4. S. Rajalakshmi, P. M. Rao, D. S. R. Sarma, *Biochem. Biophys. Res. Commun.* **81**, 936 (1978).
5. U. Bachrach and G. Eilon, *Biochim. Biophys. Acta* **179**, 494 (1969).
6. P. E. Brown, *Radiat. Res.* **34**, 24 (1964).
7. P. S. Mamont, M. Duchesne, A. Joder-Ohlenbusch, J. Grove, in *Enzyme-Activated Irreversible Inhibitors*, N. Seler, M. J. Jung, J. Koch-Weser, Eds. (Elsevier/North-Holland, Amsterdam, 1978), pp. 43–53; J. Seidenfeld, J. W. Gray, L. J. Marton, *Exp. Cell Res.* **131**, 209 (1981).
8. D. T. Hung, D. F. Deen, J. Seidenfeld, L. J. Marton, *Cancer Res.* **41**, 2783 (1981); S. M. Oredsson, D. F. Deen, L. J. Marton, in preparation; S. M. Oredsson, A. E. Pegg, D. F. Deen, L. J. Marton, in preparation.
9. L. C. Erickson, M. O. Bradley, J. M. Ducore, R. A. G. Ewig, K. W. Kohn, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 467 (1980); R. J. Weinkam and H.-S. Lin, *J. Med. Chem.* **22**, 1193 (1980).
10. S. M. Oredsson, D. F. Deen, L. J. Marton, *Cancer Res.* **42**, 1296 (1982).
11. P. J. Tofilon, S. M. Oredsson, D. F. Deen, L. J. Marton, *Science* **217**, 1044 (1982).
12. P. Pohjanpelto and S. Knuutila, *Exp. Cell Res.* **141**, 333 (1982).
13. R. E. Chapman, L. C. Klotz, D. S. Thompson, B. H. Zimm, *Macromolecules* **2**, 637 (1969); L. C. Klotz and B. H. Zimm, *J. Mol. Biol.* **72**, 779 (1972); *Macromolecules* **5**, 471 (1972); R. Kavenoff and B. H. Zimm, *Chromosoma* **41**, 1 (1973).
14. B. H. Zimm, *J. Chem. Phys.* **24**, 269 (1956).
15. E. L. Uhlenhopp, *Biophys. J.* **15**, 233 (1975); E. S. Chase and R. H. Shafer, *ibid.* **28**, 93 (1979); R. H. Shafer and E. S. Chase, *Cancer Res.* **40**, 3186 (1980); ———, K.-L. W. Wun, *Photochem. Photobiol.* **33**, 335 (1981); R. H. Shafer, E. S. Chase, J. Eisenach, *Radiat. Res.* **85**, 47 (1981).
16. K.-L. W. Wun and R. H. Shafer, *Radiat. Res.* **90**, 310 (1982).
17. H. Dertinger and H. Jung, *Molecular Radiation Biology* (Springer-Verlag, New York, 1970).
18. V. A. Bloomfield, D. M. Crothers, I. Tinoco, Jr., *Physical Chemistry of Nucleic Acids* (Harper & Row, New York, 1974).
19. We thank J. Hoyt for technical assistance and N. Buckley for editorial assistance. Supported in part by program project grant CA-13525 and grants CA-19658 and CA-27343 from the National Institutes of Health and by American Cancer Society grant RD-137.
* Address reprint requests to L.J.M.

12 October 1982; revised 26 November 1982

An Altered aroA Gene Product Confers Resistance to the Herbicide Glyphosate

Abstract. *The hypothesis that the herbicide glyphosate (*N*-phosphonomethylglycine) acts on plants and microorganisms by inhibiting synthesis of 5-enolpyruvyl-3-phosphoshikimate, a precursor to aromatic amino acids, was tested.* Salmonella typhimurium *was treated with ethyl methanesulfonate, and mutants mapping at the aroA locus, which encodes 5-enolpyruvyl-3-phosphoshikimate synthetase, were isolated by selection for glyphosate resistance. One of the mutants results in the synthesis of a 5-enolpyruvyl-3-phosphoshikimate synthetase that is resistant to inhibition by glyphosate. The mutant aroA gene and the corresponding wild-type allele were cloned. The mutation confers high resistance to glyphosate when introduced in* Escherichia coli *in the presence or absence of the wild-type aroA allele.*

The herbicide phosphonomethylglycine (common trade name: glyphosate) is used as a broad spectrum weed killer. It appears to inhibit the shikimic acid pathway (*1–4*), which in plants and bacteria provides a precursor for the synthesis of aromatic amino acids. The glyphosate-sensitive step is the conversion of phosphoenolpyruvate and 3-phosphoshikimic acid to 5-enolpyruvyl-3-phosphoshikimic acid catalyzed by the enzyme 5-enolpyruvyl-3-phosphoshikimate synthetase (E.C. 2.5.1.19).

We now describe the isolation of glyphosate-resistant mutants in *Salmonella typhimurium* that map in the locus, aroA, coding for 5-enolpyruvyl-3-phosphoshikimate synthetase. The aroA gene from one of the mutants was cloned in *Escherichia coli* and the mutation or mutations characterized as a structural alteration of the enzyme encoded by the aroA locus. The properties of this mutant gene make it potentially useful for the introduction and expression of herbicide resistance in plant cells, which is our long-range goal.

If the aroA gene product is the primary target of glyphosate inhibition, glyphosate-resistant mutants which map in the aroA locus may be isolated by selection on glyphosate. Such mutations were identified by tight cotransduction of glyphosate resistance and aroA into the aroA-deficient *S. typhimurium* strain A1 (*5*). Ethyl methanesulfonate mutagenesis (*6*) was used to increase the frequency of glyphosate-resistant mutations, large numbers of which were screened by preparing mixed P22 lysates from thousands of pooled mutants (Table 1).

Strain CTF3 is a representative mutant obtained by mutagenesis and selection at a glyphosate concentration of 350 μg/ml. Although resistance of this mutant to glyphosate was not very high, it cotransduced 97 percent with the aroA mutation of *Salmonella* strain A1. A second cycle of mutagenesis on CTF3 and selection at 1000 μg/ml yielded a series of aroA related mutants very resistant to glyphosate. They grew well on glyphosate at 2000 μg/ml. All cotransduced 95 to 99 percent with the independent *S. typhimurium* aroA-deficient mutations aroA1, aroA124 (*7*), and aroA148 (*5*). One of these mutants, designated CT7, was chosen for further characterization.

Resistance to glyphosate mediated by a mutation at the aroA locus could result from altered regulation leading to overproduction of 5-enolpyruvyl-3-phosphoshikimate synthetase or to structural changes in the enzyme. To distinguish between these possibilities we analyzed enzyme preparations from the mutant strain CT7 and an isogenic wild-type strain, STK1, prepared by transducing the wild-type aroA allele from *S. typhimurium* strain TA831 into strain A1.

At saturating substrate concentrations, CT7 enzyme preparations had twice the specific activity of those from STK1 (*8*). Also, they were more resistant to glyphosate than those from STK1 over a range of different substrate and inhibitor concentrations. Neither the mutant nor the wild-type enzyme was overproduced in response to glyphosate-induced stress.

To study the mutation or mutations in the aroA locus of mutant strain CT7, we cloned in *E. coli* both mutant and wild-type alleles. A cosmid bank was constructed from each strain in the low-copy cosmid vector pVK100 (*9*). The chromosomal DNA was partially digested with restriction endonuclease Sau 3A, ligated

Table 1. Frequency of glyphosate-resistant mutants. Strain TA831 is a *his*$^-$ derivative of *S. typhimurium* LT2; strain CTF3 is a mutant strain originated from experiment 2, resistant to glyphosate at 350 μg/ml and mapping in the aroA locus. It was constructed by transducing an aroA-glyphosate–resistant allele from experiment 2 in strain Al (*5*). EMS, ethyl methanesulfonate.

Experiment	Strain	EMS	Glyphosate* (μg/ml)	Frequency	
				Glyphosate	aroA-Glyphosate resistance†
1	TA831	−	350	10^{-8}	$< 10^{-9}$
2	TA831	+	350	10^{-4}	10^{-6}
3	CTF3	+	1000	10^{-6}	10^{-9}

*A commercial preparation of glyphosate was used. †Glyphosate-resistant mutations mapping at the aroA locus were identified by cotransduction. Since lysates were prepared from pooled groups of mutants the ratio of glyphosate-resistant aroA cotransductants to the total number of aroA transductants was taken as a close estimate of the ratio of aroA-encoded to aroA-unrelated mutations in the original mutagenized culture. To assure that the estimates were meaningful, random cotransductants were further tested for tight cotransduction.

to pVK100 partially digested with Bgl II (to avoid excision of the cos site), and packaged in phage lambda heads (*10*). Since it was unknown whether glyphosate resistance was conferred in a merodiploid situation, we decided to complement the aroA-deficient *E. coli* mutant strain LC3 (an hsdR derivative of strain AB2829). By complementation of aroA in strain LC3, cosmids carrying wild-type and mutant alleles of aroA were isolated from the respective gene banks. We chose pAROA1, a cosmid carrying the wild-type aroA allele, and pPMG1, which carries the mutant aroA gene, for further characterization (Fig. 1). The presence of both markers on a 5.5-kb Bgl II–Hind III DNA fragment (pPMG1) supports, with our transduction data, that aroA codes for the gene conferring glyphosate resistance. Plasmid pPMG1, and its derivatives pPMG5, pPMG6, and pPMG11, conferred resistance to glyphosate both in aroA-deficient and aroA$^+$ backgrounds. By conjugation (*11*), pPMG1 was reintroduced into *S. typhimurium* strains carrying, respectively, the aroA1, aroA124, and aroA148 mutation, complementing all three and conferring the same glyphosate resistance as originally observed in strain CT7. Enzyme activity from LC3 harboring pPMG1 showed the same resistance to glyphosate inhibition as the one from strain CT7.

Growth kinetics of both *S. typhimurium* and *E. coli* strains with wild-type and mutant aroA loci were investigated. In minimal medium, strains of either *S. typhimurium* or *E. coli* harboring only the glyphosate-resistant aroA allele exhibited a 15 percent lower growth rate than the isogenic line harboring either the wild-type allele, or both wild-type and mutant alleles. At a glyphosate concentration of 100 μg/ml, wild-type *E. coli* showed 40 percent inhibition in growth rate, whereas glyphosate at 1000 μg/ml prevented any growth. The aroA *E. coli* strain LC3 harboring pPMG1 was not inhibited significantly at 2000 μg/ml.

We now provide conclusive genetic evidence that at least in enteric bacteria, the 5-enolpyruvyl-3-phosphoshikimate synthetase enzyme is the biochemical target for the herbicide glyphosate. This was accomplished by isolating and characterizing a mutation (or mutations) that has the following properties: it maps in the aroA locus; it confers high-level resistance to glyphosate; and it results in the production of an altered form of 5-enolpyruvyl-3-phosphoshikimate synthetase. A mutant was identified that confers high (2000 μg/ml) resistance to glyphosate. Since most glyphosate-resistant mutations do not map in the aroA locus, it appears that more than one locus can be altered to confer resistance. In theory, three types of glyphosate-resistant mutants other than the aroA mutants could be obtained: perhaps due to alteration in permeability, shifts in metabolic pools, and de novo recruitment of glyphosate-modifying enzymes.

Resistance conferred by a mutant aroA allele could be due to either overproduction of 5-enolpyruvyl-3-phosphoshikimate synthetase or to a structural modification of this enzyme. The properties from mutant CT7 are consistent with both possibilities: (i) enzyme activity is twice as high in the mutant as in the wild-type and (ii) the mutant enzyme is less inhibited by glyphosate. Since two cycles of mutagenesis were used, most likely two independent mutations were introduced in the aroA locus and each could contribute to either mechanism of resistance. However, the differential inhibition by glyphosate indicates that the enzyme has been structurally altered.

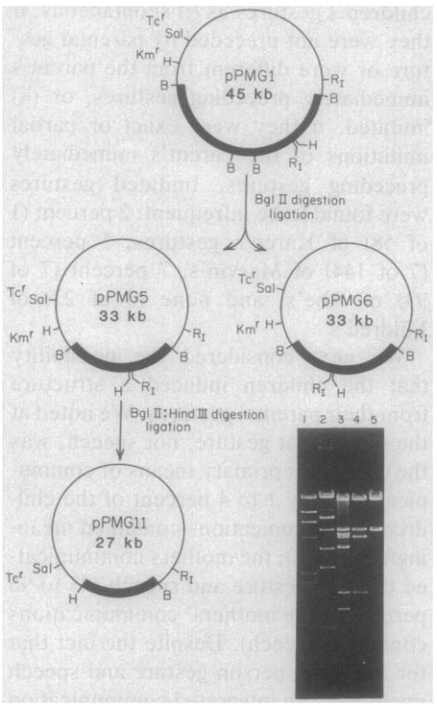

Fig. 1. Subcloning of the DNA segment that confers the aroA-glyphosate–resistant traits. Plasmid pPMG1, which contains three Bgl II fragments, was digested with Bgl II and ligated in vitro. The DNA was transformed to *E. coli* strain LC3, and colonies selected for an aroA$^+$ phenotype. Plasmids pPMG5 and pPMG6 complemented aroA mutants and contained a 10-kb Bgl II fragment in both orientations relative to the vector pVK100. Plasmid pPMG5 was digested with Bgl II and Hind III and ligated in vitro and the DNA was transformed to *E. coli* strain LC3. Plasmid pPMG11, which contains the 5.5-kb Hind III–Bgl II fragment, was selected. Restriction endonuclease sites on the plasmids for Bgl II, Eco RI, and Hind III are designated B, RI, and H, respectively. Km^r and Tc^r denote the drug resistance markers kanamycin and tetracycline. The bold lines refer to *S. typhimurium* DNA segments. In the inset gel, plasmid DNA's were digested with Bgl II and Hind III, subjected to electrophoresis on a 0.7 percent agarose gel, and stained with ethidium bromide. (Lane 1) Lambda DNA digested with Hind III. Fragment sizes are 23 kb, 9.7 kb, 6.8 kb, 4.3 kb, 2.2 kb, 1.9 kb, and 0.5 kb, respectively. (Lane 2) pAROAI DNA. (Lane 3) pPMG1 DNA. (Lane 4) pPMG5 DNA. (Lane 5) pPMG11 DNA. All plasmids contain the common 5.5-kb Bgl II–Hind III DNA segment.

Our results indicate that a structural alteration of the enzyme is the main determinant of resistance in mutant strain CT7. We have demonstrated that the primary site of action of glyphosate in enteric bacteria is the enzyme 5-enolpyruvyl-3-phosphoshikimate synthetase. This enzymatic target can be genetically altered by structural mutations to resist the inhibitive action of glyphosate. The cloning and characterization of a dominant gene conferring glyphosate resistance should facilitate considerably the long-range goal of introduction and expression in plant cells of this determinant to achieve herbicide resistance in crop plants.

LUCA COMAI
LOUVMINIA C. SEN
DAVID M. STALKER
Calgene, Inc., 1910 Fifth Street, Davis, California 95616

References and Notes

1. E. G. Jaworski, *J. Agric. Food Chem.* **20**, 1195 (1972).
2. H. Hollander and N. Amrhein, *Plant Physiol.* **66**, 823 (1980).
3. N. Amrheim *et al.*, *ibid.*, p. 830.
4. H. C. Steinruecken and N. Amrhein, *Biochem. Biophys. Res. Commun.* **94**, 1207 (1980).
5. Y. Nishioka, M. Demerec, A. Eisenstark, *Genetics* **56**, 341 (1967).
6. J. H. Miller, *Experiments in Molecular Genetics* (Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1972), pp. 431–433.
7. E. Gollub, H. Zackin, D. B. Sprinson, *J. Biol. Chem.* **242**, 5323 (1967).
8. Cells were grown for 24 hours at 37°C in M9 broth, harvested by centrifugation at 4°, washed twice with M9 salts, resuspended in 0.01*M* tris-HCl (*p*H 8.2), and sheared with a French press. The homogenate was centrifuged at 16,000*g* for 40 minutes, and the supernatant was treated with 2 percent protamine sulfate (1.0 ml of 2 percent protamine sulfate per 35 mg of protein). The precipitate was removed by centrifugation at 18,000*g* for 35 minutes. The supernatant was used for enzyme assays. Activity of the enzyme was determined by measuring the rate of release of inorganic phosphate (*12*). A typical assay mixture contained 0.1*M* malic acid buffer (*p*H 5.6), $2.4 \times 10^{-3}M$ phosphoenolpyruvate, $3.4 \times 10^{-3}M$ 3-phosphoshikimate, and enzyme fraction in a total volume of 1.5 ml. Protein concentrations were determined as described (*13*).
9. V. Knauf and E. Nester, *Plasmid* **8**, 45 (1982).
10. L. Enquist and N. Sternberg, *Methods Enzymol.* **68**, 281 (1979).
11. G. Ditta, S. Stanfield, D. Corbin, D. Helinski, *Proc. Nat. Acad. Sci. U.S.A.* **77**, 7347 (1980).
12. J. K. Heinonen and R. J. Lahti, *Anal. Biochem.* **133**, 313 (1981).
13. O. H. Lowry, N. J. Rosebrough, A. L. Farr, R. J. Randall, *J. Biol. Chem.* **193**, 265 (1951).
14. We thank C. Schilling and J. Klemencic for technical assistance and E. Nester for the 3-phosphoshikimic acid.

4 February 1983; revised 9 May 1983