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Selection of spontaneous mutants of *Yarrowia lipolytica* by inositol-less death

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SUMMARY

A procedure was developed for the selection of spontaneous mutants of the yeast *Yarrowia lipolytica*. An inositol-requiring mutant of a wild-type *Y. lipolytica*, YB 3-122, was derived by mutagenesis and screening. The mutant had a reversion frequency of less than 6×10^{-9}. A mutant selection procedure based on inositol-less death was then developed using this mutant strain. The selection procedure killed growing *Y. lipolytica* cells and enriched for mutants yielding cultures that consisted of 60–98% spontaneous mutants after two rounds of inositol-less death. The procedure enriched for four classes of mutants, strains that were auxotrophic, metabolite analog sensitive, temperature sensitive, or unable to grow on citric acid as the sole carbon source. Since strain YB 3-122 is now available to yeast researchers, inositol-less death will be useful for the routine isolation of spontaneous mutants of *Y. lipolytica*.

INTRODUCTION

The heterothallic dimorphic yeast *Yarrowia lipolytica* is of interest to industrial microbiologists because it has the capacity to produce organic acids, efficiently secrete enzymes, and assimilate hydrocarbons as the sole carbon source. *Y. lipolytica*, formerly known as *Saccharomycopsis lipolytica*, is the sexual form of *Candida lipolytica* [29]. *Y. lipolytica* can produce citric acid from carbohydrates or alkanes [6], and industrial-scale processes for citric acid production with *C. lipolytica* from C_{12}–C_{18} alkanes have been patented [22,24,25]. *C. lipolytica* [1,20,23] and *Y. lipolytica* [19] also produce extracellular proteases, and *C. lipolytica* secretes a lipase [16].

Mutants of *Y. lipolytica* have been valuable in both basic and applied research [5,9,21]. Such mutants have been derived by mutagenesis and screening or mutagenesis and then selection with the antibiotic nystatin [17,26]. While such methods effectively generate or enrich for mutants of *Y. lipolytica* [18,28], they are not applicable in situations where spontaneous mutations are desired. The selection of spontaneous mutations is preferred be-

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cause it minimizes general cell damage and may generate novel classes of mutations [12].

The selection of spontaneous mutants by inositol starvation, termed inositol-less death, has been successful with *Neurospora crassa* [15], *Ustilago maydis* [14], and *Saccharomyces cerevisiae* [8,12]. Inositol-less death describes the finding that inositol-requiring cells lyse and die when starved for inositol in medium that is otherwise sufficient for growth [8,12]. The mutant selection procedure depends on the selective death of those cells that lack secondary auxotrophic mutations (other than the inositol defect) and thereby continue to grow in minimal (unsupplemented) medium. Cells carrying secondary auxotrophic mutations cannot actively proliferate under such conditions and are spared from inositol-less death. Hence, the procedure enriches for cells that cease growth and survive by virtue of a secondary mutation that blocks metabolism or growth. Here we report the derivation of an inositol-requiring strain of *Y. lipolytica* and the selection of this yeast by inositol-less death. A preliminary report of this mutant enrichment procedure has appeared elsewhere [7].

MATERIALS AND METHODS

Yeast strains

Y. lipolytica strains ATCC 18943 (also known as NRRL YB-423-3) and ATCC 18944 (also known as NRRL YB-423-12) were obtained from the American Type Culture Collection, Washington, DC. The former is of the A mating type and the latter is of the B mating type. These strains grow in yeast nitrogen base minimal medium (Difco Laboratories, Detroit, MI) containing glucose without any additional supplements. Strain YB 12-81, a uracil requiring mutant, was derived from strain YB-423-12 after mutagenesis with ethyl methanesulfonate (EMS) and then enrichment in the presence of nystatin.

Media and culture methods

Since yeast nitrogen base medium contains inositol, an inositol-free minimal medium was prepared according to Henry et al. [12]. Inositol (Sigma Chemical Co., St. Louis, MO) was supplemented at a concentration of 2.0 mg/l when desired. Other nutrients (Sigma Chemical Co., St. Louis, MO) were added when necessary. Solid medium containing 2% agar (Difco Laboratories, Detroit, MI) was used routinely. Cultures were incubated at 28°C. Liquid cultures were grown in 13 × 100 mm test tubes on a Model 7637 rotator (Cole Parmer Instrument Co., Chicago, IL). Yeast strains were maintained on YEPD agar containing 1% yeast extract, 2% peptone, 2% agar (Difco Laboratories, Detroit, MI) and 2% D-glucose (Sigma Chemical Co., St. Louis, MO).

Mutagenesis

EMS (Eastman Kodak, Rochester, NY) mutagenesis was performed according to the method of Fink [10].

Mutant enrichment procedure

Spontaneous mutants of *Y. lipolytica* YB 3-122 were derived according to a variation of a procedure developed by Henry et al. [12]. The procedure shown in Table 1 describes the two rounds of prestarvation, starvation, and recovery in appropriate media. Recovery medium was YEPD when the entire spectrum of possible auxotrophic mutants was examined. Recovery medium was supplemented minimal medium when a specific auxotrophic mutant was desired. Mutants were characterized by replica plating to appropriate selective media.

The efficiency of the enrichment procedure (Table 1) was dependent on several factors. The prestarvation steps were essential since they depleted cells of endogenous pools of nutrients and metabolites. Log phase cells were critical in the prestarvation step for effective killing by inositol starvation. The actively growing cells for this step had to be adequately washed with prestarvation medium to prevent the introduction of nutrients from the rich medium. An inoculum of 5×10^4 to 5×10^5 cells/ml into prestarvation medium was suitable and did not lead to significant cross-feeding of non-growing cells by lysed cells during inositol-less death. An incubation time of 30 h at 28°C was optimal for mu-

Table 1

Procedure for Selection of Spontaneous Mutants by Inositol-less death

Media

Nutrient medium — yeast extract, peptone, dextrose (YEPD)
Prestarvation medium — defined minimal with inositol
Starvation medium — defined minimal without inositol

Procedure

Growth of inoculum
Inoculate fresh colony into YEPD and incubate on rotator for 18 h at 28°C.
Prestarvation I
Harvest log phase cells and wash 3 times with prestarvation medium.
Inoculate prestarvation medium to a density of 5×10^4 to 5×10^5 cells/ml. Incubate cells at 28°C for 30 h.
Starvation I
Wash cells 3 times with starvation medium. Inoculate with 1×10^4–1×10^5 cells/ml. Incubate at 28°C for 30 h.
Recovery I
Allow starved cells to recover in YEPD for 24–48 h. Log cells are essential for round II.
Prestarvation II
As above
Starvation II
As above
Recovery II
As above
Cell plating, replica plating, mutant analysis

tant enrichment. The starvation, prestarvation, and recovery steps could all be conveniently carried out in 2.0 ml of culture medium in test tubes inserted in a small rotator. The same culture tubes could be used during both rounds of selection.

RESULTS AND DISCUSSION

Derivation of inositol mutant

An inositol-requiring strain of *Y. lipolytica* was derived by mutagenesis with EMS, plating on nutrient medium, and then replica plating on minimal agar with and without 2.0 mg/l inositol. Among 20 500 colonies of strain ATCC 18943 only one mutant unable to grow on minimal medium lacking inositol was derived. The inositol dependent mutant was named YB 3-122. No inositol-dependent mutants of strain ATCC 18944 were obtained after examination of 26 800 colonies.

Spontaneous revertants of YB 3-122 could not be obtained after plating 1.6×10^8 cells on minimal medium lacking inositol at 28°C. No inositol independent colonies were observed after prolonged incubation at 28°C. The reversion frequency for mutant YB 3-122 therefore must be less than 6×10^{-9}. Plating of 9.6×10^7 cells mutagenized with EMS also failed to yield any revertants. The low reversion frequency for the inositol defect suggests that several mutations or perhaps a deletion is responsible for the inositol requirement. This characteristic makes this strain ideal for a mutant enrichment procedure by inositol-less death. It also obviates the need for derivation of a double mutant, as was used in experiments with *S. cerevisiae* [12].

Efficiency of inositol-less death

The viability of mutagenized and non-mutagenized cells during inositol starvation was monitored by periodically removing cells from cultures in starvation medium and plating on YEPD medium. Six independent cultures of *Y. lipolytica* YB 3-122 were used in both cases. Cultures of strain YB 3-122 inoculated with unmutagenized cells lost viability during round 1 (Fig. 1) and round 2 (Fig. 2) of inositol deprivation. Viability decreased by at least 2 orders of magnitude in most experiments. Similar results were obtained with mutagenized cells. In the case of both mutagenized and unmutagenized cell cultures, the majority of the cells were auxotrophic mutants after two rounds of inositol starvation and then a recovery period in rich medium (Table 2). When mutagenized cells were used for mutant selection about 1% of the cells were auxotrophic mutants after round 1 and 90–100% were mutants after round 2 based on 10 experiments. When unmutagenized cells were used for inositol-less death about 1% of the cells were spontaneous mutants after round 1 and 60–98% after round 2 based on 10 experiments. Clearly, the selection of auxotrophic mutants by inositol-less death using *Y. lipolytica* YB 3-122 is extremely efficient.

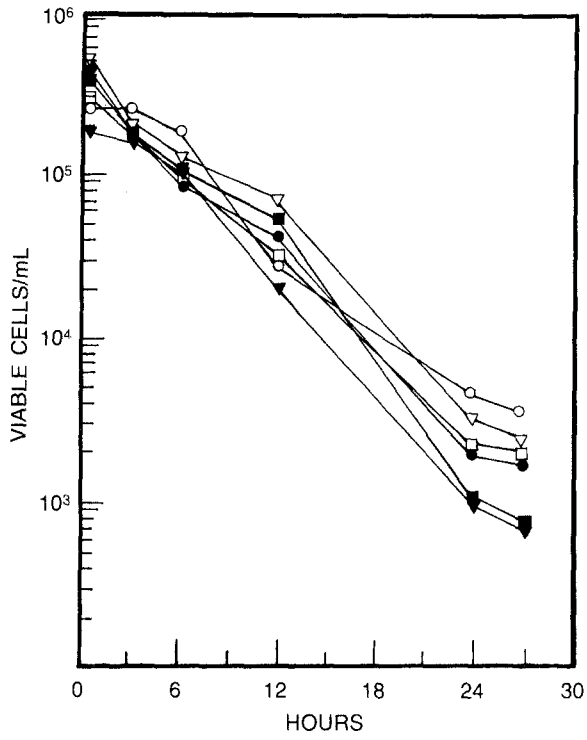

Fig. 1. Loss of cell viability of 6 independent cultures of *Y. lipolytica* YB 3-122 in starvation medium after one round of inositol-less death.

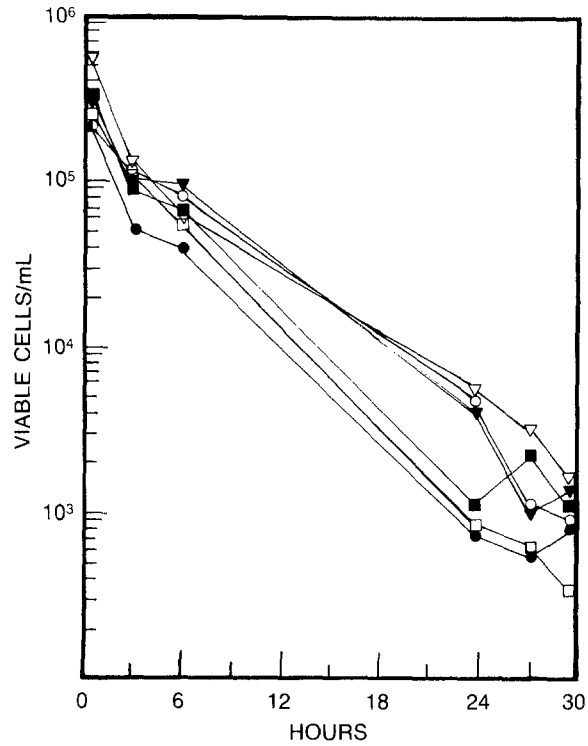

Fig. 2. Loss cell viability of 6 independent cultures of *Y. lipolytica* YB 3-122 in starvation medium after a second round of inositol-less death.

DERIVATION OF SPONTANEOUS MUTANTS

A variety of spontaneous mutants that required amino acids or pyrimidines were obtained when cells were recovered in YEPD medium. For example, a study of 136 auxotrophs from one enrichment experiment revealed mutants that required uridine (4), arginine (45), histidine (9), isoleucine-valine (6), methionine (42), proline (8), lysine (19), tryptophan (1), or all three aromatic amino acids (2). Complementation groups were not determined for each auxotrophic requirement. After some enrichment experiments, the specific requirements of some mutants could not be identified; they did not require any of the standard amino acids, purines, pyrimidines, vitamins, or combinations of these.

Temperature sensitive strains could also be se-

Table 2

Efficiency of inositol-less death with *Y. lipolytica* YB 3-122

Inoculum		Surviving fraction	% Mutants
I. Mutagenized Cells	Round 1	0.012	~1%
	Round 2	0.003	98.2[1]
II. Unmutagenized Cells	Round 1	0.096	~1
	Round 2	0.007	85.7[2]

[1] Mean of 10 experiments; standard deviation of 8.8. [2] Mean of 10 experiments; standard deviation of 13.4.

lected with high efficiency by performing the starvation steps at 34°C and recovering the cells at 25°C. Replica plating on minimal medium demonstrated that these mutants were unable to grow at the non-permissive temperature (34°C). Almost 100% of the surviving cells were temperature sensitive after 2 rounds of selection and then recovery in rich medium at 25°C.

Metabolite analog sensitive mutants could be isolated after inositol-less death by performing the starvation step in the presence of an analog. Cells were allowed to recover in minimal medium with inositol according to the protocol in Table 1. For example, using this procedure spontaneous mutants that are sensitive to 0.1% (w/v) fluoroacetic acid were obtained. Inositol-less death generated survivors 90–100% of which were unable to grow in liquid or solid medium containing 0.1% analog. Since Akiyama et al. [2] have reported that fluoroacetic acid sensitive mutants of *C. lipolytica* efficiently produce citric acid from *n*-paraffins, spontaneous mutants of this class could be useful in this regard.

Other spontaneous mutants of *Y. lipolytica* useful in the derivation of citric acid producing strains were also obtained. Citric acid non-utilizing mutants, which have been reported by Fukuda et al. [11] to be important in citric acid production, were generated. One round of inositol-less death in the presence of 2.7% (w/v) citric acid as the sole carbon source yielded survivors 50% of which were mutants unable to grow on citric acid. Such mutants were incapable of growing on minimal agar containing 0.3% citric acid as the sole carbon source, conditions which supported growth of wild-type strains or the inositol-requiring strain, YB 3-122.

The inositol requirement of YB 3-122 could easily be moved into other strains. Mating of this strain with another of the opposite mating type that had a genetic marker and then random spore plating permitted such contructions. For example, a cross of strain YB 3-122 with strain YB 12-81, which requires uridine, yielded mutants that required both inositol and uridine. Progeny cells were examined and 32 of 151 were found to require both nutrients. The remainder required only inositol (28) or uridine (30), or were prototrophs (61) that did not appear to sporulate.

Mechanism of Y. lipolytica cell death

The mechanism of cell death of *Y. lipolytica* YB 3-122 during inositol starvation is likely similar to that proposed for inositol auxotrophs of *S. cerevisiae* [12,27], which involves unbalanced growth and cytoplasmic membrane damage leading to cell death. In fact, in this study, inositol-starved cells of the *Y. lipolytica* inositol-requiring mutant were found to have an altered morphology. They were clumpy and resembled some *ino* mutants of *S. cerevisiae* after inositol deprivation. Studies by Beckerich et al. [5] indicate that membrane alterations lead to eventual cell death of *Y. lipolytica* inositol auxotrophs during inositol starvation. They have shown that such inositol auxotrophs release small amounts of a large number of metabolites into the medium during inositol starvation. Furthermore, the phosphatidylinositol level of starved cells is only 36% of that of the inositol-independent strain.

Industrial applications

Since strain YB 3-122 is now available to yeast researchers, inositol starvation will be a useful method for the routine isolation of spontaneous *Y. lipolytica* mutants. The procedure is convenient, does not require antibiotics and is unaffected by the spectrum of action of mutagenic agents. The inositol-requiring mutant described in this work should also be valuable for the isolation of novel classes of *Y. lipolytica* mutants, some with possible applications in industrial fermentations. Four classes of mutants were derived in this study: auxotrophic, temperature sensitive, metabolite analog sensitive, and carbon utilization mutants. The derivation of specific mutants of these categories perhaps could be applied to the commercial production of primary metabolites using hydrocarbons as feedstock. Moreover, the approach discussed in this work provides a rapid means for advancing the genetics of this still poorly characterized yeast of considerable practical interest.

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