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Identification of mutations preventing n-hexadecane uptake among 26 n-alkane non-utilizing mutants of *Yarrowia (Saccharomycopsis) lipolytica*

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Summary. Genetic analyses of n-alkane non-utilizing mutants of the yeast *Yarrowia (Saccharomycopsis) lipolytica* were continued. By analyses of inter-mutant complementation and recombination a total of 26 genetic loci have been identified. Mutations representing these loci have phenotypes characteristic of defects in substrate uptake or in one or more of the enzymatic activities making up the hydroxylase complex. Tests of ^{14}C n-hexadecane uptake by a set of alkane-negative mutants representing the 26 loci show that 16 of the mutations cause a significant reduction in n-alkane uptake. N-alkane uptake by *Y. lipolytica* is shown to be inducible and to be inhibited by the metabolic poisons 2–4 dinitrophenol and KCN. The latter observation indicates that n-alkane uptake of *Y. lipolytica* is due to active transport.

Key words: *Y. lipolytica* – n-Alkanes – Mutants – Uptake

Introduction

The first three enzymatic reactions of n-alkane catabolism by *Y. lipolytica* and other hydrocarbon utilizing yeasts have been shown to catalyze the oxidation of an n-alkane substrate into a fatty acid of the same chain length (Liu and Johnson 1971; Gilewicz et al. 1978; Bassel and Mortimer 1982; Yorifugi 1978). The enzymes involved in these reactions are n-alkane hydroxylase (cytochrome p450, NADPH cytochrome p450 reductase) alcohol dehydrogenase and aldehyde dehydrogenase. These enzymes are found as an inducible microsomal multi-enzyme complex and can be assayed in vitro by following the converson of a ^{14}C n-alkane into its corresponding fatty acid (Bassel and Mortimer 1982; Yorifugi 1978). The multi-enzyme aggregate oxidizing n-alkanes to fatty acids has been termed the hydroxylase complex (Bassel and Mortimer 1982).

In a previous report 21 UV induced mutants of *Y. lipolytica*, unable to utilize n-decane as a growth substrate but able to utilize long-chain fatty acids, were shown to represent 17 or 18 different genes (Bassel and Mortimer 1982). The large numbers of genes conferring this phenotype suggests that many steps are involved in n-alkane uptake since only a limited number of mutant loci would be expected to abolish one or more of the enzymatic activities making up the hydroxylase complex. This study reports on the isolation and genetic analysis of several new alkane non-utilizing, fatty acid utilizing, mutants and confirms that a majority of the alkane non-utilizing mutants isolated so far are defective in n-alkane uptake.

Materials and methods

Strains. Yarrowia (Saccharomycopsis) lypolytica is an ascosporogenous yeast that is the subject of an accumulating body of research. Many phenotypic classes of mutants have been analyzed by tetrad dissection and random spore analysis. The current genetic map of *Y. lipolytica* consists of 5 linkage groups on which *29* mutations have been located (Ogrydziak et al. 1978; Ogrydziak et al. 1982).

The genetic studies of *Y. lipolytica* have included mutant phenotypes that are not obtainable in the genetically well characterized yeasts: *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. These mutants have been isolated in the course of studies on the secretion of extracellular alkaline protease (Ogrydziak and Mortimer 1977) and the metabolism of n-alkanes (Bassel and Mortimer 1982).

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Two haploid strains of *Y. lipolytica* were utilized in this study; their genotypes are as follows: CX39-74B *trp1, B,* CX39-74C *ura1, A.* A and B denote bi-polar mating type (Bassel et al. 1973). These strains were developed during an inbreeding program starting with sporulating strains of yeast originally identified as *Candida lipolytica* (Ogrydziak et al. 1978).

Media. The following media were used for culture maintenance, mating, sporulation, auxotrophic marker scoring and the detection of extra-cellular protease: YM, RG, SM, SC, OM and SKM. The compositions of these media have been described (Ogrydziak et al. 1978). The following media were used to test for the utilization of n-alkanes and n-alkane oxidation products: ALK, ALC, ALD, FA and AC. The compositions of these media have been described (Bassel and Mortimer 1982).

RG (restrictive growth) medium enhances mating frequency in *Y. lipolytica* and is used to grow cells prior to and during mating (Ogrydziak et al. 1978). A recent reformulation of the medium (RG^+) has been shown to markedly enhance mating frequencies in several crosses (unpublished data). The composition of RG^+ is as follows: 0.025% yeast extract (Difco), 0.025% peptone (Difco), 0.025% glucose, 2% agar plus amino acids and bases as described for SC medium.

Genetic techniques. The genetic techniques utilized for mating, sporulation and mutagenesis by UV light have been described (Ogrydziak et al. 1978). The alkane non-utilized mutants described in this study were analyzed genetically by inter-mutant complementation and by recombination. Recombination was studied in randomly isolated ascospores. The techniques utilized in the analyses of complementation and recombination have been described (Bassel and Mortimer 1982).

Growth and induction of mutant and wild type strains. 500 ml baffled flasks containing 30 ml of SC plus 1% glucose medium were inoculated with approximately 10^9 cells from 24 h YM plate cultures. The cells were then incubated in a rotary shaker (320 RPM) and harvested in their logarithmic growth phase (20 h 30 c). The cells were harvested by centrifugation and washed once with sterile distilled water. Next, the cells were resuspended in 30 ml of SC + 0.3% n-decane (500 ml flasks) and reincubated with shaking for 8 h at 30 °C. The cells were then reharvested by centrifugation and washed twice with distilled water. Full induction of the n-alkane oxidizing enzymes has been found to require at least a 6 h exposure to the inducer, n-decane (Bassel and Mortimer 1982).

^{14}C hexadecane uptake. Induced, washed cells were resuspended in 10 ml of SC medium less carbon source and the optical density of each suspension was determined by employing a Klett-Summerson Colorimeter (Green filter). Next, the optical densities of the cell suspensions, typically four per experiment, were equalized by dilution with fresh SC less carbon source to within 20 Klett units (0.04 OD). The cell suspensions in a volume of 10 ml were transferred to 25 ml Erlenmeyer flasks and to each was added 5 µl of ^{14}C hexadecane (200 µCi/ml, Amersham 99% + purity). Although *Y. lipolytica* grows equally well on n-decane and n-hexadecane, n-decane has been shown to induce a higher level of hydroxylase complex activity (Bassel and Mortimer 1982). ^{14}C hexadecane was used in the uptake experiments because commercially available ^{14}C n-decane proved to be too volatile for this purpose. The flasks were incubated in a New Brunswick rotary shaker water bath at 30 °C or 24 °C at 200 RPM. One ml samples of the cell suspensions were withdrawn and filtered on glass fiber filters (Whatman, 2.4 cm) at 0, 20, 40, 60, 80 and 100 min. In order to remove the unabsorbed ^{14}C hexadecane, the cells were washed with two 5 ml aliquots of ice-cold ethanol. Ethanol has been shown to be an efficient solvent for this purpose (Yorifugi 1978). The filter papers and cells were placed in scintillation vials and 15 ml of Betaflour (National Diagnostics) was added. The samples were counted on a Packard Tricarb model 460c Scintillation Counter. Counting efficiencies were determined by the sample channel ratio technique. Uptake is expressed as DPM ^{14}C hexadecane incorporated per mg dry wt cells. Dry weight was calculated from the observation that one gram dry wt. cells per liter equals 250 Klett units (Ogrydziak and Mortimer 1977).

Results

Isolation and substrate utilization analyses of n-alkane non-utilizing mutants

The abilities of alkane-negative mutants to utilize intermediates in the pathway of alkane catabolism is an indication of the location of their metabolic defects. The alkane hydroxylase complex oxidizes alkane substrates to fatty acids of the same chain length. Thus, one would expect alkane non-utilizing, fatty acid utilizing mutants to be defective either in substrate uptake or in one or more of the enzymatic activities included in the hydroxylase complex (n-alkane hydroxylase, alcohol dehydrogenase, aldehyde dehydrogenase). Fatty acid positive, alkane negative mutants unable to utilize long chain alcohols or aldehydes as carbon sources suggest defects in alcohol or aldehyde dehydrogenase activities or in substrate uptake. The fatty acids resulting from alkane oxidation are broken down further by β-oxidation to acetate and oxidation is completed via the TCA cycle and the glyoxylate shunt (Kawamoto et al. 1978; Armitt et al. 1976).

Twenty-five mutants unable to utilize n-decane as a carbon source were isolated following ultraviolet irradiation of strain CX39-74C *ura1 A.* Table 1 presents the phenotypic classification of these mutants by substrate utilization tests. Strain CX39-74C complements and mates with strain CX39-74B *trp1 B* in which 44 alkane-negative mutants were previously isolated. These 44 strains were the subject of an earlier report and have been classified by substrate utilization tests identical to those of Table 1 (Bassel and Mortimer 1982). In this report, as in the previous one, only those mutants with phenotypes A and C are genetically analyzed. A block in the β-oxidation of fatty acids to acetate is indicated by phenotype D although uptake mutants could also show this phenotype (Kawamoto et al. 1978). The acetate-negative (phenotype E) strains probably represent mutations affecting the glyoxylate shunt or gluconeogenesis (Armitt et al. 1976).