THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 260, No. 8, Issue of April 25, pp. 4724-4728, 1985
Printed in U.S.A.

A Single Amino Acid Substitution in the Enzyme 5-Enolpyruvylshikimate-3-phosphate Synthase Confers Resistance to the Herbicide Glyphosate*

(Received for publication, September 14, 1984)

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The enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EC 2.5.1.19), encoded by the *aroA* locus, is a target site of glyphosate inhibition in bacteria. A glyphosate-resistant *aroA* allele has been cloned in *Escherichia coli* from a mutagenized strain of *Salmonella typhimurium*. Subcloning of this mutant *aroA* allele shows the gene to reside on a 1.3-kilobase segment of *S. typhimurium* DNA. Nucleotide sequence analysis of this mutant gene indicates a protein-coding region 427 amino acids in length. Comparison of the mutant and wild type *aroA* gene sequences reveals a single base pair change resulting in a Pro to Ser amino acid substitution at the 101st codon of the protein. A hybrid gene fusion between mutant and wild type *aroA* gene sequences was constructed. 5-Enolpyruvylshikimate-3-phosphate synthase was prepared from *E. coli* cells harboring this construct. The glyphosate-resistant phenotype is shown to be associated with the single amino acid substitution described above.

The enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EC 2.5.1.19) catalyzes the reversible transfer of the enolpyruvate moiety of phosphoenolpyruvate to the 5-hydroxy group of shikimate 3-phosphate. The product, 5-enolpyruvylshikimate 3-phosphate (EPSP[1]), is an intermediate of the biosynthetic pathway leading to chorismate and thus to aromatic amino acids and related aromatic compounds. In enteric bacteria, the enzyme is encoded by the *aroA* locus (1). EPSP synthase preparations from bacterial and plant sources (2–4) have been shown to be inhibited by the broad spectrum, postemergence herbicide glyphosate (phosphonomethylglycine, "Roundup"). In a previous communication (5), we presented physical and genetic evidence that EPSP synthase was indeed a site of glyphosate inhibition by isolating ethylmethanesulfonate-induced mutations in the *Salmonella typhimurium aroA* gene which conferred a glyphosate-resistant phenotype. A mutant *S. typhimurium aroA* gene was cloned via a broad host range cosmid vector and shown to confer glyphosate resistance to *Escherichia coli* (5). Resistance to glyphosate can also be achieved by overexpression of EPSP synthase in *E. coli* (6) and in plant cells (3).

In this study, we describe further subcloning and characterization in *E. coli* of the *S. typhimurium* mutant *aroA* locus conferring glyphosate resistance. The nucleotide sequence of the wild type *S. typhimurium aroA* gene is presented. When this sequence was compared to the DNA sequence of the ethylmethanesulfonate-induced glyphosate-resistant *aroA* gene, a single base pair change was revealed. That this base change is responsible for the glyphosate-resistant phenotype is supported by experiments in which the portion of the mutant *aroA* gene harboring the mutation is replaced by the corresponding wild type DNA sequence, resulting in a glyphosate-sensitive phenotype. Assay of purified EPSP synthase preparations revealed that the mutant enzyme is less sensitive to inhibition by glyphosate than the wild type enzyme. The reconstructed hybrid gene produces a protein identical to the wild type enzyme. This detailed analysis of the *aroA* gene is carried out with the express goal of introduction and expression of glyphosate resistance in plant cells.

EXPERIMENTAL PROCEDURES

Materials—All chemicals used were of reagent grade quality. DEAE-cellulose and phosphocellulose were obtained from Whatman Products (Kent, England). Isopropylthio-β-galactoside and 5-bromo-4-chloro-3-indolylgalactose were purchased from Bachem (Torrance, CA). Boehringer Mannheim was the source for deoxy and dideoxy nucleotide triphosphates, while [α-^{32}P]dCTP (3000 Ci/mmol), [γ-^{32}P]ATP (3000 Ci/mmol), and [^{35}S]L-methionine (800–1100 Ci/mmol) were obtained from the Radiochemical Centre (Amersham, United Kingdom). Purified phosphonomethylglycine (glyphosate) and shikimate 3-phosphate were the kind gifts of Monsanto Co. (St. Louis, MO) and Dr. Gregory Thompson, respectively. Restriction endonucleases were purchased from Bethesda Research Laboratories and used according to the supplier's specifications. New England Biolabs (Beverly, MA) was the source of *Bal*31 nuclease and *Bam*HI linkers, while Promega Biotec (Madison, WI) provided T_4 DNA ligase. Avian myeloblastosis virus reverse transcriptase was obtained from Life Sciences Inc. (Gainesville, FL).

Bacterial Strains—*E. coli* strains utilized were AB1321 (*proA2, aroA2, his4, thi, lacY, galK, xyl, mtl, tsx, supE44*), AB2829 (*aroA354, supE42*), 71-18 (*lac/pro, thi,* Su2, Flac$^+$ Iq^z Δ M15), C600 (*thr, leu, thi, lacY,tonA, supE44*), and YS1 (*thr, leu, thi, str, minA, endI*). Strains LC2 and LC3 are *hsdR* derivatives of AB1321 and AB2829, respectively (5).

Plasmids and DNA Manipulations—Plasmids pACYC184, pUC9, and pUC8 have been described elsewhere (7, 8). *E. coli* strains carrying plasmids were grown in L broth with appropriate antibiotic selection and covalently closed circular DNA forms purified from either Sarkosyl (9) or sodium dodecyl sulfate/alkaline (10) lysates by two centrifugations in CsCl/ethidium bromide gradients. The plasmid DNAs were precipitated with ethanol and dissolved in TE buffer (0.02 M Tris-HCl, pH 8.0, 0.1 mM EDTA) prior to restriction analysis. Rapid DNA preparations (clone analysis DNAs) were prepared from Triton lysates as described previously (11). Purified DNA fragments used for cloning experiments were electroeluted from 0.7 or 1.0% agarose gels. DNA fragments to be end-labeled for chemical sequencing reactions were purified from 6% (w/v) polyacrylamide gels and eluted with a buffer containing 0.5 M ammonium acetate, 0.01 M magnesium acetate, 0.01 M EDTA, and 0.1% (w/v) sodium dodecyl

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[1] The abbreviations used are: EPSP, 5-enolpyruvylshikimate 3-phosphate; kb, kilobase; Pmg^r, phosphonomethylglycine-resistant phenotype.

sulfate. All purified DNA fragments were precipitated with 2 volumes of ethanol, dried, and resuspended in TE buffer.

Ligation and Transformation of Plasmid DNA—Ligation reactions were performed in a volume of 20 μl containing 0.02 M Tris-HCl, pH 7.5, 0.03 M sodium chloride, 0.01 M magnesium chloride, 0.01 M dithiothreitol, 0.5 mM ATP, and 1 unit of T_4 DNA ligase. *E. coli* strains were transformed with plasmid DNA as described (12).

DNA Sequence Determinations—DNA segments from various plasmid restriction enzyme digests were cloned into the modified M13 phages M13mp8 and M13mp9 (13). After selection on 5-bromo-4-chloro-3-indolylgalactoside, putative clones were size screened by electrophoresis of the single-strand templates on 0.7% agarose gels. Dideoxy sequence reactions utilizing reverse transcriptase were carried out as previously described (14), and the reaction products were fractionated on 8% (w/v) and 6% (w/v) polyacrylamide/urea sequencing gels. For chemical sequencing, chemical degradations were carried out accordingly (15), except methylene blue was used as reagent for guanine modifications (16). Degradation products were run on 25% (w/v), 12% (w/v), and 8% (w/v) polyacrylamide/urea sequencing gels. Gels were dried and subjected to autoradiography.

Purification and Assay for EPSP Synthase—*E. coli* strain LC3 containing plasmids pAROA8, pPMG31, and pAROA9, respectively, was grown aerobically to midlog phase in M9 salts and 0.2% (w/v) glucose, collected by centrifugation, and stored frozen until used. Cell paste was thawed in 20 ml of buffer A (0.01 M Tris-succinate, pH 6.8, 0.1 mM dithiothreitol) and disrupted by passage through a French pressure cell. Cell debris was removed by centrifugation at 27,200 × *g* for 15 min, and the supernatant was fractionated essentially as described (17). Following the initial passage over DE52 DEAE-cellulose, fractions containing EPSP synthase activity were pooled and concentrated by addition of ammonium sulfate to 90% saturation. After centrifugation at 12,000 × *g* for 15 min, the precipitate was dissolved in 10 mM potassium citrate, pH 6.0, and 0.4 mM dithiothreitol, dialyzed overnight against this same buffer, and fractionated by phosphocellulose chromatography as described (18). Fractions containing peak enzyme activity were pooled, dialyzed against buffer A, and analyzed on denaturing polyacrylamide gels (19).

Activity of the enzyme was determined by measuring the rate of release of inorganic phosphate (20). A typical assay mixture contained 0.1 M malic acid buffer, pH 5.6, 0.001 M phosphoenolpyruvate, 0.003 M shikimate 3-phosphate, and enzyme fraction in a total volume of 1.5 ml. Protein concentrations were determined as described (21).

RESULTS

Subcloning of the aroA-Pmg^r Gene—Plasmid pPMG11 (5) contains a 5.5 kb segment of *S. typhimurium* DNA harboring the mutant *aroA* locus. This plasmid confers resistance to glyphosate up to concentrations of 2 mg/ml and complements *aroA*-deficient *E. coli* strains. Plasmid pPMG11 was digested with the restriction endonucleases *Bgl*II and *Sal*I, and the fragments were ligated into the *Bam*HI/*Sal*I cut cloning vector pACYC184. The DNA was transformed to the *aroA*-deficient *E. coli* strain LC2, and resulting clones were selected for an ampicillin-resistant, tetracycline-sensitive phenotype and complementation of *aroA* activity. As depicted in Fig. 1, the resultant clones harbored a 6.5-kb plasmid (pPMG17) containing a 2.7-kb segment of *S. typhimurium* DNA. When mapped with restriction enzymes, the 2.7-kb *Bgl*II/*Sal*I DNA segment was found to contain four *Cla*I and two *Nar*I restriction sites.

Further reduction in size of the 2.7-kb fragment was carried out by partially digesting plasmid pPMG17 with the restriction enzyme *Hpa*II and cloning the fragments into the unique *Acc*I site of pUC9. Ampicillin-resistant transformants that complemented the *aroA*-deficient *E. coli* strain LC2 were scored, and the smallest plasmid species was selected after clone analysis. The smallest plasmid obtained (pPMG31) is 4.2 kb in size and contains a 1.55-kb insert of *S. typhimurium* DNA. This plasmid complements both *aroA E. coli* mutants AB1321 and AB2829 and confers glyphosate resistance at 3 mg/ml. Nucleotide sequencing from the pPMG31 *Hin*dIII site located a putative open reading frame beginning 200 base pairs from the *Hin*dIII site extending past the initial *Nar*I restriction site.

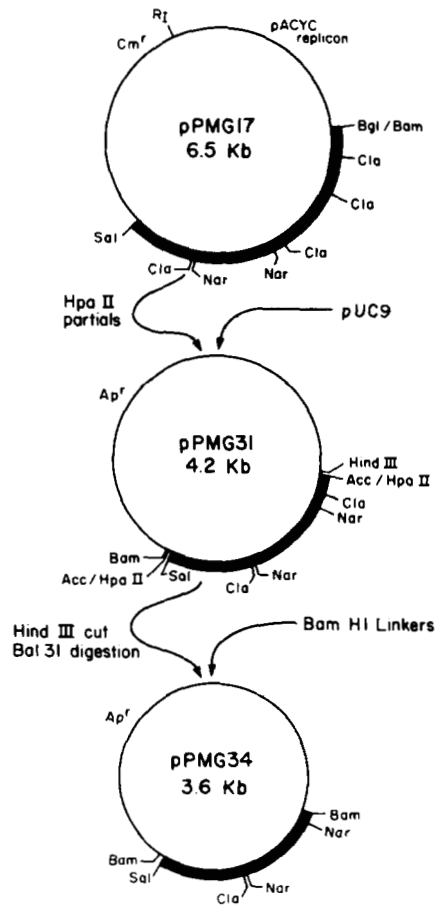

FIG. 1. **Subcloning of the *S. typhimurium aroA*-Pmg^r gene.** Construction of the plasmids shown is described in the text. Relevant restriction endonuclease sites are indicated. *Cm^r* and *Ap^r* refer to chloramphenicol- and ampicillin-resistant determinants, respectively. The *bold lines* refer to cloned *S. typhimurium* DNA segments. Plasmid pPMG34 contains 26 5′-nucleotides and 22 3′-nucleotides flanking the *aroA*-Pmg^r structural gene. These flanking bases are shown in the sequence in Fig. 2.

The *S. typhimurium* DNA segment was further reduced in size by linearizing pPMG31 at the unique *Hin*dIII site and limit-digesting the DNA with the double-strand exonuclease *Bal*31. *Bam*HI linkers were ligated onto the ends, the DNA preparation was recircularized, and the mixture was transformed to *E. coli* strain LC2. Ampicillin-resistant clones were selected, and the DNA was fractionated on acrylamide gels and screened for the presence of the internal *Nar*I fragment. The majority of plasmids containing the intact *Nar*I fragment complemented *aroA E. coli* strains. One of these clones, designated pPMG34, was a 3.6-kb plasmid containing a 1.3-kb insert of *S. typhimurium* DNA. Sequence analysis of the *Bal*31 deletion indicated the presence of an open reading frame with 26 5′-flanking nucleotides. All plasmids containing *Bal*31 deletions which lost the *Hin*dIII proximal *Nar*I site from pPMG31 were incapable of complementing *aroA*-deficient *E. coli* strains, suggesting the putative open reading frame to be the 5′ terminus of the *aroA* structural gene.

Nucleotide Sequence Analysis of the S. typhimurium aroA Gene—Plasmid pPMG31 (Fig. 1) was digested with *Taq*I, *Nar*I, and *Cla*I, respectively, the resulting DNA fragments were cloned into the unique *Acc*I site of the M13 phage vectors mp8 or mp9, and the nucleotide sequence of the inserts was

determined. Sequences from the ends of the *S. typhimurium* insert DNA were obtained by sequencing from the unique *SalI*/*Hin*dIII sites located in pPMG31. Sequences from the 3′ ends of the unique *SalI* and *Hin*dIII sites were determined by chemical degradations. The cloning strategy was very efficient as six internal *TaqI* fragments were identified within the *S. typhimurium* DNA insert. These *TaqI* fragments, when sequenced, could easily be overlapped with sequences obtained from the *ClaI* and *NarI* fragments. This strategy facilitated the sequencing of each nucleotide on both strands of the *S. typhimurium* DNA insert in plasmid pPMG31. Any regions of ambiguity appearing in the dideoxy sequencing gels were resequenced by chemical degradations until the nucleotides in that region were resolved. A similar strategy was employed to sequence the wild type *aroA* locus in plasmid pAROA8.

The antisense strand and putative amino acid sequence of the wild type *S. typhimurium aroA* gene are displayed in Fig. 2. The putative amino acid sequence of the *S. typhimurium aroA* protein is 427 codons in length with a molecular weight of 46,100. This value is in good agreement with the value obtained for the protein product synthesized in *E. coli* and in exact agreement with the length and molecular weight of the EPSP synthase obtained from the sequence of the *E. coli aroA* gene (22). The putative EPSP synthase of *S. typhimurium* is slightly acidic, containing 46 acidic residues, 42 basic residues, and 136 hydrophobic amino acids.

Identification of the Mutation in the S. typhimurium aroA Gene Conferring Glyphosate Resistance—Comparison of the nucleotide sequence of the mutant and wild type *aroA* genes identified a single base pair difference between the two. This change at nucleotide position 1703 resulted in a Pro to Ser amino acid substitution in the open reading frame. To confirm that this mutation was responsible for the glyphosate-resistant phenotype, a restriction fragment spanning that region was isolated from the wild type *aroA* gene and substituted for the identical DNA fragment of the glyphosate-resistant *aroA* gene as described in the legend to Fig. 3. Plasmid pAROA8 contains a cloned 2.7-kb *Bgl*II/*Sal*I DNA fragment subcloned from the cosmid pAROA1 (5). The 2.7-kb *Bgl*II/*Sal*I fragment of pAROA8 encodes a wild type EPSP synthase and is analogous to the fragment contained in pPMG17 (Fig. 1). Plasmid pAROA8 was digested with *Nar*I, and the 497-base pair *Nar*I fragment was isolated from agarose gels as described under "Experimental Procedures." Concurrently, plasmid pPMG34 was partially digested with *Nar*I, religated, and transformed to *E. coli* strain AB1321. Ampicillin-resistant *aroA*-deficient colonies were selected, and the plasmids were screened for the presence of two *Bam*HI and *Nar*I restriction sites, respectively. One of the plasmids, designated pΔ3, contained a deletion of the 497-base pair *Nar*I fragment from the *aroA*-Pmg^r allele. Plasmid pΔ3 was partially digested with *Nar*I, mixed with the 497-base pair *Nar*I fragment purified from the wild type *aroA* gene, ligated, and transformed to the *aroA*-deficient strain AB1321. Ampicillin-resistant colonies complementing the *aroA* deficiency were selected, and plasmids were screened for the presence of the 497-base pair *Nar*I fragment. One of these clones was designated pAROA9 (Fig. 3). Plasmid pAROA9 contains 497 base pairs from the wild type *S. typhimurium aroA* gene and 834 base pairs of the *aroA*-Pmg^r allele. *E. coli* AB1321 cells harboring pAROA9 showed similar levels of glyphosate sensitivity as cells containing plasmid pAROA8 in both liquid and solid media (data not shown). This observation suggests that the mutation specifying a glyphosate-resistant phenotype was located within the 497-base pair *Nar*I fragment of the *aroA* structural gene.

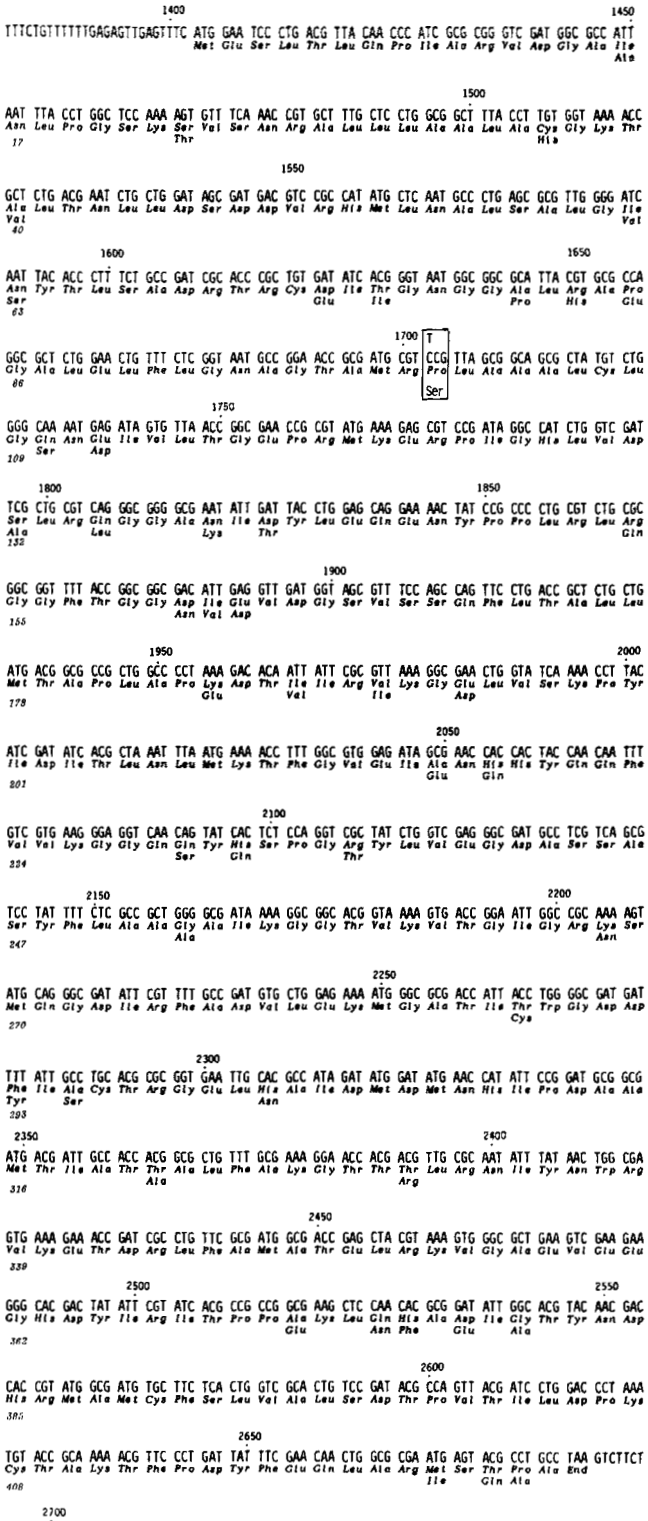

FIG. 2. **Antisense strand of the 1331-base pair sequence of the wild type *S. typhimurium aroA* gene.** Codon-specified amino acids are shown *below* the sequence. Amino acid substitutions found in the *E. coli aroA* protein are placed *underneath* the deduced *S. typhimurium aroA* protein sequence. Base pairs and amino acids are numbered accordingly. The ethylmethanesulfate-induced mutation which results in a glyphosate-resistant enzyme is shown at nucleotide 1703. The amino acid substitution (Pro to Ser) resulting from the mutation is indicated by the *box*.

To further confirm that replacing the *Nar*I fragment of the *aroA*-Pmg^r gene resulted in production of a wild type EPSP synthase, purified EPSP synthases were obtained, as de-

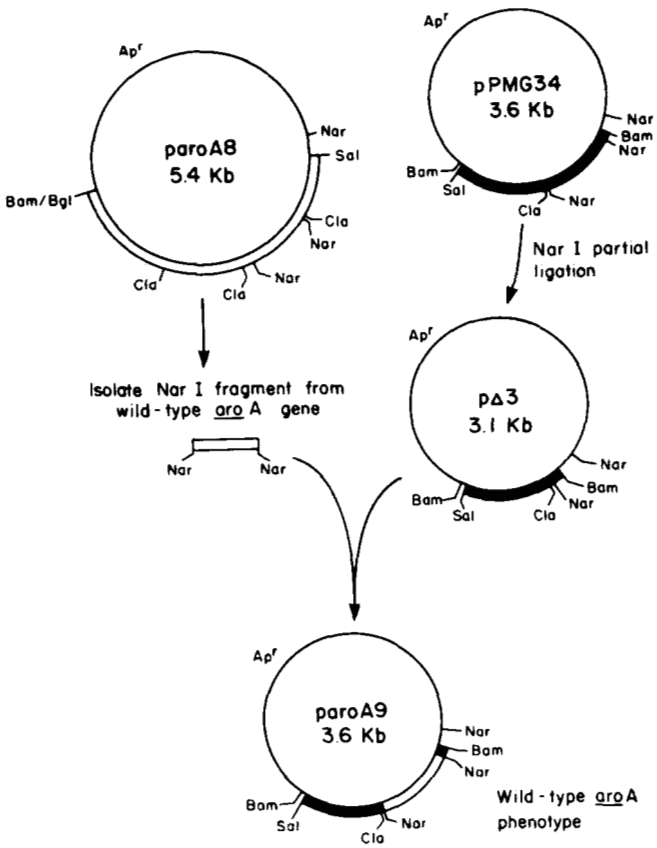

FIG. 3. **Determination of the DNA segment in the *aroA*-Pmg^r gene that confers a glyphosate-resistant phenotype.** Construction of plasmids is described in the text, and relevant restriction endonuclease sites are indicated. *Ap^r* refers to the ampicillin-resistant determinant. *Solid bold lines* indicate *S. typhimurium* DNA segments cloned from the glyphosate-resistant *S. typhimurium* strain CT7, while *bold open lines* refer to cloned DNA sequences from the wild type *S. typhimurium* strain STK1 (5).

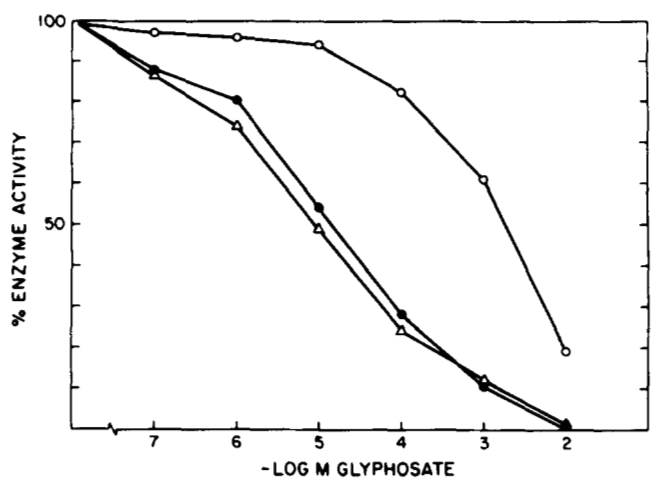

FIG. 4. **Glyphosate inhibition of EPSP synthases purified from *E. coli* strain LC3 containing plasmids pAROA8, pPMG31, and pAROA9, respectively.** The enzymes were purified and assayed as described under "Experimental Procedures." ○——○, EPSP synthase encoded by pPMG31; ●——●, EPSP synthase encoded by pAROA8; △——△, EPSP synthase encoded by pAROA9.

scribed under "Experimental Procedures," from *E. coli* strain LC3 (*aroA*-deficient) harboring plasmids pAROA8, pPMG31, and pAROA9, respectively. The three EPSP synthase preparations exhibited comparable specific activities and were judged greater than 95% homogeneous by analysis on denaturing polyacrylamide gels, the major component being a protein of $M_r \sim 42{,}000$ (data not shown). As described in the legend to Fig. 4, EPSP synthases encoded by plasmids pAROA8 (wild type *aroA* gene), pPMG31 (glyphosate-resistant *aroA* gene), and pAROA9 (*Nar*-I-substituted *aroA* gene) were assayed for the conversion of shikimate 3-phosphate to 5-enolpyruvylshikimate 3-phosphate in the presence of varying glyphosate concentrations. At 10 mM glyphosate, EPSP synthases encoded by plasmids pAROA8 and pAROA9 are fully inhibited, while the enzyme specified by plasmid pPMG31 retains 20% activity. Inhibition at 50% for the pAROA8- and pAROA9-encoded enzymes occurs at 0.01 mM. The EPSP synthase encoded by plasmid pPMG31 is 50% inhibited at a glyphosate concentration of 1.2 mM. These data support evidence that the mutation conferring a glyphosate-resistant phenotype is located within the 497-base pair *Nar*I fragment of the glyphosate-resistant *aroA* gene. The data in Fig. 4 also show the mutant EPSP synthase to be more resistant to glyphosate than the wild type enzyme encoded by pAROA8 at the substrate concentrations tested.

Again, sequencing of both strands of the 497-base pair *Nar*I fragment and comparison to the mutant gene sequence showed only the single nucleotide difference at position 1703 of the sequence (Fig. 2). This nucleotide change results in a substitution of the amino acid serine for proline at that codon. These data indicate that a single amino acid substitution (Pro to Ser) at the 101st codon in the *S. typhimurium aroA* gene is responsible for a glyphosate-resistant EPSP synthase.

DISCUSSION

In this study, we describe the characterization of a mutation in the enzyme 5-enolpyruvylshikimate-3-phosphate synthase that confers resistance to the herbicide glyphosate. Nucleotide sequence analysis of the *aroA*-Pmg^r gene has revealed a single open reading frame 427 amino acid residues in length coding for a $M_r = 46{,}100$ protein. Comparison of the *E. coli* (22) and *S. typhimurium aroA* genes indicates a 21% divergence in the nucleotide sequence and an 11% difference in the amino acid sequence. These values compare favorably with values obtained comparing the nucleotide and amino acid sequences of genes in the tryptophan operons of *E. coli* and *S. typhimurium* (23, 24). Of the nucleotide differences between the *aroA* genes of *E. coli* and *S. typhimurium* which represent synonymous codon changes, 65% are in the third position, while 7.8% are first or second position substitutions. A number of highly conserved amino acid stretches exist between the *E. coli* and *S. typhimurium* EPSP synthases. The 15 N-terminal residues of the two polypeptides are identical, and there is a highly conserved region from amino acid residue 86 through residue 131. In the C-terminal region of the two proteins, highly homologous regions exist in the peptide regions 302–371 and 381–422, respectively. The 5 residues at amino acid positions 81–85 are interesting in that the proline residue positioned at amino acid 81 in the *E. coli* enzyme is lost in the *S. typhimurium* sequence. However, a proline residue now appears at position 85 in the *S. typhimurium* EPSP synthase. All other proline residues are exactly conserved when comparing the two proteins except for the proline residue occurring at amino acid position 426 at the C-terminal end.

The site of the glyphosate-resistant mutation was identified by sequencing the wild type and mutant *aroA* genes, which revealed a single nucleotide change (position 1703) resulting in a Pro to Ser amino acid substitution at the 101st residue of the amino acid sequence. Further evidence was obtained from glyphosate inhibition curves on EPSP synthase preparations purified from *E. coli* strains containing plasmids

pARO8, pPMG31, and pAROA9, respectively. The purified enzyme encoded by pAROA9 was inhibited by glyphosate at concentrations identical to those derived for the purified wild type EPSP synthase. Nucleotide sequence information and phenotypic analysis of purified EPSP synthases strongly suggest the C to T transition mutation, resulting in a single amino acid substitution (Pro to Ser), to be responsible for the glyphosate-resistant phenotype. It is also interesting that the site of the mutation resides within one of the highly conserved peptide regions of the *E. coli* and *S. typhimurium* enzymes (amino acids 86–131).

We originally reported (5) that two rounds of ethylmethanesulfonate mutagenesis were required to obtain a *S. typhimurium* strain resistant to high concentrations of glyphosate. Nucleotide sequence analysis of the operon containing the *aroA* gene revealed the first mutation to reside in the promoter region of the operon.[2] This regulatory mutation conferred low levels (350 μg/ml) of glyphosate resistance by elevating the wild type EPSP synthase levels 2- or 3-fold. The second mutation, described in this study, results in a structurally altered EPSP synthase resistant to high glyphosate concentrations.

Glyphosate is known to be a competitive inhibitor of phosphoenolpyruvate (17). Preliminary kinetic analysis[3] of purified wild type and mutant enzymes indicates that the altered EPSP synthase, while having a reduced affinity for glyphosate, has an increased affinity for phosphoenolpyruvate and a slightly increased affinity for shikimate 3-phosphate. These results suggest that the Pro to Ser amino acid substitution described above does not result in a kinetically deficient enzyme. In fact, an increase in affinity for substrate by the mutant enzyme may contribute further resistance to glyphosate.

In summary, a single point mutation in the gene encoding EPSP synthase has been characterized which confers a glyphosate-resistant phenotype. It is hoped that this genetically altered EPSP synthase may provide herbicide resistance when the mutant *aroA* gene is expressed in plant cells.

[2] D. M. Stalker, W. R. Hiatt, and L. Comai, manuscript in preparation.

[3] G. Thompson and L. J. Huang, manuscript in preparation.

Acknowledgments—We wish to thank L. J. Huang, K. McBride, S. Burger, and T. Friedemann for excellent technical assistance and John Caton for help with the nucleotide sequence analysis.

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