A CR1, a gene encoding a protein related to mitochondrial carriers, is essential for acetyl-CoA synthetase activity in <i>Saccharomyces cerevisiae</i>

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**Abstract.** The utilization of ethanol via acetate by the yeast <i>Saccharomyces cerevisiae</i> requires the presence of the enzyme acetyl-coenzyme A synthetase (acetyl-CoA synthetase), which catalyzes the activation of acetate to acetyl-coenzyme A (acetyl-CoA). We have isolated a mutant, termed <i>acr1</i>, defective for this activity by screening for mutants unable to utilize ethanol as a sole carbon source. Genetic and biochemical characterization show that, in this mutant, the structural gene for acetyl-CoA synthetase is not affected. Cloning and sequencing demonstrated that the <i>ACRI</i> gene encodes a protein of 321 amino acids with a molecular mass of 35,370 Da. Computer analysis suggested that the <i>ACRI</i> gene product (ACR1) is an integral membrane protein related to the family of mitochondrial carriers. The expression of the gene is induced by growing yeast cells in media containing ethanol or acetate as sole carbon sources and is repressed by glucose. <i>ACRI</i> is essential for the utilization of ethanol and acetate since a mutant carrying a disruption in this gene is unable to grow on these compounds.

**Key words:** Acetyl-CoA synthetase – Mitochondrial carriers – Sequence – Disruption – Yeast

**Introduction**

The yeast <i>Saccharomyces cerevisiae</i> can use ethanol as a sole carbon source. In this pathway the enzyme alcohol dehydrogenase first transforms ethanol to acetaldehyde, which is then metabolized to acetate by the action of aldehyde dehydrogenase. The activation of acetate to acetyl-CoA is carried out by acetyl-CoA synthetase. Acetyl-CoA is further metabolized through the glyoxylate pathway or the tricarboxylic acid cycle (see Gancedo and Serrano 1989; Heinisch and Hollenberg 1993 for recent reviews on intermediary metabolism).

In recent years we have been interested in ethanol metabolism in <i>S. cerevisiae</i>. We have isolated mutants lacking isocitrate lyase activity, which were used to clone the structural gene by functional complementation (Fernández et al. 1992). The deletion of this gene from the yeast genome leads to cells that are unable to grow on ethanol, indicating that only one gene encoding isocitrate lyase is active under these metabolic conditions (Fernández et al. 1992). This is not the case for alcohol dehydrogenase (Ciric 1975), aldehyde dehydrogenase (Seegmiller 1955; Tamaki and Hama 1982) and malate synthase (Hartig et al. 1992; Fernández et al. 1993), where several isoenzymes have been identified.

Early biochemical investigations on acetyl-CoA synthetase from <i>S. cerevisiae</i> led to contradictory conclusions. While Klein and Jahnke (1979) and Satyanarayana et al. (1980) reported two enzymes, one located in mitochondria and the other in the microsomal fraction, Frenkel and Kitchens (1977) found only one enzyme. A gene encoding acetyl-CoA synthetase (<i>ACS1</i>) from <i>S. cerevisiae</i> has been cloned recently, and the nucleotide sequence predicted a product of 79.2 kDa (De Virgilio et al. 1992). The presence of a VKL sequence at its C-terminal end was interpreted as an indication that the enzyme is likely to be located in peroxisomes. The fact that null mutants constructed in the latter study still grow on ethanol (for which acetyl-CoA synthetase activity is essential, see above), and have residual activity, also suggests the existence of two isoenzymes. The genes encoding the corresponding enzymes from <i>Aspergillus nidulans</i> and <i>Neurospora crassa</i> have been sequenced and showed high homology with the one from <i>S. cerevisiae</i>. It was demonstrated that their synthesis is induced by acetate (Thomas et al. 1988; Sandeman and Hynes 1989; Connerton et al. 1990).

In this paper we describe the isolation of a mutant unable to grow on ethanol, having only basal levels of acetyl-CoA synthetase activity. Genetic and biochemical characterization indicate that the mutant is not affected in the structural gene encoding acetyl-CoA synthetase. Cloning of the gene by complementation of the mutant
phenotype and sequence analysis suggest that it encodes a new member of the family of mitochondrial carriers.

Materials and methods

Strains and plasmids. The S. cerevisiae strain 10.7-11A (MATa leu1 MAL3 suc3-3) was used for the isolation of mutants and YTGT-1A (MATa trp1 mal0) for genetic analyses (Rodicio and Zimmermann 1985). Strains AMW-13C+ (MATa trp1 fs ura3 fs leu2-3,112 his 3-11,15 can1 (fs stands for frameshift mutations); a cir+ derivative of a strain kindly provided by M. Whiteway) and eth19-1D (MATa acr1 trp1 fs ura3 fs leu2-3,112 his3-11,15; this work) were used in transformation experiments. The expression studies were performed in addition to the mutant strains JS87.11-1A (MATa trp1-289 cat1::HIS3 MAL2-8'MAL3 SUC3) and JS87.15-2B (MATa ura3-52 trp1-289 cat3::LEU2 MAL2-8'MAL3 SUC3), kindly provided by K.D. Entian. For genetic manipulations in Escherichia coli, strains HB101 (Bolivar and Backman 1979) and DH5αF (Gibco/BRL) were used.

The ACR1 gene was cloned from a yeast genomic library constructed by Nasmyth and Tatchell (1980) in the multicopy vector YEp13, which carries LEU2 as a selection marker. Vectors YRp7 (Struhl et al. 1979) and pUK21 (Vieira and Messing 1991) were used in the construction of the disruption plasmid.

Media, growth conditions and enzymatic analysis. Rich medium was based on 1% yeast extract and 2% peptone (YPE). As carbon sources either 2% glucose (D), 3% ethanol (E) or 3% potassium acetate (A) were added. Synthetic medium consisted of 0.67% yeast nitrogen base without amino acids (SC) and was supplemented with 2% glucose (D), 3% ethanol (E), 3% potassium acetate (A) or 3% pyruvate (P). Amino acids and uracil were added as required. When either acetate or pyruvate were used as carbon sources, the pH of the medium was adjusted to 6.5.

For preparation of crude extracts, cells were grown on YEPD and then transferred to YEPE for 8 h to allow for induction of the enzymes. Acetyl-CoA synthetase was tested following the method of Jones and Lippmann (1955) but potassium fluoride was not added to the reaction mixture. Isocitrate lyase and malate synthase were assayed according to Dixon and Kornberg (1959). Protein concentrations were determined by the method described by Lowry et al. (1951). Specific activities are expressed as nmol substrate consumed per min per mg protein in crude extracts.

Isolation of mutants defective in acetyl-CoA synthetase activity. The mutant defective in acetyl-CoA synthetase activity was obtained as described by Fernández et al. (1992) for the isolation of mutants affected in the isocitrate lyase structural gene. Basically, cells of strain 10.7-11A treated with ethylmethanesulfonate were plated on SCP medium and then replica-plated onto SC medium. Colonies that were not able to utilize ethanol as a sole carbon source were analyzed.

DNA manipulations. E. coli cells were transformed by the method of Hanahan (1985) and yeast cells by the method of Klebe et al. (1983). Plasmid DNA was isolated from bacteria as described by Birnboim and Doly (1979). Chromosomal and plasmid DNA were prepared from yeast according to Hoffman and Winston (1987). Total RNA was isolated from yeast as described by Sherman et al. (1986). Southern and Northern analyses were performed by the standard methods described in Maniatis et al. (1982).

Disruption experiments. To construct a strain carrying a disruption in the ACR1 gene, plasmid pACR-d was used (see Fig. 5, top, for a restriction map of the insertion). The 2.2 kb DNA fragment from the XhoI site of the insert in pACR1.1 (Fig. 1) to the HindIII site of the vector was first cloned into pUK21. The resulting plasmid was linearized with EcoRI and ligated to the 1.45 kb EcoRI fragment of YRp7 containing the TRP1 gene to yield pACR-d. Plasmid pACR-d was then double-digested with XhoI and BssHII and transformed into strain AMW-13C+. Transformants were selected on synthetic medium with glucose as a carbon source and omitting tryptophan.

Results

Isolation of mutants defective in acetyl-CoA synthetase activity

In order to investigate ethanol metabolism in S. cerevisiae we isolated mutants that grow on pyruvate media but are unable to utilize ethanol as a sole carbon source (Materials and methods). Since this yeast has several isoenzymes for alcohol dehydrogenase and aldehyde dehydrogenase, this screen should yield only mutants lacking acetyl-CoA synthetase, malate synthase or isocitrate lyase activities. To identify the enzymatic defect in each of the mutants isolated, the specific activities of these enzymes were tested after growth on ethanol medium. One mutant, termed acr1 (acetyl-CoA-synthetase regulation) for reasons explained below, showed very low acetyl-CoA synthetase activity as compared to the wild-type strain, while the levels of isocitrate lyase and malate synthase activities were similar to wild type (Table 1). The acr1 mutant was genetically characterized by tetrad analysis. Thus, in a cross to a wild-type strain a 2:2 segregation was found for ethanol utilization showing that the mutation had occurred at a single locus. This result was confirmed by testing the acetyl-CoA synthetase activity in two complete tetrads. For each tetrad two of the spores had wild-type activity while the other two had only the basal levels expected for the mutant allele.

To ascertain whether the isolated mutant was affected in the structural gene encoding acetyl-CoA synthetase or