



transgenes provide the sole means of conferring tolerance to this herbicide. In such plants, glyphosate accumulates in the meristems, where it may interfere with reproductive development and may lower crop yield.

*N*-Acetylglyphosate is not herbicidal and is a very poor inhibitor of EPSPS. A soluble enzyme with high affinity for glyphosate, and that would rapidly acetylate this secondary amine, would be a strong alternative candidate for conferring tolerance, without product accumulation. No such enzyme had been identified until 2004, when Castle and coworkers reported the successful creation of such an “optimized” enzyme by directed evolution. These researchers screened hundreds of isolates of *Bacillus* species for the ability to acetylate glyphosate. Cultures were grown to stationary phase, permeabilized, and incubated with glyphosate and acetyl-CoA (Figure 11.7). Supernatants were analyzed for *N*-acetylglyphosate by a sensitive mass spectrometry method. Three strains (ST401, B6, and DS3) of the common saprophytic bacterium *Bacillus licheniformis* exhibited *N*-acetylglyphosate transferase (GAT) activity. The responsible enzymes were cloned and expressed in *E. coli* and characterized. These 146-residue soluble enzymes were 94% identical in sequence. The acetylation of glyphosate by these enzymes had a rate constant ( $k_{\text{cat}}$ ) of 1.0 to 1.7 min<sup>−1</sup>. Their affinity for glyphosate was low ( $K_{\text{m}}$  1.2 to 1.8 mM at pH 6.8 and 21°C) but high for acetyl-CoA ( $K_{\text{m}}$  1 to 2 μM), indicating that acetyl-CoA is the native acetyl substrate. Their average  $k_{\text{cat}}/K_{\text{m}}$  was 0.81 min<sup>−1</sup> mM<sup>−1</sup>. Expression of these enzymes in transgenic tobacco or *Arabidopsis* did not confer herbicide tolerance. Improvement in GAT catalytic properties was then sought through directed evolution.

BLAST search of databases for sequences related to the three *B. licheniformis* GAT enzymes revealed a number of homologs in other bacteria. The three *B. licheniformis* genes were then subjected to 11 iterations of DNA shuffling. During the shuffling process, at the end of several cycles, amino acid diversity from the predicted sequences of four other hypothetical *N*-acetyltransferase proteins ranging in identity with GAT from 59% to 28% (Table 11.4) was incorporated into the library. The best GAT enzyme obtained by this directed molecular evolution process had a  $k_{\text{cat}}/K_{\text{m}}$  of 8320 min<sup>−1</sup> mM<sup>−1</sup>, an improvement in enzyme efficiency of about 10,000-fold over the *B. licheniformis* ST401 GAT. The sequence of the evolved enzyme differed in more than 30 positions from that of the parent enzyme. The evolved enzyme conferred high glyphosate tolerance to *E. coli*, *Arabidopsis*, tobacco, and maize, and caused no adverse symptoms.

Gene Site Saturation Mutagenesis

The term *gene site saturation mutagenesis* describes a technique whereby each amino acid of a protein is replaced with each of the other 19 naturally

**FIGURE 11.7**  
*N*-Acetylation of glyphosate catalyzed by a glyphosate acetyltransferase (GAT).