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Evidence for the control of a mutation in lysine catabolism by the mating type in *Yarrowia lipolytica**

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Summary. Reversions of a mutation lyc1.5 blocking the first step of the lysine catabolic pathway were isolated. These reversions mapped inside the LYC1 locus. They exhibited an alteration of the regulation of the lysine pool after nitrogen and phosphorus starvation. This phenomenon did not appear in sexual diploids homozygous for the reversion. Diploids homozygous for the mating type were constructed by protoplast fusion. They displayed a pattern similar to that of the haploids. We conclude that the expression of this mutation is under the control of the physiological state of the mating type.

Key words: ROAM mutation – *Yarrowia lipolytica* – Lysine catabolism – Regulation of lysine pool

Introduction

Among yeasts, *Yarrowia lipolytica* displays the unusual property of being able to utilize lysine as carbon source as well as nitrogen source. Its lysine catabolic pathway was described by Gaillardin et al. (1976). The first step is the acetylation of lysine into N-ε-acetyllysine. This step is controlled by the gene LYC1. Here we describe a new type of mutation affecting the gene LYC1.

Material and methods

Strains and culture media. Strains used in this work are described on Table 1. Strain 15901.7, 15901.8 and 14701.10 were given by C. M. Gaillardin. Culture media used in maintenance as well as growth and mating assays have been described by Gaillardin et al. (1973). Liquide culture media were buffered with 50 mM Na citrate, citric acid pH 5.0 buffer.

Genetic techniques. Methods of copulation and crossing were those set up by Gaillardin et al. (1973).

For mutagenesis, the cells were harvested in stationary phase of growth from an agar slant and suspended in distilled water at a density of 2×10^7 cells/ml. They were UV irradiated at 500 ergs/cm^2. The survival rate was about 10% to 20%. In the case

Table 1. List of the strains

Strain	Genotype
15901.7	A ura$^-$2 LYS1.5 lyc$^-$1.5
8052.9	A lys$^-$1.13 LYC$^+$1
15901.8	A ura$^-$2 LYS1.5 LYC$^+$1
01.18	B ura$^-$2 LYS1.5 LYC$^+$1.018 coming from 15901.7
DML15	A his$^-$1 URA$^+$2 LYC$^+$1.018
	B HIS$^+$1 ura$^-$2 LYC$^+$1.018 LYS1.5
49.78	B his$^-$1 URA$^+$2 nic$^-$1 ARG$^+$1 LYC$^+$1.018
	B HIS$^+$1 ura$^-$2 NIC$^+$1 arg$^-$1 LYC$^+$1.018
49.81	B his$^-$1 URA$^+$2 nic$^-$1 ARG$^+$1 LYC$^+$1.018
	B HIS$^+$1 ura$^-$2 NIC$^+$1 arg$^-$1 LYC$^+$1.018
58.32	B his$^-$1 URA$^+$2 nic$^-$1 PAN$^+$1 LYC$^+$1.018
	B his$^-$1 URA$^+$2 NIC$^+$1 pan$^-$1 LYC$^+$1.018
60.04	B his$^-$1 URA$^+$2 nic$^-$1 PAN$^+$1 LYC$^+$1.018
	B his$^-$1 URA$^+$2 NIC$^+$1 pan$^-$1 LYC$^+$1.018
81.14	A HIS$^+$1 ura$^-$2 NIC$^+$1 PAN$^+$1 LYC$^+$1.018
	A his$^-$1 URA$^+$2 arg$^-$2 LYS$^+$ LYC$^+$1.018
	A HIS$^+$1 ura$^-$2 ARG$^+$2 lys$^-$ LYC$^+$1.018
81.17	A his$^-$1 URA$^+$2 arg$^-$2 LYS$^+$ LYC$^+$1.018
	A HIS$^+$1 ura$^-$2 ARG$^+$2 lys$^-$ LYC$^+$1.018

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Table 2. Estimation of the pools of free lysine and free arginine in strains 15901.7 (A ura$^-$2 LYS1.5 lyc$^-$1.5) and 01.18 (B ura$^-$2 LYS1.5 LYC 1.018). Pools are expressed in nanomoles of lysine or arginine * by mg of dry weight

Strain	Growth medium	Pool
15901.7	MM	256
	MM + lys 20 mM	682
	MM + arg 20 Mm	74*
	MM + homoarg 5 mM	119*
01.18	MM	34
	MM + lys 20 mM	80
	MM + arg 20 mM	54*
	MM + homoarg 20 mM	182*

of auxotrophic mutant induction, a nystatin selection was performed. After an overnight expression phase, cells were starved in a nitrogen free minimal medium for 8 h. Then $(NH_4)_2SO_4$ (5 g/l) was added and 6 h later, cells had resumed growth and were treated for 90 min with nystatine (10 mg/l). To stop this treatment, the cells were washed by filtration.

Selection of mutants altered in amino acid pool was devised from the method set up by Cramer and Davis (1979) in *Neurospora crassa*. After UV mutagenesis, cells were plated on complete medium plates and replicated on a suitable minimal medium containing 1 g/l lysine. After 3 to 4 days incubation, each replica dish was treated by 2 ml of permeabilizing solution (ethanol/toluene 3/1 v/v). A disk of Whatman P81 ion exchanger paper was put thoroughly on the plate and then a disk of Whatman n° 1 filter paper. The papers were pressed with a replica block during 5 min. Filters were dried in an oven at 95 °C. A mixture of acid nynhydrine (methylcellosolve/0.6 N HCl in water, 5/1 v/v, 7.5% nynhydrine w/v) was sprayed with a gas propeller. Coloration was revealed by incubating 15 min at 95 °C in an oven. This coloration is specific of lysine (derived from Vogel and Shimura 1971).

Study of lysine accumulation in the intracellular pool. Cells were grown overnight in buffered minimal medium. After washing, they were transferred in nitrogen free, phosphate free minimal medium and starved during 5 h. Then ammonium sulfate (5 g/l) and lysine (25 mM) were added. Aliquotes were taken from time to time. Lysine pool was determined according to the procedure of Gaillardin et al. (1975). Arginine dosage was derived from Van Pilsum (1956).

Method for protoplast formation. The protocol of protoplastisation was derived from those set up in Wiemken's laboratory (personal communication). Cells were grown overnight in supplemented minimal medium. After washing, they underwent a reducing treatment (40 mM dithiothreitol, 10 mM EDTA, 100 mM pH 8.0 Tris HCl in an osmotic stabilizer) in proportion of 5 ml for 1 g of cell wet weight during 15 min at 30 °C. The osmotic stabilizer was in some cases 0.6 M KCl and in other cases 1.2 M sorbitol. The cells were washed with 20 mM pH 6.0 MES Tris buffer with osmotic stabilizer. They were protoplastised in the same buffer plus 50 mg/ml cytohelicase (I. B. F. Gennevilliers, France) and 5 mg/ml zymolyase 5000 (Seikagaku Kogyo Co. Ltd. Japan) in proportion of 5 ml/mg wet weight. Incubation varied according to the strain from 30 min to more than 1 h to obtain more than 99% protoplasts (see Table 3). The protoplasts were washed 3 times in OSB buffer (10 mM $CaCl_2$, 10 mM pH 7.4 Hepes Tris in osmotic stabilizing buffer) and counted with a hemacytometer.

The procedure of protoplast fusion was adapted from those of Fournier et al. (1977) and Stahl (1978). 5×10^7 protoplasts of each strain were mixed together. 4 ml 40% PEG 4000 in OSB buffer were added to 200 µl of cells. After mixing, the tubes were allowed to stand for 20 min at room temperature. Then 4 ml OSB buffer were added to improve sedimentation and the cells were pelleted. They were poured into top agar (3% agar purified, Difco) on selective medium. Fusion products colonies appeared after 5 to 10 days of incubation at 30 °C.

To check the fusion products, haploidization was performed by growing cells overnight in liquid complete medium containing 1.5 mg/l benomyl. Then, cells were plated on complete medium and then replicated on selective media.

To study fusion products, DNA dosage was performed according to Burton (1956) on cells taken in early stationary phase.

Results

Induction of low lysine pool mutants

To isolate strains altered in lysine accumulation, we adapted the method of Cramer and Davis (1979) to the selection of clones with low lysine pools. As a parent, we used the strain 15901.7. It carried LYS1.5 and lyc1.5. LYS1.5 is a feedback mutation in the structural gene of the first enzyme of the biosynthetic pathway (Gaillardin and Heslot 1979); this mutation confers resistance to S-2-aminoethyl cysteine (aec) (100 mg/l), a lysine antagonist. The lyc1.5 mutation prevents lysine utilization as a nitrogen source. The combination of both mutations led to a high intracellular accumulation of free lysine (Table 2). After UV mutagenesis, among about 2,500 colonies, 37 putative low pool clones were selected (see protocol in Material and methods). They were screened in a high lysine pool strain to prevent selection of lysine uptake mutants and on a medium containing 1 mM lysine to avoid obtention of bradytrophic mutations. However they displayed a large array of free lysine contents on minimal medium supplemented with 1 mM lysine, from 80 to 240×10^{-9} M lysine/mg dry weight. As expected for strains with a low lysine pool, they were aec sensitive. One of them appeared deficient in polyphosphates pool (cf. Beckerich et al. 1981). All the others had regained the ability to use lysine as a nitrogen source. More strikingly, all these strains had switched mating type from A to B. We focused our further studies on one of these mutants: 01.18 which presented one of the lowest pools.

Genetic studies

The reversion of lyc1.5 in 01.18 was dominant. We tested the linkage between the "suppressor" of lyc$^-$1.5