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Cloning of the isocitrate lyase gene (*ICL1*) from *Yarrowia lipolytica* and characterization of the deduced protein

Gerold Barth, Thomas Scheuber

Department of Microbiology, Biozentrum, University of Basel, Klingelbergstr. 70, CH-4056 Basel, Switzerland

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Abstract. The *ICL1* gene encoding isocitrate lyase was cloned from the dimorphic fungus *Yarrowia lipolytica* by complementation of a mutation (*acuA3*) in the structural gene of isocitrate lyase of *Escherichia coli*. The open reading frame of *ICL1* is 1668 bp long and contains no introns in contrast to currently sequenced genes from other filamentous fungi. The *ICL1* gene encodes a deduced protein of 555 amino acids with a molecular weight of 62 kDa, which fits the observed size of the purified monomer of isocitrate lyase from *Y. lipolytica*. Comparison of the protein sequence with those of known pro- and eukaryotic isocitrate lyases revealed a high degree of homology among these enzymes. The isocitrate lyase of *Y. lipolytica* is more similar to those from *Candida tropicalis* and filamentous fungi than to *Sacharomyces cerevisiae*. This enzyme of *Y. lipolytica* has the putative glyoxysomal targeting signal S-K-L at the carboxy-terminus. It contains a partial repeat which is typical for eukaryotic isocitrate lyases but which is absent from the *E. coli* enzyme. Surprisingly, deletion of the *ICL1* gene from the genome not only inhibits the utilization of acetate, ethanol, and fatty acids, but also reduces the growth rate on glucose.

Key words: Glyoxylate cycle – Fungus – Yeast – Peroxisome, glyoxysome – Gene disruption

Introduction

The glyoxylate cycle is present in pro- and eukaryotic microorganisms, protozoa, molluscs, insects and plants but only in some tissues at certain stages of development in mammals (Vanni et al. 1990). This pathway is required for the utilization of acetate, ethanol, and fatty acids as carbon sources. It replenishes intermediates of the tricarboxylic acid cycle and forms glyoxylate, from which glycine and serine are synthesized. This bypass is very weakly expressed in most organisms during growth on carbon sources such as hexoses. Isocitrate lyase is one of the enzymes of this pathway which is being intensively studied in several organisms (Vanni et al. 1990). The structural gene encoding this enzyme has been cloned from *Escherichia coli* (El-Mansi et al. 1987; Matsuoka and McFadden 1988; Rieul et al. 1988), the yeasts *Candida tropicalis* (Atomi et al. 1990) and *Saccharomyces cerevisiae* (Fernandez et al. 1992), the filamentous fungi *Aspergillus nidulans* (Ballance and Turner 1986; Gainey et al. 1992), *Coprinus cinereus* (Mellon et al. 1987), and *Neurospora crassa* (Gainey et al. 1992), and the three plants *Brassica napus* (Comai et al. 1989), *Gossypium hirsutum* (Turley et al. 1990), and *Ricinus communis* (Beeching and Northcote 1987).

The *ICL* genes of plants and filamentous fungi contain several introns, which are not present in the *ICL1* gene of the yeast *S. cerevisiae* (Gainey et al. 1992; Fernandez et al. 1992). Furthermore isocitrate lyases of plants, *A. nidulans*, *N. crassa*, and *C. tropicalis* are detected in organelles called glyoxysomes, although isocitrate lyases of filamentous fungi do not contain any known glyoxysomal targeting signals (Gainey et al. 1992). In contrast, the isocitrate lyase of the yeast *S. cerevisiae* has thus far not been localized to glyoxysomes and its carboxy-terminus sequence is V–K–K, which differs from the glyoxysomal targeting consensus sequence S/A–K/R–L/M (Gould et al. 1990). Therefore it has been suggested that another targeting signal must be present in the isocitrate lyase sequence, assuming that glyoxysomal localization of the enzyme occurs in *S. cerevisiae* (Fernandez et al. 1992).

The fungus *Yarrowia lipolytica*, which forms both yeast cells and hyphae, is specialized for the utilization of alkanes, fats, fatty acids, acetate, and ethanol. High levels of glyoxylate cycle activity are observed in cells grown in such media (Kujau et al. 1992). However it is not known where the enzymes of this cycle are localized in this dimorphic fungus. Mutants in the structural gene for isocitrate lyase (*ICL1*) and in regulatory genes (*ICL3*, *GPR1*) from *Y. lipolytica* have been isolated and

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Correspondence to: G. Barth

characterized (Barth 1985; Barth and Weber 1987; Kujau et al. 1992).

This report describes the cloning and sequencing of the *ICL1* gene from the dimorphic ascomycetous fungus *Y. lipolytica*. The aim of this study was to investigate whether introns occur in the *ICL1* gene of this yeast and to determine whether a glyoxysomal targeting signal like S–K–L is present at the carboxy-terminus of the enzyme. Additionally the expression of this gene was studied, and a comparison of all hitherto known protein sequences of isocitrate lyase was performed to identify characteristic features.

Materials and methods

Strains and plasmids. Y. lipolytica strain B204–12C (*MATA met6-1 spol-1;* Kujau et al. 1992) was used as source of DNA for the gene library. Strains B204–12C–108 (*MATA icl1–108 met6–1 spol–1)* and B511–1 (*MATA icl1–108 leu2–20 met6–1 spol—1)* were used as recipients for transformation and expression studies of the *ICL1* gene. Plasmid pBR322 (Bolivar et al. 1977) was used for the construction of a gene library of *Y. lipolytica*. Vector pINA237 (Fournier et al. 1991) containing the *LEU2* gene, the *ars18* region, and a centromere region of *Y. lipolytica* was used for subcloning the fragment containing the *ICL1* gene. Plasmid pINA237 was also used for extrachromosomal expression of the *ICL1* gene. Plasmid pYLI132 was constructed during this work and contains the *ICL1* gene in the *Bam*HI site of pINA237.

E. coli strain SK1590 (*endA gal hsdR4 recA thi1 sbcB15 Sm*^{r}; Kushner 1978) was used as recipient for construction of the gene library of *Y. lipolytica*. Strain HB101 (*supE44 hsdS20 recA13 ara-14 proA2 lacY1 galK2 rpsl20 xyl-5 mtl-1;* Sambrook et al. 1989) was used for comparison of isocitrate lyase activities. The Acu$^-$ mutant K8-5m (*aceA3 galK2 iclR13 lac met-24 thi;* Maloy and Nunn 1982) of *E. coli* was used for selection of the *ICL1* gene of *Y. lipolytica* by complementation of the *aceA3* mutation.

Media and culture conditions. The complete medium (YPD) and the synthetic media (MMT) with 2% glucose (MMT–G) or 0.4% sodium acetate (MMT–A) and culture conditions used for growth of *Y. lipolytica* are described by Barth and Weber (1983). Acetate-induced cells were prepared according to Barth (1985). Amino acids were supplied at final concentrations of 50 mg/l in MMT. Cultivation of *Y. lipolytica* cells took place at 28° C. Cultivation of *E. coli* cells was performed in LB medium or in synthetic medium containing 0.4% sodium acetate (M9–A; Sambrook et al. 1989).

Preparation of cell-free extracts and enzyme assay. Preparation of cell-free extracts and assay of isocitrate lyase (threo-D-isocitrate glyoxylate lyase, EC4.1.3.1) was done as described by Barth and Weber (1987). The protein content was determined by the method of Lowry et al. (1951).

Nucleic acid preparation, Southern hybridization, and DNA sequencing. Y. lipolytica strain B204–12C was cultivated in YPD for 24 h, harvested and transferred into fresh MMT–A at a density of about $2–3 \times 10^7$ cells/ml. This culture was shaken at 28° C for 10 h, harvested, and washed with water. DNA was isolated from these cells according to the method of Cryer et al. (1975). Plasmid DNA was isolated from *E. coli* as described by Birnboim and Doly (1979). Southern hybridization and non-radioactive detection of DNA fragments was performed with the ECL Kit from Amersham. DNA sequencing was performed using the T7 sequencing kit of Pharmacia LKB Biotechnology with double-stranded DNA preparations as templates. The sequence was obtained from both strands after subcloning of fragments into pUCBM20 (Boehringer Mannheim) using M13 primers and synthetic oligonucleotides. The sequence has been deposited in the EMBL database in Heidelberg under accession number: 72848.

Isolation of RNA and Northern hybridization. For RNA preparation cells of *Y. lipolytica* B204–12C were precultivated in YPD for 18 h, collected by centrifugation, washed with MMT and resuspended in MMT. One hundred millilitres of MMT–G and MMT–A were inoculated with these cells at a density of 4×10^7 cells/ml and shaken at 28° C for 4 h. Cells were collected by filtration (millipore filter, pore size 1.2 µm), washed with 2 ml MMT and frozen at −70° C. Extraction and purification of RNA was done according the method of Domdey et al. (1984). Twenty micrograms of RNA were electrophoresed on a formaldehyde-containing agarose (1%) gel in MOPS buffer for 3.5 h (Sambrook et al. 1989). Washing of the gel, transfer of RNA onto nylon membrane (Biodyne A, Pall AG), hybridization, and detection were done as recommended by the protocol provided with the ECL kit (Amersham). The 2.3 kb *Bam*HI fragment containing the entire *ICL1* gene was used as a probe for hybridization.

Construction of the gene library and transformation of E. coli *and* Y. lipolytica. DNA of *Y. lipolytica* B204–12C was partially digested with *Sau3*A and fragments of 2–10 kb were collected after sucrose gradient centrifugation according to Sambrook et al. (1989). These fragments were ligated into the vector pBR322 which was digested with *Bam*HI and dephosphorylated. About 20 000 transformants were harvested after transformation of *E. coli* SK1590. The transformants were pooled and the plasmids isolated; the average sizes of the inserts in the pBR322 vector were found to be about 4–8 kb. Transformation of *E. coli* cells was done either by the $CaCl_2$ method (Sambrook et al. 1989) or by electroporation according to the protocol of Bio-Rad. Transformation of *Y. lipolytica* cells was carried out either by the lithium acetate method (Davidow et al. 1985) or by electroporation according to the protocol of Bio-Rad.