

GENETIC ANALYSIS OF MUTANTS OF *SACCHAROMYCES CEREVISIAE* RESISTANT TO THE HERBICIDE SULFOMETURON METHYL

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Manuscript received July 13, 1984

Revised copy accepted September 10, 1984

ABSTRACT

Sulfometuron methyl (SM), a potent new sulfonylurea herbicide, inhibits growth of the yeast *Saccharomyces cerevisiae* on minimal media. Sixty-six spontaneous mutants resistant to SM were isolated. All of the resistance mutations segregate 2:2 in tetrads; 51 of the mutations are dominant, five are semidominant and ten are recessive. The mutations occur in three linkage groups, designated *SMR1*, *smr2* and *smr3*. Several lines of evidence demonstrate that the *SMR1* mutations (47 dominant and four semidominant) are alleles of *ILV2* which encodes acetolactate synthase (ALS), the target of SM. First, *SMR1* mutations result in the production of ALS enzyme activity with increased resistance to SM. Second, molecular cloning of the *ILV2* gene permitted the isolation of mutations in the cloned gene which result in the production of SM-resistant ALS. Finally, *SMR1* mutations map at the *ILV2* locus. The *smr2* mutations (four recessive, two dominant and one semidominant) map at the *ptr1* (pleiotropic drug resistance) locus and show cross-resistance to other inhibitors, typical of mutations at this locus. The *smr3* mutations (six recessive and two dominant) define a new gene which maps approximately midway between *ADE2* and *HIS3* on the right arm of chromosome XV.

SULFOMETURON methyl (SM), *N*-[(4,6-dimethylpyrimidin-2-yl) aminocarbonyl]-2-methoxycarbonylbenzenesulfonamide, is one of a new group of herbicides designated sulfonylureas which are notable for very high herbicidal activity and low mammalian toxicity. A number of observations with plants suggested that these herbicides inhibit a basic cellular function rather than the more common herbicidal target, photosynthesis (RAY 1982a,b). Isolation and genetic characterization of tobacco mutants resistant to these herbicides demonstrated that a single nuclear gene mutation could confer resistance at the whole plant level (CHALEFF and RAY 1984).

It has recently been discovered that SM inhibits growth of several bacterial species including *Salmonella typhimurium* (LAROSSA and SCHLOSS 1984). LAROSSA and SCHLOSS used the bacterial system to identify the molecular target of SM as acetolactate synthase (ALS) (EC 4.1.3.18) isozyme II, encoded by *ilvG*. This enzyme, also known as acetohydroxyacid synthase, catalyzes the first

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TABLE 1

List of strains

Name	Genotype	Source
FY138	α <i>aas3 his4 ura3-50</i>	S. C. FALCO, Massachusetts Institute of Technology (M.I.T.)
DBY640	a <i>ade2-1</i> (s288c genetic background)	D. BOSTEIN, M.I.T.
DBY917	α <i>his4 lys2</i> (s288c genetic background)	M. CARLSON, M.I.T.
CG379	α <i>ade5 his7-2 leu2-3 leu2-113 trp1-289 ura3-52</i>	C. GIROUX, University of Chicago
DBY890	α <i>arg1 his7 lys7 met6 gal4</i>	G. KAWASAKI, University of Washington
JG204	a <i>cyh3 leu2 his3 gal2 can1</i>	J. GOLIN, University of Oregon
FY242	α <i>his3 trp1 ura3</i>	S. C. FALCO, E. I. du Pont de Nemours and Company

common step in the biosynthesis of isoleucine and valine. Genetic and biochemical studies indicate that ALS is the target of the two structurally similar herbicides, chlorsulfuron and SM, in plants as well (CHALEFF and MAUVAIS 1984; RAY 1984).

The yeast *Saccharomyces cerevisiae* provides an ideal eukaryotic model system to investigate the action of sulfonylurea herbicides at the cellular and molecular levels. In this paper we report the isolation and characterization of yeast mutants resistant to the herbicide SM. We show that resistance mutations arise at three different genetic loci. We demonstrate that the molecular target of the herbicide in yeast, as in bacteria and higher plants, is the amino acid biosynthetic enzyme ALS. We also describe the molecular cloning of the wild-type *ILV2* gene, which encodes ALS, and the isolation of several mutant alleles of *ILV2*, which encode SM-resistant variants of this enzyme.

MATERIALS AND METHODS

Yeast strains and culture media: The yeast strains used in this study are listed in Table 1. Formulations used for media (YEPD for nonselective growth; SD with appropriate supplements for selective growth and scoring of nutritional markers) are given in SHERMAN, FINK and LAWRENCE (1974). Sporulation media contained 2% potassium acetate; 0.1% glucose; 0.25% Bacto-yeast extract (Difco) and 1.5% Bacto-agar (Difco).

SM was provided by the Du Pont Agricultural Chemicals Department. For addition to solid media, SM (molecular weight 364) was dissolved in acetone at a concentration of 2 mg/ml and added to the media, to the desired concentration, just prior to pouring into Petri dishes.

Isolation of SM-resistant mutants: Single colonies of haploid strain DBY640, an s288c derivative, grown nonselectively on YEPD agar, were picked and suspended in sterile H₂O. Cells were spread on minimal medium (SD plus adenine) containing SM at a concentration of 3 μ g/ml or 10 μ g/ml to select spontaneous resistant mutants. Between 2×10^6 and 1×10^7 cells were spread per plate and incubated at 30°. A single resistant clone derived from each single colony was picked for further characterization to assure the independent origin of mutants.

Genetic manipulations: Putative mutants were mated to strain DBY917 for dominance tests. Matings were carried out by cross-streaking strains on YEPD. Diploids were obtained by replicating onto selective (SD) plates and purified by single-colony isolation on YEPD plates. For

meiotic segregation analysis diploids were incubated on sporulation plates for 3 days at 30°. Asci were digested in glusulase and spores were separated by micromanipulation, grown up on YEPD and replica-plated onto various minimal media to ascertain phenotype(s). Complementation tests of recessive mutants and genetic linkage of mutations were accomplished as described (SHERMAN, FINK and LAWRENCE 1974) using mutants of opposite mating types derived from crosses. Rapid chromosome mapping of the cloned *ILV2* gene was done as described previously (FALCO and BOTSTEIN 1983).

Assay of ALS: The assay is essentially as described in MAGEE and DE ROBICHON-SZULMAJSTER (1968a,b). Each reaction (0.5 ml) contains 0.1 M potassium phosphate buffer, pH 8.0, 50 mM sodium pyruvate, 5 mM MgSO₄, 25 µg/ml of thiamine pyrophosphate and toluene-permeabilized cells. Permeabilized cells were prepared from cultures in exponential phase ($1-2 \times 10^7$ cells/ml) grown in SD media supplemented with nutrients. Cells were harvested by centrifugation, washed one time in 0.1 M potassium phosphate buffer, pH 8.0, and resuspended in one-twentieth the original culture volume of 0.1 M potassium phosphate, pH 8.0, 25% (v/v) glycerol and 1 mM EDTA, pH 8.0. Reagent grade toluene was added to a concentration of 10% (v/v), and the suspension was mixed vigorously for 30 sec and then incubated for 2 min at 30° in a waterbath. The cells were then kept on ice until needed. Total protein was measured by the Bradford protein assay (BRADFORD 1976) using the Bio-Rad protein assay reagent. Fifty microliters of permeabilized cells, containing 10–30 µg of total protein, were used per reaction. SM was dissolved at 10 µg/ml in 0.1 M potassium phosphate, pH 8.0, or 2 mg/ml in acetone and added to reactions at the desired concentrations.

DNA preparation: Plasmid DNA was prepared from *E. coli* by a rapid method (RAMBACH and HOGNESS 1977) or by the CsCl equilibrium density gradient method (DAVIS, BOTSTEIN and ROTH 1980) when large scale, pure preparations were required. DNA was isolated from yeast as described previously (FALCO *et al.* 1982). The construction of the yeast recombinant plasmid libraries has been described previously (CARLSON and BOTSTEIN 1982).

Yeast transformation: Transformation of yeast was done by a modification of the method of HINNEN, HICKS and FINK (1978): glusulase treatment was done in 1 M sorbitol containing 1% mercaptoethanol and buffered to pH 5.8 using 0.1 M citrate. The concentration of glusulase was 2%, and the spheroplasting treatment was 3 h at 30°.

Measurement of marker stability in yeast: Strains were grown nonselectively, as isolated single colonies, on solid YEPD medium. Single colonies were picked and diluted in water; we estimate that these colonies contained between 10^7 and 10^8 cells, corresponding to about 25 generations of growth from the single cell deposited on the plate. The dispersed, diluted cells were replated on YEPD so that 100–300 colonies appeared per plate. These plates were then replica-plated to minimal media containing supplements such that the various phenotypes could be scored.

Restriction endonuclease mapping and construction of deletion mutations: Single and double restriction endonuclease digests of plasmid pCP2-4 and the parent vector YEp24 were prepared. The sizes of the DNA fragments resulting from the digests were determined by agarose gel electrophoresis. An unambiguous map of the sites for the enzymes shown in Figure 2 was derived from the fragment sizes.

The deletion mutations shown in Figure 2 (from the upper to the lower) were constructed as follows: pCP2-4 was digested with *Bgl*II and reclosed, deleting the two internal *Bgl*II fragments; pCP2-4 was digested with *Pvu*II and reclosed, deleting from the internal *Pvu*II site shown to the single *Pvu*II site in the vector YEp24 (BOTSTEIN *et al.* 1979); pCP2-4 was digested with *Eco*RV and reclosed, deleting from the internal *Eco*RV site shown to the *Eco*RV site in the *URA3* gene of the vector; the *Kpn*I site of pCP2-4 was converted to a *Bam*HI site with an adaptor and the *Bam*HI to *Hind*III internal fragment was subcloned into a high copy plasmid derived from YIp5 (BOTSTEIN *et al.* 1979) by addition of the 2.1-kb *Eco*RI to *Hind*III fragment from the B form of the yeast 2µ circle.

RESULTS

Isolation and characterization of SM-resistant mutants: Growth of *S. cerevisiae* is inhibited by low concentrations (3 µg/ml) of the herbicide SM on minimal

but not on rich medium. Spontaneous resistant mutants were selected in the strain DBY640, which is a derivative of s288c. Mutants arose at a frequency of approximately one per 10^7 cells on plates containing 3 $\mu\text{g}/\text{ml}$ of SM and at about ten-fold lower frequency when selected at 10 $\mu\text{g}/\text{ml}$ of SM. A total of 66 independent mutants were isolated and further characterized.

The mutants were crossed to the SM-sensitive strain CG379; all of the mutations segregated 2:2 in tetrads indicative of single nuclear gene mutations. Diploids were constructed by crossing each mutant to the SM-sensitive s288c derivative, DBY917, to determine the dominance relationship of each mutation to wild type. Fifty-one of the mutations were dominant, five semidominant and ten recessive.

Since the great majority of the mutations were dominant, complementation analysis was not generally useful for grouping them into cistrons. Therefore, genetic linkage analysis was undertaken. Linkage was determined by crossing each successive mutation by a tester from each linkage group which was established from previous crosses. Between 10 and 20 tetrads were analyzed from each cross. Each of the mutations was found to reside in one of three genetic linkage groups designated *SMR1*, *smr2* and *smr3* (for sulfometuron methyl resistant).

Most of the mutations (47 dominant and four semidominant), including all that resulted in resistance to high levels of SM (30 $\mu\text{g}/\text{ml}$), were in the *SMR1* linkage group. Seven mutations (four recessive, two dominant and one semidominant) which result in resistance at 3–10 $\mu\text{g}/\text{ml}$ of SM were in the *smr2* linkage group and eight mutations (six recessive and two dominant) which result in resistance at 3–10 $\mu\text{g}/\text{ml}$ of SM were in the *smr3* linkage group.

Complementation tests were performed with the recessive mutations in *smr2* and *smr3*. Mutations in each linkage group complemented those in the other group. However, within both linkage groups complementation was seen. Mutation *smr2-27* complemented the other three recessive mutations in this group. The six recessive mutations in the *smr3* group were divided by complementation into two groups of three mutations each. These results may indicate more than one gene at each locus or reflect intragenic complementation.

SMR1 mutations are in the ILV2 gene which encodes ALS: Evidence that the enzyme ALS is the target of SM in bacteria and plants has been reported recently (LAROSSA and SCHLOSS 1984; CHALEFF and MAUVAIS 1984; RAY 1984). Therefore, ALS from wild-type and SM-resistant yeast mutants was assayed in the presence and absence of SM from toluene-permeabilized cells. Acetolactate is formed at a linear rate for at least 90 min under the reaction conditions used (Figure 1A). Wild-type ALS is very sensitive to SM; 50% inhibition is seen at a concentration of 45 ng/ml (0.12 μM) SM (Figure 1B). This result, along with the observation that growth inhibition by SM only occurs on minimal media and is reversed by addition of the branched chain amino acids, suggests that ALS is the target of SM in yeast.

This conclusion is supported by ALS enzyme activities of the mutants. All mutations in the *SMR1* linkage group (and only the mutations in this group, see Table 2) lead to the production of altered enzyme activity. All *SMR1*

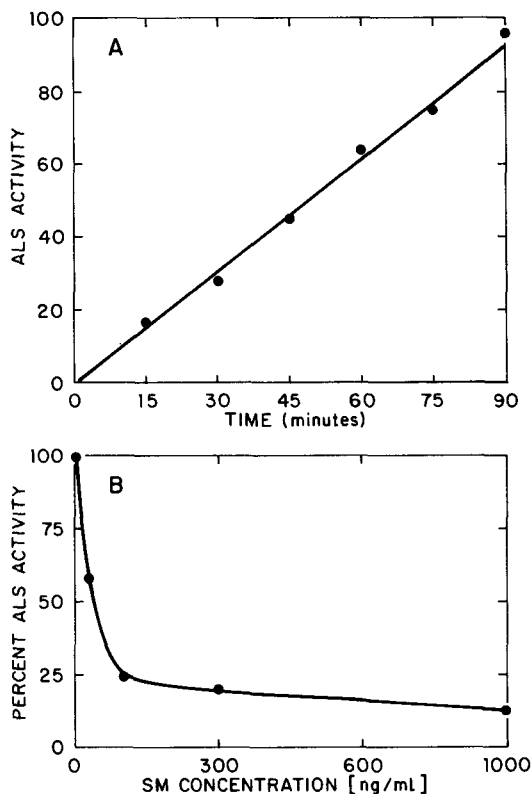


FIGURE 1.—ALS activity in toluene-permeabilized yeast cells. DBY640 was grown to midlog phase; cells were collected and ALS activity was measured as described in MATERIALS AND METHODS. A, Assay time course: ALS activity is expressed as product absorbance at 530 nm/mg of total protein. B, Inhibition of ALS by SM: ALS activity was measured in a 60-min reaction at varying SM concentrations.

mutants show an increase in the percent of enzyme activity remaining at 0.8 μM SM, a concentration at which wild-type ALS is 90% inhibited. In some cases up to nearly 100% of the activity of the mutants is resistant, *e.g.*, *SMR1-1*, *SMR1-6* and *SMR1-7*. In many mutants increased resistance to SM is associated with a decrease in specific activity, *e.g.*, *SMR1-6*, *SMR1-7* and *SMR1-15*, suggesting that the mutations have adversely affected enzyme function. These results suggest that *SMR1* mutations are in *ILV2*, the structural gene encoding ALS (MAGEE and DE ROBICHON-SZULMAJSTER 1968a). Several phenotypically distinct *SMR1* mutations have been identified (see examples in Table 2), indicating that many alterations of ALS which render the enzyme resistant to SM while leaving sufficient enzyme activity for amino acid biosynthesis are possible.

To prove that the *SMR1* mutations were in *ILV2*, the gene was cloned. Because no *ilv2* mutations were available, the strategy used to isolate the *ILV2* gene depended upon overproduction of ALS resulting from increased gene dosage of *ILV2* due to its presence on a high copy number plasmid (RINE *et al.* 1983). Overproduction of ALS was then detected by increased resistance to SM. The strain FY138 was transformed to uracil prototrophy with three

TABLE 2

Properties of SM-resistant mutants

Mutation	Maximum concentration of SM for growth ($\mu\text{g/ml}$)	Dominance	ALS specific activity: % of wild-type	% ALS activity resistant to $0.8 \mu\text{M}$ SM
+	1		100	10
<i>smr3-21</i>	3	R	87	9
<i>smr2-5</i>	3	R	85	10
<i>SMR1-3</i>	3-10	D	16	75
<i>SMR1-4</i>	10	D	100	33
<i>SMR1-15</i>	10	D	38	43
<i>SMR1-1</i>	>30	D	100	100
<i>SMR1-6</i>	>30	D	50	100
<i>SMR1-7</i>	>30	D	12	100
<i>SMR1-8</i>	>30	R/D	15	90

R, recessive; D, dominant.

independent yeast genomic libraries in the high copy vector YE_p24 (BOTSTEIN *et al.* 1979). More than 10,000 Ura⁺ transformants from each library were pooled and spread at a concentration of approximately 1×10^6 cells per plate on media lacking uracil and including SM at the minimal inhibitory concentration of 1 $\mu\text{g/ml}$. Colonies resistant to SM appeared at a frequency of 1-5/ 10^4 transformants, the approximate frequency that other single-copy genes have been isolated from the libraries (S. C. FALCO, unpublished results).

Six resistant colonies, two derived from each library, were picked and tested for instability of the resistance phenotype. Segregants that were Ura⁻, due to loss of the plasmid, were isolated from each, following growth on nonselective media. In four of the six clones all Ura⁻ segregants were also SM sensitive, indicating that the SM resistance determinant was plasmid borne. ALS activity was assayed from each of these transformants. One (YT571) consistently showed a four- to five-fold increase in ALS-specific activity over the untransformed parent. The plasmid present in YT571, designated pCP2-4, was isolated by transformation into the *E. coli* strain HB101, from which plasmid DNA was prepared. When this plasmid was transformed back into yeast strain FY138 all transformants were SM resistant.

A restriction endonuclease map of the 5.6-kb yeast genomic DNA fragment carried on plasma pCP2-4 is shown in Figure 2. To localize the resistance determinant on this fragment, several deletion mutations were constructed *in vitro*. The deletion derivatives of pCP2-4 were then introduced into strain FY138 by transformation and resistance or sensitivity of the transformants to SM was determined. The results of this analysis, shown in Figure 2, indicate that the putative *ILV2* gene is located in a 3.0-kb region at the right-hand end of the insert of pCP2-4.

Two additional experiments demonstrate that pCP2-4 carries the *ILV2* gene. For the first, plasmid pKD3-4 was constructed as shown in Figure 3; pKD3-4 is an integrating plasmid carrying a deletion mutation that inactivates the SM

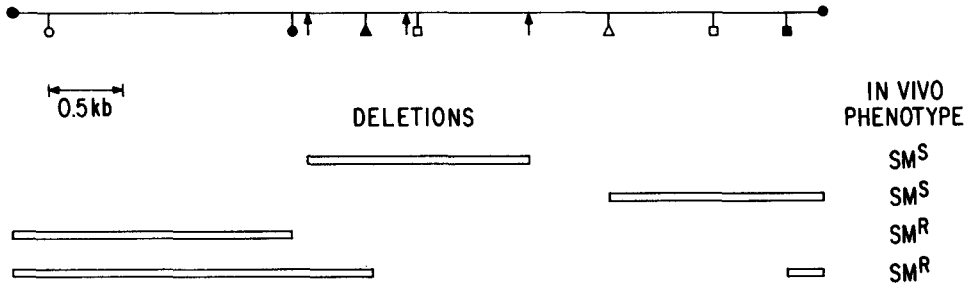


FIGURE 2.—Restriction enzyme map of yeast genomic segment cloned in pCP2-4. ○, *Cla*I; ●, *Eco*RV; ↑, *Bgl*II; ▲, *Kpn*I; □, *Eco*RI; △, *Pvu*II; ■, *Hind*III. Also shown are four *in vitro* constructed deletion mutations and the *in vivo* phenotype of FY138 transformed by the deletion plasmid.

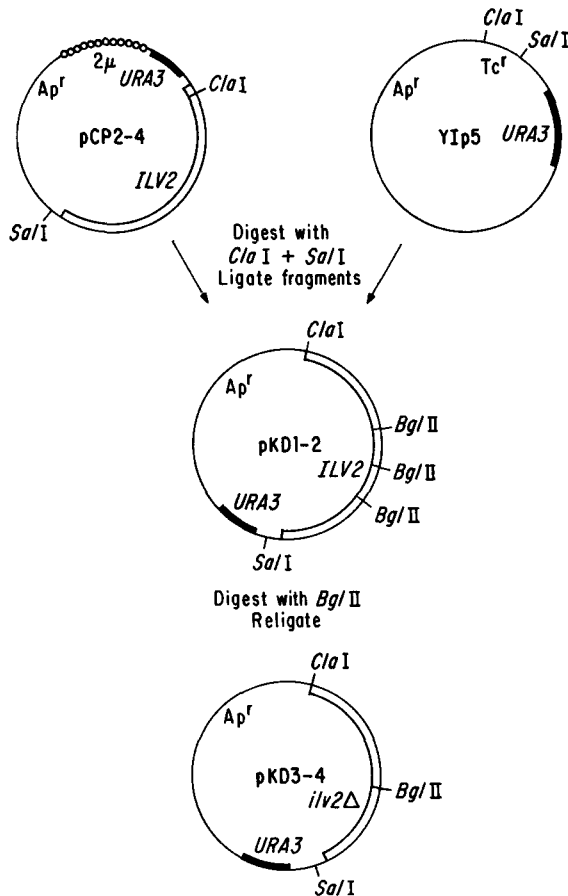


FIGURE 3.—Construction of integrating plasmids. Vector YIp5 has been described previously (BOTSTEIN *et al.* 1979). pCP2-4 and YIp5 were digested with restriction enzymes *Cla*I and *Sal*I; the desired DNA fragments were purified and ligated; *Ap^r*, *Tc^r* bacterial transformants were isolated, plasmid DNA was prepared and screened by restriction enzyme analysis for the desired plasmid pKD1-2. pKD1-2 was digested with *Bgl*II and religated to yield the deletion plasmid pKD3-4.

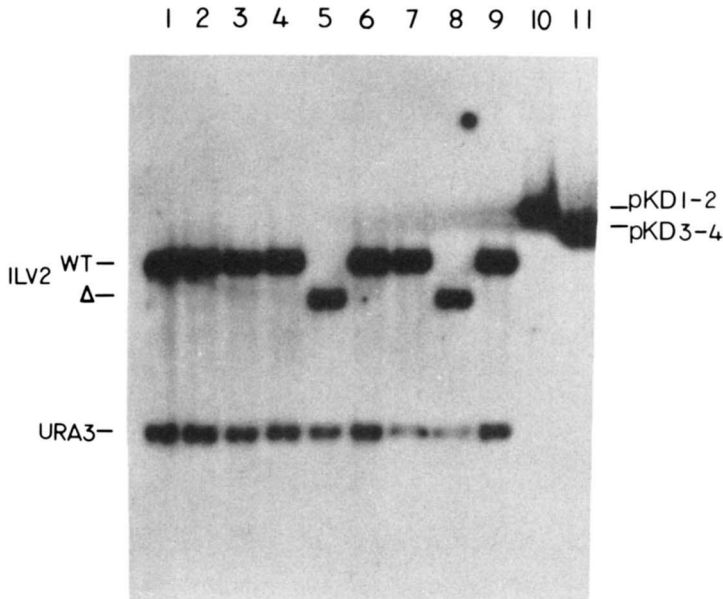


FIGURE 4.—Analysis of Ura^- segregants derived from transformants of strain FY138 with pKD3-4. DNA was isolated as indicated in MATERIALS AND METHODS, digested with restriction endonuclease *Cla*I and the resulting fragments were size separated by agarose gel electrophoresis. The DNA was transferred to nitrocellulose by the method of SOUTHERN (1975) and hybridized to 32 P-labeled pKD1-2 DNA prepared by nick translation as described by DAVIS, BOTSTEIN and ROTH (1980). Hybridizing DNA fragments were detected by autoradiography. Lane 1, FY138; lanes 2–9, Ura^- segregants 51 to 58, respectively; lane 10, pKD1-2; lane 11, pKD3-4.

resistance determinant of pCP2-4 (see Figure 2). This plasmid was used to transplace the deletion mutation into the yeast genome (SCHERER and DAVIS 1979). Ura^+ transformants of strain FY138 with plasmid pKD3-4 were selected, and the genomic DNA of the transformants was analyzed using the method of SOUTHERN (1975) to demonstrate that the plasmid integrated at the desired locus (not shown).

Rare Ura^- segregants which result from loss of the plasmid by recombination between the direct repeats formed by the integration event were then isolated by replica-plate screening of colonies grown nonselectively. Thirteen Ura^- segregants were isolated from 60,000–70,000 colonies. Total genomic DNA was prepared from the Ura^- segregants and analyzed by the Southern method. Results for eight segregants are shown in Figure 4. Six of the eight are identical with the wild-type parent strain FY138 (as are the other five Ura^- segregants not shown), indicating that the integrated plasmid has indeed been lost, leaving the normal genomic segment intact. Two of the eight, S4 and S7, show a 1.5-kb smaller fragment instead of the wild-type fragment, indicating that the wild-type gene has been replaced by the deletion mutation. Analysis of these segregants with a second restriction enzyme, *Hind* III (not shown), is in agreement with this interpretation.

TABLE 3

SM-resistant ALS activity due to plasmid-borne mutations

Plasmid	% ALS activity resistant to 0.8 μ M SM
None	7
YEP24	9
pCP2-4	4
pCP2-4-5	72
pCP2-4-8	70
pCP2-4-10	100
pCP2-4-11	77
pCP2-4-13	85
pCP2-4-15	83

The strain in all cases was FY138. ALS was assayed as described in MATERIALS AND METHODS. Assay time was 30 min.

When tested for growth requirements, the two segregants with the deletion mutation, but none of those that carry the wild-type DNA fragment, were found to require isoleucine and valine. ALS enzyme activity was undetectable in the segregants carrying the deletion, providing strong evidence that the deletion disrupts the *ILV2* gene.

In the second experiment, mutants of strain FY138 carrying pCP2-4 were selected on media lacking uracil and including SM at high concentration (30 μ g/ml). (The presence of pCP2-4 in FY138 confers SM resistance only to a concentration of 3 μ g/ml.) Six independently isolated spontaneous, high level resistant mutants were obtained. The mutations were shown to be plasmid borne by instability and by isolation of plasmid DNA and retransformation of strain FY138 to high level SM resistance.

The ALS activity of strains carrying each of these mutant plasmids was assayed in the presence and absence of 0.8 μ M SM. The data shown in Table 3 indicate that each plasmid mutation leads to a large increase in the percent of ALS activity resistant to SM. These results provide additional evidence that confirms the conclusion that pCP2-4 carries the *ILV2* gene.

To determine whether the *SMR1* mutations were alleles of *ILV2*, plasmid pKD1-2 (Figure 3) was used to integrate the *URA3* gene as a genetic marker at the *ILV2* locus. Strain FY138 was transformed to Ura⁺ with pKD1-2 yielding YT617. This transformant was crossed with strain FY249 which carries *SMR1*-103; the diploid was sporulated, and tetrads were analyzed. All tetrads were parental ditype (Table 4, genetic cross 1), indicating that *SMR1* is tightly linked to *ILV2*. Since *SMR1* mutations result in the production of SM-resistant ALS activity and map at the *ILV2* locus, it is concluded that they are alleles of *ILV2* and that ALS is the target of SM in yeast.

Genetic mapping of SMR1, smr2 and smr3: A rapid mapping method for cloned yeast DNA segments (FALCO and BOTSTEIN 1983) was used to determine the chromosomal location of the cloned *ILV2* gene (and thus *SMR1*). The method relies on specific chromosome loss induced by the presence of 2 μ circle DNA in the chromosome of interest. Chromosomal integration of 2 μ

TABLE 4

Meiotic mapping of SMR1, smr2 and smr3

Genetic cross	Gene pair	PD	NPD	T	Conclusion					
1	<i>URA3</i> (at <i>ILV2</i>)— <i>SMR1</i>	31	0	0	<i>SMR1</i> is tightly linked to <i>ILV2</i>					
2	<i>LYS7</i> — <i>SMR1</i>	39	1	44	<i>SMR1</i> is 30 cM from <i>LYS7</i>					
3 ^a	<i>TRP1</i> — <i>SMR1</i>	21	20	91	<i>SMR1</i> is not centromere linked					
4 ^a	<i>TRP1</i> — <i>smr2</i>	47	54	22	<i>smr2</i> is 8.9 cM from centromere					
5	<i>cyh3</i> — <i>smr2</i>	24	0	0	<i>smr2</i> is tightly linked to <i>cyh3</i>					
6	<i>ade2</i> — <i>smr3</i>	42	0	66	<i>smr3</i> is 30 cM from <i>ade2</i>					
	<i>his3</i> — <i>smr3</i>	67	2	39	<i>smr3</i> is 24 cM from <i>his3</i>					
	<i>ade2</i> — <i>his3</i>	33	3	77	<i>ade2</i> is 42 cM from <i>his3</i>					
Genetic cross 1:	<u>YT617</u>	α	<u><i>his4</i></u>	<u><i>aas3</i></u>	<u><i>ura3</i></u>	+	<u><i>pKD1-2 (URA3) at ILV2</i></u>			
	FY249	a	+	+	<u><i>ura3</i></u>	<u><i>ade2</i></u>	<u><i>SMR1</i></u>			
Genetic cross 2:	<u>DBY890</u>	α	<u><i>arg1</i></u>	<u><i>his7</i></u>	<u><i>lys7</i></u>	<u><i>met6</i></u>	<u><i>gal4</i></u>	+	+	+
	FY249	a	+	+	+	+	+	<u><i>ura3</i></u>	<u><i>ade2</i></u>	<u><i>SMR1</i></u>
Genetic cross 3:	<u>CG379</u>	α	<u><i>leu2</i></u>	<u><i>ade5</i></u>	<u><i>his7</i></u>	<u><i>trp1</i></u>	<u><i>ura3</i></u>	+	+	
	DBY640- <i>SMR1</i>	a	+	+	+	+	+	<u><i>ade2</i></u>	<u><i>SMR1</i></u>	
Genetic cross 4:	<u>CG379</u>	α	<u><i>leu2</i></u>	<u><i>ade5</i></u>	<u><i>his7</i></u>	<u><i>trp1</i></u>	<u><i>ura3</i></u>	+	+	
	DBY640- <i>smr2</i>	a	+	+	+	+	+	<u><i>ade2</i></u>	<u><i>smr2</i></u>	
Genetic cross 5:	<u>JG204</u>	a	<u><i>leu2</i></u>	<u><i>gal2</i></u>	<u><i>can1</i></u>	<u><i>his3</i></u>	+	<u><i>cyh3</i></u>		
	SR17-34a	α	<u><i>leu2</i></u>	+	+	+	<u><i>ade5</i></u>	<u><i>smr2</i></u>		
Genetic cross 6:	<u>FY242</u>	α	<u><i>his3</i></u>	+	+	<u><i>trp1</i></u>	<u><i>ura3</i></u>			
	SR41	a	+	<u><i>smr3</i></u>	<u><i>ade2</i></u>	+	+			

^a Results are summed from many crosses of *SMR1* and *smr2* mutations.

circle DNA at the locus to be mapped results from recombination between DNA carried on the vector and the homologous DNA on the chromosome. Results from this procedure indicated that *ILV2* mapped on chromosome *XIII*, either on the left arm or distal to *LYS7* on the right arm (data not shown). Meiotic linkage of *SMR1* and *LYS7* is shown in Table 5 (cross 2); a genetic map distance of 30 cM is calculated. *SMR1* is not centromere linked (Table 5, cross 3); therefore, it is concluded that *SMR1* is located 30 cM centromere distal to *LYS7* on the right arm of chromosome *XIII*. This is in agreement with a previous report of the map position of *ILV2* (PETERSEN *et al.* 1983).

Crosses of *smr2* mutations to strain CG379 revealed that these mutations were centromere linked (Table 5, cross 4). On the yeast genetic map near the centromere of chromosome *VII* are a number of recessive drug resistance markers including *cyh3*, *ant1*, *oli1*, *til1* (MORTIMER and SCHILD 1980). To test whether *smr2* maps at this locus it was crossed by a *cyh3* mutation (Table 4, cross 5) and found to be tightly linked. A separate cross of *smr2* by *leu1* (not shown) yielded the expected linkage. In addition, *smr2* mutations conferred resistance to cycloheximide and the *cyh3* mutation conferred resistance to SM. Finally, the *cyh3* mutation failed to complement two *smr2* mutations. Thus, it is concluded that mutations at the *smr2* locus are allelic to *cyh3*. Evidence that suggests that all of the mutations at this locus are alleles of the same gene, designated *pdr1* (pleiotropic drug resistance), has been reported (RANK and

TABLE 5
SM resistance mutations of yeast

Gene	Alternate gene designation	Dominance	Maximum SM tolerated	Map position	Effect on ALS activity	Gene function
<i>SMR1</i>	<i>ILV2</i>	47 Dominant 4 Semidominant	31, >30 µg/ml 8, 10-30 µg/ml 7, 10 µg/ml 5, 3-10 µg/ml	XIII R, 30 cM distal to <i>LYS7</i>	Altered; increased resistance to SM	Encodes ALS
<i>smr2</i>	<i>pdr1</i> <i>cyh3</i>	2 Dominant 1 Semidominant 4 Recessive	All, 3-10 µg/ml	VII L, 8.3 cM from centromere	None	Affects cell permeability to inhibitors
<i>smr3</i>		2 Dominant 6 Recessive	All, 3-10 µg/ml	XV R, 30 cM distal to <i>ADE2</i> , 24 cM proximal to <i>HIS3</i>	None	Unknown

BECH-HANSEN 1973; RANK, ROBERTSON and PHILLIPS 1975; COHEN and EATON 1979; SAUNDERS and RANK 1982).

Crosses of the *smr3* mutations to strain CG379 revealed no centromere linkage, but fortuitously, the mutations were seen to be linked to *ade2*. To position the *smr3* locus on chromosome XV a three-factor cross involving *ade2 his3* and *smr3* was analyzed (Table 4, cross 6). The results shown indicate that *smr3* is between *ade2* and *his3*. Analysis of double recombinants from this cross confirms that *smr3* is the middle marker. No other yeast genetic marker maps at this position, suggesting that *smr3* mutations defined a previously unknown yeast gene.

DISCUSSION

The potent new herbicide, SM, is also a potent growth inhibitor of the yeast *S. cerevisiae*. The results presented in this report demonstrate that SM blocks yeast growth by inhibition of the amino acid biosynthetic enzyme, ALS. The herbicide has been shown to block growth via inhibition of ALS from diverse sources including bacteria (LAROSSA and SCHLOSS 1984), fungi (this report) and plants (CHALEFF and MAUVAIS 1984; RAY 1984).

Sixty-six yeast mutants resistant to SM have been isolated. A summary of the properties of the SM resistance mutations is shown in Table 5. Most of the mutations (including all that confer resistance to high concentrations of SM) are dominant and are tightly linked, identifying a genetic locus designated *SMR1*. Strains carrying *SMR1* mutations produce ALS activity that is less sensitive to SM than the wild-type enzyme, suggesting that these are mutations in the *ILV2* gene. The *SMR1* mutations have been shown to map at the same genetic locus as a cloned yeast genomic DNA segment which carries the *ILV2* gene, providing strong support for this conclusion.

At least ten phenotypically distinct *SMR1* mutations have been identified. The procedure used to select these mutants required that they be resistant to SM but still produce enough ALS to grow in the absence of the branched chain amino acids. Apparently, a level of ALS activity considerably lower than the wild-type level is sufficient to supply these amino acids, since a number of SM-resistant mutants produce only 10–15% normal activity. Other *SMR1* mutations do not significantly affect ALS activity but reduce or eliminate inhibition by SM. The mechanism through which SM inhibits ALS is not known. LAROSSA and SCHLOSS (1984) have shown that SM binds tightly but reversibly to the bacterial ALS II isozyme. The *SMR1* mutations should prove useful in the analysis of SM action as well as in the determination of structural domains important for ALS activity.

SM resistance mutations were isolated at two additional genetic loci, designated *smr2* and *smr3*. All mutations at these loci confer resistance only to low levels of SM. Although most *smr2* and *smr3* mutations are recessive, it seems unlikely that they represent loss of function mutations for several reasons. Mutations at either of these loci were much less frequently obtained than *SMR1* mutations. Since *SMR1* mutations must represent very specific, and, therefore,

relatively rare, alterations of the *ILV2* gene, they would be expected to appear less rather than more frequently than loss-of-function mutations. In addition, dominant mutations were identified at both *smr2* and *smr3*; dominant loss of function mutations, although not unprecedented, are rare. Thus, it is probable that the mutations at these loci result in specific functional changes that confer resistance to SM.

In contrast to *SMR1* mutations, *smr2* and *smr3* mutations affect neither the level nor the SM sensitivity of ALS. The mechanism by which these mutations lead to resistance is not yet known. In the case of the *smr2* mutations evidence was presented that indicates that these mutations are alleles of the *pdr1* gene. Evidence that the *pdr1* mutations lead to resistance by reducing permeability of the plasma membrane to inhibitors has been reported (RANK, ROBERTSON and PHILLIPS 1975). The molecular basis for the reduced permeability is unknown. If reduced permeability is the basis for resistance of *smr2* mutations, they may provide insight into the mechanism by which these compounds enter cells.

The yeast *ILV2* gene was cloned by its ability to confer low level resistance to SM when carried on a high copy number plasmid. Resistance results from a four- to six-fold increase in ALS activity in cells carrying many copies of the gene. Proof that the cloned segment includes the *ILV2* gene was provided by two additional experiments. First, the putative *ILV2* gene was localized to a 3.0-kb region within the 5.6-kb cloned DNA segment by *in vitro* constructed deletion mutations. A deletion that inactivated the gene was used to replace the wild-type chromosomal gene. The resulting strain required isoleucine and valine for growth and produced no detectable ALS enzyme activity. Second, mutations in the cloned DNA segment which cause high level resistance to SM were selected. Strains containing the plasmids bearing these mutations produce ALS which is insensitive to SM.

The isolation of the yeast *ILV2* gene provides an example of resistance to an inhibitor resulting from overexpression of the target due to increased gene dosage. Several analogous cases in yeast have recently been reported (FOGEL and WELCH 1982; RINE *et al.* 1983). It will be interesting to determine whether SM-resistant mutants which result from tandem duplication of the yeast *ILV2* gene, as has been found for the *CUP1* gene mediating copper resistance (FOGEL and WELCH 1982), can be isolated. Since the level of resistance to SM provides a sensitive and selectable phenotype, the cloned *ILV2* gene can be used to screen for promoter-up mutations following site-specific mutagenesis. In addition, the SM-resistant mutants of the cloned *ILV2* gene that have been isolated provide a dominant selectable genetic marker which has been used for the transformation of commercial strains of yeast (S. C. FALCO, unpublished results). These mutant genes may ultimately prove useful as selectable markers for transformation of other fungi and for the transfer of herbicide resistance to crop plants as well.

We thank ROY CHALEFF, DEBORAH CHALEFF, JOHN GOLIN, BOB LAROSSA, KEN LIVAK, BARBARA MAZUR and JOHN SCHLOSS for many helpful discussions and critical reading of the manuscript. We thank VARETTA MANLOVE for preparation of the manuscript.

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Communicating editor: D. BOTSTEIN