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Evidence for Mutations in the Structural Gene for Homocitrate Synthase in *Saccharomycopsis lipolytica**

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Summary. Eight strains devoid of homocitrate synthase activity were found among lysine requiring mutants of the yeast *Saccharomycopsis lipolytica*. Genetic analysis of these strains showed that they were all affected at the same locus *LYS 1*. Three lines of evidence suggest that this locus defines a structural gene for homocitrate synthase. First, the mutations show various degrees of intragenic complementation; it could be shown in some cases that the hybrid enzyme formed in vivo displayed modified properties in vitro. Second, reversion of some of these mutations can result in a modified enzyme (desensitized). Third, a feedback mutant of homocitrate synthase was directly isolated from the wild type strain, and shown to carry a single mutation at or near *LYS 1*.

We also present here the first attempts at genetic fine mapping in *Saccharomycopsis lipolytica*.

Introduction

The biosynthesis of lysine in yeasts and fungi occurs via the α-aminoadipate pathway (Strassman and Weinhouse, 1953). The first step of this pathway, the synthesis of homocitric acid from 2-oxoglutaric acid and acetylcoenzyme A, is catalyzed by homocitrate synthase (EC.4.1.3.21). This enzyme is feedback inhibited by lysine in yeast (Tucci and Ceci, 1972) and in *Penicillium* (Demain and Masurekar, 1974). In *Saccharomyces cerevisiae*, an extensive search for lysine-less mutants has been conducted, but no strain lacking homocitrate synthase has so far been found (Bhattacharjee and Sinha, 1971). This has been ascribed by Tucci and Ceci 1972) to the existence of isoenzymes, presumably coded for by independent genes.

In *Saccharmoycopsis lipolytica,* we found no evidence for the occurence of two homocitrate synthases (Gaillardin et al., 1976a), and strains devoid of this activity have been obtained. The possibility nevertheless remained that these mutants actually had resulted from a mutation in (e.g.) a regulatory rather than structural gene. The existence of multiple loci controlling a single enzymatic step has been well documented in yeasts (see for instance Bhattacharjee and Sinha, 1971; Masselot and Surdin-Kerjan, 1977).

We therefore set out for better characterizing our mutants on a physiological and genetical basis.

Material and Methods

Origin and Nomenclature of the Strains

All strains derive from two wild type isolates, W29 and Z30 of mating types *A* and *B*. Auxotrophs derived from them were backcrossed 4 to 5 times to inbred strains previously described (Gaillardin et al., 1973) and we used following "standard" strains: 8051-9 and 8051-10 (*A* and *B, lys1.13)*; 8051-11 and 8051-13 (*A* and *B, his1.*); 105-9 and 105-1 (*A* and *B, ura2.*); 13131-6 and 13131-8 (*A* and *B, ade1.)*; 103-1 (*B, arg1.*). The isolation of mg-5 (ATCC number 20462) from W29 has been reported earlier (Gaillardin et al., 1975): homocitrate synthase of mg-5 is no longer sensitive to feedback inhibition by lysine.

Most of the lysine-less strains used here were derived from W29 and Z30, before inbred strains were available (see Table 1). The conditions for mutagenesis were described previously (Gaillardin et al., 1973), along with the procedure for auxotrophs enrichment using the fungicide nystatine. These *lys*⁻ strains were crossed at least 3 times against the "standard" strains, both to increase spore viability and mating frequency, and to introduce the auxiliary auxotrophic requirements needed for complementation studies (see below). For the sake of simplicity, these strains will only be described by their known genotype where they appear in text and tables. Diploids are designated by the genotypes of their parents,

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Abbreviations used: lys: lysine; arg: arginine; ade: adenine; ura: uracile; TDL: 4,5-transdehydrolysine; *Sm.: Saccharomycopsis;* KR: kilorads.

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e.g.: *A, ade1./B,his1.* for a diploid resulting from a cross between 13131-6 and 8051-13.

Media and Culture Conditions

We described previously the following media (Gaillardin et al., 1973): complete yeast extract medium (YE), minimal glucose-ammonia medium (GN), mating medium (YM) and sporulation medium (V8). GN medium was supplemented as required with aminoacids and bases, each 100 mg/l (e.g. GNlys); for lysine requiring diploids, V8 medium was supplemented with 100 mg/l lysine (V8lys). The lysine analog 4,5-transdehydrolysine was filter sterilized (this product was synthetized on request by the Ecole Supérieure de Chimie, Mulhouse, France). Cultures were usually grown at 28° C, except were noted.

Complementation Tests

Due to poor mating frequency, the diploids had to be selected for by prototrophy, using auxiliary auxotrophic requirements. The parents were grown as parallel streaks on master plates of YE medium and cross-relica plated on an YM plate (20 to 25 crosses could be conducted an the same YM plate) After 4 days at 25° C, the YM plates were replicated on selective GN medium: the diploids appeared after 3–4 days at 30° C, usually as a confluent lawn at the intersection of complementing strains.

To test for complementation among lys^- strains, we used strains carrying an additional auxotrophy (*ura2., ade1., his1.)*: the diploids were first selected on GNlys medium, and complementation was tested by replica on GN medium. As a safety, complementing diploids were restreaked on GNlys for single colonies and tested for complementation and sporulation. The complementation map shown on figure 1 was drawn following Gillie (1966).

Sporulation and Meiotic Mapping

Sporulating diploids turn dark brown after 8 days on V8 medium. Random spore analysis was performed as already described (Gaillardin et al., 1973; Ogrydziak et al., 1978). Spore preparations were checked under the microscope for clumping, and those showing very low or no clumping were used.

For intragenic recombination studies, the spore suspension was plated at appropriate dilutions on GN medium, with or without lysine and supplemented for all other requirements. Lys^+ colonies appearing on the lysine less medium were checked for color on V8 medium, so as to get an estimate of the frequency of contaminating vegetative cells. The ratio of lys^+ haploids to the total number of viable spores was used as an estimate of the recombination frequency. A map unit was defined as 1 lys^+ spore/10^4 viable spores.

Gamma Rays Induced Mitotic Conversion

This was performed according to the procedure described by Korch and Snow (1973) using the cobalt-60 source of the Institut du Radium (Paris). At least 3 single colonies of each diploid to be tested were run in parallel; the cells were not aerated during exposure to increasing doses of gamma rays (0, 1, 2, 3, 4 and 6 KR). The survival at 6 KR was higher than 95% (estimated by plating on YE medium). Treated cells were plated on GN and GNlys media at appropriate dilutions. The number of lys^+ diploids was scored after 4 days; lys^+ diploids present on the O rad control plate were substracted from the number of colonies appearing in the treated samples.

With heteroallelic non complementing diploids, a linear response was observed for the appearance of converted lys^+ diploids when they were subjected to increasing doses of gamma rays. No correction was made to account for gamma rays induced reversion: we did not find a significant effect of this treatment on the frequency of reversion of homoallelic diploids (less than 1 reversion/10^6 cells/KR). A map unit was defined as 1.0 induced prototroph/10^6 cell/KR; for a triad of alleles, an additivity coefficient was calculated, as the sum of the two smaller distances divided by the largest.

Osmotic Remediability and Revertibility

To test for osmotic remedial mutants, the method described by Denis-Duphil and Lacroute (1971) was used on solid GN medium. The plates were incubated at 10, 22 and 32° C and the growth recorded after one week.

Revertibility was tested by plating the mutants on GN medium (10^6 to 10^7 cells/plate) and treating the cells with an UV source at 1,000 ergs/mm^2. An untreated sample served as control and the plates were compared after 4 days.

Enzymatic Assays

Homocitrate synthase activity was determined on crude extracts as described previously (Gaillardin et al. 1976a). The activity is expressed in laboratory units (optical density increase at 412 nm/min/mg of protein). Proteins were estimated according to Lowry et al. (1951) using lysozyme as a standard.

Results

Characterization of LYS1. Mutants

No homocitrate synthase activity could be detected in 9 out of 22 independent lys^- mutants tested. Following physiological characteristics of these strains were investigated (see Table 1): leakiness, temperature and osmotic sensitivity, occurence of feedback modified reversions (these show an increased resistance toward the lysine analog 4,5-transdehydrolysine or TDL^R phenotype, see below).

These mutants, except lys6 which did not mate, were repeatedly crossed against ade^-, ura^-, or his^- strains: in each case, a single recessive mutation seemed to be involved in the lysine requirement. All possible confrontations among the 8 mutants were realized, and the diploids were subjected to random spore analysis: less than 2% lys^+ spores were recovered, suggesting that the 8 mutations defined a single locus, hereafter refered to as *LYS1.*

Complementation Data

Since isogenic strains of *Sm. lipolytica* were not available we repeated each type of cross with at least