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Genetic and Biochemical Studies of N-Alkane Non-Utilizing Mutants of *Saccharomycopsis lipolytica*

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Summary. Alkane non-utilizing mutants of the yeast *Saccharomycopsis lipolytica* were induced by ultraviolet light. Thirtyfour of the mutants were found to be alkaline-negative and fatty acid-positive (Phenotypes A and C) indicating a defect in n-alkane uptake or in hydroxylase complex activity. The hydroxylase complex is a microsomal aggregate composed of the first three enzymes of n-alkane catabolism. Leaky and non-mating mutants were eliminated leaving 21 mutants which were analyzed genetically. All 21 of the mutations showed a 1:1 pattern of segregation indicating they are chromosomal and all but one were recessive. Analyses of inter-mutant complementation and recombination showed that the 21 mutations represent 18 genes.

Several of the mutants had pleiotropic phenotypes in addition to alkane non-utilization. These phenotypes included a loss of mating function, an inability to sporulate, a changed colony and cellular morphology, osmotic sensitivity and a lack of extracellular protease.

The hydroxylase complex activities of mutants and wild type were assayed in cell-free extracts prepared by protoplast lysis. A small amount of detergent was necessary for the extraction of hydroxylase complex activity. The hydroxylase complex was inducible by n-decane and incubation was complete by 6 h. Hydroxylase complex activities in the mutants varied from 2.8% to 46.5% of wild type. The hydroxylase complex activities of two temperature sensitive mutants were as stable as wild type at the non-permissive temperature. These mutants showed temperature sensitive induction suggesting that the uptake of n-alkanes is temperature dependent in these strains.

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Introduction

After the discovery of sporulating strains of *Candida lipolytica* (Wickerham 1970) genetic studies were initiated because this organism has unique properties not found in other genetically well characterized yeast. *C. lipolytica* utilizes hydrocarbons, excretes a variety of extracellular enzymes including extracellular protease, RNAse and lipase and is di-morphic. Di-morphic yeast exhibit mycelial or yeast phase cell morphologies depending on the conditions of cultivation (Johanides 1972). The strains of *C. lipolytica* exhibiting sexuality were designated *Saccharomycopsis lipolytica* (Yarrow 1972).

The initial genetic studies established that *S. lipolytica* has stable haploid and diploid growth phases and is heterothallic with two mating types, designated A and B (Wickerham et al. 1969; Bassel and Mortimer 1973). Later studies have developed genetically useful strains by in-breeding and have also developed techniques for mating, sporulation, ascospore dissection and random spore analysis (Gaillardin et al. 1973; Ogrydziak et al. 1978). Mapping studies involving several phenotypic classes of mutants have identified six linkage groups (Ogrydziak et al. 1978). Genetic and enzymological investigations of specific mutant phenotypes have included studies of mutants deficient in the secretion of extracellular protease (Ogrydziak and Mortimer 1977) and of mutants defective in lysine catabolism (Gaillardin 1977). Preliminary studies on the isolation of n-alkane non-utilizing mutants of *S. lipolytica* have de-

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monstrated the segregation of such mutants in genetic crosses (Bassel and Mortimer 1973) and identified 6 complementation groups (Bassel and Ogrydziak 1978).

Biochemical studies involving *S. lipolytica* and other species of n-alkane utilizing yeast have shown that the first three enzymatic reactions of n-alkane catabolism oxidize n-alkane to fatty acids of the same chain length and that these reactions can be carried out in vitro by an inducible, microsomal enzyme aggregate (Liu and Johnson 1971; Gilewicz et al. 1978; Yorifuji 1978). The enzymes involved in these oxidative reactions are n-alkane hydroxylase, alcohol dehydrogenase and aldehyde dehydrogenase. n-alkane hydroxylase is a mixed function oxygenase composed of an electron transport flavoprotein (NADPH-cytochrome P450 reductase) and a hemoprotein (cytochrome P450). The particulate enzyme aggregate which oxidizes n-alkanes to fatty acids is termed the hydroxylase complex.

The purpose of this study was to isolate and characterize n-alkane nonutilizing mutants of *S. lipolytica* deficient in hydroxylase complex activity and/or n-alkane uptake. The mutants were first characterized by substrate utilization tests and subsequently by genetic and enzymological analyses.

Materials and Methods

Strains. The parental strains used in this study (CX39-74B *trp*1 B, CX161-1B *ade*1 *A*) were developed by inbreeding of strains (*Candida lipolytica* YB-421 and YB 423-12) obtained from Wickerham. *A* and *B* denote bipolar mating type (Bassel et al. 1973).

Media. The compositions of the media designated as YM, OM, SC and SM have been described (Ogrydziak et al. 1978). The compositions of the additional media used in this study are as follows:

ALK: Substrate is present in the vapor phase. 1 cc of n-decane (Aldrich, 99%) is added to a 9 cm filter paper disc in the lid of a Petri dish. The inverted plates are sealed with parafilm or placed in a plastic bag before incubation. The basal medium is SC less carbon source.

ALC, ALD: Substrates are present in the vapor phase. 20 µl of dodecanol (ALC) or dodecyl aldehyde (ALD) are added to a 9 cm filter paper in the lid of a Petri dish containing SC medium less carbon source. Larger amounts of these compounds were inhibitory to wild type. The plates were incubated in closed plastic bags. Dodecanol and dodecyl aldehyde were purchased from the Aldrich Chemical Co.

FA: The substrate is 0.1% n-hexadecanoic acid (Aldrich) solubilized in 2% of the detergent Tergitol NP40 (Sigma). Tergitol and n-hexadecanoic acid are added to the SC basal medium before autoclaving.

AC: SC basal medium plus 0.5% sodium acetate.

Scoring Extracellular Protease Production. This technique has been described (Ogrydziak and Mortimer 1977).

Genetic Techniques. The following techniques have been described (Orgydziak et al. 1978): mating, sporulation, ascus dissection and mutagenesis by UV light.

Random Spore Analysis: Suspensions of sporulated diploid cells were spread on YM plates at a concentration of 50 to 100 cells per plate as previously described (Ogrydziak et al. 1978). The YM plates were then incubated for 9 days at 24 °C to permit sporulation. Sporulated diploids turn a brown color and can be differentiated from white haploid colonies. Haploid spore colonies can be selected by their color and are greatly enriched by this technique.

Complementation Analysis: Alkane-negative mutants derived in CX39-74B by UV mutagenesis were crossed with CX161-1B and diploids selected on minimal medium. The diploids were sporulated and spores were isolated by either dissection or a random spore technique. Two alkane-negative spore clones from each cross, one with *trp*1 *A* markers and the other a *ade*1 *B* were used in the complementation analysis. The alkane-negative mutants were crossed with themselves (control crosses) and with each other. Before being replica plated to ALK media all putative diploids were confirmed by testing for sporulation. A positive complementation response was indicated by confluent growth on ALK plates after 48 h at 30 °C. Non-complementing mutants showed no growth after 5 days at 30 °C.

Analysis of Inter-Mutant Recombination: The diploids previously tested for complementation were sporulated and random spores were analyzed for the segregation of the *trp*1 *and ade*1 markers and the alkane-negative phenotype. The auxotrophic markers were scored on OM plates and alkane utilization on ALK media. In the absence of selection one would expect 25% of the haploid psores derived from a cross between two unlinked alkane-negative mutants to be alkane-positive. In order to rule out the possibility of scoring complementing diploids as alkane-positive segregants, only those spore clones expressing the recessive markers *ade*1 and/or *trp*1 were used in this analysis.

Growth in Liquid n-alkane Media. Baffled 500 ml flasks containing 100 ml of SC plus 1% glucose were inoculated heavily with cells from a 24 h YM agar culture of CX39-74B. The flasks were incubated overnight at 30 °C on an orbital shaker. The cells were harvested by centrifugation, washed once with distilled water, diluted and added as inocula to 500 ml baffled flasks containing SC plus 0.5% n-decane or n-hexadecane. The flasks were incubated at 30 °C with shaking (320 RPM). Growth was followed by measuring turbidity with a Klett-Summerson Colorimeter with a green filter. One ml samples were removed periodically and diluted up to 5 ml with water. Another technique calls for dilution with propionic acid as a means of reducing turbidity (Yorifuji 1978) but this proved to be unnecessary since water gave a clear, non-turbid cell suspension. The low concentration of n-alkanes (0.5%) may account for the lack of turbidity atributable to the n-alkane substrates.

Preparation of Cell Free Extracts by Protoplast Lysis. Baffled 500 ml flasks containing 100 ml of SC plus 1% glucose medium were inoculated heavily with cells of the wild type strain or the alkane-negative mutants grown for 24 h on YM agar plates. After about 20 h incubation at 30 °C on an orbital shaker (320 RPM) the cells were harvested in log phase (200 to 875 Klett units). One gram dry weight of cells per liter yields a value of 250 Klett units (Ogrydziak and Mortimer 1977). In SC plus 1% glucose medium cells of the wild type strain, CX39-74B, were found to reach early stationary phase at a turbidity of 950