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Overproduction of Threonine by *Saccharomyces cerevisiae* Mutants Resistant to Hydroxynorvaline

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In this work, we isolated and characterized mutants that overproduce threonine from *Saccharomyces cerevisiae*. The mutants were selected for resistance to the threonine analog α-amino-β-hydroxynorvalerate (hydroxynorvaline), and, of these, the ones able to excrete threonine to the medium were chosen. The mutant strains produce between 15 and 30 times more threonine than the wild type does, and, to a lesser degree, they also accumulate isoleucine. Genetic and biochemical studies have revealed that the threonine overproduction is, in all cases studied, associated with the presence in the strain of a *HOM3* allele coding for a mutant aspartate kinase that is totally or partially insensitive to feedback inhibition by threonine. This enzyme seems, therefore, to be crucial in the regulation of threonine biosynthesis in *S. cerevisiae*. The results obtained suggest that this strategy could be efficiently applied to the isolation of threonine-overproducing strains of yeasts other than *S. cerevisiae*, even those used industrially.

In *Saccharomyces cerevisiae*, threonine and methionine are synthesized from aspartate through a common metabolic sequence that leads to homoserine in three steps. Homoserine is the branching point from which the pathways for methionine and threonine diverge. The regulation of this route takes place at a number of steps, both at the level of enzyme synthesis and at the level of activity (for a review, see reference 8). Threonine seems to be the primary compound that regulates the carbon flow into and through the common pathway, specifically at the level of aspartate kinase and homoserine dehydrogenase, the first and third enzymes of this route, respectively. Moreover, the flow into each specific arm is controlled by end product inhibition of the corresponding first enzymes: threonine inhibits the homoserine kinase, while methionine inhibits the homoserine *O*-acetyltransferase.

The regulation of an amino acid biosynthetic route can be investigated by the use of amino acid analogs. Mutants that are resistant to a toxic analog are good candidates to be altered in their ability to regulate the synthesis of the natural amino acid. In fact, many analogs have been used to isolate amino-acid-overproducing strains, both in prokaryotes (1) and in eukaryotes (15).

α-Amino-β-hydroxyvaleric acid (hydroxynorvaline) is a threonine analog known to inhibit bacterial growth mainly because it mimics threonine and hinders the activity of the threonine feedback-inhibited enzymes (4). As a consequence, cells growing in the presence of hydroxynorvaline produce smaller quantities of the amino acids synthesized through this pathway, which can even become limiting for growth. Hydroxynorvaline-resistant mutants have been isolated from bacteria such as *Escherichia coli* and *Brevibacterium* and *Corynebacterium* spp. (13). In these mutants, one of the threonine-sensitive enzymes has become insensitive to the feedback control by this amino acid and, as a consequence, the mutants overproduce to a certain degree and even excrete threonine into the medium.

To our knowledge, hydroxynorvaline has never been tested before in any yeast species. If the situation in this organism was similar to that in bacteria, one would expect that, in a certain hydroxynorvaline-resistant yeast strain, one or more steps of the pathway would have been released from threonine inhibition. Some of these mutants could also overproduce and excrete threonine. The frequency at which each kind of mutant arises and the degree of overproduction of the amino acid would be indicative of the relative importance of that step in the control of the pathway. Additionally, hydroxynorvaline resistance could also arise by a defect in the permease that transports it into the cell.

In this work, we have isolated hydroxynorvaline-resistant mutants from *S. cerevisiae* and have selected from among them those able to excrete threonine into medium, which indicates threonine overproduction. We have studied the accumulation of threonine and other amino acids in the mutant cells and characterized their defect both biochemically and genetically.

MATERIALS AND METHODS

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

Enzymes and chemicals. Hydroxynorvaline was a gift from Degussa A.B.M. (Frankfurt, Germany); borrelidin was a kind gift from K. Poralla; lactate dehydrogenase (EC 1.1.1.27), pyruvate kinase (EC 2.7.1.40) from rabbit muscle, phosphoenol pyruvate, *o*-phthalaldehyde, and β-mercaptoethanol were from Sigma Chemical Co. (St. Louis, Mo.); *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) was from Aldrich Co. (Dorset, United Kingdom); all other biochemicals were purchased from Sigma. Inorganic compounds were from Merck AG (Darmstadt, Germany).

Media. Complete (YPD) medium, minimal (SD) medium, and presporulation and sporulation media were prepared by the methods of Sherman et al. (20). Minimal proline (SDP) medium is identical to SD medium, except that L-proline (0.1%) is used instead of ammonium sulfate (0.5%) as the sole nitrogen source (6). Hydroxynorvaline toxicity was

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

Strain(s)	Genotype or phenotype	Source[a]
D273-11A	*MATα ade1 his1*	CSH
F4	*MATa thr4*	LCC
MMY1	*MATα ura3D52* Cyh^R	SERI
XCR13-5A	*MATa trp1-1 lys1-1 ura4 leu2*	DGS
XCR28-4A	*MATa ura3 arg6 hom3 his1*	DGS
AHV1 to AHV6	*MATa trp1-1 lys1-1 ura4 leu2* Ahv^R Thr^E	This work
YAHV1	*MATa ura4 HOM3-R1*	This work
YAHV2	*MATa HOM3-R2*	This work
YAHV3	*MATa HOM3-R3*	This work
YAHV5	*MATa ura3 HOM3-R5*	This work
YAHV6	*MATa ura3 HOM3-R6*	This work

[a] Abbreviations: CSH, Cold Spring Harbor Laboratory (Cold Spring Harbor, N.Y.); LCC, La Cruz del Campo S.A. (Seville, Spain); SERI, Solar Energy Research Institute (Golden, Colo.); DGS, Departamento de Genética y Biotecnia, Universidad de Sevilla (Seville, Spain).

tested in SDP plates containing 10 mM hydroxynorvaline. Under these growth conditions, the MIC for the wild type was found to be 2.5 mM. When necessary, minimal media were supplemented with the appropriate requirements (20).

Mutagenesis. Early-stationary-phase cells of strain XCR13-5A grown in YPD medium at 30°C were treated with NTG as recommended by Calderón and Cerdá-Olmedo (3). Under these standard conditions, the survival rate of this strain was approximately 40%.

Cross-feeding test for threonine excretion. Cells of the Thr^- strain F4 were grown to the stationary phase in YPD medium, centrifuged, washed twice, and suspended in water. A sample of this cell suspension, containing about 10^6 cells, was spread onto SD plates. The strains to be tested were streaked onto this cell lawn with toothpicks, and the plates were incubated at the temperatures stated for different periods of time. The presence of a halo of F4 colonies growing around a streak was considered a positive result.

Genetic analysis. Conventional procedures were used for strain mating, diploid selection, and tetrad analysis (20).

Partial purification of the aspartate kinase. Aspartate kinase was partially purified by the method of Ramos et al. (16). The method is basically that described as follows. Late-exponential-phase cells, grown at 30°C in SD medium, were harvested and resuspended in AT buffer (40 mM phosphate [pH 7.2], 0.1 M KCl, 5 mM $MgCl_2$, 2 mM EDTA, 1 mM dithioerythritol 1 mM L-threonine [19]) and disrupted in a Braun homogenizer with 0.5-mm-diameter glass beads. Cell debris was removed by two consecutive centrifugations at 4°C, the first for 20 min at 8,000 × *g* and the second one for 2 h at 105,000 × *g*. Protamine sulfate (5 mg/g [wet weight]), neutralized to pH 7 with NaOH, was added to the crude extract, and, after 1 h of stirring at 4°C, the pellet was removed by centrifugation. Ammonium sulfate was added, and the fraction precipitating between 30 and 45% saturation that contained the aspartate kinase activity was redissolved in BT buffer (identical to AT buffer except that the phosphate concentration was 20 mM), dialyzed extensively against the same buffer, and stored at −80°C.

Aspartate kinase assay. Aspartate kinase activity was assayed as previously reported (16) by monitoring ADP production by use of the pyruvate kinase-lactate dehydrogenase coupled assay described for the *E. coli* homoserine kinase (2, 21), except that 10 mM ATP and 4 mM L-aspartate were used as the substrates. One unit of activity was defined as the amount of partially purified extract required to convert 1 μmol of ATP to ADP per min at 30°C. The standard assay mixture contained the components described previously (19), with minor modifications. The reaction was initiated by adding L-aspartate to the mixture. Protein concentrations were determined by the method of Lowry et al. (9).

Amino acid determination. Amino acids were analyzed by reverse-phase liquid chromatography by the method of Martínez-Force and Benítez (10). The system consisted of a Waters high-performance liquid chromatograph with a Novapack C-18 column (18 by 100 mm), two pumps, and a Waters absorbance detector. An IBM AT computer with a Baseline 810 chromatography workstation was used as the controller, integrator, and data collector. The gradient program consisted of two separate solvent mixtures. Solvent A consisted of 20 mM sodium phosphate (pH 7.2), 1.5% tetrahydrofuran, and 93.5% distilled water (by volume); solvent B consisted of 10 mM sodium phosphate (pH 7.2), 55% acetonitrile, and 42.5% distilled water (by volume).

Amino acid samples were prepared as described previously (5). Samples (40 ml) of a late-exponential-phase culture (optical density at 660 nm, approximately 0.7), were centrifuged. The supernatant was freeze-dried and resuspended in 1 ml of deionized water, and the suspension was filtered through a 0.45-μm-pore-size Millipore filter and used to measure excreted amino acids. To determine intracellular amino acids, the pellet was resuspended in 2 ml of deionized water and boiled for 15 min. A clear supernatant was obtained after a brief centrifugation and a filtration as described above.

Amino acids were derivatized by mixing 25 ml of each suspension with 75 ml of the following mixture: 0.4 M sodium borate (pH 10), 4.5 ml; 54 mg of *o*-phthalaldehyde per ml of methanol, 0.5 ml (10).

RESULTS

Conditions for hydroxynorvaline toxicity test. Preliminary experiments showed that hydroxynorvaline is toxic in media with a poor nitrogen source such as proline but not in media with a good nitrogen source such as ammonium sulfate (data not shown). This is probably due to the fact that this compound enters the yeast cell mainly through the general amino acid permease (11), whose activity is both inhibited and repressed by ammonium (6). Consequently, all of the media in which hydroxynorvaline toxicity was to be tested carried proline as the sole nitrogen source.

It was also found (data not shown) that the addition of different amino acids such as threonine, homoserine, asparagine, and citrulline to media containing hydroxynorvaline counteracted its toxicity. The degree of this effect was variable, probably depending on the mechanism involved in each case. As expected from the proposed hydroxynorvaline mode of action (4), threonine and homoserine, a threonine precursor, improved the strain growth. It is very likely that the threonine or homoserine addition bypasses the need for the enzymes acting in this biosynthetic pathway, some of which are inhibited by this drug (16). Consistent with this view, it was found that, even though methionine alone had no effect, the simultaneous addition of threonine and methionine completely restored growth. Besides, these and other amino acids may exert their influence at the transport level. Citrulline, for instance, may compete with hydroxynorvaline for the general amino acid permease, while others that serve as good nitrogen sources (e.g., asparagine) may affect the

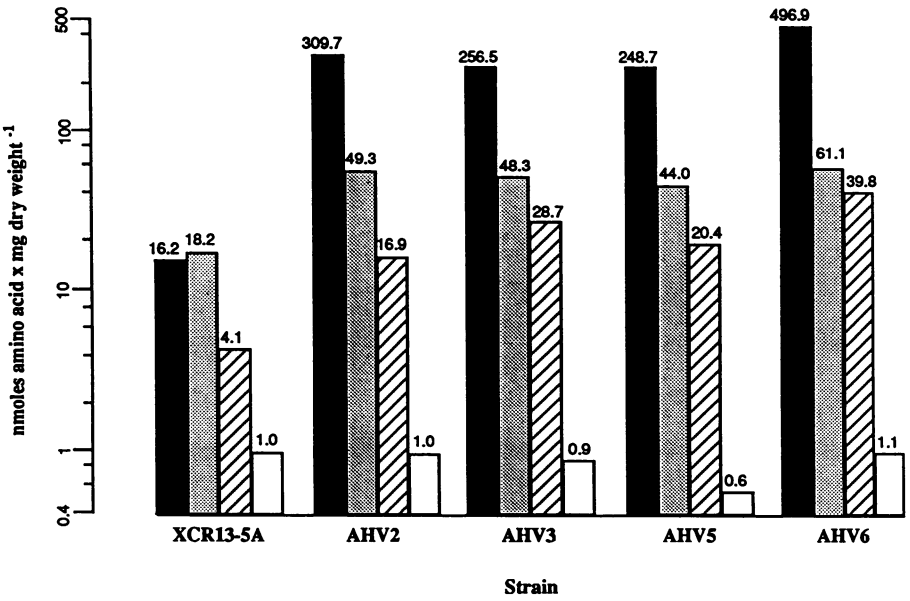

FIG. 1. Quantities of amino acids accumulated (inside) and excreted to the medium (outside) by different Ahv^R Thr^E strains. Symbols: ■, threonine inside; ▩, threonine outside; ▨, isoleucine inside; □, methionine inside.

general amino acid permease activity, thus hindering its entrance into the cell.

Isolation of hydroxynorvaline-resistant (Ahv^R) mutants. Cells of the wild-type strain XCR13-5A were plated, either directly or after nitrosoguanidine treatment, onto SDP medium containing 10 mM hydroxynorvaline, and the plates were incubated for 5 days. Spontaneous and NTG-induced mutants arose among the survivors at frequencies of 1.5×10^{-6} and 5.0×10^{-5}, respectively.

In *S. cerevisiae*, the excretion of an amino acid by a certain strain usually indicates overproduction of this amino acid. This criterion was applied to select candidates to become threonine overproducers from the Ahv^R strains. Thus, all Ahv^R colonies obtained were subjected to the threonine cross-feeding test described in Materials and Methods. While 60 of the 128 NTG-induced Ahv^R mutants tested were threonine excretors (Thr^E), none of the 60 spontaneous Ahv^R colonies were Thr^E. We do not have a plausible explanation for this difference. The Ahv^R Thr^E strains (designated by AHV followed by a number) were used in further studies.

Dominance or recessiveness test. The dominance or recessiveness of the alleles responsible for the Ahv^R and Thr^E phenotypes with respect to the wild-type alleles was investigated by crossing the mutant strains with strain MMY1. All of the resulting diploids were able to grow on SDP medium containing 10 mM hydroxynorvaline, after 4 days of incubation at 30°C, proving the dominance of the mutant alleles. All of them were also able to cross-feed the Thr^- strain, i.e., to excrete threonine, when incubated for 4 days at 37°C. However, when incubated at 22°C, they showed a variety of responses in the cross-feeding test: no diploid excreted before 4 days of incubation, and while some presented an excretion halo after 7 days, others did not, even after 11 days of incubation. Five AHV strains whose respective AHV/MMY1 diploids showed different cross-feeding times at 22°C were chosen for further characterization. The selected strains were AHV1 (cross-feeding time, 7 days), AHV2 and AHV3 (cross-feeding times, 9 days), AHV5 (cross-feeding time, 11 days), and AHV6 (no cross-feeding observed after 11 days of incubation).

Accumulation of amino acids by the mutant strains. To establish whether the Ahv^R Thr^E strains were, in fact, threonine overproducers, the quantities of this and other amino acids accumulated and excreted into the medium by these strains were investigated. The results (Fig. 1) show that all of them accumulate 15 to 30 times more threonine and excrete about 3 times more threonine than the parental strain. They also contain more isoleucine, an amino acid that derives from threonine, than the control does. Both in the mutants and in the parental strain, the accumulated isoleucine seems to be proportional to that of threonine (about 1:5 to 1:3). However, the amount of methionine, whose biosynthetic pathway shares several steps with that of threonine, is similar to that of the control in all mutants.

Genetic characterization. The following experiments were designed to determine how many genes are responsible for the Ahv^R and Thr^E phenotypes and to try to establish what gene(s) is responsible for the threonine overproduction phenotype. Previous kinetic studies demonstrated that the aspartate kinase plays a very important role in the regulation of the pathway at the enzymatic activity level (16). Thus, we first tried to prove if, at least in some cases, threonine overproduction depends on the presence in the cell of a mutant allele of the *HOM3* gene, which codes for this enzyme. This kind of test is usually carried out by meiotic analysis of crosses between the mutant strain and a strain carrying a known allele, in this case, *hom3*. However, such a cross provides only partial information since the $Hom3^-$ spores require the presence of homoserine in the medium. This would interfere severely with the hydroxynorvaline resistance test. Consequently, only $Hom3^+$ spores are susceptible to such an analysis.

As stated above, the presence of amino acids in the medium interferes with the hydroxynorvaline resistance test. Thus, it was found necessary to eliminate as many genetic markers as possible from the cross. AHV/MMY1 diploids were sporulated, and the resulting tetrads were

TABLE 2. Genetic analysis of Ahv^R Thr^E strains

Cross	Markers	No. of tetrads[a]		
		PD	NPD	T
YAHV2 × D273-11A	Thr^E-*HIS1*	26	0	0
	Ahv^R-*HIS1*	26	0	0
	Ahv^R-Thr^E	26	0	0
YAHV5 × D273-11A	Thr^E-*HIS1*	19	0	0
	Ahv^R-*HIS1*	19	0	0
	Ahv^R-Thr^E	19	0	0
YAHV6 × D273-11A	Thr^E-*HIS1*	13	0	1
	Ahv^R-*HIS1*	13	0	1
	Ahv^R-Thr^E	14	0	0

[a] PD, parental ditype; NPD, nonparental ditype; T, tetratype.

TABLE 3. Effect of L-threonine and hydroxynorvaline on the aspartate kinase activity of the Ahv^R Thr^E strains[a]

Strain	Sp act (mU mg of protein^{-1})	Inhibition (%) by:	
		20 mM L-Threonine	50 mM Hydroxynorvaline
XCR13-5A	188	90	91
AHV1	38	0	0
AHV2	45	0	0
AHV3	135	0	32
AHV5	37	0	0
AHV6	44	50	21

[a] Each value represents the mean of at least two experiments; the standard deviation was, in all cases, around 15%.

dissected. Meiotic descendants from these diploids showing an Ahv^R Thr^E phenotype and either a Ura^+ or Ura^- phenotype were chosen and called YAHV (followed by the same number as that of their parents). When analyzing this cross, it could already be stated that, in all cases, the excretion of threonine segregated in a 2+:2− fashion, indicating the monogenic nature of this character.

Strains YAHV1, YAHV2, and YAHV5 were crossed with strain XCR28-4A (*hom3*). About 20 tetrads deriving from each cross were dissected and analyzed. In these crosses, the excretion of threonine also segregated in a 2+:2− fashion. Moreover, all of the Thr^E spores were $Hom3^+$ and no recombinant $Hom3^+$ Thr^{NE} (threonine-nonexcreting) spore was observed. This result suggests that *hom3* and the three mutations are allelic. Therefore, these mutations have been designated *HOM3R-1*, *HOM3R-2*, and *HOM3R-5*.

This conclusion was further proven when Ahv^R Thr^E mutants were crossed with a strain carrying a *his1* allele. The genes *HIS1* and *HOM3* are located in the *S. cerevisiae* map at a distance of 2.5 centimorgans (12). This cross allowed us to carry out whole-tetrad analysis.

Table 2 presents the results of the genetic analysis of the crosses between the strains YAHV and D273-11A. It clearly shows the close linkage between the markers studied. Thus, we can infer that the overproduction of threonine depends on the presence in these strains of mutant alleles of the *HOM3* gene.

Aspartate kinase activity in the mutants. Studies carried out to determine the kinetic parameters of the aspartate kinase showed that both substrates, ATP and L-aspartate, displayed normal Michaelis-Menten saturation kinetics, with K_ms of 3.6 and 4 mM, respectively (16). It has also been established that threonine very effectively inhibits the aspartate kinase activity, 3 mM being the concentration required for half-maximal inhibition (16). Of other amino acids tested, only hydroxynorvaline inhibits this activity (16). These and other studies suggest that the regulation of the aspartate kinase of *S. cerevisiae* takes place mainly through feedback inhibition by threonine (16). Thus, an alteration in this enzyme, such as making possible the activity of the enzyme but not its regulation by threonine, could cause overproduction of this amino acid. To test whether this was the case with the Ahv^R Thr^E mutants, their aspartate kinase was partially purified and the effects of threonine and hydroxynorvaline on the activity were assayed. The results (Table 3) show, in fact, that all mutants tested have an altered pattern of inhibition by both amino acids.

DISCUSSION

In this article, we describe the isolation and characterization of *S. cerevisiae* mutants resistant to the threonine analog hydroxynorvaline. Among them, those able to cross-feed a threonine auxotroph were chosen as candidates for threonine overproduction. This criterion has proven to be useful since all selected strains are, in fact, threonine overproducers. However, a negative result in this biological test does not necessarily mean that the strain produces the same amount of threonine as, or less than, the wild type. It is possible that even if a large amount of threonine is produced per cell, either it is not excreted into the medium or the amount produced and excreted is not enough to feed the tester strain. It is also possible that the growth rate of the strain is so low that the final biomass, and thus the total production of threonine, is small. If so, one would expect a continuous distribution of the Ahv^R mutants with respect to threonine production; only those mutants producing and excreting a certain level of threonine would be detected as such in the test. In fact, when the cross-feeding test was performed at 22°C, the heterozygotic Ahv^R Thr^E/wild type diploids behaved very differently. However, this behavior does not correlate with the threonine production of the correspondent haploid strain; in fact, the AHV6/MMY1 diploid is the slowest excretor, despite the fact that AHV6 is the best threonine producer. Moreover, no correlation was found between the amount of threonine produced and the amount excreted by the mutants: the amount of accumulated threonine varied between 15 and 30 times more than that in the wild type, while the quantity of excreted threonine was, in all of the mutants, only about 3 times higher than that in the wild type (Fig. 1). Thus, we can infer that the variety found among the heterozygotic diploids in the cross-feeding test is not due to true differences in excretion but is due to differences in other factors such as growth rate or lysis of the strain at that temperature. Since one of the parents of the diploids is, in all cases, strain MMY1, such differences should reflect differences between the AHV mutants. These mutants may differ not only in their *HOM3* allele but also in the genetic background due to the indiscriminate action of the nitrosoguanidine.

S. cerevisiae mutants able to excrete amino acids have been isolated previously (15). In all cases studied, excretion was shown to be a direct consequence of overproduction. Two genes specifically affecting threonine excretion (*TEX1* and *TEX2*) have been described (5). These genes are linked neither to each other nor to *HOM3* (5). Laboratory strains seem to have different combinations of mutant and wild-type

TEX alleles. The close relationship between overproduction and excretion found in all of the genetic crosses carried out in this work suggests that all strains used are isogenic with respect to the *TEX* alleles, and, therefore, that this factor is irrelevant to the interpretation of the results.

All Ahv^R Thr^E mutants examined so far have an aspartate kinase that is totally or partially insensitive to feedback inhibition by threonine. Other authors, using different strategies, have found similar results. Nass and Poralla (14) isolated mutants resistant to the macrolide antibiotic borrelidin. Their mutants fell into four groups. Only one of them, *BOR1*, corresponds to strains that overproduce threonine; all *BOR1* strains bear mutations in the *HOM3* gene. Thus, borrelidin is not adequate to obtain threonine-overproducing strains, since most of the resistant mutants isolated do not accumulate this amino acid. As expected, AHV mutants are also resistant to 20 μM borrelidin (data not shown), and, conversely, the *BOR1* mutants tested (BOR1-1 and BOR1-III [see reference 14]) are resistant to hydroxynorvaline (data not shown).

Delgado et al. (5) showed that it is possible to directly isolate threonine-overproducing strains by reverting a $Hom3^-$ mutant; all of the isolated strains had a feedback-insensitive aspartate kinase. Given the mode of action of hydroxynorvaline (4), one would expect to find, among the Ahv^R Thr^E strains, mutants in the enzymes susceptible to feedback inhibition by threonine, namely, aspartate kinase, homoserine dehydrogenase, and homoserine kinase. However, it has been reported that yeast aspartate kinase is much more sensitive to inhibition by hydroxynorvaline than homoserine kinase (16). Therefore, it is logical that only mutants in the aspartate kinase were isolated. On the other hand, no correlation was found between the degree of sensitivity to feedback inhibition by threonine or hydroxynorvaline of the aspartate kinase and the production of threonine (Table 3). This result could mean that the conditions used to measure the inhibition do not totally reflect the "in vivo" activity of the enzymes. Table 3 also shows that the specific activity of the aspartate kinase in crude extracts of mutants is smaller than that of the wild type. This effect could be due to repression by threonine of the *HOM3* gene, but other causes cannot be dismissed. Thus, it appears that the presence in the cell of a threonine-insensitive aspartate kinase overcomes other constraints imposed on its activity, that is, the regulation takes place mainly at the enzymatic activity level.

AHV mutants accumulate homoserine to about the same level as *BOR1* mutants do (18), i.e., about 10 times more than the wild type (data not shown). They also overproduce isoleucine but to a lesser degree than threonine does (Fig. 1). The regulation of the pathway leading from threonine to isoleucine relies mainly on the *ILV1* gene and its product (L-threonine deaminase), which catalyzes the conversion of threonine into α-ketobutyrate (7). Isoleucine overproduction in the mutants is probably due to the induction by threonine of the gene *CHA1* coding for a catabolic L-serine (L-threonine) deaminase (17). This enzyme can carry out the same reaction as the anabolic threonine deaminase but is not sensitive to feedback inhibition.

The mutants do not accumulate more methionine than the wild type does, which indicates that the methionine-specific part of the route is strictly controlled, as expected from the reported strong inhibition and repression of the homoserine-*O*-transacetylase exerted by methionine and *S*-adenosylmethionine (8). Conversely, and on the basis of the results presented above, the last part of the threonine pathway seems to play a secondary role in the regulation of the biosynthesis of this amino acid.

The facts that all Ahv^R Thr^E mutants isolated are threonine overproducers and that the *HOM3-R* alleles are dominant suggest that this strategy could be efficiently used for the isolation of threonine-overproducing strains of industrial yeast species.

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