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Current Topics

Rapid Evolution of Bacterial Catabolic Enzymes: A Case Study with Atrazine Chlorohydrolase†

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Evolutionary biologists compare organismal and geological changes that occurred on coincident time scales by calibrating molecular sequence information with the fossil record. The fossil record allows estimates of organismal divergence. Prokaryotes and eukaryotes separated on the order of 2.5 billion years ago (*1*, *2*); mammals diverged more than hundreds of millions of years ago (*3*), and modern humans originated within the past 100 000 years (*4*). A similar time scale must be covered in studies on molecular evolution. DNA or protein sequences are used to generate evolutionary trees that represent millions of years of divergence. This provides a context for the most effective use of the large-scale genome sequencing projects.

As of 2000, GenBank contained 11 billion nucleotides, and this body of information is still growing exponentially. Some protein families now consist of more than 1000 homologous sequences. Despite this large volume, homologous proteins compared during genome annotation are usually less than 80% identical in amino acid sequence and thus have been separated by millions of years of evolution. It would be insightful to observe a greater number of intermediate sequences of proteins that have not yet been characterized, particularly when the homologous proteins display different biological functions. Many of these intermediates will likely not be discovered due to the complexity of the biosphere. More than 10 000 prokaryotic species have been described to occur in a single gram of soil, with more than 99% of these undefined (*5*) and carrying approximately 2×10^{12} prokaryotic genes. In contrast, on the order of 10^4 proteins have been described. Clearly, this is only a small fraction of the total proteins that exist on earth. In addition, it is not known to what extent the known proteins represent the range of biological functions existing in the global protein pool. The continual discovery of novel catabolic reactions provides evidence that many enzyme reaction types remain to be discovered (*6*, *7*).

This review discusses examples in which it is possible to sift through the complexity of the biosphere to find related enzymes which display distinct functions. The clearest example to date is atrazine chlorohydrolase, an enzyme which is shown to have evolved for the function of catabolizing atrazine. More than 2 billion pounds of the herbicide atrazine have been applied to soils globally, and this has provided selective pressure for the evolution of new metabolism. The amino acid sequence of atrazine chlorohydrolase is shown to be 98% identical with that of melamine deaminase, an enzyme that catalyzes deamination reactions. The chlorohydrolase is shown to be firmly linked with a major amidohydrolase protein superfamily.

Protein Superfamily and Enzyme Catalytic Diversity

With sequence divergence, enzymes can acquire distinctly new biological functions. A new function is defined here as the catalysis of a different reaction that results from changes

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Table 1: Enzymes That Catalyze Newly Discovered Reactions with Industrial Chemicals as Substrates

enzyme	UM-BBD enzyme ID[a]	Pfam designation[b]	family or homologue (% identity)
tetrachloroethene reductive dehalogenase	e0271	PF00037: 4Fe−4S	hypothetical proteins (20−28%)
cyanamide hydrolase	e0426	none	hypothetical proteins (17−33%)
pentaerythritol tetranitrate reductase	e0035	PF00724: NADH:flavin oxidoreductase/NADH oxidase	87.7% *N*-ethylmaleimide reductase (P77258) (30−50%) reductases
phosphotriesterase	e0054	PF02126: phosphotriesterase family	amidohydrolase superfamily (19−70%)
tetrachlorohydroquinone reductive dehalogenase	e0251	PF00043: glutathione *S*-transferase	glutathione *S*-transferase (19−30%)
styrene epoxide isomerase	e0196	none	40.5% *p*-cymene methyl hydrolase (O84920)
benzylsuccinate synthase	e0259	PF00037: 4Fe−4S; PF02143: radical activating protein	glycyl radical protein (30−50%)

[a] University of Minnesota Biocatalysis and Biodegradation Database enzyme number. [b] Pfam (Protein family database of alignments).

in the amino acid sequence of the enzyme. These changes in function often allow recruitment of the new enzyme to perform a different metabolic role. Clustering proteins that have diverged in function but retained identifiable sequence and structural identity are the basis of superfamilies. Because of the common evolutionary history of its members, analysis of superfamilies has been very informative for functional and structural studies. One example of a superfamily of enzymes that has a great deal of functional diversity is the enolase superfamily, which consists of a conserved TIM barrel structure and contains homologous enzymes that catalyze hydration, isomerization, dehalogenation, decarboxylation, hydrolysis, ring closure, and a retro-Claison reaction (*8*, *9*). The enolase superfamily is broadly represented in proteins identified in three kingdoms of life during genome sequence annotation. These proteins may hold additional activities not yet attributed to the superfamily. For example, genome annotation predicts the presence of 24 probable enoyl-CoA hydratases/isomerases in *Mycobacterium tuberculosis*, but it seems unlikely that all of the enzymes actually carry out this single reaction (*8*).

Another well-characterized superfamily is the crotonase superfamily. Bacterial enoyl-CoA hydratase (crotonase) and 4-chlorobenzoyl-CoA dehalogenase are members of this superfamily and are 56% identical in amino acid sequence pairwise comparison. Although neither enzyme will catalyze the other's reaction, comparing the X-ray crystal structure for these two enzymes (*10*, *11*) revealed conserved and different amino acids that were important in each respective reaction pathway. Subsequently, site-directed mutagenesis was used to transform 4-chlorobenzoyl-CoA dehalogenase into a catalyst with crotonase activity via the addition of two mechanistically essential glutamate residues normally present in crotonase enzymes (*12*).

Diversity in the Environment

The greatest enzyme diversity may reside in soil bacteria. In the soil, relatively low levels of environmental nutrients make for strong selective pressure, favoring bacteria that can rapidly adapt by oxidizing alternative nutrient sources. *Pseudomonas* species have been described which are capable of utilizing hundreds of different compounds as their source of carbon and energy (*13*). On the order of 100 000 distinct, natural product, organic structures are proposed to exist in a given sediment (*14*), and more than 10 million organic compounds have been synthesized by organic chemists (*15*). With the large number of new synthetic chemicals that are being introduced into the environment, the evolution of novel catabolic activities has been suggested to have occurred recently, over a period of decades. Table 1 lists newly discovered reactions involved in the transformation of synthetic compounds. For example, the dry cleaning solvent, tetrachloroethene, was once considered to be nonmetabolizable, but is now known to be a substrate for tetrachloroethene reductive dehalogenase and serves as the final electron acceptor in microbial metabolism occurring in contaminated anaerobic sediments (*16*, *17*).

Tetrachloroethene reductive dehalogenase is a 4Fe−4S protein that also contains cobalamin for catalyzing the reductive cleavage of carbon−chlorine bonds within chloroethenes. A search for homologous proteins in the databases yielded only hypothetical proteins identified in sequencing projects. Thus, there is currently little information about the evolutionary origins of tetrachoroethene reductive dehalogenase. More information exists on the origins of the tetrachlorohydroquinone reductive dehalogenase (TCHQ).[1] On the basis of sequence analysis, this enzyme, from *Sphingomonas chlorophenolica* ATCC 39723 (formerly *Flavobacterium*), was found to be a member of the glutathione *S*-transferase superfamily (*18*). More recently, the enzyme was shown to possess significant maleylacetone isomerase activity; the k_{cat} was 0.8 s^{-1} (*19*). Yet TCHQ functions, in concert with pentachlorophenol monooxygenase (PcpB) and dichlorohydroquinone dioxygenase (PcpA), to provide a metabolic route for funneling the wood preservative pentachlorophenol into TCA cycle intermediates. The observed isomerase activity might be vestigial, or it may represent a dual function for TCHQ.

Atrazine Catabolism and the Amidohydrolase Superfamily

The chlorinated herbicide atrazine was once considered to be poorly biodegraded in soils. The major metabolites detected in soils and groundwaters suggested that the herbicide underwent nonspecific oxidative dealkylation reactions (Figure 1). A cytochrome P450 monooxygenase from *Rhodococcus* strains TE1, N186/221, and B30 was subsequently discovered to catalyze this reaction (*20*−*24*). The bacterial cytochrome P450 was shown to degrade other

[1] Abbreviations: AtzA, atrazine chlorohydrolase; AtzB, hydroxyatrazine ethylaminohydrolase; AtzC, *N*-isopropylammelide *N*-isopropylaminohydrolase; TriA, melamine deaminase; TrzA, *s*-triazine hydrolase; TCHQ, tetrachlorohydroquinone reductive dehalogenase; PcpA, dichlorohydroquinone dioxygenase; PcpB, pentachlorophenol monooxygenase; TCA cycle, citric acid cycle.

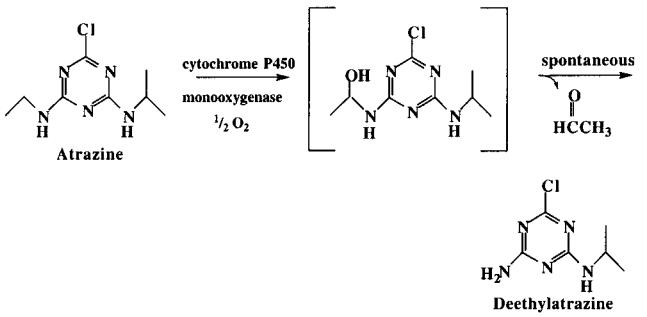

FIGURE 1: Atrazine catabolism via nonspecific monooxygenation.

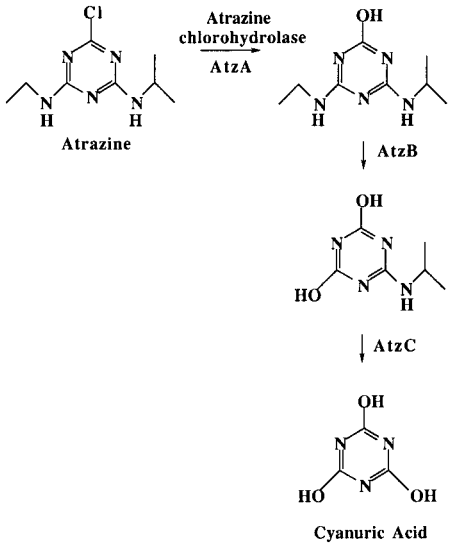

FIGURE 2: Atrazine catabolic pathway from *Pseudomonas* sp. ADP.

herbicides structurally unrelated to atrazine and is likely functioning as a nonspecific oxygenative catalyst rather than an enzyme that has evolved specifically to catabolize atrazine. Starting in 1993, however, numerous bacteria were ascertained to initiate atrazine metabolism via a hydrolytic dechlorination reaction (Figure 2). More recently, the genes encoding the chlorohydrolase have been shown to be essentially identical in different genera of bacteria independently isolated from four continents by different researchers (*25*). This suggests that the ability to dechlorinate atrazine arose since the introduction of atrazine and that this phenotype spread quickly around the globe.

The enzymes responsible for the first three steps of the atrazine dehalogenation pathway were initially identified in *Pseudomonas* sp. strain ADP (Figure 2). The enzymes that catalyze these steps are atrazine chlorohydrolase (AtzA, EC 3.8.1.8), hydroxyatrazine ethylaminohydrolase (AtzB, EC 3.5.99.3), and *N*-isopropylammelide *N*-isopropylaminohydrolase (AtzC, EC 3.5.99.4), respectively. Sequence comparisons revealed that all three enzymes belong to the amidohydrolase superfamily (*26*). Amidohydrolase superfamily members for which structures are defined have an $(\alpha\beta)_8$ barrel structure (*27*, *28*). Moreover, they share conserved features of the reaction mechanism in which one or two divalent metals are coordinated by the enzyme and serve to activate water for nucleophilic attack on the respective substrate. The amino acids serving as metal ligands are maintained across the superfamily. The majority of reactions catalyzed by the superfamily involve the hydrolytic removal of amino groups from purine and pyrimidine rings,

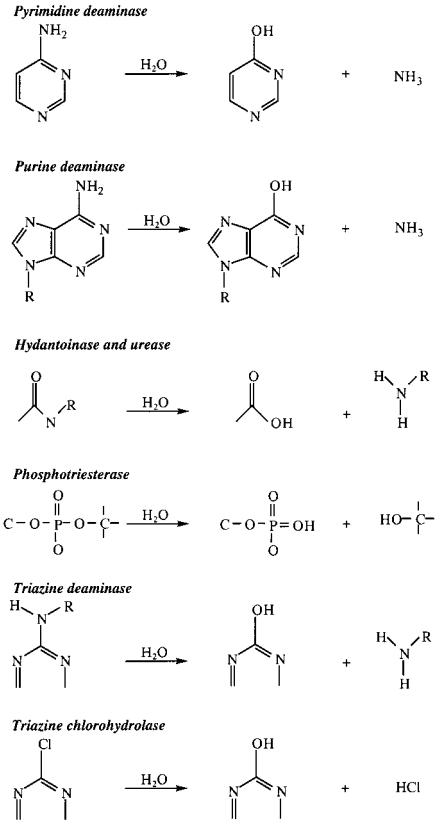

FIGURE 3: Diversity of substrates and catalytic activities of amidohydrolase superfamily members.

or amide bond hydrolysis reactions (Figure 3). The former reactions are represented by enzymes such as adenosine deaminase. The latter are illustrated by urease and cyclic amidases such as hydantoinase.

Recent studies have expanded the range of reactions that are known to be catalyzed by amidohydrolase superfamily members (Figure 3). Some of the existing enzymes catabolize synthetic organic compounds (Table 1). Phosphotriesterase, for instance, catalyzes the cleavage of a phosphorus−oxygen bond of the pesticide parathion (*29*). It has been speculated that the true substrate for phosphotriesterase from *Pseudomonas dismuta* is yet to be discovered. But it is also plausible that the enzyme has evolved under selective pressure to hydrolyze phosphotriester insecticides since their introduction some decades ago.

The *s*-triazine ring compounds on which atrazine chlorohydrolase acts closely resemble the pyrimidine ring compounds found in intermediary metabolism. In fact, amidohydrolase family members are important throughout biological systems for catalyzing pyrimidine and purine deamination. In adenosine deaminase, the enzyme-coordinated zinc atom is implicated in activating water for nucleophilic attack on the substrate (*30*). Moreover, the enzyme is proposed to protonate a nitrogen adjacent to the ring carbon undergoing nucleophilic substitution. These represent plausible mechanistic features that could translate to atrazine dechlorination. The *s*-triazine ring is even more activated than the pyrimidine ring system toward nucleophilic aromatic substitution (*31*). Moreover, chloride is an excellent leaving group, and it is known that calf intestinal mucosa adenosine deaminase catalyzes dehalogenation of chloropurines (*32*, *33*).

Atrazine Chlorohydrolase

Atrazine chlorohydrolase from *Pseudomonas* sp. ADP has been purified to homogeneity. The monomeric unit is predicted, on the basis of the DNA sequence, to have a molecular weight of 52 421, and the holoenyzme is estimated to have a molecular weight of 240 000, based on gel filtration chromatography (*34*). Thus, the subunit stoichiometry is likely either α_4 or α_5 under the chromatography conditions. Initially, metal dependence was not reported. Since that time, divalent metal salts of iron, manganese, and cobalt have been shown to stimulate enzyme activity. Metal chelators greatly depress activity, and the activity of the inactive apoenzyme can subsequently be restored by the same metals that stimulate activity (H. McTavish and J. L. Seffernick, unpublished observations). The overall reaction proceeds via a hydrolytic mechanism as demonstrated in experiments using $H_2{}^{18}O$ (*34*). Other hydrolytic dehalogenases are known; however, none belong to the amidohydrolase superfamily or are proposed to require a metal for activity. In fact, the structures of haloalkane dehalogenase (*35*, *36*), 4-chlorobenzoyl-CoA dehalogenase (*10*), and L-2-haloacid dehalogenase (*37*) have been determined, and the initial nucleophile which displaces the halide is an enzyme carboxylate in all of these examples. An enzyme catalyzing dechlorination of chlorohydroquinol was initially described as a metal-containing hydrolase (*38*), but this enzyme is now known to be an oxygenase (*39*, *40*). At this time, atrazine chlorohydrolase is a unique metal-dependent halohydrolase.

It was considered that atrazine chlorohydrolase might catalyze one or more reactions unrelated to herbicide dechlorination. This was plausible given the evolutionary history of the protein family to which it belongs and the fact that chloride displacement from an *s*-triazine ring is a fairly facile reaction (*31*). In this context, the substrate specificity of atrazine chlorohydrolase was investigated. Only chorine and fluorine leaving group substituents underwent hydrolytic cleavage. An analogue of atrazine that contains an amino group in place of the chlorine substituent was shown not to undergo hydrolysis (*41*). Furthermore, pseudohalides such as cyano, azido, and methoxy groups were not hydrolytically removed by atrazine chlorohydrolase. Additionally, atrazine chlorohydrolase is very specific for *s*-triazine ring substrates. It is less restrictive with respect to the *N*-alkyl substituents. For chloride hydrolysis to occur, at least one of the two nitrogen side chain atoms must contain an alkyl group, but the allowable steric bulk can vary from a methyl to *tert*-butyl group. Overall, the enzyme was specific for the triazine ring and the chloride leaving group, consistent with the idea that it evolved under selective pressure to catalyze dechlorination of *s*-triazine herbicides, such as atrazine and simazine.

Other data support the view that the *Pseudomonas* AtzA evolved under selective pressure and was maintained in soil microbial populations to metabolize *s*-triazine herbicides. The *atzA* gene was not found in randomly chosen laboratory strains but was detected in most bacteria recently isolated for their ability to metabolize atrazine (*25*). It is present with other genes, *atzB* and *atzC*, which encode enzymes that metabolize the AtzA reaction product in *Pseudomonas* sp. ADP *Ralstonia*, *Alcaligenes*, and *Agrobacterium* strains (Figure 2) (*25*). The atrazine catabolism genes are found on large catabolic plasmids in those same strains (*42*).

Melamine Deaminase and s-Triazine Hydrolase

Perhaps the best evidence that *atzA* is a recently evolved gene derives from its relationship with genes identified for the catabolism of melamine, or 2,4,6-triamino-1,3,5-triaizine. Melamine is an industrial product used since the early 1900s. Melamine was considered nonbiodegradable in the 1930s but was then reclassified as slightly biodegradable in the 1960s when atrazine was first introduced (*43*). Today, it is considered to be readily biodegradable in soil. Among the bacteria that metabolize melamine is *Acidovorax avenae citrulli* 12227 (formerly *Pseudomonas* sp. strain NRRL B-12227) (*44*). The first two metabolic reactions are sequential hydrolytic deamination reactions catalyzed by the same enzyme, melamine deaminase (TriA). The *triA* gene has recently been cloned and sequenced. The protein shows a remarkable identity to atrazine chlorohydrolase from *Pseudomonas* sp. ADP; *it is the same in 466 of 475 amino acids* (Figure 4) (*45*). It is also unusual that the nine nucleotide differences between *triA* and *atzA* give rise to these nine amino acid changes. The small number of changes and the absence of silent mutations are consistent with an intense selective pressure operating over a short evolutionary time period (*46*, *47*). The k_{cat}/K_m of atrazine chlorohydrolase with atrazine is 1.5×10^4 s^{-1} M^{-1} per subunit. In our most recent study, the deamination activity of this enzyme was found to be undetectable (*48*). Melamine deaminase, however, exhibits the opposite specificity. It catalyzes deamination reactions at rates comparable to dechlorination rates of atrazine chlorohydrolase. Moreover, it shows dechorination activity 2 orders of magnitude lower than the deamination activity with comparable triazine substrates. In total, these data suggest that the nine amino acid changes represent a short evolutionary trajectory between the two activities.

The sequence of a related amidohydrolase superfamily member, *s*-triazine hydrolase or TrzA (*49*), is 41 and 42% identical with the sequences of atrazine chlorohydrolase and melamine deaminase, respectively. It catalyzes both deamination and dechlorination reactions. TrzA catalyzes the deamination of nonalkylated triazines such as melamine and the dechlorination of mono-*N*-alkylated triazines. The k_{cat} for deamination of melamine is 243 s^{-1}, while that for the dechlorination of desisopropylatrazine is 2.2 s^{-1}. This is an approximately 100 times greater preference for demination over dechlorination and is consistent with the enzyme acting physiologically as a deaminase with a fortuitous dechlorination activity. This is not surprising given that chloride displacement is more facile, and adenosine deaminase is known to catalyze fortuitous halopurine dehalogenation. That TriA and AtzA discriminate between chloro and amino substrates so well despite their sequences being 98% identical is remarkable.

DNA Shuffling

It is possible that fewer than nine amino acid changes are required to interconvert melamine deaminase and atrazine chlorohydrolase activities. There are 510 possible site-directed mutants bridging the two, a large set to generate, sequence, purify, and assay. In this context, DNA shuffling

```
TriA   1 MQTLSIQHGTLVTMDQYRRVLGDSWVHVQDGRIVALGVHAESVPPPADRV 50
AtzA   1 MQTLSIQHGTLVTMDQYRRVLGDSWVHVQDGRIVALGVHAESVPPPADRV 50

                                            84       92

51 IDARGKVVLPGFINAHTHVNQILLRGGPSHGRQLYDWLFNVLYPGQKAMR 100
51 IDARGKVVLPGFINAHTHVNQILLRGGPSHGRQFYDWLFNVVYPGQKAMR 100

                                125

101 PEDVAVAVRLYCAEAVRSGITTINDNADSAIYPGNIEAAMAVYGEVGVRV 150
101 PEDVAVAVRLYCAEAVRSGITTINENADSAIYPGNIEAAMAVYGEVGVRV 150

151 VYARMFFDRMDGRIQGYVDALKARSPQVELCSIMEETAVAKDRITALSDQ 200
151 VYARMFFDRMDGRIQGYVDALKARSPQVELCSIMEETAVAKDRITALSDQ 200

                    217 219

201 YHGTAGGRISVWPAPAITPAVTVEGMRWAQAFARDRAVMWTLHMAESDHD 250
201 YHGTAGGRISVWPAPATTTAVTVEGMRWAQAFARDRAVMWTLHMAESDHD 250

     253 255

251 ERLHWMSPAEYMECYGLLDERLQVAHCVYFDRKDVRLLHRHNVKVASQVV 300
251 ERIHGMSPAEYMECYGLLDERLQVAHCVYFDRKDVRLLHRHNVKVASQVV 300

                                328 331

301 SNAYLGSGVAPVPEMVERGMAVGIGTDDGNCNDSVNMIGDMKFMAHIHRA 350
301 SNAYLGSGVAPVPEMVERGMAVGIGTDNGNSNDSVNMIGDMKFMAHIHRA 350

351 VHRDADVLTPEKILEMATIDGARSLGMDHEIGSIETGKRADLILLDLRHP 400
351 VHRDADVLTPEKILEMATIDGARSLGMDHEIGSIETGKRADLILLDLRHP 400

401 QTTPHHHLAATIVFQAYGNEVDTVLIDGNVVMENRRLSFLPPERELAFLE 450
401 QTTPHHHLAATIVFQAYGNEVDTVLIDGNVVMENRRLSFLPPERELAFLE 450

451 EAQSRATAILQRANMVANPAWRSL* 475
451 EAQSRATAILQRANMVANPAWRSL* 475
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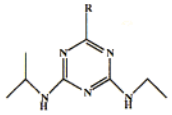
FIGURE 4: Comparative sequence alignment of atrazine chlorohydrolase and melamine deaminase. Nine differences are highlighted in color: blue for TriA and red for AtzA residues.

Table 2: Relative Activity and Amino Acid Sequence of the Nine Variant Positions in Shuffled Mutants with Maximal Activity for the Designated Substrate[a]

 R =	Activity Relative to TriA[b]	Activity Relative to AtzA[c]	Amino Acids at Each of Nine Variant Positions[e]								
			84	92	125	217	219	253	255	328	331
Cl	270	1.4	F	V	E	T	T	L	G	N	S
NH_2	3.6	[d]	L	L	D	T	T	L	W	D	S
OCH_3	80	[d]	F	L	E	T	T	I	G	D	C
$NHCH_3$	20	[d]	L	L	E	T	T	L	W	D	C
SCH_3	80	[d]	L	L	E	T	T	L	W	D	S

[a] Clones with additional PCR-derived mutations were not used for this analysis. [b] Relative is defined as a value of 1.0 being equivalent to that of wild-type TriA. [c] Relative is defined as a value of 1.0 being equivalent to that of wild-type AtzA. [d] No detectable hydrolysis by AtzA. [e] Residues in wild-type TriA are blue and wild-type AtzA are red.

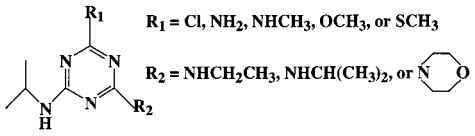


FIGURE 5: Substrate library used for screening a DNA shuffled library generated from the initial protein parents atrazine chlorohydrolase and melamine deaminase.

was conducted and the clonal variants were screened against a chemical library of substrates using high-throughput mass spectrometry (*48*). The chemical library of 15 substrates varied the leaving group and the side chains (Figure 5). Mutant enzymes were obtained that varied with respect to their activities against the different substrates. The sequences of daughter enzymes exhibiting the greatest activity for hydrolysis of atrazine analogues are displayed in Table 2. The activities of the shuffled clones were normalized to the activity of each parental enzyme. The clone with the best dechlorination activity was 1.4 times as fast as atrazine chlorohydrolase, and the clone with the best deamination activity was 3.6 times better than melamine deaminase. The small increases observed in activity upon shuffling suggest

that atrazine chlorohydrolase and melamine deaminase have among the most optimal sequences for dechlorination and deamination activities, respectively.

It is also of potential evolutionary significance that shuffled mutants were obtained with 80-fold enhanced activities with substrates containing methyl thioether and methoxy substituents. These represent the commercially relevant herbicides ametryn and atraton, respectively. An enzyme purified from a *Nocardioides* sp. was shown to hydrolyze ametryn, but it was not tested with atraton or other methoxy-functionalized herbicides (*50*). DNA from the *Nocardioides* sp. did not hybridize to an *atzA* probe, suggesting that the enzyme does not closely resemble atrazine chlorohydrolase from *Pseudomonas* sp. ADP. However, the data in Table 2 suggest that enzymes capable of metabolizing ametryn, atraton, and related triazine herbicides could be derived from *triA* or closely homologous genes in nature.

With respect to the sequences that favor dechlorination versus deamination, the data show a trend in that residue 328 appears to largely control leaving group specificity. Asn_{328} tracks with narrow specificity enzymes that largely catalyze dechlorination. Asp_{328} tracks with broader specificity enzymes which catalyze deamination and the displacement of $-NCH_3$, $-OCH_3$, and $-SCH_3$ groups. The hypothesis that this residue is crucial to the observed specificity difference between melamine deaminase and atrazine chlorohydrolase is currently being addressed with site-directed mutagenesis studies.

Conclusions

Nature must continually fine-tune enzyme substrate specificities and reaction rates over time under the aegis of biological need, usually called selective evolutionary pressure. This enzyme variability is particularly marked with soil bacteria due to their enormous numbers, large evolutionary span of 3.6 billion years, rapid reproductive rates, and great competition for scarce nutrient resources. Enzyme plasticity is important in this context, but this confounds genome annotation efforts where gene function is assigned on the basis of finding the homologue with the most identical sequence. As discussed here, enzymes with sequences that are 98% identical can catalyze different reactions. It will be imperative to flesh out a broader range of microbial enzymatic reactions, particularly for microbial catabolic enzymes where the diversity of enzymes will likely be great.

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