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Yeast Sequencing Report

Isolation of an acetyl-CoA synthetase gene (*ZbACS2*) from *Zygosaccharomyces bailii*

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Abstract

A gene homologous to *Saccharomyces cerevisiae ACS* genes, coding for acetyl-CoA synthetase, has been cloned from the yeast *Zygosaccharomyces bailii* ISA 1307, by using reverse genetic approaches. A probe obtained by PCR amplification from *Z. bailii* DNA, using primers derived from two conserved regions of yeast ACS proteins, RIGAIHSVVF (ScAcs1p; 210–219) and RVDDVVNVSG (ScAcs1p; 574–583), was used for screening a *Z. bailii* genomic library. Nine clones with partially overlapping inserts were isolated. The sequenced DNA fragment contains a complete ORF of 2027 bp (*ZbACS2*) and the deduced polypeptide shares significant homologies with the products of *ACS2* genes from *S. cerevisiae* and *Kluyveromyces lactis* (81% and 82% identity and 84% and 89% similarity, respectively). Phylogenetic analysis shows that the sequence of Zbacs2 is more closely related to the sequences from Acs2 than to those from Acs1 proteins. Moreover, this analysis revealed that the gene duplication producing Acs1 and Acs2 proteins has occurred in the common ancestor of *S. cerevisiae*, *K. lactis*, *Candida albicans*, *C. glabrata* and *Debaryomyces hansenii* lineages. Additionally, the cloned gene allowed growth of *S. cerevisiae Scacs2* null mutant, in medium containing glucose as the only carbon and energy source, indicating that it encodes a functional acetyl-CoA synthetase. Also, *S. cerevisiae* cells expressing *ZbACS2* have a shorter lag time, in medium containing glucose (2%, w/v) plus acetic acid (0.1–0.35%, v/v). No differences in cell response to acetic acid stress were detected both by specific growth and death rates. The mode of regulation of *ZbACS2* appears to be different from *ScACS2* and *KlACS2*, being subject to repression by a glucose pulse in acetic acid-grown cells. The nucleotide sequence of a common 5269 bp fragment has been deposited in the EMBL Data Library under Accession No. AJ314837.

Keywords: ***Zygosaccharomyces bailii*; acetyl-CoA synthetase; acetic acid metabolism**

Introduction

Species of *Zygosaccharomyces* are commonly associated to food and beverages spoilage due their capacity to survive under acidic stress environments (Hocking, 1996; Schuller *et al*., 2000; Sousa *et al*., 1998; Thomas and Davenport, 1985; Wium *et al*., 1990).

It has been reported in several yeast species that acetate enters the cell by a mediated transport system (Casal *et al*., 1996; Cassio *et al*., 1987; Sousa *et al*., 1996) and the first metabolic step is its conversion into acetyl-CoA by acetyl-CoA synthetase. This enzyme has been studied in detail in *Saccharomyces cerevisiae*, highlighting its physiological importance (DeVicenzy and Klein, 1970; van den Berg *et al*., 1996; van den Berg *et al*.,

1995; Zeeman *et al*., 2003). Not only is the function of acetyl-CoA synthetase associated with the metabolism of C2 compounds, but the production of acetyl-CoA is essential for overall carbon metabolism in *S. cerevisiae* (Flikweert *et al*., 1996; van den Berg *et al*., 1995). Two structural *ACS* genes have been cloned from *S. cerevisiae* and *Kluyveromyces lactis*, encoding two isoforms of the acetyl-CoA synthetase (van den Berg *et al*., 1996; Zeeman *et al*., 2003). In *S. cerevisiae,* the *ACS1* gene encodes a protein with high affinity for acetate ($K_m = 0.32$ mM; van den Berg *et al*., 1996), which is subject to glucose inactivation (de Jong-Gubbels *et al*., 1997). In addition, transcription of the gene is strongly repressed by glucose. The *ScACS2* gene encodes a protein with a lower affinity for acetate ($K_m = 8.8$ mM; van den Berg *et al*., 1996) and is expressed in the presence of glucose (de Jong-Gubbels *et al*., 1997; van den Berg *et al*., 1995). This isoenzyme seems to be responsible for the production of acetyl-CoA in the cytosol, an essential precursor for fatty acids synthesis (van den Berg *et al*., 1995). The transcriptional regulation of both genes and the fast inactivation of ScAcs1p by glucose explain the inability of the *acs2* null mutant of *S. cerevisiae* to grow in glucose medium. On the other hand, the *Scacs1* null mutant of *S. cerevisiae* only shows an increased lag-phase in synthetic medium with glucose, ethanol or acetate, while the specific growth rates are not affected (van den Berg *et al*., 1995). A double mutant of *S. cerevisiae* or *K. lactis* in both structural genes seems not to be viable, at least on the carbon and energy sources tested by the authors (van den Berg *et al*., 1995; Zeeman *et al*., 2003). For *K. lactis*, the *KlACS1* and *KlACS2* genes showed similar transcriptional regulation to their homologues in *S. cerevisiae*. However, the *K. lactis Klacs1* null mutant displays a 50% reduction of the specific growth rate on acetate as the sole carbon and energy source, when compared to the parental strain (Zeeman *et al*., 2003).

To investigate the physiological role of acetyl-CoA synthetase(s) in *Z. bailii* ISA 1307, the *Z. bailii ACS* gene(s) needed to be identified. Here, we report the isolation of one gene encoding acetyl-CoA synthetase from a *Z. bailii* genomic library. Preliminary studies on transcriptional regulation of the isolated gene were performed in batch cultures. Tests of resistance to acetic acid were performed in a *S. cerevisiae* strain expressing *ZbACS2* on a centromeric vector.

Materials and methods

Microorganisms

Zygosaccharomyces bailii ISA 1307 was used (Wium *et al*., 1990). The strains BY4742 (*MATα*; *his 3Δ 1*; *leu 2Δ 0*; *lys 2Δ 0*; *ura 3Δ 0*; EUROSCARF, Accession No. Y10 000) and GG625 (*MAT***a***/MATα HO/HO*, *ScACS1/ScACS1*, *Scacs2::Tn5BLE/Scacs2::Tn5BLE*); (van den Berg *et al*., 1995) of *Saccharomyces cerevisiae* were used for tests of acetic acid resistance and complementation experiments, respectively. *Escherichia coli* XL1-Blue was used as the bacterial host for plasmids (Bullock *et al*., 1987).

Growth conditions and media

E. coli strains were grown in Luria-Bertani medium (LB) at 37 °C supplemented with 100 μg/ml ampiciline when required (Sambrook *et al*., 1998). Yeast strains were grown in YPD (5 g/l yeast extract, 10 g/l peptone, 20 g/l glucose). For selective purposes, a synthetic medium was used, supplemented when required with the respective bases and amino acids (Sherman, 1991). The strain GG625 of *S. cerevisiae* was grown in YPE (5 g/l yeast extract, 10 g/l peptone, 20 ml/l ethanol). Studies on plasmids were performed as follows: GG625 cells were grown in YPE, until the stationary phase, and plated on the same medium. The colonies obtained were then replica-plated onto YPD (agar). Cells unable to grow on YPD media were considered as having lost the plasmid containing *ZbACS2* gene.

DNA manipulations

For plasmid isolation from *E. coli* and yeast, a Qiaprep spin miniprep kit (Qiagen) was used (Rodrigues *et al*., 2001). Agarose gel electrophoresis, DNA sub-cloning, restriction site mapping and hybridizations were performed according to standard methods (Sambrook *et al*., 1998). Yeast chromosomal DNA was isolated as described previously (Holm *et al*., 1986). Probes were labelled radioactively by using ^{32}P-dCTP and a commercial oligo labelling kit (Pharmacia). Competent cells of *E. coli* and *S. cerevisiae* were prepared as described elsewhere (Gietz and Schiestl, 1995; Inune *et al*.,

1990). Total mRNA was isolated and analyzed as published elsewhere (van den Berg *et al*., 1996). Hybridizations were performed at high-stringency conditions (65 °C) using the *ZbACS2* ORF as probe.

Cloning and sequence analysis of the *ZbACS2* gene

The *ZbACS2* gene was cloned by homologous hybridization of the *Z. bailii* ISA 1307 genomic library (Rodrigues *et al*., 2001) using standard conditions (Sambrook *et al*., 1998). Colonies were transferred to nitrocellulose membranes, and plasmids were fixed to the filter as described elsewhere (Woods, 1984). Degenerate primers were designed based on conserved sequences of known acetyl-CoA synthetases, with the sequences; 5′-CGN AT(T/A/C) GGN GCN AT(T/A/C) CA(C/T) TCN GTN GTN TT(T/C)-3′ and 5′-NCC NGA NAC (A/G)TT NAC NAC (G/A)TC (G/A)TC NAC (T/C)CT-3′. PCR was performed in standard buffer (Goldstar), and amplification was performed using the following conditions: 95 °C, 3 min; 42 °C, 90 s; 72 °C, 2 min followed by 30 cycles of 95 °C, 1 min; 42 °C, 90 s; 72 °C, 2 min. A fragment of 1.1 kb was obtained by PCR amplification on genomic DNA of *Z. bailii* ISA 1307 using the degenerate primers. The fragment obtained was ligated into pGEM-T vector (Promega) and sequenced by BaseClear (The Netherlands). The cloned fragment was used as probe for genomic library screening. The insert contained in the vector pACS139 was completely sequenced. To further extend the sequence upstream of *ZbACS2*, the vector pACS 221 was partially sequenced, starting from sequences known in the pACS139 insert. The DNA sequence was determined by BaseClear (The Netherlands) and each base was covered at least three times.

The ClustalW (Thompson *et al*., 1994) algorithm provided in the Dnaman package was used to align the sequences and construct a neighbour-joining tree with 5000 bootstrap iterations.

Results and discussion

Cloning of a *ZbACS* gene from *Zygosaccharomyces bailii*

Comparison of the putative amino acid sequences encoded by the known acetyl-CoA synthetase genes revealed two conserved regions separated by 300 amino acid residues. The conserved regions located at amino acids position 210–270 and 570–630 in Acs1p from *Saccharomyces cerevisiae*, were used to find a short sequence with the highest degree of homology. From this *in silico* study the sequences RIGAIHSVVF (ScAcs1p; 210–219) and RVDDVVNVSG (ScAcs1p; 574–583) were identified and used to design degenerated primers. PCR amplification on genomic DNA from *Z. bailii*, *Kluyveromyces lactis* and *S. cerevisiae* revealed a DNA fragment of about 1.1 kb, as expected. The DNA fragment obtained from PCR on genomic DNA from *Z. bailii* ISA1307 was cloned using the pGEM-T vector system and the insert was sequenced. In the fragment sequenced, an incomplete ORF was found. The DNA sequence showed a high degree of similarity with acetyl-CoA synthetase ORFs from other sources (data not shown). Therefore, the PCR product was used as a probe to screen a previously published genomic library from *Z. bailii* ISA 1307 (Rodrigues *et al*., 2001). Nine strongly hybridizing colonies were detected. Restriction analysis showed that the inserts of the clones isolated, range 3–7 kb, were partially overlapping. The clone pACS139, containing an insert of around 3.7 kb, was used for sequencing. Southern blot analysis of digested genomic DNA of *Z. bailii* ISA 1307 using the putative *ZbACS* as probe was performed under high-stringency hybridization conditions (data not shown). None of the hybridizing bands contradicted the sequence data, indicating that the gene is present in a single copy per haploid genome and that the isolated fragments originated from *Z. bailii*. In addition to the pACS139 insert, an extension of the upstream region of the putative *ZbACS* gene was sequenced in the vector pACS221 (see Materials and methods). In total, 5269 nucleotides were sequenced. This sequence has been deposited in the EMBL Database under Accession No. AJ314837.

An ORF of 2027 nucleotides was found, running from 1849 bp to 3876 bp, with 74.7% homology to *ScACS2* from *S. cerevisiae*. The ORF encodes a putative protein of 675 amino acid residues with a calculated molecular mass of 74.4 kDa. A comparison of the deduced aminoacid sequence revealed high similarity to acetyl-CoA synthetase from other sources. Figure 1 shows the results of a phylogenetic analysis performed with the full-length sequences of this putative protein and

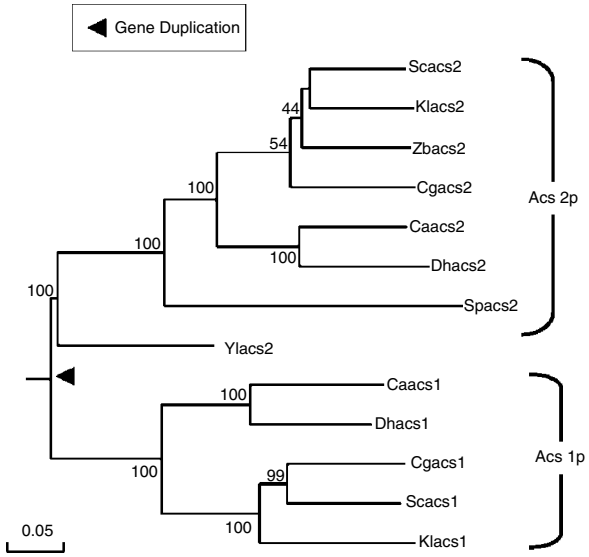

Figure 1. Neighbour-joining tree showing the phylogenetic placement of ZbAcs2p based on the differences in protein sequence of the acetyl-CoA synthetase (Zb, *Zygosaccharomyces bailii*; Sc, *Saccharomyces cerevisiae*; Kl, *Kluyveromyces lactis*; Ca, *Candida albicans*; Cg, *C. glabrata*; Dh, *Debaryomyces hansenii*; Yl, *Yarrowia lypolitica*; Sp, *Schizosaccharomyces pombe*) deposited in Génolevures, EMBL and GenBank databases. The percentage bootstrap values were obtained from 5000 iterations. Bar, 5% sequence divergence

all available yeast acetyl-CoA synthetase proteins (November, 2003). The results presented indicate that the sequence of the putative protein encoded by the isolated gene is more closely related to sequences from Acs2 than to those from Acs1 proteins. The isolated ORF was therefore named *ZbACS2*. In addition, the phylogenetic tree in Figure 1 shows the gene duplication producing Acs1 and Acs2 proteins occurred in the common ancestor of *Saccharomyces cerevisiae*, *K. lactis, Candida albicans, C. glabrata* and *Debaryomyces hansenii* lineages. This can be inferred because the acs1 sequences from these species cluster together with high support to the exclusion of the Acs2p sequences (Figure 1). Figure 2 shows the amino acids alignment of Zbacs2p with the Acs2p from *S. cerevisiae* and *K. lactis*, since these three proteins belong to the some cluster in the phylogenetic tree.

The analysis of the *ZbACS2* promoter region revealed three putative TATA boxes, with the sequence TATAA founded at positions 1211, 1517 and 1527. A search for regulatory sequences was performed in the 1 kb region upstream of the isolated gene and none of the sequences described by van den Berg *et al*. (1998) as putative regulatory elements was found. Additionally, the alignment of 1000 nucleotides upstream of *ZbACS2, ScACS2* and *KlACS2* did not show any regulatory conserved region. On studying the adjacent downstream region of the *ZbACS2*, an additional ORF was found. This incomplete ORF encodes a protein with homology to the one encoded by the ORF YLR152c from *S. cerevisiae*. The latter is a putative membrane protein (with seven putative transmembrane domains) of unknown function, present on chromosome XII of *S. cerevisiae*, encoded by a gene downstream of *ScACS2*.

To test whether the cloned fragment containing the intact ORF did indeed include a functional *ZbACS2* gene, the plasmid pACS139 was used to functionally complement the *S. cerevisiae Scacs2* null mutant (GG625; van den Berg *et al*., 1995). This mutant is unable to grow on glucose media and it is a prototrophic strain. Therefore, the transformants were selected on medium containing glucose. Many colonies (10^6 colonies/μg plasmid DNA) were indeed obtained that could grow on glucose. Replica-plating showed that the ability to grow on glucose was plasmid-linked, since it was lost in around 15% of the 1740 clones tested, after one round of growth in non-selective (ethanol) medium.

Expression studies of the *ZbACS2* gene

The mRNA levels of *ZbACS2* were measured during exponential growth of *Z. bailii* ISA 1307 in synthetic media with glucose (2%, w/v), or acetic acid (0.5%) as carbon and energy sources (Figure 3A, B). *ZbACS2* mRNA was detectable in exponentially growing cells in glucose. During exponential growth in acetic acid medium, the mRNA levels of this gene were higher than in glucose medium (Figure 2C). This result is in agreement with the absence of the regulatory sequence CCCGAGRGGA that is present in acetate-repressed promoters (van den Berg *et al*., 1998). To investigate the effects of glucose on *ZbACS2* transcription, glucose (2%, w/v) was added to cells growing exponentially in acetate medium (glucose pulse). Within 4 h the *ZbACS2* mRNA level was reduced to that of glucose-grown cells (Figure 3). These results are in apparent contrast to previous reports by Zeeman *et al*. and van

Figure 2. Comparative alignment of *Zygosaccharomyces bailii* (AJ314837), *Kluyveromyces lactis* (AF134491) and *Saccharomyces cerevisiae* (S79456) Acs2p amino acid sequences. Black box underlines the conserved sequences used to design the primers

den Berg and Steensma, in *K. lactis* and *S. cerevisiae* (van den Berg *et al*., 1995; Zeeman *et al*., 2003), where glucose induced *ACS2* expression.

ZbACS2 expression in *S. cerevisiae*

Finally, we also investigated the behaviour of *S. cerevisiae* cells expressing *ZbACS2*. Therefore, the strain BY 4742 of *S. cerevisiae* (*ScACS2*) was transformed with the plasmid pACS139, and its response to acetic acid in the presence of glucose, at pH 3.0, was analysed. The effects of the acetic acid on growth were tested by measuring the specific growing rates of the strain expressing *ZbACS2* and the strain harbouring the empty vector (pRS316) on glucose (2%, w/v) with different concentrations of acetic acid (0–0.4%). The specific growth rates of *ZbACS2* expressing cells did not differ from the control cells. Additionally, a small decrease in the

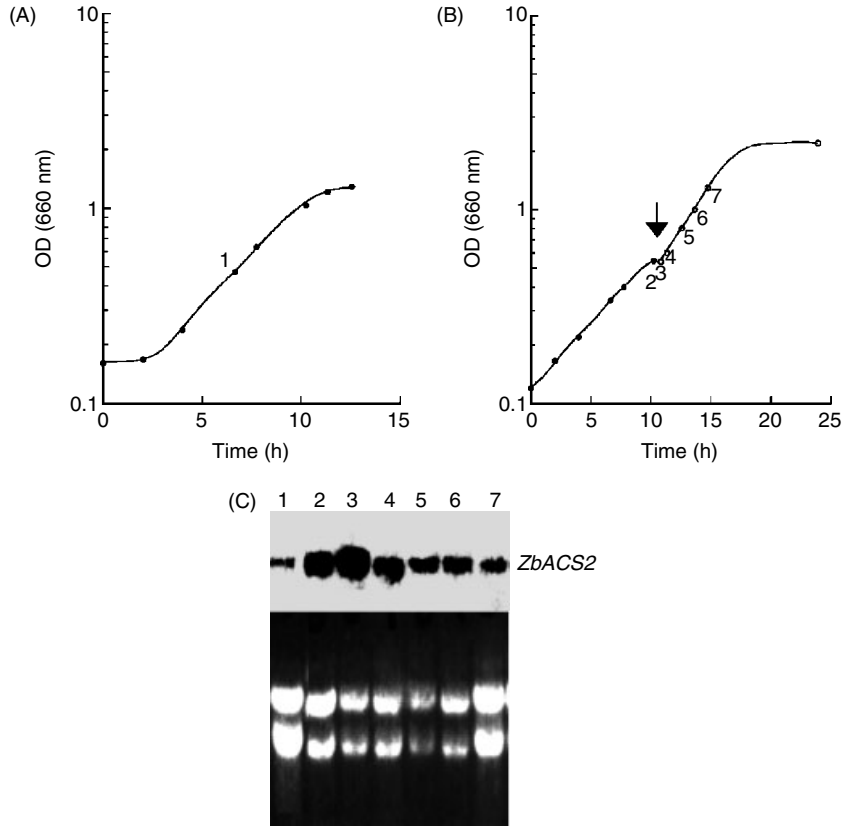

Figure 3. Growth and transcription of *ZbACS2* in *Zygosaccharomyces bailii* ISA 1307. (A) Growth on 2% w/v glucose. (B) Growth on 0.5% acetate. At the time indicated by the arrow, 125 mM glucose was added. (C) Northern blot hybridizations with *ZbACS2* mRNA. Ethidium bromide staining shows the abundance of RNA loaded in the gel. Lanes 1–7 correspond to the samples shown in (A) and (B)

lag time was observed for concentrations of acetic acid (0.1–0.35%) (Figure 4). For acid concentrations higher than 0.35%, the *ZbACS2*-expressing cells displayed a significant higher lag time (about 5 h). Loss of viability of glucose-grown cells of *S. cerevisiae* harboring the vector pACS139 or pRS316 (control cells), induced by acetic acid was measured at pH 3.0 and 26 °C. Typically, a deathless initial period was followed by a period of exponential death (Pinto *et al.*, 1989). The specific death rates for acetic acid were dependent on the acid concentration in the medium (0.5–2.0%, v/v); however, no differences were observed in both strains. In conclusion, our results show little effect of the orthologous expression of *ZbACS2* in *S. cerevisiae* with respect to resistance to acetic acid. The effect on lag phase, for concentrations lower than 0.35% acetic acid, may be attributed to the conversion of acetic acid into acetyl-CoA, for biosynthetic and/or energetic purposes. In addition, for higher concentrations of acetic acid the increase of the lag phase, in *ZbACS2*-expressing cells, can be related with ATP depletion. The higher intracellular concentration of acetic acid may lead to a rapid conversion of acetate into acetyl-CoA that cannot be further metabolized due to the overflow of respiration or its transport to mitochondria (de Jong-Gubbels *et al.*, 1998). The confirmation of these hypotheses needs further experiments, such as measurements of ATP levels and/or the study of acetate-carbon flow by ^{13}C-NMR experiments.

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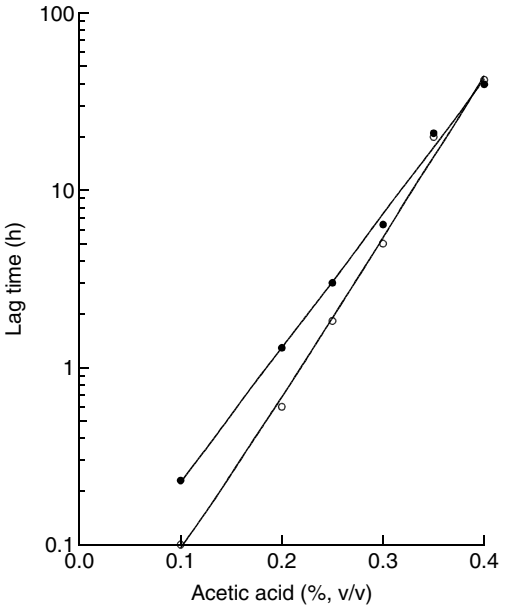

Figure 4. The effect of acetic acid (pH 3.0) on the lag time of *Saccharomyces cerevisiae* BY 4742 harbouring the plasmid pACS139 (open circles) or pRS316 (closed circles). Values obtained from three independent experiments did not differ more than 3%

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