Curr Genet (1985) 10:119–124

Current Genetics

Genetic regulation of isocitrate lyase in the yeast *Yarrowia lipolytica*

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Summary. Isocitrate lyase-less (Icl^-) mutants were selected from genetically defined strains of *Yarrowia lipolytica* to investigate the regulation of synthesis of isocitrate lyase (ICL). Eighteen mutations were localized in the gene *ICL1*, which is most probably the structural gene of ICL in this yeast. A *trans*-acting positive element (*ICL2*) could be identified. This gene is not linked to *ICL1*. One mutation was detected in another *ICL* gene (*ICL3*) which is linked to gene *ICL1*. This mutation lowered the ICL activity in *cis* but not in *trans* position to gene *ICL1*.

Key words: *Yarrowia lipolytica* – Isocitrate lyase – Structural gene – Regulatory genes

Introduction

In many organisms the glyoxylate cycle operates as an anaplerotic pathway to replenish the tricarboxylic acid cycle with C_4 compounds. During growth of microorganisms on C_2 compounds, like acetate, or on alkanes the enzymes of this cycle are detectable at high concentrations in contrast to growth on sugars, such as glucose. The key enzyme isocitrate lyase (ICL) is the strongestly regulated enzyme of this cycle in many organisms (Kornberg 1967; Flavell and Woodward 1971; Sariaslani et al. 1975; Gonzalez 1977; King and Casselton 1977; McCullough and Roberts 1980; O'Connell and Paznokas 1980). The genetic regulation of ICL activity has been intensively investigated in *E. coli* (Maloy and Nunn 1982), *Neurospora crassa* (Flavell and Woodward 1971; Leckie and Fincham 1971), *Aspergillus nidulans* (McCullough and Roberts 1980) and *Coprinus cinereus* (King and Casselton 1977). In these organisms the structural genes of ICL are known and several regulatory genes have been described; however, the regulatory mechanism is not fully understood in any microorganism.

In the ascomycetous yeast *Yarrowia* (formerly *Saccharomycopsis*) *lipolytica* Matsuoka et al. (1980, 1984) described four acetate nonutilizing mutants of a wild-type strain, which express very low levels of ICL activity. By analyzing temperature-sensitive revertants, protoplast fusion and haploidization these authors could show that these four mutations are localized in one gene, which represents most probably the structural gene coding for the ICL enzyme. They also described a mutant without any activity of acetyl-coA-synthetase ($acos^-$), in which high levels of ICL and malate synthase could not be induced.

In this paper we describe the identification of other ICL loci upon newly isolated ICl^- mutants derived from genetically improved strains of *Y. lipolytica*.

Material and methods

Yeast strains. In this study genetically improved strains of *Y. lipolytica* were mainly used. Besides these strains several mutants kindly supplied by S. Aiba (Osaka University, Osaka), J. B. Bassel (Donner Laboratory, Berkeley), C. Kurischko (our laboratory), have been included for certain investigations. The strains used in this study are listed in Table 1.

Media and culture conditions. Complete medium (YEPD), synthetic minimal media (MMT) with 2% glucose (MMT-G) or 0.7% sodium acetate (MMT-A), and conjugation medium (YM) were prepared as described by Barth and Weber (1983). Diploid strains were induced to sporulate on citrate sporulation medium (CSM) described by Barth and Weber (1985). Amino acids and bases were supplied at final concentrations of 50 mg/l in MMT. In some experiments cells were precultivated in MMT-G supplemented with a mixture of L-amino acids (alanine, arginine, asparagine, cysteine, glutaminic acid, glycine, lysine, methionine, phenylalanine, tryptophane, and tyrosine).

Table 1. List of strains of *Y. lipolytica. acos x*, very low activities of acetyl CoA synthetase, isocitrate lyase, and malate synthase; gene locus unknown. *icl x*, no activity of isocitrate lyase; mutation most probably in the structural gene coding for ICL

Strain	Genotype	Utilization of acetate (Acu)	Source or reference
B157-6B	A *met* A	Acu^+	Barth and Weber 1985
B157-6B-2	A *met* A *can* 1	Acu^+	Barth and Weber 1985
B157-6A-10	B *leu* A *nys* A	Acu^+	Barth and Weber 1985
37-K139	B *arg* A *leu* A	Acu^+	Kurischko 1983
MT4	Prototrophic	Acu^+	Matsuoka et al. 1980
MX8-1	Prototrophic *acos x*	Acu^-	Matsuoka et al. 1980
MX9-11	Prototrophic *icl x*	Acu^-	Matsuoka et al. 1980
YB268	B *trp1 alkD*	Acu^+	Bassel and Mortimer 1982
YB300	B *trp1 alkD*	Acu^+	Bassel and Mortimer 1982
YB357	B *trp1 alkD*	Acu^+	Bassel and Mortimer 1982
YB362	B *trp1 alkD*	Acu^+	Bassel and Mortimer 1982
YC87	A *ura1 alkD*	Acu^+	Bassel and Mortimer 1982
YB343	B *trp1 alkE*	Acu^-	Bassel and Mortimer 1982
YB348	B *trp1 alkE*	Acu^-	Bassel and Mortimer 1982
YB349	B *trp1 alkE*	Acu^-	Bassel and Mortimer 1982
YC69	A *ura1 alkE*	Acu^-	Bassel and Mortimer 1982

Cultures were grown in 500 ml flasks containing 100 ml liquid medium on a rotary shaker at 28 °C.

Acetate induced cells were prepared as follows: cells were transferred from a YEPD agar slant into liquid YEPD medium and shaken for 22 h. Of this suspension 1 ml was inoculated into 100 ml liquid MMT-G and incubated for 18 h. Cells were centrifuged, washed with MMT without carbon source and resuspended in 100 ml MMT-A at a concentration of about $1-2 \times 10^7$ cells per ml. These cultures were incubated on a rotary shaker for 6 h or 8 h before harvesting.

Mutant isolation. Mutagenesis by ultra-violet light and isolation of Acu^- mutants was carried out as described by Barth and Weber (1983) without enrichment procedure. The frequency of Acu^- mutants at the UV dosage applied (about 90% killing) was about 0.1–0.25% of survivals. Among all Acu^- isolates mutants without or with low levels of isocitrate lyase on acetate were chosen for further analysis.

Conjugation and complementation analysis. Conjugation and complementation analysis of Icl^- mutants were performed by the method described by Barth and Weber (1984a). Alkane-nonutilizing mutants (alkD and alkE mutants) and the mutants MX8-1 and MX9-11 were included in this complementation analysis. Surprisingly, strain MX8-1 exhibited mating type A and strain MX9-11 mating type B, though they are mutants of the same wild-type strain MT4 (Matsuoka et al. 1980). The conjugation frequencies of these strains were very low. Therefore, the acos-mutation of MX8-1 and the icl-mutation of MX9-11 were introduced into genetically improved strains. All Icl^- mutants were crossed with one Icl^+ strain of opposite mating type with different auxotrophic markers. Prototrophic Acu^+ diploids were selected, plated on CSM and spores were isolated by the nystatin technique (Barth and Weber 1984b). Acu^- spore clones of opposite mating types and various auxotrophic markers were selected from each diploid. These Acu^- (Icl^-) strains were crossed among themselves (control crosses) and also with the segregants of other diploid strains. Resultant diploid strains were tested for prototrophy and acetate utilization at 28 °C. Noncomplementing mutants showed no growth after 7 days on MMT-A.

Meiotic recombination analysis. In diploid strains previously tested for complementation sporulation was induced on CSM and spores were isolated by the nystatin method. Spore clones were replica-plated on MMT-A after 2 days to obtain a number of Acu^+ segregants. One would expect 25% of the haploid spores derived from a cross between strains with mutations in two unlinked ICL genes to be Acu^+. In the case of linkage, the frequency of Acu^+ clones should be lower. In order to rule out the possibility of scoring complementing diploid strains as Acu^+ segregants, the spore clones were additionally replica-plated on CSM. Diploid colonies could be easily distinguished from haploid ones by brown colour after 4–5 days.

Preparation of cell free extracts. Cell free extracts were prepared according to Hoenes (1983). Cells were harvested by centrifugation and stored at −20 °C at least for 24 h. These frozen cells (about $1-3 \times 10^9$ cells) were resuspended in 5 ml 0.05 M Tris-HCl buffer + $MgCl_2$ (pH 7,0) and added together with glass beads into a cooled steel tube (10 ml volume) of a vibration homogenizer, wherein cells were desintegrated. After separation of glass beads by filtration the crude extract was centrifuged (30 min at 4 °C and 16,000 x g), diluted if necessary, and used for enzyme assays.

Enzyme assay. Isocitrate lyase (threo-D-isocitrate glyoxylate-lyase, EC 4.1.3.1.) was assayed by the combinations of methods of Roche et al. (1970) and McFadden and Howes (1960) in phosphate buffer at pH 6.0. One unit of the enzyme activity was defined as the amount of enzyme capable of forming 1 μmol of product per min at 30 °C. Protein content was determined by the method of Lowry et al. (1951).

Results

Activities of isocitrate lyase in auxotrophic acetate utilizing (Acu^+) strains

Auxotrophic Acu^+-strains of *Y. lipolytica* were used for selection of Icl^--mutants. Hitherto, data on ICL-activi-