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Genetics

Single amino acid substitutions in the enzyme acetolactate synthase confer resistance to the herbicide sulfometuron methyl

(mutations/bacteria/yeast/amino acid biosynthesis/sulfonylurea)

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ABSTRACT **Sulfometuron methyl, a sulfonylurea herbicide, blocks growth of bacteria, yeast, and higher plants by inhibition of acetolactate synthase (EC 4.1.3.18), the first common enzyme in the biosynthesis of branched-chain amino acids. Spontaneous mutations that confer increased resistance to the herbicide were obtained in cloned genes for acetolactate synthase from *Escherichia coli* and *Saccharomyces cerevisiae*. The DNA sequence of a bacterial mutant gene and a yeast mutant gene revealed single nucleotide differences from their respective wild-type genes. The mutations result in single amino acid substitutions in the structurally homologous amino-terminal regions of the two proteins, but at different positions. The bacterial mutation results in reduced levels of acetolactate synthase activity, reduced sensitivity to sulfometuron methyl, and unaltered resistance to feedback inhibition by valine. The yeast mutation results in unaltered levels of acetolactate synthase activity, greatly reduced sensitivity to sulfometuron methyl, and slightly reduced sensitivity to valine.**

Considerable effort has been devoted to studies on the mode of action of herbicides because of their agricultural importance. The molecular targets of a number of herbicides—e.g., amitrole (1), glyphosate (2–4), atrazine and diuron (5, 6), phosphinothricin (7, 8), sulfonylureas (9–12), and imidazolinones (13)—have been determined. Sulfometuron methyl (SM), *N*-[(4,6-dimethylpyrimidin-2-yl)aminocarbonyl]-2-methoxycarbonylbenzenesulfonamide, is a potent sulfonylurea herbicide that inhibits growth of bacteria and yeast, as well as higher plants, by blocking the activity of acetolactate synthase [ALS; acetolactate pyruvate-lyase(carboxylating), EC 4.1.3.18], which catalyzes the first common step in the biosynthesis of branched-chain amino acids (9–12). Interestingly, ALS is also the target of a structurally unrelated class of herbicides, the imidazolinones (13).

ALS catalyzes two reactions, the condensation of two pyruvate molecules to yield acetolactate and the condensation of pyruvate and ketobutyrate to yield acetohydroxybutyrate. Catalytic activity requires FAD (flavin adenine dinucleotide), thiamin pyrophosphate, and a divalent cation (14, 15). The requirement for FAD is unusual because the reactions catalyzed by ALS involve no net reduction or oxidation. Inhibition of ALS by SM is competitive with pyruvate, but binding of SM is apparently facilitated by binding of the first pyruvate molecule to the enzyme (14).

Three major isozymes of ALS have been identified in enteric bacteria. Isozymes I and III, but not II, are sensitive to feedback inhibition by valine (16). Isozymes II and III, but not I, are sensitive to SM (17). The three bacterial isozymes are composed of two different subunits. Nucleotide sequences of the genes for the two subunits of isozymes I, II, and III in *Escherichia coli* indicate that they are organized as operons, *ilvBN* (18, 19), *ilvGM* (20–22), and *ilvIH* (20), respectively. Comparison of the deduced amino acid sequences of the large subunits of the *E. coli* isozymes shows three regions with about 50% conserved amino acids, comprising about two-thirds of the proteins and separated by sequences sharing little discernible homology. Amino acid sequence conservation, though less extensive, is also evident among the small subunits of the bacterial isozymes (18–20).

In the yeast *Saccharomyces cerevisiae*, a single gene, *ILV2*, essential for ALS activity has been identified (12, 23–26). The DNA sequence of the yeast *ILV2* gene has revealed that the polypeptide encoded by it is homologous to the large subunit of the bacterial ALS isozymes (23). It is not known if yeast ALS is composed of two different subunits. At the amino acid sequence level the *ILV2* protein shows the same structural organization and degree of amino acid sequence conservation as the large subunits of the bacterial isozymes, except for about 90 amino acids at the amino terminus, likely to be involved in the translocation of the protein into mitochondria (23, 27).

Mutants of *Salmonella*, yeast, and tobacco resistant to SM have been shown to produce SM-resistant forms of ALS. In *Salmonella* and yeast the mutations have been genetically mapped to the structural genes for *ilvGM* and *ILV2*, respectively (9, 12). In tobacco the mutations cosegregate in genetic crosses with the production of SM-resistant ALS (10).

To establish the molecular basis for SM resistance we have determined the nucleotide sequence of two mutant genes that encode SM-resistant ALS, one derived from *E. coli* and the other from yeast. We have also characterized the ALS activity of the bacterial and yeast mutant enzymes with respect to their catalytic activity, sensitivity to SM, and sensitivity to feedback inhibition by valine.

MATERIALS AND METHODS

Strains and Media. The following *E. coli* K-12 strains were used in this study: HB101 (*proA2, leuB6, thi-1, recA13, hsdR, hsdM, supE44, lacZ4, strA*); MI162 (*ilvG603, thr-10, car-94, relA1, thi-1*) (28), obtained from the *E. coli* Genetic Stock Center (Yale University); MF2000 (*ilvB800*::Mu1, *Bgl32, ilv-115, thi-1, argE3, rpsL31,* Δ[*ara, leu, ilvHI*]*863, mtl-1, xyl-5, galK2, lacY1, recA1*) (29) obtained from M. Freundlich; JM101 (Δ*lac–pro, supE, thi,* F′ *traD36, proAB, lacI*^{q}*–Z*Δ*M15*) (30). Bacterial cells were grown in LB medium (31), 2XYT medium (30), or M9 minimal medium (32) supplemented, when required, with the necessary nutrients, ampicillin (50 μg/ml), chloramphenicol (25 μg/ml), and valine (0.5 mg/ml). Stock solutions of SM in 0.1 M NaOH were used to make plates. Yeast strain DBY947 (*matα, ura3-52, ade2-101*) was used in this study and grown as described previously (12).

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Abbreviations: SM, sulfometuron methyl; ALS, acetolactate synthase; kb, kilobase(s); bp, base pair(s); Tricine, *N*-[tris(hydroxymethyl)methyl]glycine.

Isolation of SM-Resistant Mutants with Mutations in *ilvG603*. HB101(pB1) cells were grown to late logarithmic phase in LB medium, harvested, washed once and resuspended in equal volume of 1× minimal salts, and plated (at 100 μl per plate) onto minimal plates containing ampicillin, valine, and SM at 20 μg/ml. After 3 days at 37°C, 5–10 resistant colonies appeared per plate. All independent mutants tested contained plasmid-borne mutations.

Construction of Yeast Strain YT689. The yeast plasmid pCP2-4-10, which contains the *ILV2-410* mutation, was isolated as described previously (12). A 5.9-kilobase (kb) *Cla* I to *Sal* I fragment carrying the *ILV2-410* mutation was cloned in the integrating vector YIp5. Uracil-independent transformants of DBY947 were obtained and the structure of the integrated plasmid was verified by Southern blot hybridization (33). Uracil-dependent segregants were selected on SD minimal medium (40) containing 5-fluoroorotic acid (400 μg/ml) supplemented with adenine and uracil. Several uracil-dependent SM-resistant segregants were isolated and tested for the presence of a single chromosomal *ILV2* gene and no plasmid DNA by Southern blotting, yielding strain YT689.

Reagents. FAD, thiamin pyrophosphate, *N*-[tris(hydroxymethyl)methyl]glycine (Tricine), pyruvate, and amino acids were obtained from Sigma. SM was provided by DuPont. All restriction endonucleases and DNA-modifying enzymes and linkers were from Bethesda Research Laboratories or New England Biolabs and were used as suggested by the suppliers. DNA sequencing was performed by the dideoxy method (34), using a DNA sequencing kit from New England Biolabs.

RESULTS

Wild-type *E. coli* K-12 has a defective *ilvG* gene and, therefore, does not produce ALS II. Because ALS isozymes I and III are sensitive to valine inhibition, valine blocks growth of wild-type *E. coli* K-12 by causing starvation for isoleucine (28). *E. coli* K-12 strain MI162 carries a frameshift mutation, *ilvG603*, in the cryptic *ilvG* gene that restores its function. This allows growth of this strain in the presence of valine, since ALS II is insensitive to it (28, 35). The functional *ilvG603* gene and its gene product will be referred to as wild type in this paper. The *ilvG603* gene was isolated by inserting MI162 chromosomal DNA into vector pBR328 and selecting for valine resistance in strain HB101, which lacks a functional *ilvG* gene, yielding plasmid pCR17. Isolation of the *ilvG603* gene, on a 4.8-kb *Hin*dIII fragment in recombinant plasmid pCR17, was confirmed by the ability of the cloned gene to produce a valine-insensitive ALS enzyme and by a comparison of its physical map with that of the published DNA sequence of *ilvG* gene (22, 35). While strain MI162 is sensitive to SM at 33 μg/ml in the presence of valine at 0.5 mg/ml on minimal plates, HB101(pCR17) can tolerate SM at 150 μg/ml, presumably because the increased copy number of the plasmid-borne *ilvG603* gene results in the production of higher levels of ALS II. This tolerance to the high concentration of SM, which is near the solubility limit of the compound, was reduced by replacing the promoter of the *ilvG603* gene with the promoter region of the nopaline synthase gene of *Agrobacterium tumefaciens*. The nopaline synthase promoter region acts as a weak promoter in *E. coli* (unpublished observation). The resultant plasmid pB1 (Fig. 1) carries a functional *ilvG603* gene because when used to transform HB101 it confers valine resistance and when used to transform MF2000, which lacks all ALS activity, it restores isoleucine and valine prototrophy. HB101(pB1) can grow on minimal plates containing valine at 0.5 mg/ml in the absence but not in the presence of SM at 20 μg/ml.

Several independent spontaneous mutants of HB101(pB1) resistant to SM at 20 μg/ml (in the presence of valine) were isolated. Plasmid DNA from five independent SM-resistant

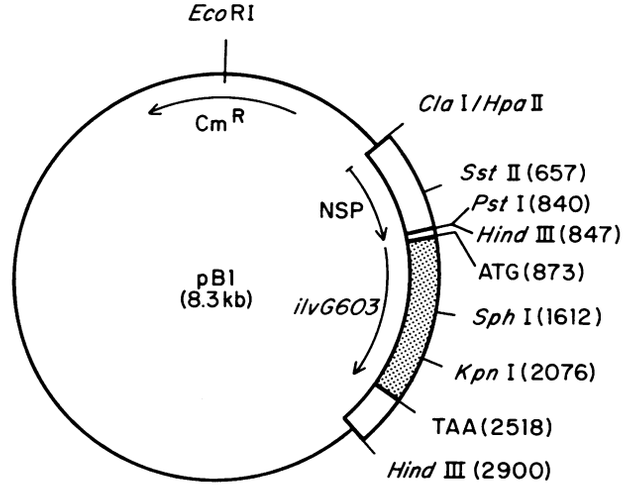

FIG. 1. Physical map of plasmid pB1. The 847-base-pair (bp) *Hpa* II–*Hin*dIII sequence carrying the nopaline synthase promoter (NSP) was constructed by addition of a *Pst* I/*Hin*dIII linker at the initiation codon of the nopaline synthase gene (36, 37). The 2.06-kb *Hin*dIII fragment carrying the *ilvG603* gene without its promoter was derived from the 4.8-kb *Hin*dIII fragment of pCR17 by the creation of a *Hin*dIII site at the *Hin*fI site 20 bp upstream of the *ilvG603* initiation codon (15, 22). The vector is a derivative of pBR325 lacking a *Pst* I site in the ampicillin resistance gene. The numbers in parentheses refer to the distance in bp from the *Hpa* II site of the nopaline synthase promoter fragment. The stippled region is the coding sequence of *ilvG603*, bounded by ATG and TAA codons as indicated. Cm^R, chloramphenicol resistance.

mutants, when used to transform HB101 cells, conferred resistance to SM at 20 μg/ml. An SM resistance mutation in a plasmid designated pB1SUR10 was localized by restriction fragment substitutions to a 772-bp *Pst* I–*Sph* I fragment, containing the first 739 bp of the *ilvG603* coding sequence (Table 1). The nucleotide sequences of the *Pst* I–*Sph* I fragments from both pB1 and pB1SUR10 were determined. Comparison of these sequences revealed a single nucleotide change (C to T) that results in substitution of valine for alanine at amino acid residue 26 (Fig. 2). Comparison of these sequences with the published sequence of the *E. coli ilvG* gene (22) revealed a silent T-to-C change at nucleotide 642 in both wild-type and mutant fragments.

The molecular cloning of the yeast *ILV2* gene, isolation of spontaneous mutations in the cloned gene that confer SM resistance and result in the production of SM-resistant ALS, and the nucleotide sequence of the wild-type gene have been previously described (12, 23). Localization of the mutation in one of the mutant genes, *ILV2-410*, was accomplished by *in vivo* recombination between the wild-type gene and restriction fragments derived from the mutant gene. The presence of the mutation on a particular DNA fragment was estab-

Table 1. Localization of bacterial SM-resistance mutation

Donor fragment	Recipient vector	Response to SM
Pst I–*Kpn* I/pB1	pB1SUR10	25/25 sensitive
Pst I–*Kpn* I/pB1SUR10	pB1	25/25 resistant
Sst II–*Sph* I/pB1	pB1′	16/16 sensitive
Sst II–*Sph* I/pB1SUR10	pB1′	13/13 resistant
Sph I–*Kpn* I/pB1	pB1′	15/15 sensitive
Sph I–*Kpn* I/pB1SUR10	pB1′	30/30 sensitive

pB1′ is a derivative of pB1 in which the *Sph* I site in the *ilvG603* gene is unique. The indicated donor fragments and recipient vectors were isolated from agarose gels after electrophoretic separation, ligated, and used to transform HB101 cells to valine resistance. Transformants were tested for growth in the presence of SM (20 μg/ml) plus valine.

FIG. 2. Deduced amino acid sequences (standard one-letter notation) of wild-type and SM-resistant yeast ALS and *E. coli* ALS isozyme II. The sequences of the wild-type proteins have been described previously (21–23). The | indicates conserved amino acid residues. The arrows indicate amino acid substitutions that result in SM-resistant ALS.

lished by the appearance of SM-resistant recombinants in yeast cells cotransformed with a plasmid-borne wild-type gene and the DNA fragment from the mutant gene. This marker rescue indicated that the mutation was located in the first 630 nucleotides of the gene. DNA sequencing revealed a C-to-T transition mutation at nucleotide 574. The complete sequence of the *ILV2-410* gene, determined by using previously described oligonucleotide primers (23), confirmed that this mutation was the only difference between the mutant and wild-type genes. The mutation results in a proline-to-serine change in the amino acid sequence of yeast ALS (Fig. 2).

For ALS assays, plasmid pB1 and its derivative pB1SUR10 were used to transform MF2000. Since MF2000 lacks all ALS isozymes, the ALS activity in the transformants is due solely to the introduced plasmids. The ALS activity in extracts of MF2000(pB1SUR10) is about one-fourth of that in the extracts of MF2000(pB1) (Table 2). This may be due to reduction in the catalytic activity or reduced levels of the protein. Although the activity of the mutant enzyme is reduced, it is about 4-fold more resistant to 0.1 mM SM than the wild-type enzyme (Table 2). The mutant enzyme is resistant to feedback inhibition by valine, as is the wild-type enzyme (Table 2).

To compare the properties of the mutant and wild-type yeast ALS enzymes, yeast strain YT689, in which the chromosomal *ILV2* gene was replaced by *ILV2-410*, was constructed as described in *Materials and Methods*. This results in the production of ALS from a single, stable *ILV2-410* gene with no background from the wild-type gene product. Total ALS activity levels measured in toluene-permeabilized yeast cells carrying the wild-type or the mutant *ILV2* gene are the same, suggesting that the mutant enzyme has no major change in catalytic activity. In contrast, there is a large difference in the sensitivities of the wild-type and mutant enzymes to SM (Fig. 3*A*). Wild-type ALS activity is inhibited to 50% at about 0.12 μM SM, whereas the ALS produced by the *ILV2-410* gene is unaffected by 25-fold higher concentrations. Specific feedback inhibition of yeast ALS with valine has been previously observed and shown to be a labile property of the enzyme (39). The effect of valine on wild-type and mutant yeast ALS differs in two ways (Fig. 3*B*). First, higher concentrations of valine are required to achieve the same level of inhibition of the mutant enzyme as seen for the wild type; second, the maximum inhibition obtained with the mutant enzyme is about 50% compared to 75% for the wild type.

DISCUSSION

The sulfonylurea herbicide SM blocks growth of bacteria, yeast, and higher plants by inhibiting ALS (9–12). SM-

Table 2. ALS activity in extracts of *E. coli* MF2000 containing plasmid pB1 or plasmid pB1SUR10

Culture	ALS specific activity, ΔA_{530} per mg protein: No addition	Valine	SM + valine	% of uninhibited activity
MF2000/pB1	27.0	30.0	6.2	21
MF2000/pB1SUR10	7.8	8.8	6.7	76

Derepressed cultures of MF2000(pB1) and MF2000(pB1SUR10) grown as described (26) were harvested, washed, and resuspended in cold 50 mM Tricine/NaOH, pH 7.0/0.2 mM dithiothreitol. The cells were sonicated and centrifuged at 11,000 × *g* at 4°C, and the resultant supernatant was used for enzyme and protein assays essentially as described in refs. 9 and 38, respectively. L-Valine and SM, when present, were at 1 mM and 0.1 mM, respectively. The enzyme reaction was at 37°C for 1 hr.

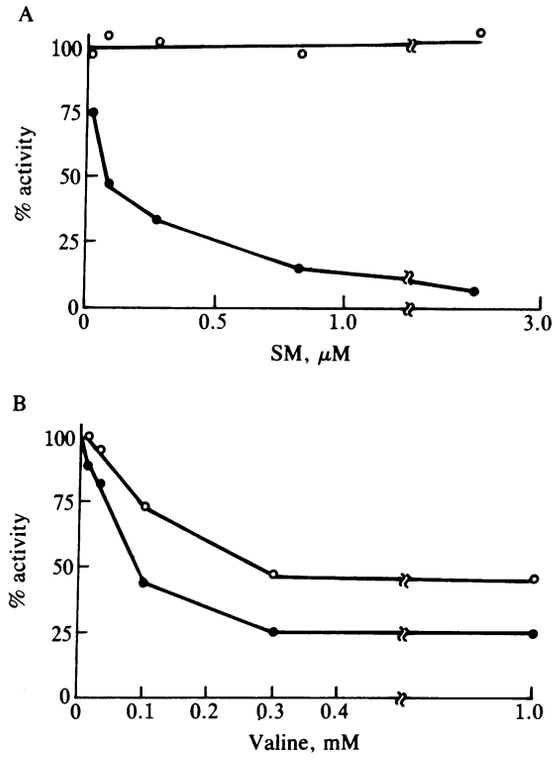

FIG. 3. Effect of SM or valine on wild-type and mutant yeast ALS. ALS activity was measured in 60-min fixed-time assays from toluene-permeabilized yeast cells as described (12). The 100% activity was approximately 110 ΔA_{530} per mg of protein for both the wild-type (●) and mutant (○) ALS. (*A*) ALS activity at various concentrations of SM. (*B*) ALS activity at various concentrations of valine.

resistance mutations in *Salmonella typhimurium* (9), *E. coli* (this paper), and yeast (12) produce herbicide-resistant ALS and map at the loci of the structural genes that encode ALS. We report the molecular basis of two SM-resistance mutations, one in the *E. coli ilvG603* gene, which encodes the large subunit of ALS isozyme II, and one in the *ILV2* gene, which encodes the corresponding polypeptide in the yeast *S. cerevisiae*. Both mutations are single nucleotide changes resulting in single amino acid substitutions in the proteins. The mutant enzymes show reduced sensitivities to SM but are otherwise phenotypically very different.

The *E. coli* and the yeast SM-resistance mutations described above result in amino acid substitutions in the 160-residue amino-terminal conserved region of the two proteins (Fig. 2). This may be coincidental or may indicate that this region is particularly important in the binding of the herbicide. Sequence analysis of additional mutations may provide further evidence indicating a unique role for the amino-terminal conserved region in the binding of SM to the enzyme.

The mutation in the bacterial gene changes an alanine residue to valine in the middle of a highly conserved stretch of amino acids (Fig. 4*A*). The valine-for-alanine substitution, although generally considered a conservative change in amino acid sequence, results in an enzyme not only with more resistance to SM inhibition but also with considerably reduced catalytic activity. Interestingly, *E. coli* ALS isozyme I, the only natural ALS enzyme known to lack an alanine at this position (18, 19), is also resistant to SM (17). However, isozyme I differs from the other ALS enzymes at many other positions, so the effect of this substitution remains to be determined. The mutation in yeast *ILV2* changes a proline residue to serine in another stretch of conserved amino acids (Fig. 4*B*). In contrast to the discussion above, *E. coli* isozyme I, which is resistant to SM, has a proline at this position as the sensitive yeast wild-type enzyme does, whereas *E. coli* isozyme II, which is sensitive to SM, has a serine. This points out the importance of the context of the substitutions in determining the properties of the particular enzyme. Ultimately structural analysis of the ALS–SM complex is needed to determine the details of the herbicide–protein interaction.

The SM-resistant ALS enzymes that result from these mutations have very different characteristics. The mutant bacterial ALS II has reduced activity in crude extracts, about one-fourth that of the wild-type enzyme, but the activity of

	Enzyme	Sequence
A		
	E. coli ALS II	V F G Y P G G A I m P V Y D A L
	Mutant ALS II	V F G Y P G G v I m P V Y D A L
	E. coli ALS I	V t G i P G G s I L P V Y D A L
	E. coli ALS III	V F G Y P G G A v L d i Y D A L
	Yeast ALS	V F G Y P G G A I L P V Y D A i
B		
	Yeast ALS	T G Q V p t S a I G T D A F Q E
	Mutant yeast ALS	T G Q V s t S a I G T D A F Q E
	E. coli ALS I	T G Q V p a S m I G T D A F Q E
	E. coli ALS II	T G Q V s a p f I G T e A F Q E
	E. coli ALS III	s G Q V a t S l I G y D A F Q E

FIG. 4. Comparisons of deduced ALS amino acid sequences in the vicinity of SM-resistance mutations. The sequences of *E. coli* ALS isozymes I, II, and III and yeast ALS have been described previously (18–20, 22, 23). Lowercase letters indicate nonconserved amino acids, and underlines indicate amino acid substitutions in mutant enzymes. (*A*) Region near SM-resistance mutation in *E. coli* ALS isozyme II. (*B*) Region near SM-resistance mutation in yeast ALS.

the mutant enzyme is about four-fold more resistant to SM than the wild-type enzyme. If the *in vitro* enzyme assays reflect the *in vivo* enzyme activities, the herbicide-resistance mutation would be expected to confer only a marginally more SM-resistant growth phenotype, as is observed. In contrast, the *ILV2* mutation described above encodes an ALS enzyme that shows no detectable reduction in activity and is unaffected by SM at a concentration 25-fold higher than that which results in 50% inhibition of the wild-type enzyme. Strains carrying this mutation can grow at a concentration of SM 30-fold higher than that sufficient to inhibit wild-type growth. The sensitivity of the mutant yeast enzyme to end-product inhibition by valine is also reduced, but not so dramatically.

The mutant ALS enzymes in four bacterial strains bearing *ilvG603* SM-resistance mutations showed similar phenotypes to the mutant enzyme described above. Among about 50 SM-resistance mutations in the yeast *ILV2* gene, at least 10 phenotypically distinct alleles have been isolated (12). It is possible that *E. coli* ALS II, in contrast to the yeast ALS, can be mutated to a very limited number of only slightly SM-resistant forms. Alternatively, differences in the genetic selections used to identify the SM-resistant mutants in *E. coli* and yeast, such as the presence of valine in bacterial selection plates, may have been an important determinant in the mutations selected. In any case, the ability to obtain SM-resistant ALS enzymes with different phenotypic properties will be useful for elucidating the details of the herbicide–enzyme interaction, allosteric inhibition, and structural features important to catalytic activity. In addition, different resistant forms of ALS provide alternatives for genetic engineering of herbicide-resistant crops.

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