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Evolution of a microbial acetyltransferase for modification of glyphosate: a novel tolerance strategy†

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Abstract: *N*-Acetylation is a modification of glyphosate that could potentially be used in transgenic crops, given a suitable acetyltransferase. Weak enzymatic activity ($k_{cat} = 5$ min^{-1}, $K_M = 1$ mM) for *N*-acetylation of glyphosate was discovered in several strains of *Bacillus licheniformis* (Weigmann) Chester by screening a microbial collection with a mass spectrometric assay. The parental enzyme conferred no tolerance to glyphosate in any host when expressed as a transgene. Eleven iterations of DNA shuffling resulted in a 7000-fold improvement in catalytic efficiency (k_{cat}/K_M), sufficient for conferring robust tolerance to field rates of glyphosate in transgenic tobacco and maize. In terms of k_{cat}/K_M, the native enzyme exhibited weak activity (4–450% of that with glyphosate) with seven of the common amino acids. Evolution of the enzyme towards an improved k_{cat}/K_M for glyphosate resulted in increased activity toward aspartate (40-fold improved k_{cat}), but activity with serine and phosphoserine almost completely vanished. No activity was observed among a broad sampling of nucleotides and antibiotics. Improved catalysis with glyphosate coincided with increased thermal stability.

Keywords: glyphosate tolerance; directed evolution; *Bacillus licheniformis*; *N*-acetyltransferase

1 INTRODUCTION

Glyphosate (*N*-phosphonomethylglycine) is a widely used herbicide that acts non-selectively through inhibition of 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase in the aromatic biosynthesis pathway.[1] Its efficacy against all plant species, low cost, low mammalian toxicity and benign environmental impact highly favor its use in crops that have a transgenic tolerance mechanism.[2] The only such mechanism in current commercial use is a transgene encoding a bacterial EPSP synthase that is insensitive to inhibition by glyphosate.[2] By analogy with species of *Streptomyces* that detoxify phosphinothricin with a specific acetyltransferase,[3] an alternative mechanism for glyphosate tolerance in crops might be *N*-acetylation of the herbicide, provided that a suitable acetyltransferase were available. Here, we review the discovery of a weak glyphosate acetyltransferase, its 7000-fold improvement through directed evolution, and the kinetic properties of native and evolved enzymes, published earlier.[4] New data on substrate specificity and thermal stability are included.

2 METHODS

2.1 *gat* gene discovery

Bacterial strains were grown in LB medium, permeabilized by incubation with 1% toluene and resuspended in 5 mM morpholine/acetic acid buffer, pH 8, 5 mM glyphosate and 200 μM acetyl coenzyme A (AcCoA). After overnight incubation, culture filtrates were analyzed for *N*-acetylglyphosate by mass spectrometry. After negative electrospray ionization, the *m*/*z* 124 daughter ion of the *m*/*z* 210 parent was detected. Details are given in Castle *et al*.[4]

2.2 Purification of GAT

Escherichia coli Cast & Chalm cells were lysed in 25 mM Hepes, pH 7.2, 100 mM KCl and 10% methanol (termed HKM) containing 2 mg ml^{-1}

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Protease Inhibitor cocktail (Sigma-Aldrich, P8465) and 1 mg ml^{-1} lysozyme (Sigma-Aldrich). The soluble extract was desalted by gel-filtration through Sephadex G25 equilibrated with HKM with KCl concentration reduced to 50 mM, and then passed through a column of CoA Agarose (Sigma-Aldrich; 1 ml bed volume for the extract from a 250-ml culture) equilibrated with HKM (50 mM KCl). After washing, elution was effected with 1 mM AcCoA in HKM. Final purification was with gel-filtration through Superdex 75 (Amersham Biosciences) equilibrated with HKM.

2.3 Assay procedures

2.3.1 DTNB end-point assay

For surveys of substrate specificity, enzyme, AcCoA and amino substrate were incubated in 25 mM Hepes, pH 6.8, 100 ml liter^{-1} ethylene glycol in the wells of a 96-well polystyrene plate. After 30 min, reactions were stopped by the addition of 30 µl of 10 mM 5, 5′-dithiobis-2-nitrobenzoate (DTNB) in 500 mM Tris, pH 7.5. After 2 min, absorbance was read at 412 nm in a Spectramax Plus^{384} plate reader (Molecular Devices, Sunnyvale, CA).

2.3.2 Continuous spectrophotometric assay

Apparent K_M values for glyphosate were determined at saturating AcCoA (167 µM) in a continuous absorbance assay at 235 nm, which monitors the breaking of the thioester bond of AcCoA (extinction, M^{-1} cm^{-1} = 3400). Enzyme was combined with AcCoA and 25 mM Hepes, pH 6.8, 100 ml liter^{-1} ethylene glycol, and reactions were started with the addition of a 294-µl aliquot to wells of a 96-well UV assay plate (Corning) containing 6 µl of a 50-fold concentrated stock solution of glyphosate. The decrease in absorbance at 235 nm, was recorded at 5-s intervals for 90 s. The Spectramax software, SoftMax Pro, was programmed to convert change in absorbance to reaction velocity, µM min^{-1}, and to return parameters of the Lineweaver–Burke transformation of the Michaelis–Menten equation. k_{cat} was calculated from the V_{max} values returned by SoftMax Pro and the concentration of GAT in the assay. Protein concentrations were calculated from measurements of absorbance at 205 nm (Ex, mg ml^{-1} = 30.5) after buffer exchange (NAP-5 column, Amersham-Pharmacia Biotech) into 50 mM Na_2SO_4. The extinction coefficient was determined by the procedure of Scopes:[5] extinction (mg ml^{-1} cm^{-1}) = $27 + 120(A_{280}/A_{205})$.

Apparent K_M for AcCoA was determined with the same mass spectrometric assay used for discovering GAT activity. A series of injections was made from each reaction mixture, which contained a fixed, saturating concentration of ammonium glyphosate, purified enzyme, AcCoA ranging from 1 to 20 µM, 10 mM morpholine/acetic acid buffer, pH 6.8 and 100 ml liter^{-1} methanol. Initial rates were determined by plotting retention time versus peak area.

3 RESULTS

3.1 *gat* gene discovery and isolation

Our novel approach for glyphosate tolerance, in which the herbicide is covalently modified, required an enzyme that catalyzes the acetyltransferase reaction shown in Fig 1. Because of the diversity of secondary metabolism exhibited within the genus *Bacillus*, we screened an in-house collection of *Bacillus* strains for their ability to catalyze production of *N*-acetylglyphosate from glyphosate and AcCoA. We used a high-throughput mass spectrometric assay that could detect as little as 200 nM *N*-acetylglyphosate.[4] The product was found in the reaction mixtures of several strains of *Bacillus licheniformis* (Weigmann) Chester, a common saprophytic bacterium. To isolate the gene that codes for GAT activity, *B licheniformis* genomic DNA was fragmented and expressed in *E coli*.[4] Pools of individual *E coli* transformants were subjected to the same screening procedure as used for discovery. Pools exhibiting activity were plated for isolation of single colonies, and the screen repeated. The nucleotide sequences of fragments coding for GAT activity were determined. A region was found that was common to all active fragments, and contained a single open-reading frame of 438 base pairs coding for a protein of 17 kD. Primers constructed from this sequence enabled the isolation, from several closely related strains, of three major alleles of *gat* genes that were 93% identical.

3.2 Characteristics of *gat* genes and protein

Position-specific iterated BLAST search and analysis indicates that GAT shares homology with members

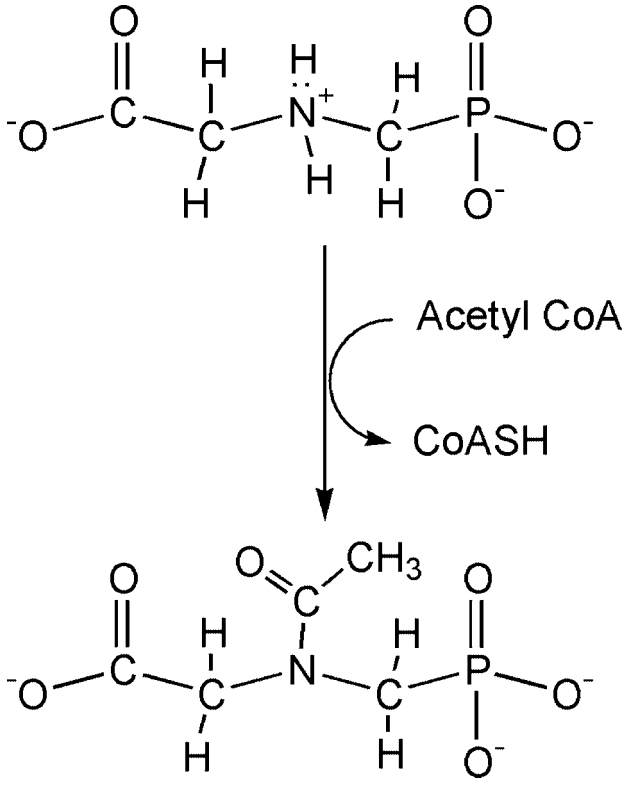

Figure 1. The glyphosate acetyltransferase reaction.

of the GCN5-related *N*-acetyltransferase (GNAT) super-family.[6,7] Preliminary analysis of X-ray diffraction data obtained from a GAT crystal confirm that GAT does indeed have the GNAT fold (data not shown). The GenBank accession most closely related to GAT is a hypothetical *N*-acetyltransferase (NAT) protein predicted from the genomic sequence of *B subtilis* (Ehrenburg) Cohn, and is termed *yitI* (GenBank accession Y09 476). The gene is 63% identical to *gat* and the hypothetical protein sequence (CAA70 664) is 59% identical and 66% similar to GAT. The *E coli*-expressed enzyme coded by *yitI* was capable of producing *N*-acetylglyphosate, detectable by mass spectroscopy, but was much less active than the *B licheniformis* enzyme. Further searching among diverse species yielded seven other hypothetical but uncharacterized proteins that are related to GAT, with identities to GAT ranging from 28 to 49%.[4]

Once *gat* genes were cloned, and the enzymes expressed in *E coli*, GAT enzymes were easily purified in two steps, without an epitope tag. The high affinity of GAT for AcCoA (Table 1) afforded highly effective affinity purification on CoA Agarose (Fig 2). Remaining contaminants were removed by gel-filtration on Superdex 75. The three GAT enzymes discovered in *B licheniformis* strains have similar properties (Table 1). The comparison with phosphinothricin acetyltransferase shows that GAT is a very poor catalyst for acetylation of glyphosate,

Table 1. Kinetic properties of three native GAT enzymes in comparison with those of phosphinothricin acetyltransferase (PAT)[a]

	Native GAT	PAT
k_{cat}, (min^{-1})	5–8	470
$K_{M\ gly\ or\ PPT}$, (mM)	1–1.7	0.023
k_{cat}/K_M, (min^{-1} mM^{-1})	4	20 400
K_{MAcCoA}, (μM)	1–2	130

[a] PAT was obtained from an *Eschericia coli* strain (M15[pREP4,pQE60:pat) expressing the *pat* gene as a 6xHis fusion. PAT was purified from cell lysates using affinity chromatography on Nickel-NTA (Qiagen).

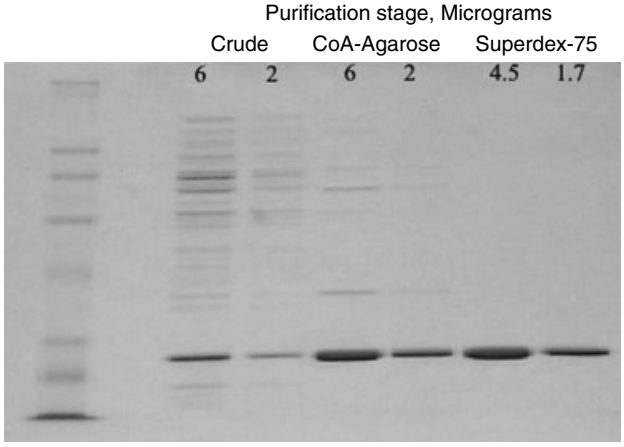

Figure 2. Purification of GAT. Numbers indicate μg of protein loaded. The molecular weight markers above and below the GAT band are 21 kD and 14 kD, respectively.

and that an effective tolerance gene would need to be improved 5000-fold over the parental genes. Native GAT conferred no tolerance to glyphosate, even in transformed *E coli* cells expressing high levels of soluble enzyme.

3.3 Directed evolution of GAT

Because the K_i value of glyphosate for inhibition of EPSP synthase is 0.4 μM,[1] the derivatization of glyphosate by GAT in the treated plant may need to be nearly quantitative and rapid, requiring that the reaction velocity (v) be appreciable at low concentrations of substrate. The kinetic parameter targeted for improvement, therefore, was k_{cat}/K_M, the rate constant for the reaction of free enzyme with substrate,[8]

$$v = k_{cat}/K_M[E][S]$$

Thus, k_{cat}/K_M is a parameter expressing overall catalytic efficiency. In practice, application of selection pressure for improved k_{cat}/K_M in the screen was effected by maintaining a concentration of glyphosate below the $K_{M\ gly}$ value of the parental enzymes.

The approach we used to create libraries of variants from which to identify improved individuals was DNA shuffling, a technique for molecular recombination between genes.[9,10] This technique has been used to alter properties of proteins such as kinetics, substrate specificity, pH optimum, thermal stability and solubility, in a direction defined by the screen.[11,12] The parental GAT genes were subjected to fragmentation-based, multi-gene shuffling as described elsewhere.[4] Shuffled variants were expressed in *E coli* and screened for activity with the same mass spectrometric assay used for discovery and cloning. AcCoA was present at 200 μM in each iteration of the process, while the concentration of glyphosate was adjusted downward as the $K_{M\ gly}$ value of the parental genes for each generation decreased. The best variants in the mass spectrometric assay were selected for purification and kinetic characterization. Those variants exhibiting outstanding k_{cat}, K_M or k_{cat}/K_M values were selected as parents for the next iteration of the process. At each iteration of DNA shuffling, we screened about 5000 variants in the first tier and analyzed 24–48 purified enzymes in the second tier.

Directed evolution resulted in steady improvement in k_{cat} through eight iterations (Table 2). However, after four iterations of evolution as described above, K_M for glyphosate improved 2-fold, but appeared to reach an asymptotic value at 0.5 mM. Overcoming this impasse required two advances. First, the 100-fold improvement in k_{cat}/K_M after three iterations was sufficient to confer tolerance to glyphosate in *E coli* cells expressing high levels of improved GAT. This allowed us effectively to screen much larger, more diverse libraries by including a selection step in which only those colonies that grew on minimal agar medium containing 1 mM glyphosate

Table 2. Evolution of GAT kinetic properties: values are the most optimal observed in each iteration of evolution, for each parameter; in most cases, three different variants are represented for each iteration[a,b]

Iteration	k_{cat}, (min^{-1})	K_M, (mM)	k_{cat}/K_M
Parent	8.3	1.27	4.2
1	102	0.9	72.6
2	153	0.6	135
3	948	0.45	453
4	936	0.6	607
5	1312	0.052	6345
6	1052	0.05	8342
7	1424	0.066	10 340
8	1900	0.046	12 408
9	1370	0.06	13 900
10	1650	0.04	27 000
11	1480	0.05	29 600

[a] Data were obtained with the continuous spectrophotometric assay (Section 2.3.2).
[b] The k_{cat} values presented here differ from those reported earlier[4] due to the earlier use of the Bradford protein assay. Poor color development with the ovalbumin standard relative to GAT overestimated GAT concentrations (hence, underestimated k_{cat}) by about fourfold.

were selected for screening. The second advance was the development of synthetic shuffling, in which synthetic oligonucleotides were used to toggle additional diversity into specific positions in a backbone template.[10] The genes mentioned above that putatively code for acetyltransferases related to GAT served as the source of additional diversity. The process is described in detail elsewhere.[4] The result of this novel approach was a dramatic decrease in $K_{M\ gly}$ from 0.5 to 0.05 mM (Table 2), and the eventual surpassing of the goal of 5000-fold improvement in k_{cat}/K_M. The best variant obtained to date is improved 7000-fold, combining a 200-fold increase in k_{cat} with a 35-fold reduction in K_M.

3.4 Herbicide-tolerant plants

The *gat* genes were introduced by *Agrobacterium*-mediated transformation into tobacco leaves[4] and maize embryos[4,13] for regeneration of T_0 plants. In all cases, transformants were selected on glyphosate. T_1-transformed tobacco plants (*Nicotiana tabacum* L cv Xanthi) were selected from T_0 transformants by spraying with 1 lb acid equivalents $acre^{-1}$ ($AE\,A^{-1}$) (1.21 kg AE ha^{-1}) glyphosate. The T_1 plants expressing GAT from the 5th iteration of shuffling were subjected to a dose-response trial in the greenhouse. While control plants were killed by a dose of 0.27 lb AE A^{-1} (0.33 kg AE ha^{-1}), descendants of seven out of 40 transformation events were tolerant to 20 lb AE A^{-1} (24.2 kg AE ha^{-1}). Some plants were completely symptom free at 10 lb AE A^{-1} (12.1 kg AE ha^{-1}). All tolerant plants were fertile, with normal seed set.

In spray tests (RoundUp UltraMAX, 3 lb AE A^{-1} (3.63 kg AE ha^{-1}) on T_0 maize plants transformed with GAT genes from the 5th to 11th iteration of shuffling, a range of tolerance was observed that correlated with the kinetic properties of the enzyme. All plants with the 5th round GAT showed stunting, and many exhibited chlorotic banding in the leaves. Half of the 7th round transformants showed no symptoms, while most 10th and 11th round transformants were asymptomatic at glyphosate application rates of 4.5 lb AE A^{-1} (5.45 kg AE ha^{-1}). Field trials for tolerance and yield are presently in progress to evaluate the commercial potential of this glyphosate tolerance trait.

3.5 Properties of selected GAT variants

3.5.1 Substrate saturation

All variants exhibited hyperbolic substrate saturation for both glyphosate and AcCoA. Values for k_{cat} and apparent $K_{M\ gly}$, measured at saturating AcCoA, are shown in Table 2. The apparent K_M for AcCoA did not measurably change from a value of 1–2 μM through 11 rounds of shuffling and selection, despite the presence of AcCoA in the screening assays at 100 times K_M.

3.5.2 Substrate specificity

In addition to glyphosate, native GAT catalyzed the acetylation of seven L-amino acids. In terms of k_{cat}/K_M, native GAT was about as active with L-aspartate, 4.7 times more active with L-serine (Table 3) and 2 times more active with phospho-L-serine (data not shown) than with glyphosate. Directed evolution of the enzyme resulted in a 40-fold increase in activity with aspartate, entirely through

Table 3. Specificity of selected GAT variants for amino substrates

				k_{cat}/K_M		
GAT variant	Substrate	k_{cat}(min^{-1})(±SE)	K_M (mM) (±SE)	(min^{-1} mM^{-1})	Fold improvement	% of glyphosate
Native	Glyphosate	5.35 (±0.043)	1.27 (±0.0144)	4.21		
R7		1150 (±27.6)	0.251 (±0.0041)	4573	1086	
R11		1480 (±65.4)	0.05	29 600	7031	
Native	L-Aspartate	24.1	6.7	3.6		85.5
R7		435 (±11.3)	2.95 (±0.162)	148		3.24
R11		702 (±12.4)	4.56 (±0.112)	154		0.520
Native	L-Serine	854	43	19.8		471
R7		242 (±15.8)	60.1 (±1.68)	4.04		0.0882
R11		388 (±18.5)	154 (±10.7)	2.53		0.00 855

improved k_{cat}, but almost complete loss of activity with serine and phosphoserine. In addition to aspartate and serine, activity (ie detection of free CoA in reaction mixtures containing enzyme, AcCoA and test substrate) with native enzyme at 3% or more of that towards glyphosate when present at 1 mM was observed with L-threonine (250%), L-glutamate (51%), L-asparagine (27%), and L-cysteine (4%). Activity with the other protein amino acids was either nil or less than 3% of that of glyphosate. Despite its ability to acetylate glyphosate's secondary amine, the *N*-methyl derivative of L-aspartate (2 mM) supported no detectable activity. Among antibiotics, no detectable activity was seen with 10 mM fosmidomycin, 2 mM D,L-phosphinothricin or 1 mM kanamycin, erythromycin, carbenacillin, spectinamycin, streptomycin, chloramphenicol or ampicillin. Other biological amines that supported no activity were 1 mM D-glucosamine, 10 mM serotonin, 10 mM anthranilate, 10 mM ornithine, 1 mM purine and pyrimidine bases, nucleosides, nucleotides, histone and tRNA.

Several commercially available derivatives of coenzyme A were tested to probe the specificity of native GAT and a 7th round variant (R7) for the acyl donor. No detectable activity was observed with the DTNB assay when 1 mM of the malonyl, crotonyl, succinyl or 3-hydroxy-3-methylglutaryl derivatives of coenzyme A were incubated with 5 mM glyphosate and either native GAT present at 1.14 μM or R7 at 0.42 μM. The only alternative acyl group donor exhibiting activity in the DTNB assay was propionyl CoA, which, with the native enzyme, supported 3% of the acylation rate of AcCoA. More detailed analysis of the kinetics with propionyl CoA was carried out for enzyme R7 using the continuous spectrophotometric assay. The concentration of glyphosate was varied against a series of fixed concentrations of propionyl CoA. The k_{cat} value with propionyl CoA was 345 min^{-1} and K_M was 100 μM, for a k_{cat}/K_M of 3.45 min^{-1} μM^{-1}. The corresponding values for AcCoA are 1160 min^{-1}, 1.5 μM and 773 min^{-1} μM^{-1}. Thus, k_{cat}/K_m with propionyl CoA is only 0.5% of that with AcCoA.

3.5.3 Thermal stability

Stability of native and evolved GAT enzymes was determined several ways. First, we measured enzymatic activity at 22 °C that survived incubation for 15 min over a range of temperatures from 30 to 60 °C (Fig 3). While native enzymes lost half their activity in the range 42–44 °C, this parameter was increased in all evolved enzymes tested to the range 47–54 °C. Second, in measurements of activity remaining when enzymes were incubated at 37.5 °C, evolved variants had half-lives that were 2- to 5-fold longer than native GAT (Table 4). Finally, using the DTNB end-point assay, we measured GAT activity at a temperature range of 10–50 °C. In a 3-min assay, activity increased linearly through the whole temperature range for variants R8, R9-2 and R9-3 (data not shown). Activity for native GAT increased linearly from 10 to 47 °C, above which temperature activity decreased sharply, as a consequence of instability at these temperatures.

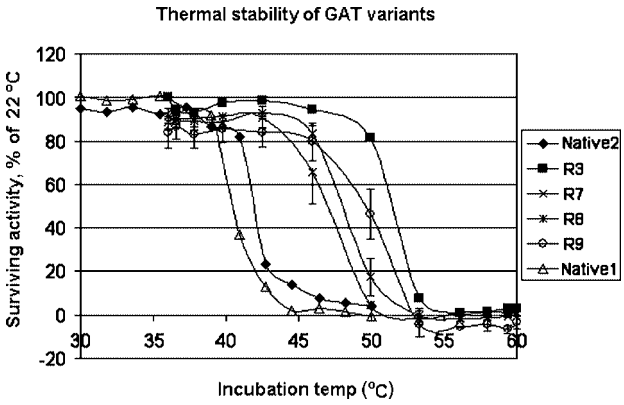

Figure 3. Thermal stability of parental GAT and shuffled variants. Enzymes were distributed to 200-μl-strip PCR tubes and incubated in a gradient thermocycler at the indicated temperature for 15 min. Precipitated protein was removed by centrifugation, and surviving activity was measured at 22 °C by the continuous spectrophotometric assay with saturating concentrations of glyphosate and AcCoA.

Table 4. Half-lives of native GAT and shuffled variants at 37.7 °C[a]

Enzyme	Iteration	Half-life (h)
Native1	Parent	14
R7	7	45
R8	8	54
R9-1	9	67
R9-2	9	26
R9-3	9	43

[a] Enzymes in a matrix of HKM were incubated at 37.5 °C. At various times, aliquots were withdrawn and assayed in triplicate at 22 °C by the continuous spectrophotometric assay. Standard errors at each time point averaged 2.9%. Activity was plotted versus incubation time, and the data fitted to a curve for exponential decay ($y = e^{-x}$), from which half-lives were calculated.

4 DISCUSSION

This work illustrates a new paradigm for agricultural biotechnology, in which a product concept defines a desired enzymatic activity, highly sensitive methods are used to identify a source of the activity, and directed evolution is used to optimize the properties for commercial levels of performance. There were several keys to our success. First, a highly sensitive mass spectrometric assay enabled detection of very weak activity. Second, the initial 100-fold improvement obtained by fragmentation-based shuffling of the original gene family was sufficient for growth of *E coli* on minimal medium containing glyphosate, enabling us to query much larger libraries. Finally, the development of synthetic shuffling allowed for the incorporation of diversity outside the native GAT family, resulting in breakthrough reduction in K_M.

The survey of substrate specificity was undertaken in part to determine the physiological substrate of the native enzyme. No compound among a broad sampling of amino acids, antibiotics and nucleotides exhibited a k_{cat}/K_M of greater than 20 min^{-1} mM^{-1} (333 s^{-1} M^{-1}), from which we conclude that the physiological role of GAT is still unknown.

The increase in thermal stability already seen in the first iteration of shuffling was fortuitous. A reasonable concern was whether an enzyme obtained from a soil microbe might, when transferred into a crop species, be denatured on hot days. We did not intentionally select for thermal stability, but the temperature used for growth of the host *E coli*, 37 °C, likely is warm relative to the ambient temperature range of the native host. Another possibility is that increased stability simply coincided with refinement of the catalytic properties.

REFERENCES

1 Franz JE, Mao MK and Sikorski JA, *Glyphosate: a unique global herbicide*, American Chemical Society, Washington, DC, pp 521–615 (1997).

2 Padgette SR, Re DB, Barry GF, Eichholtz DE, Delanny X, Fuchs RL, Kishore GM and Fraley RT, New weed control opportunities: development of soybeans with a Roundup Ready gene, in *Herbicide-resistant crops: agricultural, economic, environmental, regulatory and technological aspects*, ed by Duke SO, CRC/Lewis Publishers, Boca Raton, FL, pp 53–84 (1995).

3 Hoerlein G, Glufosinate (phosphinothricin), a natural amino acid with unexpected properties, *Rev Environ Contam Toxicol* **138**:73–145 (1994).

4 Castle LA, Siehl DL, Gorton R, Patten PA, Chen YH, Bertain B, Cho H-J, Duck N, Wong J, Liu D and Lassner M, Discovery and directed evolution of a glyphosate tolerance gene, *Science (Washington)* **304**:1151–1154 (2004).

5 Scopes RK, *Protein purification, principles and practice*, Springer, New York (1994).

6 Dyda F, Klein DC and Hickman AB. GCN5-related *N*-acetyltransferases: a structural overview, *Annu Rev Biomol Struct* **29**:81–103 (2000).

7 Neuwald AF and Landsman D, GCN5-related histone *N*-acetyltransferases belong to a diverse super family that includes the yeast SPT10 protein, *Trends Biochem Sci* **22**:154–155 (1997).

8 Fersht A, *Enzyme structure and mechanism*, 2nd edn, WH Freeman, New York (1985).

9 Stemmer WPC, DNA shuffling by random fragmentation and reassembly: *in vitro* recombination for molecular evolution. *Proc Natl Acad Sci USA* **91**:1074–1077 (1994).

10 Ness JE, Kim S, Gottman A, Pak R, Krebber A, Borchert TV, Govindarajan S, Mundorff EC and Minshull J. Synthetic shuffling expands functional protein diversity by allowing amino acids to recombine independently, *Nature Biotechnology* **20**:1251–1255 (2002).

11 Zhang J-H, Dawes G and Stemmer WPC. Directed evolution of a fucosidase from a galactosidase by DNA shuffling and screening, *Proc Natl Acad Sci USA* **94**:4504–4509 (1997).

12 Crameri A, Whitehorn EA, Tate E and Stemmer WPC. Improved green fluorescent protein by molecular evolution using DNA shuffling. *Nature Biotechnology* **14**:315–319 (1996).

13 Zhao Z-Y, Gu W, Cai T and Pierce DA. Methods for agrobacterium-mediated transformation, US Patent 5 981 840 (1999).