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Lysine-Overproducing Mutants of *Saccharomyces cerevisiae* Baker's Yeast Isolated in Continuous Culture

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Saccharomyces cerevisiae **baker's yeast mutants which produce 3 to 17 times as much lysine as the wild type, depending on the nitrogen source, have been selected. The baker's yeast strain was grown in a pH-regulated chemostat in minimal medium with proline as the nitrogen source, supplemented with increasing concentrations of the toxic analog of the lysine *S*-2-aminoethyl-L-cysteine (AEC). The lysine-overproducing mutants, which were isolated as AEC-resistant mutants, were also resistant to high external concentrations of lysine and to α-aminoadipate and seemed to be affected in the lysine biosynthetic pathway but not in the biosynthetic pathways of other amino acids. Lysine overproduction by one of the mutants seemed to be due to, at least, the loss of repression of the homocitrate synthase encoded by the *LYS20* gene. The mutant grew slower than the wild type, and its dough-raising capacity was reduced in in vitro assays, probably due to the toxic effects of lysine accumulation or of an intermediate produced in the pathway. This mutant can be added as a food supplement to enrich the nutritive qualities of bakery products, and its resistance to α-aminoadipate, AEC, and lysine can be used as a dominant marker.**

Flours obtained from cereals and used as human or animal food are deficient in amino acids such as threonine, methionine, tryptophan, and, above all, lysine (13), which are essential for humankind. After fermentation, yeast becomes part of the food; hence, a cheap way of enriching cereal flours in essential amino acids may be the addition of yeast mutants that overproduce and accumulate these amino acids.

Lysine is synthesized in *Saccharomyces cerevisiae* baker's yeast from α-ketoglutarate via α-aminoadipate (1) in a linear pathway in which eight enzymatic reactions are involved (Fig. 1) (10, 29, 30, 34). Some of the enzymes which catalyze the first four steps are located in the mitochondria, whereas the rest are cytosolic (14, 15). Homocitrate synthase seems to be located in the nucleus (4). Lysine biosynthesis is regulated by the amino acid general control so that some of the genes are subjected to derepression (Fig. 1) by starvation for several amino acids, including lysine (8); in addition, the expression of some genes is positively regulated by the product of *LYS14* and repressed by that of *LYS80* (23).

The synthesized lysine is stored in vacuoles for protein biosynthesis but cannot be used as a carbon or nitrogen source (21, 32). When α-aminoadipate is added as a nitrogen source, the cells cannot grow (33), even if another nitrogen source is added. Therefore, a lysine overproducer needs to be resistant to high concentrations of both lysine and the intermediate metabolite α-aminoadipate, which would probably increase its concentrations in the overproducers.

In haploid strains, there are two isoenzymes which catalyze the first step of the lysine biosynthetic pathway (29, 30). One of the isoenzymes, which is encoded by the *LYS20* gene (24), seems to be nuclear (4) and is responsible for 70% of the homocitrate synthase activity. Although both isoenzymes are inhibitable by lysine, only the *LYS20* gene seems to be repressible by lysine (24). The enzyme encoded by *LYS20* is probably essential for lysine biosynthesis because it catalyzes the first specific step of the biosynthetic pathway and appears to be precisely regulated (24).

Amino acid-overproducing mutants have been isolated as resistant to toxic amino acid analogs (19), since the toxicity of the analog depends on its relative concentration with regard to that of the physiological amino acid. By increasing the analog concentration in a continuous culture controlled by pH, Martínez-Force and Benítez (19) isolated mutants which were capable of overproducing 40-fold more threonine and 160-fold more methionine than the wild type. As continuous culture favors the selection of strains that grow faster, slow-growing, unhealthy mutants are discarded. By using a similar continuous culture procedure, *S*-2-aminoethyl-L-cysteine (AEC)-resistant mutants which overproduce lysine have been isolated in this work. Their isolation and characterization are described.

MATERIALS AND METHODS

Strains. The *S. cerevisiae* strains used in this work are listed in Table 1.

Media. Yeast strain were grown in any of the following media, as indicated later in the text. Minimal medium (SD) contains 0.17% nitrogen base without amino acids or ammonium sulfate, 2% glucose, and 0.5% ammonium sulfate. Minimal medium with proline (SDP), arginine (SDA), or glutamate (SDG) as a nitrogen source has the same composition as SD but with an amino acid at 0.1% instead of ammonium sulfate. Minimal medium with AEC (SDP-AEC) is the same as SDP but with AEC added at 10 μM to 1 mM. Minimal medium with lysine (SDP-Lys) is the same as SDP but with lysine added at 30 μM to 10 mM. Minimal medium with α-aminoadipate (SDP-AA) is the same as SDP but with α-aminoadipate added at 0.5 to 10 mM. Complete medium (YPD) contains 1% yeast extract, 2% glucose, and 2% Bacto Peptone. Presporulation medium (PRE) contains 0.8% yeast extract, 10% glucose, and 0.3% Bacto Peptone. Sporulation medium (SPO) contains 0.5% potassium acetate. When necessary, media were solidified by addition of 2% agar.

Culture conditions. (i) Batch cultures. Yeast cells were inoculated into 20-ml tubes containing 5 ml of liquid SD and incubated with shaking at 30°C until the stationary phase was reached. Flasks of 1,000 ml with 250 ml of the appropriate medium were inoculated and incubated with shaking at 30°C until the mid-exponential phase was reached.

(ii) Continuous cultures. For experiments carried out in continuous culture, a New Brunswick Bio-Flo C30 chemostat fermentor (New Brunswick Scientific Co., Inc., Edison, N.J.) with a 1.5-liter culture vessel was used. The fermentor was equipped with a pH electrode in the culture vessel connected to a New Brunswick pH40 automatic pH controller. The culture vessel with 1.3 liters of SDP medium was inoculated with 10 ml of a mid-exponential-phase culture and incubated at 30°C. A 10 liter vessel with a medium supply was used to feed the culture. The nutrient pump of the fermentor was regulated to provide a dilution

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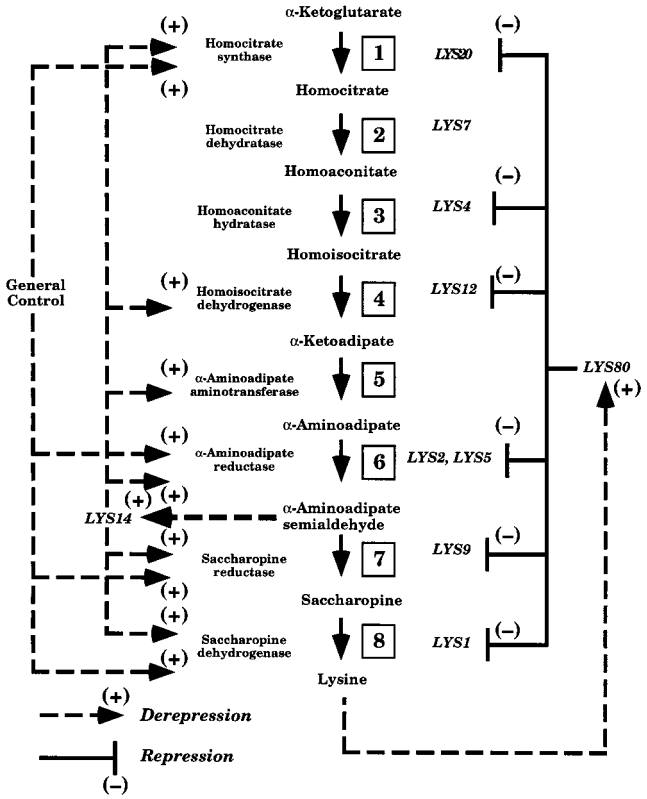

FIG. 1. Regulation of the lysine biosynthetic pathway in yeast (adapted from reference 1).

rate of 0.5 h^{-1}, which is higher than the highest growth rate of the strain used. The pump was connected to the pH controller. When the growth increased and the pH fell below a critical value, the pH controller switched on the nutrient pump and the culture was diluted with fresh medium, increasing the pH. When, after dilution, the pH rose above the critical value, the pH controller switched off the nutrient pump. This feedback system results in a semicontinuous culture, since a pulse of dilution is followed by a pulse of batch growth. However, it will be referred to as a pH-controlled continuous culture because no differences exist when the duration of the dilution pulses is very short.

Isolation of mutants resistant to AEC in continuous culture. To isolate AEC-resistant mutants, baker's yeast strain V2 was inoculated into SDP-AEC medium and the pH-controlled continuous culture was activated once the cells reached the early stationary phase. The AEC concentration was initially 10 μM and was raised gradually to 1 mM. Samples were taken periodically, diluted, and plated onto SDP-AEC.

TABLE 1. *S. cerevisiae* strains used in this work

Strain	Genotype	Source
V2	Unknown	Compañía General de Levadura, Valladolid, Spain
SL1	Unknown	This work[a]
SL2	Unknown	This work[a]
MMY1	*Matα ura3-52 Cyh*^{r}	Richard Bailey, Solar Energy Research Institute
MMY2	*MAT***a** *ura3-52 Cyh*^{r}	Richard Bailey, Solar Energy Research Institute
DS81	*MAT***a**/α *SUC2*/*SUC2 mal*/*mal gal2*/*gal2 CUP1*/*CUP1*	Yeast Genetic Stock Center, Berkeley, Calif.
C90	*MATα ura3 his3 leu2 sam1*::*URA3*	Centre National de la Recherche Scientifique

[a] Obtained in this work from the V2 strain by selection in continuous culture with AEC.

Growth. Growth was determined by measuring the exponential increase in turbidity in any medium at 660 nm with a Bausch & Lomb Spectronic 20 spectrophotometer. Previously, a linear relationship between cell number and A_{660}, ranging from 0.1 to 0.5, was observed.

Fermentative capacity. Twenty-milliliter tubes containing 6 ml of distilled water and 4 g of flour were inoculated with 0.1 g (wet weight) of biomass grown in YPD. The tubes were incubated at 30°C, and the volume increase was measured.

Enzymatic activities. Cells were cultured in SDP until the mid-exponential phase was reached. They were harvested then, washed with 20 mM phosphate buffer (pH 7.2), and homogenized with a Braun MSK Homogenizer, and, after centrifuging, the supernatant was applied to a Sephadex G-25 column; this supernatant, once eluted, was used for assay determination.

(i) Homocitrate synthase. Homocitrate synthase was measured by following the protocol of Ramos and Wiame (23). The reaction mixture contained α-ketoglutarate at 50 mM, acetyl coenzyme A at 0.4 mM, $MgCl_2$ at 1 mM, and phosphate buffer at 100 mM (pH 7.7), and when necessary, lysine at 50 mM was added to measure inhibition. The reaction was stopped with trichloroacetic acid, and the free coenzyme A was calculated by measuring the A_{412} with 5,5′-dithiobis(2-nitrobenzoic) acid (6).

(ii) α-Aminoadipate reductase. The reaction mixture contained α-aminoadipate at 12.5 mM, ATP at 10 mM, NADPH at 0.1 mM, $MgCl_2$ at 10 mM, and Tris-chloride buffer at 100 mM (pH 8.0); when necessary, lysine at 50 mM was added to measure inhibition. The reaction was determined by measuring the disappearance of NADPH at 340 nm.

(iii) Saccharopine reductase. The reaction mixture contained saccharopine at 2.5 mM, $NADP^+$ at 0.25 mM, and Tris-chloride buffer at 100 mM (pH 9.5); when necessary, lysine at 50 mM was added to measure inhibition. The reaction was determined by measuring the appearance of NADPH at 340 nm.

Sporulation and genetic analysis. Cells were sporulated as described by Codón et al. (5), by using PRE medium to culture the cells until the mid-exponential phase was reached, and then transferred into SPO medium.

Karyotypes. The basic procedure followed was that of Naumov et al. (22). The system used was a CHEF-DRII gel electrophoresis apparatus from Bio-Rad Laboratories (Richmond, Calif.). Electrophoresis was carried out at 14°C and 200 V for 15 h with a switching time of 60 s and for 11 h more with a switching time of 120 s.

Protein determination. Total protein was measured as described by Lowry et al. (16). For raw extracts, the method of Bradford (2) was used.

DNA content. The DNA content was determined with a FACScan (Becton-Dickinson) flow cytometer measuring the fluorescence at 639 nm of the DNA stained with propidium iodide as described by Martínez et al. (17).

Amino acid determination. Amino acid content was determined as described by Martínez-Force and Benítez (18), by using a Waters Chromatographer with a Waters Resolve C_{18} column (3.9 by 150 mm), an automatic injector (Waters 715 Ultra Wisp), and a fluorescence detector (Waters 420-AC).

RESULTS

Isolation of AEC-resistant mutants in a pH-regulated continuous culture. The resistance of the V2 baker's yeast strain to AEC was checked in minimal SDP medium supplemented with increasing concentrations of AEC. At 20 μM, the wild type was unable to grow.

The continuous culture was then initiated in SDP supplemented with 10 μM AEC, and the concentration of AEC was progressively increased by adding the toxic analog to the feeding tank at the times shown in Fig. 2. This addition was continued while the cells were able to grow (detected by determining the acidification of the medium). The final concentration of AEC in the culture and the samples taken are also indicated in Fig. 2. The experiment was carried out for 500 h. After this time, no mutant seemed to be able to grow at concentrations higher than 1 mM AEC, since no acidification of the medium was observed.

Samples M1 to M9, containing a mixture of the wild type and the AEC-resistant mutants, were spread on solid SDP-AEC (1 mM), and the colonies able to grow were checked twice on the same medium. Those colonies which could grow faster under these conditions were selected; 15 of them were isolated from sample 9, 10 were from sample 8, and 5 were from sample 7. No colonies from other samples could grow on 1 mM AEC. Of the 30 colonies which accumulated higher lysine concentrations than the wild type, those two which had the highest

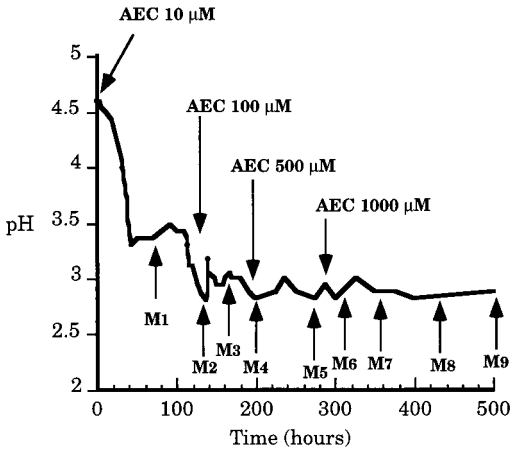

FIG. 2. Isolation of lysine overproducer mutants by pH-controlled continuous culture. The V2 baker's yeast strain was incubated in SDP in the presence of increasing concentrations of the toxic lysine analog AEC. Samples were taken at the times indicated (M1 to M9). The times at which the concentration of AEC was increased are also shown.

concentrations (SL1 and SL2) were chosen and further characterized.

Characterization of AEC-resistant mutants. (i) Electrophoretic karyotype, DNA content, and sporulation capacity. V2 is a prototrophic baker's yeast strain lacking genetic markers. To identify the AEC-resistant mutants derived from V2, the electrophoretic karyotype of the wild type and the two mutants was determined. As a control, the diploid DS81 laboratory strain was used. Figure 3 shows that the karyotype of SL1 and SL2 was very different from that of DS81 but similar to that of V2; nevertheless, some changes (indicated by arrows) seemed to have occurred. Several electrophoretic bands changed in mobility, indicating genomic rearrangements during the selective process.

Baker's yeast strains are never haploid but rather aneuploid,

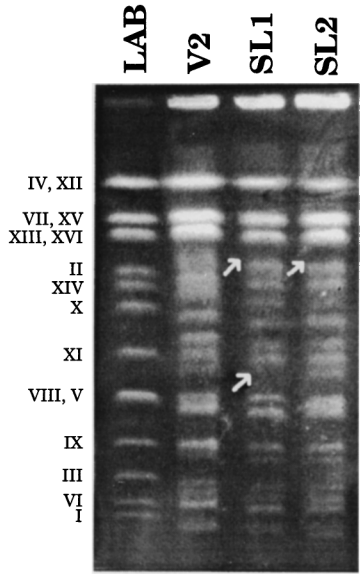

FIG. 3. Electrophoretic karyotypes of the V2 baker's yeast strain, the SL1 and SL2 lysine-overproducing mutants, and the DS81 laboratory strain. The arrows indicate band mobilities that differ from those of the V2 strain.

TABLE 2. Internal concentrations of lysine in the V2 baker's yeast strain and in the SL1 and SL2 AEC-resistant mutants when external lysine was present[a]

Concn of lysine added medium (μM)	Internal concn of lysine (nmols/10^7 cells)		
	V2	SL1	SL2
0	4	88	16
30	5	ND[b]	15
100	12	93	28
300	ND	145	ND
600	—[c]	253	231
1,000	—	191	—
10,000	—	228	—

[a] Results are averages of three independent experiments. The standard errors were less than 10%.
[b] ND, not determined.
[c] —, no growth.

and this property affects sporulation ability (5). For this reason, the DNA contents of the wild type and the mutants were measured by flow cytometry. The wild type had a DNA content of 2.7 n, whereas SL1 had 2.5 n and SL2 had 2.7 n, like the wild type. Although this difference of 7% between SL1 and the wild type was consistent and maintained in every experiment, it is within the error expected in these determinations. When the V2 parental strain was subjected to sporulation conditions, it sporulated and produced fairly viable tetrads (about 50% viability). However, none of the mutants was able to sporulate under any of the conditions used (5). This might suggest that major chromosome rearrangements impede meiosis. The mutants did not conjugate with α or **a** mating strains either.

(ii) Internal versus external concentrations of lysine. Internal lysine concentrations were measured in the wild type and in the mutants after adding different concentrations of external lysine to SDP medium; this was done to discard those mutations of lysine permeases which may account for the resistance to AEC shown by the mutants. Table 2 shows that in up to 600 mM lysine, both mutants incorporated this amino acid in concentrations proportional to the external one; this indicates that their permeases were not affected.

(iii) Internal free amino acid concentrations. The internal pool of lysine, glutamate, arginine, alanine, and aspartate was measured in the V2 strain and in the SL1 and SL2 AEC-resistant mutants grown in minimal medium with either ammonium sulfate (SD), proline (SDP), arginine (SDA), or glutamate (SDG) as the nitrogen source. As a control, the C90 laboratory strain was also included. Glutamate and arginine were chosen because of their close relationship with the lysine biosynthetic pathway, whereas the alanine and aspartate pathways are not closely related to that of lysine.

Table 3 shows that under any of the condition assayed, the SL1 mutant accumulated maximal internal concentrations of lysine (3- to 17-fold greater than those of the wild type). SL2 also accumulated higher concentrations of lysine than did the wild type but substantially (1.5- to 3-fold) less than did SL1. The internal pool of the amino acids aspartate and alanine, whose biosynthetic pathways are related to that of lysine, was almost identical to that of the wild type in the SL1 and SL2 mutants. However, the concentration of glutamate and arginine did show significant variations among the strains, depending on the nitrogen source employed.

(iv) Resistance to AEC, lysine, and α-aminoadipate. The resistance to the amino acid lysine, its toxic analog AEC, and the intermediate metabolite of the lysine biosynthetic pathway

TABLE 3. Internal amino acid concentrations in the V2 baker's strain, in the SL1 and SL2 AEC-resistant mutants, and in laboratory strain C90 cultivated in minimal medium with different nitrogen sources[a]

Amino acid	Concn (nmol/10^7 cells)												
	Ammonium				Proline			Arginine			Glutamate		
	V2	SL1	SL2	C90	V2	SL1	SL2	V2	SL1	SL2	V2	SL1	SL2
Asp	4	5	4	3	3	4	3	4	4	5	4	4	3
Glu	22	2	22	32	6	9	37	22	9	29	62	4	44
Ala	9	5	6	20	1	1	2	8	6	7	2	2	2
Arg	15	16	3	10	1	7	1	103	112	144	7	10	2
Lys	4	18	6	5	5	86	16	2	6	4	12	63	17

[a] Results are averages of three independent experiments. The standard errors were less than 10%.

α-aminoadipate, precursor of the toxic compound α-aminoadipate semialdehyde (33), was determined in the V2 strain and the SL1 and SL2 mutants. We measured the growth rates of these strains grown in minimal medium SD, and in SDP (to allow the transport of lysine, AEC, or α-aminoadipate), supplemented with different concentrations of lysine, AEC, and α-aminoadipate.

Table 4 shows that in SD and SDP, the SL1 and SL2 mutants grew slower than the wild type. However, the SL1 mutant still grew at 2 mM AEC, 10 mM lysine, or 10 mM α-aminoadipate; on the contrary, the SL2 strain stopped growing at 0.1 mM AEC or 1 mM lysine and the wild type displayed maximal sensitivity. Results indicated that both mutants were more resistant to lysine, the intermediate α-aminoadipate, and the toxic analog AEC than was the wild type and that the resistance was related to the internal concentration of lysine accumulated by the mutants and the wild type. Only the SL1 mutant was further characterized.

(v) Enzymatic activities of the V2 strain and the SL1 mutant. Homocitrate synthase (which is the first enzyme of the lysine biosynthetic pathway and is probably essential in lysine biosynthetic pathway regulation), α-aminoadipate reductase, and saccharopine reductase (the enzymes which participate in the reactions just before and after the formation of the toxic intermediate α-aminoadipate semialdehyde) were measured in the V2 strain and the SL1 mutant.

It has been reported that lysine represses the synthesis of these enzymes when yeast cells are grown in its presence and that it inactivates at least one of the homocitrate synthase isoenzymes (31) in in vitro assays. For this reason, the enzymatic activities were measured in cells grown in SD or SDP or in one of these media supplemented with lysine at 30 μg/ml. The assays were carried out with or without 10 mM lysine.

Table 5 shows that the SL1 mutant had about three times as much homocitrate synthase activity as the wild type when the cells were grown in SD with ammonium as the nitrogen source. Under these conditions, lysine is not transported and therefore no effect can be seen when SD is supplemented with this amino acid. In SDP, the SL1 mutant also had more homocitrate synthase activity than the wild type (about twice as much); the addition of lysine, however, repressed the activity in the wild type and in the mutant. Therefore, it seemed that overproduction of lysine by the SL1 mutant was in part due to overproduction of homocitrate synthase and that both the wild type and the SL1 mutant responded to repression by lysine in SDP medium. When the homocitrate synthase assays were carried out in the presence of 10 mM lysine, a strong inhibitory effect on this activity was observed in the wild type and in the mutant, yet the inhibition seemed to be stronger in the mutant.

Table 5 also shows the absolute and relative values, where 100 represents maximal homocitrate synthase activity under any condition. It is noteworthy that the activity of the SL1 mutant in SDP was twice that of the wild type, and the addition of lysine to the culture medium only reduced the activity to 85% of its value without lysine, whereas the activity was repressed to 43% in the wild type. However, when the activity was measured while adding lysine to the assay, the inactivation of the enzyme reached 75% in the mutant and only 50% in the wild type.

When the α-aminoadipate reductase and saccharopine reductase activities were measured after cultivating the cells in SDP medium with or without lysine (Fig. 4), results different from those for homocitrate synthase were obtained. No repression effect of lysine on the α-aminoadipate reductase activity was observed in the wild type. However, the activity in the SL1 mutant was higher than in the wild type (50% increase) and seemed to be repressed by lysine to the level of the wild type (Fig. 4A). This effect was systematically detected. Almost no differences were observed between the wild type and the mutant when saccharopine reductase was determined (Fig. 4B). The two strains display similar levels of specific activity in the absence of lysine, and the addition of this amino acid repressed the enzyme. This effect was slightly higher in the mutant. Identical results were obtained when both enzymes were assayed with or without lysine added, indicating that these enzymes did not undergo inactivation by this amino acid.

(vi) Fermentation ability. To simulate the fermentative capacity shown in an industrial process, the V2 baker's yeast strain and the SL1 mutant were inoculated into a mixture of flour and water and the increase in volume was monitored periodically. As a laboratory control, strain MMY1 was also included. As Fig. 5 shows, the SL1 mutant possessed the wild-type capacity to increase the volume of the dough and to reach the same maximal value. However, the V2 wild-type strain initiated dough leavening after a 20-min lag and its fermenta-

TABLE 4. Growth rates of the V2 baker's yeast strain and the SL1 and SL2 AEC-resistant mutants cultivated in different media[a]

Strain	Growth rate (h^{-1}) in:																	
	SD	SDP + AEC (μM)[b]						SDP + lysine (μM)[b]						SDP + α-aminoadipate (μM)[b]				
		0	20	50	100	200	2,000	0	30	100	600	1,000	10,000	0	500	2,500	5,000	10,000
V2	0.31	0.16	—	—	—	—	—	0.16	0.14	0.06	—	—	—	0.16	0.06	—	—	—
SL1	0.16	0.13	0.13	0.13	0.12	0.12	0.04	0.13	ND[c]	0.12	0.13	0.13	0.13	0.13	0.10	0.11	0.11	0.11
SL2	0.27	0.17	0.19	0.06	—	—	—	0.17	0.19	0.16	0.07	—	—	0.17	0.07	—	—	—

[a] Results are averages of two independent experiments. The standard errors were less than 10%.
[b] —, no growth.
[c] ND, not determined.

TABLE 5. Absolute and relative homocitrate synthase activities[a]

Strain and assay condition	Absolute (relative [%]) activity, % reduction by lysine			
	SD	SD + Lys	SDP	SDP + Lys
V2				
No lysine	70 (25), 49	70 (25), 50	275 (100), 51	118 (43), 49
With lysine[b]	36 (13)	35 (13)	134 (49)	60 (22)
SL1				
No lysine	190 (38), 72	188 (37), 71	504 (100), 77	428 (85), 75
With lysine[b]	53 (11)	54 (11)	118 (23)	107 (21)

[a] The values in parentheses are relative activities (100 is the maximal activity). Cells were cultivated in the media indicated, and assays were done with or without added lysine. Results are averages of three independent experiments. The standard errors were less than 10%. Absolute activity is reported in micromoles per hour per milligram of protein.

[b] 10 mM lysine added to the assay.

tion rate was higher. This strain reached maximal volume after an 80-min incubation, whereas the SL1 mutant did so about 1 h later. The behavior of the SL1 mutant strain under industrial conditions is not known.

DISCUSSION

The selection of amino acid overproduction mutants carried out in continuous culture with increasing concentrations of a toxic amino acid analog has been shown to be successful. Mutants which accumulated up to 150 times as much methionine and 37 times as much threonine as the wild type have been isolated (19).

The method was originally described for selection of ethanol-tolerant yeast strains in a pH-regulated continuous culture with increasing ethanol concentrations (12) but shown to function for any toxic compound. With this procedure, baker's yeast mutants resistant to increasing AEC concentrations have been isolated in this study (Fig. 2). One of them accumulated 3 to 17 times as much lysine as did the wild type (Table 3).

The electrophoretic karyotypes of the wild type and the SL1 mutant (Fig. 3) indicated that some chromosomal rearrangements occur in the mutant during the selective process. These rearrangements, however, did not imply changes in DNA content (2.7 n in the wild type and the mutants) and could not be directly related to the phenotype of the mutants either, including their inability to sporulate or conjugate.

The resistance to AEC could be due to mutations occurring in any of the permeases able to transport lysine, encoded by either the *GAP1* (11, 25), the *CAN1* (9), or the *LYP1* gene (28). However, the mutants possessed functional permeases for lysine (Table 2). This is not surprising, since the DNA content of the strain is 2.7 n and most of the mutations producing a phenotype are expected to be dominant. In fact, the resistance seemed to proceed from dilution of the toxin by the large amounts of lysine produced by the AEC-resistant mutants (Table 3). Whereas the mutants accumulated lysine and had altered concentrations of amino acids whose metabolic pathways are closely related, such as glutamate and arginine, almost no variations were detected in other amino acid, such as aspartate or alanine (Table 3). This suggested that only the lysine biosynthetic pathway was affected. In fact, the alterations of some amino acid concentrations may account for the lower growth rate of the mutants than the wild type under certain conditions (8).

The mutants isolated in this study seemed to have partially lost the repression effect of lysine on the gene which encodes the key enzyme of the biosynthetic pathway, homocitrate synthase, encoded by the *LYS20* gene (24) (Tables 4 and 5). In consequence, the mutants overproduced lysine, diluting the toxic analog. This is the reason why SL1 and SL2 were resistant to AEC (Table 4).

When homocitrate synthase, α-aminoadipate reductase, and saccharopine reductase were measured in the SL1 mutant (Fig. 4 and Table 5), the results confirmed the derepressed activity shown by homocitrate synthase (a threefold increase). α-Aminoadipate reductase increased by only 50%, and saccharopine reductase activity did not change. Neither did the regulation of these two enzymes or their insensitivity to inactivation by lysine with regard to the wild type.

Supporting our data, other authors (31) have reported that the synthesis of these three enzymes is repressed by lysine, but that only homocitrate synthase is inhibited by this amino acid (3, 26, 27). These results also suggested that homocitrate synthase is more strongly regulated and therefore is the key en-

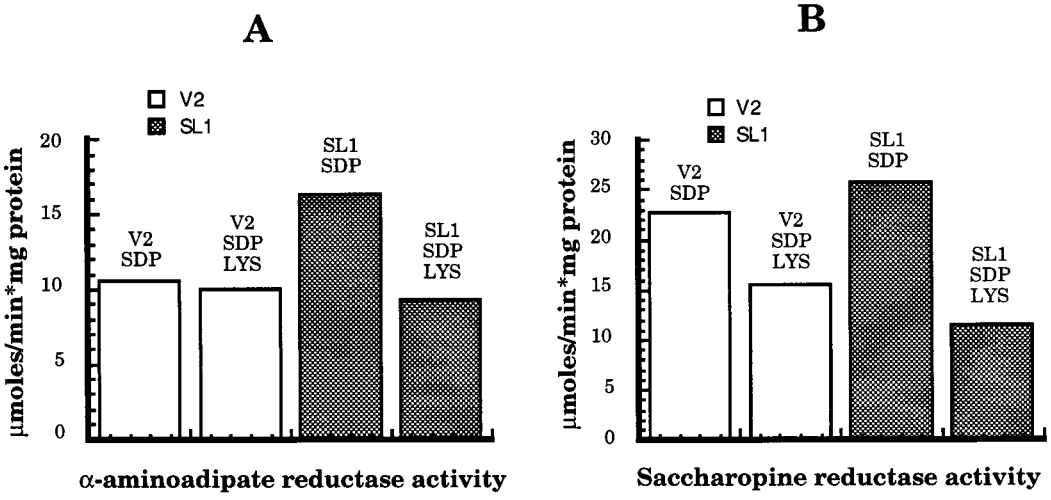

FIG. 4. α-Aminoadipate reductase (A) and saccharopine reductase (B) activities of the V2 baker's yeast strain and the SL1 lysine-overproducing mutant grown in SDP or in this medium supplemented with lysine at 60 mg/liter. No inhibitory effect exerted by lysine was observed when it was added to the assay at a final concentration of 10 mM. The results shown are averages of three independent experiments, and the standard errors were less than 10%.

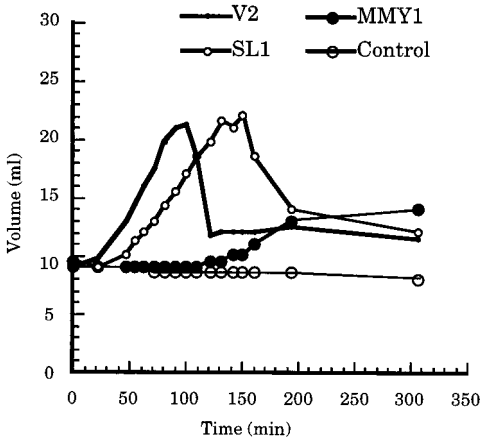

Fig. 5. Leavening ability of the V2 baker's yeast strain, the SL1 lysine-overproducing mutant, and the MMY1 laboratory strain. The control was a noninoculated mixture of flour and water. The results shown are averages of two independent experiments, and the standard errors were less than 10%.

zyme of the lysine biosynthetic pathway. The lack of α-aminoadipate reductase repression by lysine (Fig. 4) could be due to the lack of isogenicity of V2 with the strain described by Urrestarazu et al. (31). According to our data, the homocitrate synthase of the V2 strain is inhibited 50% by 10 mM lysine, whereas laboratory strains showed 90% inhibition (29, 30), probably for the same reason. Other yeast amino acid-overproducing mutants that have been described, which overproduced methionine and threonine (19), possessed key biosynthetic enzymes which have lost the inhibitory effect of the amino acid without their synthesis regulation being affected (20). However, Ramos and Wiame (23) and Gray and Bhattacharjee (7) have reported that lysine accumulation is the result of lack of homocitrate synthase repression. The origin of these differences may be the fact that lysine is located in vacuoles, whereas the lysine biosynthetic enzymes are located in the mitochondria, the cytosol, and the nucleus (1, 4).

In addition to the fact that lysine represses the synthesis of many enzymes of its biosynthetic pathway (8), the inhibitory effect on the growth rate of wild-type strains caused by lysine could be due to the accumulation of the toxic intermediate α-aminoadipate semialdehyde. Wild-type cells stopped growing when α-aminoadipate was added to the growth medium, but it had no effect on *lys2* or *lys5* mutants (Fig. 1) (33, 35).

Thus, high concentrations of lysine might sequentially stop reaction 8 (Fig. 1) and then reaction 7, until a critical concentration of α-aminoadipate semialdehyde, higher than the usual one, inhibits the cells. This accumulation might be favored by the lack of inhibition and low repression of, at least, saccharopine reductase by lysine (Fig. 1).

We did not however, find a logical explanation for the resistance to lysine and α-aminoadipate shown by the AEC-resistant mutants. The chromosomal rearrangements observed in the mutants (Fig. 3) and the selective pressure exerted on the V2 strain while the cells were incubated in the presence of AEC in the continuous culture indicated that the AEC-resistant strains had accumulated several mutations which might account for the results obtained. Finally, the accumulation of lysine in the AEC-resistant mutants results in toxicity, as reflected in their low growth rate and decreased leavening ability (Fig. 5).

With regard to the possible location of the mutation responsible for lysine overproduction in the SL1 mutant, the most reasonable mutations could be located either in the negative transcription regulator of the genes encoding enzymes involved in the lysine biosynthetic pathway (Fig. 1) and corresponding to the *LYS80* gene product (23) or in the regulatory region of the *LYS20* gene encoding the homocitrate synthase isoenzyme (24). However, *lys80* mutants have a two- to six-fold increase in several enzymes of the lysine biosynthetic pathway and not only in the homocitrate synthase pathway; these enzymes are not repressed by lysine, and mutations in the *LYS80* gene are recessive. When the SL1 mutant was analyzed in this work, only homocitrate synthase showed a clear (threefold) increase in specific activity (Table 5). This enzyme, as well as α-aminoadipate reductase and saccharopine reductase, was repressed by lysine (Fig. 4). Finally, the SL1 mutant was crossed (by protoplast fusion and appropriate selection) with the MMY1 and MMY2 laboratory strains. The hybrids behaved the same way as the SL1 mutant insofar as their resistance to lysine, α-aminoadipate, and AEC was concerned. The mutation is therefore dominant. Unfortunately, no viable meiotic product was obtained from the hybrids and it was therefore impossible to analyze the segregation of the character or to carry out allelic tests with *lys80* strains. These data allow us to discard the idea that the *LYS80* gene product is affected in the SL1 mutant. The same reasons could be given to discard the idea of a mutation in the positive regulator encoded by the *LYS14* gene, so that the SL1 mutant is probably mutated in the regulatory region of the *LYS20* gene.

During the production of bakery products, a certain amount of yeast is added to increase the nutritive properties of the product obtained. In this sense, the addition of baker's yeast could be carried out with lysine-overproducing mutants since none of the mutants isolated secretes the amino acid. Instead, they accumulate it, making it part of their biomass.

AEC resistance could also be useful as a dominant marker, which is very scarce and necessary in industrial yeasts, most of which are polyploid.

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