mode of action, on chemical similarities of the toxicant to other compounds that the organism commonly contacts, and, of course, on the intensity of selection. In this review we develop a set of necessarily broad and speculative predictions about the initial size and composition of this pool of resistance mutants, arising from the integration of biochemical and molecular classifications (table 1). Although we will discuss this basic genetic component, it is clear that this approach also lends itself to a reappraisal of the operational factors involved (Georghiou and Taylor 1977; Mani and Wood 1984), and perhaps to the addition of an ecology/life history dimension to the evolution of resistance (Cook 1981; Roush and McKenzie 1987). Such further analysis is, however, beyond the scope of this review.

Patterns of Resistance Mutations: General Considerations

The first step in predicting the rate of evolution of toxicant resistance is to estimate the number and initial population frequencies of resistance-conferring mutations. Unlike most phenotypic traits that have been studied, resistance is accessible to thorough genetic analysis. This enhances the prospect of developing a predictive theory of initial gene frequency. Several generalizations are possible based on existing knowledge.

The mode of action of toxicants involves binding to a unique major target molecule, although some pes-
Table 1
Major Mechanisms of Resistance to Toxicants, and Predictions of Relative Commonness of Different Mutations

<table>
<thead>
<tr>
<th>CLASS OF MOLECULAR MUTATION</th>
<th>BIOCHEMICAL MECHANISM</th>
<th>Target Modification</th>
<th>Metabolism or Sequestration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Structural</td>
<td>C—Single mutations that reduce toxicant affinity can give large resistance effect. Fitness costs highly variable, however. U—If multiple targets exist.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Up-regulation</td>
<td>C—Compensates for loss of target molecules to poisoning. This is less effective if toxicant binds irreversibly.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amplification</td>
<td>C—Mutation rate high, and lowest fitness costs.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cis-acting</td>
<td>U—Many mutants, but high costs of de-regulating expression.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>trans-acting</td>
<td>U—Many mutants, but high costs of pleiotropic effects.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Down-regulation</td>
<td>U—Only in cases that toxicant enhances rather than inhibits target activity.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Disruption</td>
<td>R—Many mutants, very high cost of lost function.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cis-acting</td>
<td>U—Many mutants, but high costs of reduced function.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>trans-acting</td>
<td>U—Many mutants, but high costs of pleiotropic effects.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

NOTE: C = common, U = uncommon, R = rare.

Population frequency prior to toxicant selection is determined by the balance between mutation rate to resistance and natural selection against heterozygotes relative to the average wild genotype, under equilibrium conditions and assuming that frequency of resistant homozygotes and back-mutation rate are both negligible. Dominance and fitness of resistant alleles under natural selection is, therefore, critical. Dominant deleterious alleles will be much less common than recessive alleles. To the extent that dominance can be inferred from molecular biology, we may be able to make more precise predictions. Alleles with both minor fitness costs and large resistance effects are likely to be very few, perhaps nonexistent, or we would commonly find that resistance evolution was virtually immediate for a novel toxicant. This is essentially Fisher's explanation for the predominance of micromutational change in Darwinian evolution (Fisher 1958; Orr and Coyne 1992).

Under special circumstances (table 1), resistance could derive from disruptive mutations that silence, down-regulate, or perhaps de-repress loci. There is likely to be a very large pool of such deleterious mutations (though each alone may be rare), which may be favored only with high-dose selection. Clark, Wang, and Hulleberg (1995) reported a very high rate for pleiotropic trans-acting mutations that modify metabolism for Drosophila melanogaster.

High-dose selection was once advocated as a means of defeating resistance evolution. As dominance
of resistance typically declines with increasing doses all heterozygotes can theoretically be killed by a high enough dose. The existence of a critical dose above which a large reservoir of disruptive mutations may confer resistance is reason to expect failure for such a strategy. Such disruptive mutations would be observed to increase and decline rapidly with episodes of high-dose selection. We expect a type of genetic "succession" of such resistance mutations, as distinct from accumulation of mutations into a polygenic phenotype.

Population size, if small, may allow even deleterious resistance alleles to achieve locally high frequencies by neutral drift. This is an unlikely consideration for agricultural pests, however, which as pests typically have large population sizes and migration rates.

Polyploidy has a dampening effect on allelic dominance relations. Thus, we expect that a larger load of deleterious alleles can persist in populations of polyploid organisms. A consequence may be that the pool of suitable mutations can be larger and evolution more rapid following selection by toxicants. Polyploidy is more common among plants than among animals, and thus herbicide resistance may be expected to evolve more rapidly than pesticide resistance in animals, all else equal, with the exception of mutants in the chloroplast genome. This possibility has not been examined specifically in the literature, but rapid (3–4 years) evolution of resistance in weeds is not uncommon (Powles and Holtum 1994). For viruses, mutation rates are commonly much greater than for higher organisms, and resistance evolution is expected and found to be very rapid (e.g., Kellam et al. 1994).

Although empirical data are sparse on fitness and dominance of resistance alleles in the absence of toxicant selection, molecular biology may shed more light. For example, a mutation in a target site that reduces affinity to the toxicant may also reduce turnover efficiency for the natural ligand, and thus entail some fitness cost. Whereas over 10 mutations in the quinone binding site in photosystem II can confer herbicide resistance in various laboratory systems, only one is commonly found in weeds in the field (Comai and Stalker 1986). Single amino acids are often critical for normal function of a given enzyme, such as the active-site triad of esterases. The commonly large proportions of conserved nucleotide sites across taxa suggest that modification of the few key residues is deleterious. Hence, resistance deriving from such modifications is likely to entail fitness costs.

Site-directed mutagenesis promises to provide valuable information about the distribution of fitness effects of mutants. For example, only one of five mutants at different sites in various acetylcholinesterases retained normal function, and two were inactive (Soreq et al. 1992). Similarly, numerous induced mutations in β-lactamase genes on plasmids were found to confer cephalosporin resistance to bacteria, but only a small subset of these were found in clinical isolates (Palzkill and Botstein 1992).

We have mapped a set of predictions of initial frequencies of resistance genes onto our two-dimensional scheme (table 1). In each case, we attempt no more than a crude three-level prediction: "rare," "uncommon," and "common." Predictions will be explained further below.

Patterns of Resistance Mutations: General Methods and Problems

Collection Criteria

Studies appropriate to testing the predictions of table 1 would ideally have all the following components: (1) resistant individuals sampled from diverse populations both early and late in resistance evolution, (2) single genes contributing to resistance isolated in lines backcrossed to a single isogenic reference strain, (3) toxicological classification of mutations, (4) linkage analyses and tests of allelism, (5) field-cage competition experiments to estimate fitness costs of each mutant relative to "wild types," (6) identification of molecular mutations, and (7) complementation analyses to prove function. Such studies do not exist. Instead, many independent studies have been gathered from the literature to examine the hypotheses advanced.

Papers were included only if they identified recently evolved mutations at loci with a known functional relationship to the toxicant used in selection. Loci were excluded if, clearly, they had evolved over a long period and most likely were fixed for many different mutations as a result of a long history of selection. For example, monarch butterflies, which have low Na⁺,K⁺-ATPase sensitivity to host-plant cardiac glycosides, have a substitution in the ouabain-binding site that could account for insensitivity relative to other, sensitive species (Höfzinger, Frick, and Wink 1992). This is unlikely to have been a recent event, and is usually referred to as "tolerance" rather than "resistance." There was no recorded intraspecific variation in allele frequency for this mutation, and numerous other mutations and modifiers may also have accumulated. Hence, studies such as this were excluded.

Studies of purified target or metabolizing proteins have enabled identification of several mutant molecules. A few such cases have been included when loci with known functional relationships to toxicants were involved. Although the exact molecular mutations were unknown, papers were included if mutations could reasonably be inferred to be point mutations in structural loci.

Libraries of induced mutants were common in the literature. Only papers reporting mutations at loci of known function were included. For example, screening for nitrate-reductase deficiency as chlorate resistance in mutagenized plants uncovered five mutations, all recessive and all at different loci (Schoenmakers et al. 1991). Only one was in the nitrate-reductase enzyme and the rest were at unknown loci and so this study could not be included. In a contrasting case, three cell lines of corn were selected for resistance to imidizolinones and sulfonylureas. Toxicological studies suggested that the mutations were at herbicide-binding sites in the target mol-
Biases in Discovery of Mutations

The literature is probably biased toward reports of mutations that are more readily discovered. The single greatest bias that affects our survey is the pervasive focus on toxicant target genes. Identification of resistance mutations in target molecules is laborious, but the targets are usually known for well-established toxicant classes. Mutations in factors that regulate targets or metabolizing enzymes are typically less well known, and are often no more than map positions. Such mutations are expected to be underrepresented in the literature.

Amplification mutations are easily detectable by Southern hybridization, although the task of defining distinct amplicons is more difficult, leading to a probable underestimation of the number of distinct mutational events.

Exclusion of Plasmid Mutants in Bacteria

Horizontal plasmid and other gene transfer is so pervasive among bacteria that a species concept special to prokaryotes is necessary (Cohan 1994). Resistance often involves the coordinated action of several specific loci on a plasmid. Acquisition of plasmids by horizontal transfer, sometimes in a contagious manner, can provide resistance, even to many different antibiotics. The species in which mutations originated are usually unknown. Thus it is difficult to reconstruct the stepwise evolution of such plasmids (O'Brien et al. 1985; Davies 1994).

The prevalence of horizontal transfer and the difficulty of reconstructing mutation events necessitated the virtual exclusion of the large literature on bacterial antibiotic resistance, although these are very useful for any discussion of biochemical mechanisms. Only chromosomal resistance mutations were included. Recent reviews of bacterial drug resistance offer analyses of plasmid-based mechanisms (Davies 1994; Spratt 1994).

Patterns of Resistance Mutations: Predictions and Evidence

Structural Mutations in the Target: Predictions

This was predicted to be the most common molecular mechanism of target-site resistance. The very effect of a toxicant often derives from similarity to natural ligands of target molecules, or to transition states of substrates. Toxicants affect the target either by displacing normal substrate molecules or because the target itself is converted to an inactive or otherwise disrupted form. This distinction is important, as discussed below.

It is conceivable that for any target-toxicant system, there exists as little as a single residue change that could reduce the effect of the toxicant on the target, but without greatly affecting the target's interaction with natural ligands, whether substrates or allosteric effectors. Single residue changes in the target could completely abolish the toxicant’s effect in some systems.

Such mutants would enjoy large fitness benefits in the presence of the toxicant. However, we expect there to be very few amino acid changes, perhaps none, that could selectively reduce target–toxicant affinity and also leave unchanged normal catalysis or allosteric interactions. Consequently, if the target is a highly taxonomically conserved molecule, the same mutation should be found repeatedly favored by selection with the same toxicant in diverse taxa.

Competitive inhibitors are likely to interact with some of the same amino acids in the target as does the normal substrate, and the available pool of mutations would be correspondingly small. In such cases other types of resistance are more likely to appear first. A mutation with a comparatively large fitness cost may be favored if selection is intense enough to outweigh the cost.

Structural Mutations in the Target: Evidence

The prediction of commonness is amply borne out in the literature (tables 2 and 3). We expected a research discovery bias favoring studies of target genes, but within target genes discovery of structural mutations typically involves as extensive genetic analysis as does discovery of other types of target-modifying mutations.

There is a paucity of studies that quantify relative fitness costs for such mutations under realistic field conditions (Roush and Daly 1990), and yet this is critical for estimation of natural selection against mutants and, thus, their frequency in the absence of toxicant selection. The well-studied resistance mutations in the quinone-binding subunit of photosystem II of diverse auxotrophs provide the best set of evidence for any one system. Mutations at the four positions other than 264 and 251 did not entail reduced electron transport, but provided only weak atrazine resistance (table 3). The two mutations 264 Ser to Ala or Gly were the commonest: the former for diuron, the latter for atrazine resistance. Both mutations reduced electron transport, suggesting that this residue is the critical residue for function. For toxicants that attack an essential residue, resistance evolution must incur a fitness cost that only later modifier mutations may be able to reduce.

Table 2

<table>
<thead>
<tr>
<th>Class of Molecular Mutation</th>
<th>Biochemical Mechanism</th>
<th>Target Modification</th>
<th>Metabolism or Sequestration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Structural*</td>
<td></td>
<td>80</td>
<td>3</td>
</tr>
<tr>
<td>Up-regulation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amplification*</td>
<td></td>
<td>26</td>
<td>19</td>
</tr>
<tr>
<td>cis-acting</td>
<td></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>trans-acting</td>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Down-regulation*</td>
<td></td>
<td>0</td>
<td>12</td>
</tr>
<tr>
<td>Disruption</td>
<td></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>cis-acting</td>
<td></td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>trans-acting</td>
<td></td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Table 3.
* Table 4.
* Table 5.
### Table 3
Resistance Mutations in Structural Loci

<table>
<thead>
<tr>
<th>Biochemical Mechanism</th>
<th>Selection Regime</th>
<th>Gene</th>
<th>Number of Mutations</th>
<th>Taxa</th>
<th>Toxins to Which Resistant</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Target modification...</td>
<td>Lab 16sRNA</td>
<td>3</td>
<td>Angiosperm</td>
<td>Spectinomycin: substrate mimic</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Acetolactate synthase (nucleotide synthesis)</td>
<td></td>
<td>2</td>
<td>Angiosperm</td>
<td>Sulfonyleurea substrate competitor/imidazolones noncompetitive inhibitor</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Acetylcholine esterase (neurotransmitter catabolism)</td>
<td></td>
<td>1</td>
<td>Insect</td>
<td>Organophosphates: locks enzyme in covalent complex</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Aminopeptidase-N (Bt toxin receptor)</td>
<td></td>
<td>1</td>
<td>Insect: 4 spp.</td>
<td>Bt toxins: form midgut lesion when bound to receptor</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>H+ ATPase (ion transport)</td>
<td></td>
<td>2</td>
<td>Yeast</td>
<td>Vanadate: inhibits ATP hydrolysis</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Beta tubulin (microtubule assembly)</td>
<td></td>
<td>1</td>
<td>Yeast</td>
<td>Benomyl: inhibits microtubule assembly</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Cytochrome b (respiratory e-flow)</td>
<td></td>
<td>4</td>
<td>Yeast</td>
<td>Rhizoxin: inhibits microtubule assembly</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Dihydrofolate reductase-thymidylate synthase (nucleotide catabolism)</td>
<td></td>
<td>1</td>
<td>Cyanobacterium</td>
<td>Antimycin: inhibits cytb electron transfer</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>DNA topoisomerase (DNA synthesis)</td>
<td></td>
<td>1</td>
<td>Mammal (cell)</td>
<td>Pyrimethamine: competitive inhibitor</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>DNA polymerase (DNA synthesis)</td>
<td></td>
<td>2</td>
<td>Virus</td>
<td>Pyrimethamine: competitive inhibitor</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>DNA gyrase (DNA synthesis)</td>
<td></td>
<td>1</td>
<td>Bacteria</td>
<td>Amsacrine: transition state inhibitor</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>EPSP synthase (aromatic aa synthesis)</td>
<td></td>
<td>1</td>
<td>Bacteria</td>
<td>Glyphosate: substrate competitor</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>JH-binding protein (signal transduction)</td>
<td></td>
<td>1</td>
<td>Insect</td>
<td>Methoprene: juvenile hormone analog</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>Phytoene desaturase (carotenoid synthesis)</td>
<td></td>
<td>1</td>
<td>Cyanobacteria</td>
<td>Norflurazon: inhibitor</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>Photosystem II, subunit DI (photosynthetic electron flow)</td>
<td></td>
<td>5</td>
<td>Alga</td>
<td>Metribuzin, atrazine, diuron; plastquinone competitors</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
<td>Cyanobacteria</td>
<td>Metribuzin, atrazine, diuron; plastquinone competitors</td>
<td>17</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Atrazine</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Atrazine, diuron</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Atrazine, diuron, phenylureas</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>Sodium channel &quot;sch&quot; (nerve action)</td>
<td></td>
<td>1</td>
<td>Insect</td>
<td>DDT, pyrethroids: Na+ channel activators</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td>Acetylcholine esterase (neurotransmitter catabolism)</td>
<td></td>
<td>4</td>
<td>Insect</td>
<td>Organophosphates: locks enzyme in covalent complex</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>DHFR-TS</td>
<td></td>
<td>2 (deletion)</td>
<td>Protozoan</td>
<td>Pyrimethamine: competitive inhibitor</td>
<td>23</td>
<td></td>
</tr>
</tbody>
</table>
Up-regulated Target Molecules: Predictions

Up-regulation of targets compensates for the reduction of the pool of functional target molecules by toxicant poisoning. The level of resistance obtainable by overproduction of target is likely to be lower than can be obtained by a structural mutant with reduced inhibition by the toxicant. This is especially true when the toxicant has very low $K_i$ (concentration at 50% inhibition), or when it binds irreversibly to wild-type target.

Such mutants were predicted, therefore, to be more common in lab-selected than in field-selected strains. An exception is that resistance from target overexpression can achieve this result, they are likely also to carry a fitness bias. Such mutants would, however, disappear rapidly with the appearance of less deleterious alternatives such as structural mutations. As ephemeral adaptations we expect the bias against their discovery, relative to mutations with lesser fitness costs.

Mutations in cis-acting regulatory factors were also predicted to be uncommon, and more common earlier than late in evolution of resistance, as for trans-acting mutations. In eukaryotes most genes are tissue-specific, implying that expression is regulated by a balance of activation of silencer and enhancer elements by diverse regulatory molecules (Clark and Docherty 1993). Disruptive mutations in silencer elements could result in ectopic overexpression. While many mutations could achieve this result, they are likely also to carry a fitness cost from dominant expression in inappropriate tissues. The net mutation/selection balance for such a pool of mutants, while probably at least of the order of that for structural mutations, would make this class of mutations less fit.

Mutations in trans-acting regulatory factors were predicted to be uncommon. Such mutations are likely to affect more loci than just the target and as such entail substantial pleiotropic fitness costs. Pleiotropy is fairly common for trans-acting modifiers of metabolism in Drosophila, and the mutation rate for modifiers of any one metabolic locus can be quite high (Clark, Wang, and Hulleberg 1995). We propose that such mutants, with high rates of mutational generation but also with large fitness costs, should be moderately abundant early in resistance evolution, but disappear rapidly with the appearance of less deleterious alternatives such as structural mutations. As ephemeral adaptations we expect the bias against their discovery, relative to mutations with lesser fitness costs.
Table 4

Amplified Resistance Genes

<table>
<thead>
<tr>
<th>Biochemical Selection Mechanism</th>
<th>Selection Regime</th>
<th>Gene</th>
<th>Amplicons</th>
<th>Taxa</th>
<th>Toxictants to Which Resistant</th>
<th>Reference(s)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low intensity selection... Lab</td>
<td>Adenylate deaminase</td>
<td>2</td>
<td>Mammal (cell)</td>
<td>Coformycin: substrate competitive inhibitor</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Aldoketoreductase</td>
<td>1</td>
<td>Nematode</td>
<td>Methotrexate: inhibits DHFR (AKR also a target?)</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dihydrofolate reductase - thymidylate synthase</td>
<td>3</td>
<td>Protozoan</td>
<td>Methotrexate: pyrimethamine: inhibit DHFR and dTTP production</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Field</td>
<td>EPSP synthase</td>
<td>1</td>
<td>Angiosperm (cell)</td>
<td>Glyphosate: inhibits EPSPS</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Glutamine synthetase</td>
<td>2</td>
<td>Angiosperm</td>
<td>Glyphosate</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GST-AMPD'</td>
<td>5</td>
<td>Mammal (cell)</td>
<td>Glyphosate</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>P-glycoprotein (mdr)</td>
<td>1</td>
<td>Mammal (cell)</td>
<td>Coformycin: substrate competitive inhibitor</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ribonucleotide reductase (both subunits)</td>
<td>2</td>
<td>Mammal (cell)</td>
<td>Hydroxyurea: enzyme deactivator</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Metabolism, sequestration... Lab</td>
<td>GST-AMPD'</td>
<td>1</td>
<td>Mammal (cell)</td>
<td>Various anticancer drugs</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>P-glycoprotein (mdr)</td>
<td>5</td>
<td>Insect</td>
<td>Organophosphates: inhibit AChE</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>Protozoan</td>
<td>Melquin</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>Nematode</td>
<td>Hydrophobic drugs</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Esterase E4</td>
<td>3</td>
<td>Insect</td>
<td>Hydrolyzable insecticides</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Esterase B4 and 5</td>
<td>2</td>
<td>Insect</td>
<td>Organophosphates</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Metalloclothinine</td>
<td>1</td>
<td>Insect</td>
<td>Heavy metals</td>
<td>15</td>
<td></td>
</tr>
</tbody>
</table>


Amplification mutations were predicted to be common. At moderate levels, amplification is less likely to carry pleiotropic fitness costs relative to other mechanisms of up-regulation. Also, chromosomal mutation rates such as insertions/deletions, translocations, and duplications can range as high as 10^-2 per locus per generation, much higher than the base substitution rate (Schimke 1988). Selective overreplication, as distinct from elevated transcription, may be a relatively common method of up-regulation during normal development (Schimke et al. 1986).

Unequal exchange and stable chromosomal reincorporation is more common in plants, whereas overreplication of minute extrachromosomal amplisomes is more common in mammals. These amplisomes are less frequently reintegrated into chromosomes (Schimke 1988; Pauletti, Lai, and Attardi 1990). A mammalian cell transposon has been shown to favor the amplification of dihydrofolate reductase (DHFR) and hence resistance to the DHFR inhibitor methotrexate in cell cultures (McArthur and Stanners 1991). Mutagens which produce double-minute chromosomes and stimulation of the oncogene c-myc have both resulted in rapid evolution of methotrexate resistance through enhanced rate of amplification of DHFR (Hahn, Nevaldine, and Morgan 1990; Denis et al. 1991).

We expect a bias favoring discovery of such mutations relative to nonamplification mutations because DNA amplification is easier to detect, whereas discrimination among other regulatory mechanisms requires more laborious genetic analysis.

Up-Regulated Target Molecules: Evidence

The prediction that up-regulation of targets would be moderately common in lab-selected, but uncommon in field-selected organisms is supported by the literature (tables 2, 4, and 5). Amplification was predicted to be the most common means of target overexpression, and this was borne out clearly in the literature (tables 2 and 4). While cis mutants were predicted to be uncommon but not rare, no examples were evident from the literature. Trans-acting regulatory mutations were found to be uncommon as predicted (table 5).

Down-Regulated Target Molecules: Predictions

Underexpression of target molecules was predicted to be common, but only for toxicants that aggravate rather than inhibit the normal function of the target. For example, pyrethroid and DDT insecticides lock nerve
membrane sodium channels into an open state, causing chronic sodium leakage and unregulated nerve activity (Narahashi 1986). *Bacillus thuringiensis* toxicants bind to insect midgut N-aminopeptidase target molecules, forming a destructive transmembrane pore (Knight, Glickmole, and Eller 1994; Sangadala et al. 1994). In such cases resistance can result from down-regulation of expression, posttranslational inactivation, or even silencing of the target genes.

As the down-regulated gene is expected to have indispensable functions, such mutants are likely to entail fitness costs. The frequency of any one allele is therefore likely to be low, depending on dominance in the absence of selection. However, there may be a large pool of such disruptive alleles at low frequencies and any of which may achieve some form of down-regulation or gene-silencing leading to resistance. *Trans*-acting down-regulation was predicted to be more common than simple disruption, as pleiotropic fitness costs are less likely with finer reductions in control. Down-regulating mutants are likely to be recessive, and thus relatively common, but also unlikely to provide any resistance to heterozygotes, in the case of enzymes embedded in networks of metabolism (Kacser and Burns 1981; but see Savageau 1992).

### Down-Regulated Target Molecules: Evidence

Evidence was scanty and inconclusive. Resistance to pyrethroid insecticides in tobacco budworm (Taylor et al. 1993; Taylor, Shen, and Kreitman 1995), house fly (Williamson et al. 1993; Knipple et al. 1994), and cockroach (Dong and Scott 1994) is genetically linked to the gene that encodes the putative target site, a voltage-gated sodium channel. However, whether the mutation(s) alters structural binding properties or regulation is, as yet, unresolved (Taylor et al. 1993). Lab-selected mutants at a locus that down-regulates sodium channels indirectly have been found to confer weak resistance to pyrethroids, but the occurrence of such mutants under field conditions is unknown (Hall and Kasbekar 1989).

A structural point mutation in another sodium channel has been reported for DDT resistance in another laboratory-selected *Drosophila* strain (table 3; Amichot et al. 1992).

### Structural Mutations in Metabolizing Enzyme Loci: Predictions

We predicted that mutations which tailor the affinity and turnover efficiency of a detoxification enzyme to the toxicant would be much less common than up-regulated or amplified mutations. There is likely to be a cost of changing affinity to suit a new substrate, since affinity for normal substrates may be lost. On the other hand, the pool of possible resistance mutants may be larger, especially for larger families of genes devoted to a chemical modification that could also modify the toxicant (esterases, glutathione transferases, cytochromes P450, etc.).

Only one or a few base substitutions may be capable of substantially enhancing catalytic efficiency for a novel toxicant. We propose that mutants that can better degrade the toxicant should be rarer than up-regulated mutants and should appear as modifiers later rather than

---

**Table 5**

<table>
<thead>
<tr>
<th>Biochemical Mechanism</th>
<th>Selection Regime</th>
<th>Molecular Mechanism</th>
<th>Gene Regulated</th>
<th>Mutations</th>
<th>Taxa</th>
<th>Toxicants to Which Resistant</th>
<th>Reference(s)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Target modification...</td>
<td>Lab</td>
<td><em>trans</em>-down-regulated</td>
<td>Sodium channel (para)</td>
<td>1b</td>
<td>Insect</td>
<td>Pyrethroids, DDT; sodium channel agonists</td>
<td>1</td>
</tr>
<tr>
<td>Metabolism, sequestration...</td>
<td>Lab</td>
<td>Disruption</td>
<td>Nitrate reductase 2</td>
<td>1 deletion</td>
<td>Angiotensin</td>
<td>Chlorate: substrate competitive inhibitor</td>
<td>2</td>
</tr>
<tr>
<td>Field</td>
<td><em>trans</em>-up-regulated</td>
<td>Cytochrome P450 (CYP6A1)</td>
<td>1b</td>
<td>Insect</td>
<td>DDT; sodium channel agonist</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Clinical</td>
<td>Disruption</td>
<td>Thymidylate kinase</td>
<td>1 bp deletion</td>
<td>Virus</td>
<td>Acetylovin: pyrrole mimics terminates DNA polymerization</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Disruption</td>
<td>Peroxidase-catalase (katG)</td>
<td>10+ mis-sense mutations</td>
<td>Bacterium</td>
<td>Isoniazid: catalyzed by katG to cytotoxic form</td>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>cis</em>-down-regulated</td>
<td>Ferredoxin</td>
<td>2 substitutions</td>
<td>Bacterium</td>
<td>Methotrexate reduced by ferredoxin to cytotoxic form</td>
<td>6</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>


* Precise mutational change unknown.
earlier in the history of resistance evolution for a given species and toxicant. Just such a case appears to have occurred for a P-glycoprotein locus in a mammalian tumor cell line selected with various anticancer drugs (Choi et al. 1988; Safa et al. 1990). The first mutation detected was an amplification, on one descendant of which a subsequent point mutation appeared that enhanced efflux activity for certain drugs (Gonzalez and Nebert 1990). This sequence of duplication and divergence is a logical route for evolution of new functions and avoidance of pleiotropic fitness costs. This seems to be the principal route for the diversification of large gene families (Uyenoyama 1986) such as the cytochromes P450 (Gonzalez and Nebert 1990).

Structural Mutations in Metabolizing Enzyme Loci: Evidence

As predicted, structural mutations in enzyme loci that enhance the metabolism of toxicants were less common than were up-regulating mutations (tables 2–5). In one case, a point mutation appeared after the appearance of an amplification mutation, as discussed above. Biochemical mechanisms of resistance to toxicants very commonly involve increased metabolism, with little known of the molecular mechanisms. The extent to which structural mutations or amplifications contribute remains to be seen. We would predict an extension of the pattern observed, with a predominance of amplifications.

Up-Regulated Metabolizing Enzymes: Predictions

We predicted up-regulatory mutations to be most common for metabolizing enzyme loci. The panoply of metabolizing enzymes, classified by the type of modification they catalyze (oxidases, reductases, esterases, transferases, etc.) are highly diversified into large multigene families. Substrate specificities of such metabolizing enzymes can be broad and overlapping. Thus, there is likely to be an enzyme with some activity against a novel toxicant, depending on chemical similarity to substrates commonly encountered intracellularly or in the environment (diet, soil, host cell, etc.). If such loci are available, resistance may derive from increased constitutive expression of the enzyme.

If nontarget enzymes have strong affinity to the toxicant, by chance or by similarity to target, then it is possible that up-regulation could also confer a sequestration form of resistance, rather than metabolism. Sequestration of organophosphorus insecticides by esterases following gene amplification has been observed in mosquitoes (Karunaratne et al. 1993).

Amplifications are expected to be the most common mutation in this class, as mutation rate is high and pleiotropic costs low for moderate levels of amplification. Mutations cis to the controlled locus that disrupt repressor binding sites could also achieve up-regulation of detoxification enzymes.

The arguments advanced earlier for up-regulated target molecules for the relative rarity of trans- and cis-acting mutations are also pertinent here.

Up-Regulated Metabolizing Enzymes: Evidence

The main prediction was strongly supported. Up-regulation is found to be the most common molecular mechanism of metabolic resistance, and amplification the most common form of up-regulation (tables 2–5). There were two clear cases of trans-acting up-regulation of a glutathione transferase locus and a P450 locus in resistant insects (table 5), whereas no cis-acting mutations were found.

Down-Regulated Metabolizing Enzymes: Predictions

This was predicted to be uncommon and restricted to the specific case of toxicants that are metabolized in vivo to more toxic metabolites. In such a case, resistance may derive from down-regulation or silencing of the gene for the activating enzyme. This could appear quite rapidly as a disruptive mutation in the gene for that enzyme.

Down-Regulated Metabolizing Enzymes: Evidence

The cases available were all consistent with this prediction (table 5). Acyclovir, when kinased, mimics purines and blocks DNA polymerization. A disruptive point mutation in a Herpes virus' thymidylate kinase conferred acyclovir resistance (Palu et al. 1992). Resistance of clinical isolates of Mycobacterium tuberculosis derives from numerous missense mutations in the locus for a peroxidase catalase which converts the drug isoniazid to a more toxic form (Heym et al. 1995). Ferredoxin reduces metronidazole to its cytotoxic form in Trichomonas vaginalis. Resistant strains have 40% lower transcription rate for ferredoxin apparently as a result of point mutations in the upstream untranscribed region which are known to affect binding of a regulatory protein (Quon, Doliveira, and Johnson 1992). This case is the only cis-regulatory mutation we found.

There were also a number of cases for which molecular mechanisms were less certain. Methyl paraoxon is produced in vivo by desulfuration (oxidation) of the less toxic primary insecticide methyl parathion. Resistance from impaired oxidation has been reported, although it remains to be shown whether this is a case of gene disruption, or cis- or trans-down-regulation (Korona, Hodgson, and Rauteran 1989; table 5). A complemental mutant of unknown molecular basis enhances hydrolysis of the malaoxon metabolite. Angiosperm nitrate reductase is poisoned by chlorate, which is a nitrate analog, and resistance derives from gene silencing through a disruptive mutation (Wilkinson and Crawford 1991).

Circumvention

A biochemical mechanism of resistance that is not commonly reported or discussed in the literature may be termed "circumvention." Pathways blocked by action of a toxicant can be bypassed by alternate pathways if the latter are enhanced or originated by compensating mutations. We predicted limited opportunities for such mechanisms, and thus few, if any, reports. Nevertheless, one case was found which fits this category. Amplification of a short-chain dehydrogenase gene confers...
methotrexate resistance to nematodes, probably by providing an alternate folate reductase pathway (White et al. 1988; Papadopoulou, Roy, and Quellette 1992).

**A central issue is the paucity of information about fitness costs for particular classes of mutants. Our predictions were based on plausible conjectures. There are many studies estimating fitness costs and resistance ratios for specific mutations. However, most of these studies are for target modifications and are conducted under highly artificial laboratory conditions and so suffer from the universal problem of uncertain relevance to field conditions (Rough and Daly 1990). More studies of fitness costs under realistic field conditions are needed to test such conjectures as we and others have made.**

**Regulatory Mutations**

Temperature-sensitive mutants of *D. melanogaster* at *para*, an X-linked voltage-gated sodium channel locus, and *nap*, an X-transcription-regulating locus, are also mildly resistant to pyrethroid insecticides (Kasbekar and Hall 1988; Kernan et al. 1991). Mutations at unknown trans-acting regulatory loci enhance expression of a glutathione transferase conferring DDT-resistance in a lab-selected mosquito strain (Grant and Hammoek 1992), and a cytochrome P450 linked to resistance in houseflies (Carinho et al. 1994).

While these cases, despite their indefinite status, were included (tables 3), other interesting but less definite cases were not. Scheidel and Stollar (1991) found that several mutants for resistance to ribavirin in Sindbis virus mapped not to the target, RNA guanylyl-transferase, but to a nonstructural protein involved in mRNA capping. Overexpression of *mdr* mRNA in *Entamoeba histolytica* resistant to emetine was not related to DNA amplification (Samuelson et al. 1990). Carbapenem-resistant strains of *Bacteroides fragilis* from hospitals appear to involve a single regulatory mutant that activates a previously-silent but functional broad-spectrum β-lactamase (Podglajen et al. 1992).

The logistic difficulties of locating regulatory mutations means that this class of mutations is highly likely to have been underrepresented in our sample, as discussed above. Only further work focusing on this class of mutations will generate enough cases to resolve uncertainties about fitness costs and relative commonness of regulatory mutations.

**Recombinational Limitations**

While we have discussed the crude abundance of mutations of a particular class, we have ignored the accumulation of mutations within any one locus. Recombinational accumulation of distinct resistance mutations should result in increased resistance. In *Drosophila*, roughly additive increases in resistance to acetylcholinesterase inhibitors appear to have resulted from linkage of several point mutations in the AChE locus (Pralavorio et al. 1994; Mutero et al. 1994). Transfer and recombinational coupling among plasmids may be viewed as a means of rapid evolution of bacterial drug resistance (Davies 1994). When individual mutants are relatively common and loci are unlinked, recombinational assembly is free and offers no barrier to resistance evolution. For the evolution of resistance within a locus, recombinational assembly of a highly resistant haplotype for small loci may be more of a limitation than the mutation rate. Indeed, recombinational coupling of distinct point mutations already present in the population may be less likely than a second mutation, depending on population size, gene frequency, etc. Hence, small loci are expected to evolve more slowly than large loci if several distinct mutations can contribute additively to resistance.

**Genetic “Succession”**

We have conjectured that in cases where numerous disruptive mutations can confer resistance (whether through derepression, down-regulation, or gene-silencing), high-dose selection may overcome fitness costs associated with disruption and thus favor a large pool of normally deleterious mutations. However, fitness costs also select against such alleles in the absence of toxicant selection, moderated by recessivity and drift. Hence, deleterious alleles, while they may appear sooner than the few alleles with lower fitness costs, may later be replaced by them. It may be possible to discriminate distinct classes of “pioneer” and “settler” mutations. Deleterious alleles could remain at high frequency only with the appearance of modifier mutations that reduce fitness cost. Without the appearance of modifiers, we expect a succession of resistance genes in populations.
There are a few cases which appear to support this model. An azidovudine (AZT)-resistant mutation in HIV-1 reverse transcriptase (Arg 70 Lys) that always appears early in treatment for AIDS in humans is replaced later in the viral population by other mutations (Thr 215 Tyr/Phe) (Kellam et al. 1994). Colchicine selection on mammalian cell lines first favored a P-glycoprotein (mdr) amplification and later a point mutation that enhanced colchicine turnover efficiency through reduced binding affinity (Choi et al. 1988; Safa et al. 1990).

Less clear is the case of pyrethroid insecticide resistance for the moth *Heliocoverpa armigera*. The first mechanism to appear in Australian populations was a highly resistant nerve-insensitive form of unknown genetic basis. This seems to have been replaced by a metabolic mechanism of resistance in all populations (Gunning et al. 1991; Daly and Fisk 1992). A similar succession of mechanisms may be happening for *Heliothis virescens* populations in the USA (Ottea et al. 1995). However, whether this results from a successional process cannot be determined without more knowledge of the mutations underlying the traits. To test the model adequately for other organisms, observations must be made over time of the spectrum of resistance-conferring alleles at contributing loci, not a trivial task.

**Negative Cross-Resistance**

Negative cross-resistance or "collateral sensitivity" refers to the ability of some toxicants to act selectively against mutant organisms that are resistant to another toxicant. In the case of structural mutations affecting the target site, novel compounds may be better adapted to the the mutant target. The extent to which negative cross-resistance is common will determine the extent to which reversal of the evolution of resistance can be brought about. Only a very few cases are evident at this time. N-propylcarbamates are active against organophosphate-resistant acetylcholinesterases (Yamamoto, Takahashi, and Kyomura 1983), and N-alkylamides are more active against flies with *super-kdr* resistance (Elliott et al. 1986). The Ser764 to Gly mutation in the psbA gene confers atrazine resistance in weeds, but also increased sensitivity to the herbicides bentazon and pyridate (Gronwald 1994). Negative cross-resistance has also been observed for fungicides, for instance between benomyl and N-propylcarbamates (Davidse 1987). The molecular mechanism is unknown in this last case.

**Future Research**

It could be said that development of better theory for resistance evolution, based on more precise estimates of initial frequencies of resistance mutations, is at best a stop-gap measure for agriculture. Indeed, the environmental toll and ultimate futility of the pesticide treadmill has for some time given impetus to biological control and integrated pest management (IPM). Few such alternatives exist, however, for disease control. Ironically, much serious disease is bacterial and outside the scope of this review, as bacterial resistance to drugs is often plasmid-based and thus difficult to analyze from a population–genetic perspective. More analysis of fitness costs associated with different molecular classes of resistance mutations in field conditions is a clear priority, to allow better assessment of prior frequencies in populations. Further questions that we believe need more attention in future research are:

- Are regulatory mutations really as rare in resistance evolution as we expect them to be, and is there a predominance of trans- or cis-acting mutations?
- What are the fitness costs of specific mutations under field conditions, and are they consistent with the scheme we have proposed?
- Is recombinational limitation important in resistance evolution?
- Is genetic succession important in resistance evolution?
- How common is negative cross-resistance for specific mutations and to what extent can it be exploited?

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