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Development of Genetic Techniques and the Genetic Map of the Yeast *Saccharomycopsis lipolytica*

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Summary. Genetically useful strains of the hydrocarbon-utilizing yeast *Saccharomycopsis lipolytica* were developed through extensive inbreeding. Spore viability and the percentage of 4-spored asci were increased to the point where tetrad analysis was possible. Procedures for mutant isolation and scoring, maintenance of stocks, mating, sporulation, complementation, tetrad and random spore analysis have been developed for these inbred strains. Sixty.seven mutations in fifty-eight genes have been isolated and utilized in mapping studies. Twenty-two cases of linkage have been detected among the 278 gene pairs investigated. Six linkage fragments have been established and a few genes ordered in these fragments. No centromere, linked markers have yet been detected. Evidence for gene conversion, mitotic recombination and diploidization in *S. lipolytica* is presented.

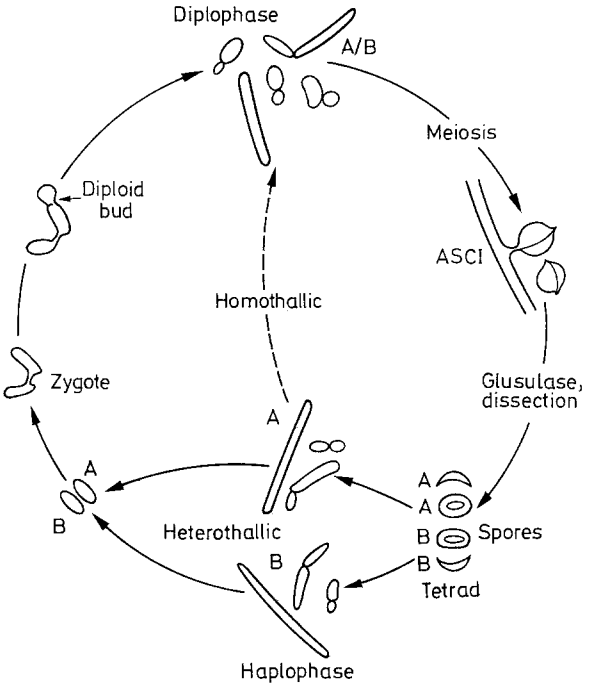

Fig. 1. Life cycle of *Saccharomycopsis lipolytica*

Introduction

Nearly ten years ago Wickerham discovered sexuality in a strain of the dimorphic, hydrocarbon yeast *Candida lipolytica* isolated from a corn processing plant (Wickerham et al., 1969). The strain Wickerham isolated, when sporulated, yielded walnut shaped asci containing 1 to 4 crescent shaped spores which when grown into haploid colonies could be re-mated to form sporulating diploids (Fig. 1). Mating was found to be heterothallic and to involve two mating types designated as *A* and *B* (Wickerham et al., 1969; Bassel and Mortimer, 1973). Strains of *C. lipolytica* exhibiting sexuality will be referred to as *Saccharomycopsis lipolytica* (Yarrow, 1972).

In 1970, mating strains were obtained in this laboratory from Wickerham for the purpose of developing genetic procedures and establishing the basic genetics of *S. lipolytica*. Early reports from this laboratory included introduction of genetic markers into wild-type and demonstrations of complementation and genetic recombination (Bassel et al., 1971) and a genetic analysis of mating type and alkane utilization (Bassel and Mortimer, 1973). Development of the genetics of *S. lipolytica* was continued in Wickerham's laboratory (Wickerham et al., 1970; Herman 1971) and was begun in Heslot's laboratory (Gaillardin et al., 1973; Gaillardin et al., 1976).

This paper includes a detailed report on the development of genetic procedures and the genetic map

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of *S. lipolytica*. It includes procedures for mutant isolation and scoring, strain improvement, culture maintenance, mating, sporulation, random spore and tetrad analysis, complementation and mitotic recombination. It also includes a compilation of mapping data obtained in this laboratory and the first genetic map of *S. lipolytica* which consists of six linkage fragments.

Materials and Methods

Strains. The parental strains CX 39-74B *trp1 B*, CX 39-74C *ura1 A* and CX 161-1B *ade1 A* were developed by inbreeding of the original strains *Candida lipolytica* YB-421 and YB-423-12 obtained from Wickerham. The *A* and *B* denote the bipolar mating type (Bassel et al., 1973).

Media, Culture Maintenance and Scoring of Genetic Markers. The following media were used:

YM Yeast extract-malt extract medium: 0.3% each of yeast extract and malt extract, 0.5% peptone, 1% glucose and 2% agar.

RG Restrictive growth medium: 0.02% yeast extract, 0.02% peptone, 0.1% glucose and 2% agar.

SM Synthetic minimal medium: 0.6% Difco Yeast Nitrogen Base without amino acids, 2% glucose and 2% agar.

SC Synthetic complete medium: SM plus the following amino acids and bases, per liter: adenine 30 mg, uracil 20 mg, L-tryptophan 60 mg, L-threonine 37.5 mg, L-histidine 20 mg, L-lysine 40 mg, L-methionine 20 mg, L-phenylalanine 50 mg, and L-tyrosine 20 mg. The threonine and tryptophan were filter sterilized and added after autoclaving. For proline supplementation, 0.3 ml of a 4.0 mg/l proline solution was spread onto each plate.

OM Omission media: SC lacking one of the amino acids or bases; e.g., *-trp* (SC minus tryptophan), *-ade* (SC minus adenine).

D Dissection medium: 5% glucose, 0.5% peptone, 0.3% beef extract, 1% yeast extract and 3.0% agar plus amino acids and bases as in SC.

SKM Skim milk medium: 1% Difco skim milk, 0.17% Difco yeast nitrogen base without $(NH_4)_2SO_4$ and amino acids, 0.24% KH_2PO_4, 0.35% Na_2HPO_4, adenine 25 mg/l, uracil 33 mg/l and 2% agar. Details of preparation of this medium are described in Ogrydziak and Mortimer (1977).

ALK Alkane medium: SC minus glucose, 1 ml of decane (Aldrich Chem. Co.) pipetted onto a 9 cm Whatman filter paper which is then placed in the lid of the petri dish and the dish is sealed with Parafilm.

YMS Yeast extract-malt extract slants: 0.5% each of yeast extract and malt extract, 0.8% peptone, 3% glucose and 2% agar.

Cultures are maintained on YMS slants at 4° C. Initially YM slants were used to store strains but diploids showed evidence of sporulation after long term storage on this medium. The time of onset of sporulation seemed to be directly related to glucose concentration. Thus YMS medium (3% glucose) is more satisfactory than YM (1% glucose) for culture maintenance. Also in some cases white sectors (indicating no sporulation) have been found upon resporulating old slant cultures of diploid strains.

Nutritional markers were scored by replica plating to appropriate OM media. The OM plates were usually scored after 2 days (23°) and the ALK plates after 3 to 4 days (23°). Temperature sensitivity (*ts*) was scored on YM at 34°. UV sensitivity (*uvs*) was scored as growth-no growth on YM after a dose of 2175 ergs/mm^2 from a germicidal lamp.

Replica plating was carried out with fresh (< 24 h) YM plates because most of the strains produce dry, convoluted colonies which become tougher and more difficult to replica plate with age. Even with fresh master plates, only 5 to 6 replicas can be made from a single master plate.

Extracellular protease production (*xpr*) was scored after 2 days (23°) by the presence or absence of a zone of clearing surrounding a colony toothpicked onto a SKM plate. The *pop1* gene was scored by the presence or absence of red pigment formation on YM after 3 to 4 days at 23°. Mating type was scored by mating the segregants with *pro1A* and *pro1B* tester strains.

Mutagenesis. Haploid strains were grown for 48 h (23°) in liquid RG medium resulting in a suspension consisting primarily of single unbudded cells in the stationary growth phase. Mutants were induced by UV irradiation from a germicidal lamp with a dose of 1450 ergs/mm^2 at the surface of the plates. This treatment results in about 30% survival of the treated cells. The treated cells were plated on YM at a concentration to yield 80 to 100 colonies per plate. For isolation of auxotrophic mutants, these YM plates were incubated for 3 to 4 days and then replica plated to SM (containing the parental growth requirements, either adenine, tryptophan or uracil). For isolation of *alk* and *ts* mutants the master plates were replica plated to ALK and YM plates, respectively. The YM plates were incubated at 34°. The extracellular protease non-producing mutants (*xpr*) were isolated by plating directly (about 40 colonies per plate) on SKM plates or on SKM plates supplemented with glucose and ammonium sulfate or glycerol, ammonium sulfate, glutamine and cysteine. Colonies not surrounded by a zone of clearing were selected.

Mating. Modifications of the procedure described by Herman (1971) were used. Due to the very low mating frequency (Gaillardin et al., 1973) matings were set up using haploids with complementary nutritional markers. The haploids to be mated are grown overnight on YM and then replica plated to RG and incubated 2 days. All steps were done at 23°. The haploids were then thoroughly mixed on the RG medium and incubated for another 2 days. The plates were then replicated to minimal medium (SM) where only the diploids or revertants should grow. The diploids appeared in 3 to 4 days and were repurified on SM medium. Mating frequency was quite low and only rarely was there confluent growth on the minimal plate. In some cases, only a single colony appeared. If the controls showed no revertants, then most often this single colony was a true diploid. For matings showing average mating frequency, as many as 6 matings per plate are possible. For more difficult matings, either more cells can be used in the mating mixture (only 1 or 2 matings on a plate) and/or more cells can be transferred from the RG to the SM plate. A promising recent modification involves cutting out the controls and mating mixtures from the RG medium and placing the RG agar pieces on minimal medium with the cells facing up. With this method all the cells are transferred to SM and enough nutrients diffuse from the SM to the RG agar pieces to support confluent growth.

Sporulation. Sporulation was done on YM medium (23°) as suggested by Wickerham et al. (1969). A heavy streak was made across the YM plate. Two or three streaks per plate are optimal. Spores are present after 4 to 5 days (even sooner on YM medium containing 0.1% glucose instead of the usual 1%). However, it is very difficult to dissect at this stage as the spores stick together quite tenaciously. After 12 to 14 days dissection is much easier. A maturation of the asci seems to be involved. Cultures can then be stored at 4° for 1 to 2 weeks with no apparent loss of spore viability.