Function and regulation of acetyl-coenzyme A synthetase in the yeast

Saccharomyces cerevisiae
Function and regulation of acetyl-coenzyme A synthetase in the yeast
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Proefschrift

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door

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doctorandus biologie

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Chapter 1

Introduction
The history of bakers' yeast, *Saccharomyces cerevisiae*, started with the first recording of bread by the ancient Egyptians, at the beginning of the third millennium BC. Until the production of pressed bakers' yeast in 1781, bakers were dependent on the brewing industry for their yeasts (Chen and Chiger, 1985). Eventually, a bakers' yeast industry independent of alcoholic beverage production was developed. Inspired by its industrial use, *S. cerevisiae* has been the subject of many physiological, biochemical and genetic studies. With the introduction of recombinant DNA techniques, the knowledge of the organism increased even more rapidly. Recently, the sequence of the entire *S. cerevisiae* genome has been completed (Bradbury, 1996). However, many questions about *S. cerevisiae* are still to be answered (Oliver, 1996). Among these are fundamental questions about the regulation of primary metabolism, i.e. glycolysis and fermentation.

**Glucose metabolism**

Glucose is by far the best studied carbon source used for growth by *S. cerevisiae*. When growing on glucose the yeast represses the synthesis of enzymes used for growth on less favoured substrates as maltose or ethanol (Wills, 1990). Glucose is degraded via the glycolysis into pyruvate (Fig. 1) with the formation of two molecules of ATP. Pyruvate is at the branchpoint of respiratory and fermentative metabolism. Under anaerobic conditions pyruvate is converted into ethanol and carbon dioxide. Degradation of sugar to ethanol is called alcoholic fermentation. Under aerobic conditions pyruvate can be completely oxidized to carbon dioxide and water via the citric acid cycle. Depending on the coupling between respiratory chain and oxidative phosphorylation this yields an additional 14 to 36 molecules of ATP per molecule of glucose. From an energetic point of view, respiration is favourable above fermentation, since the former process generates more ATP. Fermentation, however, is a very fast process in *S. cerevisiae*, whereas respiration occurs relatively slowly (Lagunas, 1979). Therefore, in the presence of excess glucose, under fully aerobic conditions, *S. cerevisiae* ferments part of the glucose to ethanol. This aerobic alcohol production, or mixed respiro-fermentative metabolism, is called the Crabtree effect (Crabtree, 1929; De Deken, 1966). Until relatively recently, several authors held the view that this was due to a limited respiratory capacity of the cells, which is not sufficient for competing effectively with fermentation for NADH (Fiechter et al., 1981; Rieger et al., 1983; Käppeli, 1986). However, this point of view has changed over the years as will be discussed below.

**The Crabtree effect**

Industrial bakers' yeast production needs the highest possible cell yield. Nowadays, fed-batch is used as the main fermentation process to produce bakers' yeast (Reed, 1982; Barford, 1987). To prevent alcoholic fermentation, which sets in at high growth rates (Rieger et al., 1983; Postma et al., 1989), the growth rate is kept below 0.2 h⁻¹ by controlling the sugar supply rate. However, it is not possible to entirely prevent ethanol formation. This is due to imperfect mixing in the huge industrial fermenters, which leads to sugar and oxygen concentration gradients. Low oxygen concentrations will limit the respiration rate and lead to
alcoholic fermentation (Sweere et al., 1988a, b). In addition, a sudden increase in the local sugar concentration, will trigger alcoholic fermentation within one minute. This transient effect is called the short-term Crabtree effect (Petrik et al., 1983; Van Urk, 1989). When cells are adapted to the fermentative state, as under steady-state conditions at high growth rates, the occurrence of alcoholic fermentation is called the long-term Crabtree-effect (Fiechter et al., 1981; Postma et al., 1989).

Using glucose-limited chemostat cultures of *S. cerevisiae*, Postma et al. (1989) have studied the appearance of the long-term Crabtree effect at increasing dilution rates. No fermentation products were observed at dilution rates (D) up to 0.30 h⁻¹ (Fig. 2B). Above D = 0.30 h⁻¹, however, pyruvate and acetate appeared in the culture vessel (Fig. 2B), the qO₂ and qCO₂ increased disproportionally and the biomass yield dropped. This is caused by an ‘overflow’ in metabolism at two levels. First, due to the large differences in K_M for pyruvate of pyruvate dehydrogenase or the mitochondrial pyruvate transporter (0.40 mM; Kresze and Ronfit, 1981) and pyruvate decarboxylase (6 mM; Postma et al., 1989), the affinity constants of these enzymes are decisive parameters for metabolic fluxes (Holzer and Goedde, 1957). Above D = 0.30 h⁻¹ the carbon flux becomes too high for complete oxidation and the internal pyruvate concentration increases to a level sufficient for pyruvate decarboxylase. In addition this enzyme becomes activated by pyruvate (Hübner et al., 1978). Therefore, the flux will be redirected via pyruvate decarboxylase, in stead of via pyruvate dehydrogenase. Together with
acetaldehyde dehydrogenase and acetyl-coenzyme A synthetase, pyruvate decarboxylase forms the so-called Pdh bypass, which can produce acetyl-coenzyme A (Fig. 1; Holzer and Goedde, 1957; Pronk et al., 1994b). At higher dilution rates acetyl-coenzyme A synthetase activity becomes limiting (Fig. 2A), and acetate will be excreted (Fig. 2B). The uncoupling effects of acetate then will increase the qO₂ and decrease the biomass yield (Postma et al., 1989). At a further increase in dilution rate, up to D = 0.38 h⁻¹, the acetaldehyde dehydrogenase activity also decreases to a level below the pyruvate decarboxylase activity (Fig. 2A). At this point

Figure 2. The long-term Crabtree effect. Both graphs are shown as a function of the dilution rate in glucose-limited cultures of S. cerevisiae. A. In vitro activity (in U·mg protein⁻¹) of Pdc (— — — ), Adh (—— ), Ald (— — ) and Acs (——— ) activity. B. Production of pyruvate (-----), acetaldehyde, (— — ), acetate (— — — ) and ethanol (—— ) in mM. Adapted from Postma et al. (1989).
acetaldehyde builds up and becomes available for alcohol dehydrogenase, which has a hundred-fold lower affinity for acetaldehyde than acetaldehyde dehydrogenase (Postma et al., 1989), and the yeasts will produce ethanol (Fig. 2B). Postma et al. (1989) have argued that the maximal \( qO_2 \) of \( S. \) cerevisiae should allow for full respiratory growth up to \( D = 0.48 \) h\(^{-1}\), but the acetic acid, produced at \( D = 0.30 \) h\(^{-1}\), triggered enhanced respiration and thereby plays a key role in the onset of alcoholic fermentation.

Immediate alcoholic fermentation upon addition of a glucose pulse to a glucose-limited continuous culture, the short-term Crabtree effect (Fig. 3), has been studied by Van Urk (1989). He has compared a group of Crabtree-positive and Crabtree-negative yeasts, which revealed several differences between the two groups (Table 1). Basically, the kinetics of glucose uptake, the rate of glycolysis and glycogen formation, the levels of the enzymes around pyruvate differ and may regulate the appearance of the Crabtree effect. In Crabtree-positive yeasts as \( S. \) cerevisiae the flux from glucose to pyruvate is fast, resulting in a high internal concentration of pyruvate, redirecting the carbon flux to the Pdh bypass. The enzyme levels of the Pdh bypass then determine the production of ethanol. Especially the ratio between pyruvate decarboxylase and acetyl-coenzyme A synthase might be important for the appearance of the Crabtree effect (Table 1). In Crabtree-negative yeasts this ratio is in favour of acetyl-coenzyme A synthetase and any flux through the Pdh bypass will be completely converted into acetyl-coenzyme A, whereas in Crabtree-positive yeasts the capacity of this enzyme seems to be insufficient, which will lead to acetate, acetaldehyde and ethanol formation (Van Urk, 1989).

![Graph](image)

**Figure 3. The short-term Crabtree effect.** A pulse of 50 mM glucose was added to a glucose-limited culture of \( S. \) cerevisiae. Concentrations of glucose (-----), ethanol (-----) and acetate (-----) are shown in mM. Adapted from Van Urk et al. (1988).
Table 1. Comparison of Crabtree-positive and Crabtree-negative yeasts, at a steady state of and during a glucose pulse to a glucose-limited continuous culture. The short-term Crabtree effect. Enzyme activities are shown in U·mg protein\(^{-1}\) (Adapted from Van Urk (1989); = only determined in *S. cerevisiae* and *Candida utilis*).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Crabtree-positive yeasts</th>
<th>Crabtree-negative yeasts</th>
</tr>
</thead>
<tbody>
<tr>
<td>At steady state ((D = 0.10 \text{ h}^{-1}))</td>
<td></td>
<td></td>
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<tr>
<td>Glucose transport</td>
<td>passive</td>
<td>active</td>
</tr>
<tr>
<td>Glucose-6-P-dehydrogenase</td>
<td>0.1</td>
<td>1.1</td>
</tr>
<tr>
<td>Phosphofructokinase</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>Fructosebisphosphatase</td>
<td>0.01</td>
<td>0.06</td>
</tr>
<tr>
<td>Pyruvate dehydrogenase (Pdh)(^{*})</td>
<td>0.09</td>
<td>0.11</td>
</tr>
<tr>
<td>Pyruvate decarboxylase (Pdc)</td>
<td>0.2-0.7</td>
<td>0.05-0.1</td>
</tr>
<tr>
<td>Alcohol dehydrogenase (Adh)</td>
<td>1.0-7.0</td>
<td>0.2-9.0</td>
</tr>
<tr>
<td>Acetaldehyde dehydrogenase (Ald)</td>
<td>0.1-1.0</td>
<td>0.1-0.7</td>
</tr>
<tr>
<td>Acetyl-coenzyme A synthetase (Acs)</td>
<td>0.05-0.2</td>
<td>0.1-0.5</td>
</tr>
<tr>
<td>Ratio Pdc:Acs</td>
<td>4.0</td>
<td>0.3</td>
</tr>
<tr>
<td>Mitochondrial volume(^{*})</td>
<td>9 ± 1% of cell vol.</td>
<td>12 ± 3% of cell vol.</td>
</tr>
<tr>
<td>Respiratory activity(^{\text{O}_2}(\text{min}\cdot\text{mg}^{-1}))</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NADH</td>
<td>0.36 (\mu\text{mol})</td>
<td>0.22 (\mu\text{mol})</td>
</tr>
<tr>
<td>NADPH</td>
<td>0.02 (\mu\text{mol})</td>
<td>0.20 (\mu\text{mol})</td>
</tr>
<tr>
<td>During pulse experiments</td>
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<td></td>
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<tr>
<td>(q_{\text{Glucose}})</td>
<td>high</td>
<td>low</td>
</tr>
<tr>
<td>Inactivation glucose carrier</td>
<td>slow</td>
<td>rapid</td>
</tr>
<tr>
<td>Accumulation of glycogen/trehalose</td>
<td>no</td>
<td>0-16% of dry weight</td>
</tr>
<tr>
<td>(q_{\text{Ethanol}})</td>
<td>high</td>
<td>zero or very low</td>
</tr>
<tr>
<td>(q_{\text{O}_2})</td>
<td>2.3-6.6 mmol/(g·h)(^{\text{I}})</td>
<td>5.1-7.0 mmol/(g·h)(^{\text{I}})</td>
</tr>
</tbody>
</table>

**Pyruvate metabolism**

As discussed above, the enzymes around pyruvate may be an important factor in the appearance of the Crabtree effect. Therefore, they will be discussed separately.

**Pyruvate dehydrogenase**-At the interface of glycolysis and citric acid cycle, pyruvate dehydrogenase converts pyruvate into acetyl-coenzyme A (Fig. 1). Yeast pyruvate dehydrogenase is a multi-enzyme complex consisting of five subunits, E1\(\alpha\), E1\(\beta\), E2, E3 and X (Reed and Ycman, 1987), of which E1\(\alpha\) catalyses the rate limiting step, i.e. the decarboxylation. In mammals the activity of the whole complex is mainly regulated via the E1\(\alpha\) subunit, which contains three phosphorylation sites (Reed, 1974; Sugden *et al.*, 1978, 1979; Ycman *et al.*, 1978). In *S. cerevisiae* there is only one site in E1\(\alpha\) which can be phosphorylated in vitro (Uhlinger *et al.*, 1986; James *et al.*, 1995). This is a serine residue which is essential for Pdh activity (Wenzel and Steensma, 1993b), but a kinase or phosphatase have not been isolated yet and hence, it is not clear whether regulation by
(de)phosphorylation plays a role in *S. cerevisiae*.

It was shown that almost the complete carbon flux in *S. cerevisiae* cells grown in glucose-limited chemostat cultures with a dilution rate of 0.10 h⁻¹ was metabolised via this enzyme (Pronk et al., 1994b). When *S. cerevisiae* cells were in excess glucose there was no actual decrease in PDH activity, in the amount of Elα protein, nor in the transcription of the *PDA1* gene (Wenzel et al., 1993a). However, under these conditions the main carbon flux was redirected via pyruvate decarboxylase (Pronk et al., 1994b).

*Pyruvate decarboxylase*-The first step of both the Pdh bypass and alcoholic fermentation is catalysed by pyruvate decarboxylase (Fig. 1). Three structural genes have been cloned, i.e. *PDC1*, *PDC5* and *PDC6* (Schmitt et al., 1993; Seeboth et al., 1990; Hohmann, 1991). Because the enzyme has a low affinity for pyruvate (K_M = 6 mM) and is inhibited by inorganic phosphate, only a small flux of carbon will go via pyruvate decarboxylase in a glucose-limited chemostat culture, when the pyruvate concentration is low and the inorganic phosphate concentration high (Van Urk et al., 1989; Postma et al., 1989). Upon transition to glucose excess the free inorganic phosphate is drastically reduced, *PDC1* transcription is induced and the accumulation of pyruvate activates the enzyme, so pyruvate will be decarboxylated to acetaldehyde (Hübner et al., 1978; Schmitt et al., 1983; Van Urk et al., 1989).

*Acetaldehyde dehydrogenase*-Two isoenzymes have been described, a mitochondrial NAD⁺-dependent and a cytosolic NADP⁺-dependent acetaldehyde dehydrogenase (Jacobsen and Bernofsky, 1974; Llorente and Núñez de Castro, 1977). The K_M for acetaldehyde of the cytosolic isozyme is much lower than the K_M of alcohol dehydrogenase for acetaldehyde, 0.006 mM versus 0.61 mM, respectively (Verduyn et al., 1988; Postma et al., 1989). Therefore, ethanol formation will not set in before the pyruvate decarboxylase reaction exceeds the acetaldehyde dehydrogenase reaction and the acetaldehyde concentration has been build up.

*Acetyl-coenzyme A synthetase*-The final step of the Pdh bypass is catalysed by acetyl-coenzyme A synthetase (Fig. 1). Two forms of the enzyme, an 'aerobic' and an 'anaerobic' form, which were immunologically different and showed different enzyme kinetics, were described (Satyanarayana and Klein, 1974; Satyanarayana et al., 1974). Furthermore, they were differentially expressed and localised, since the enzyme was located in the microsomal fraction during anaerobic and early aerobic growth and associated with the mitochondrial fraction during the later stages of aerobic growth (Klein and Jahnke, 1968, 1971). A gene, called *ACS1*, encoding an acetyl-coenzyme A synthase was cloned (DeVirgilio et al., 1992). Yeasts with a disruption in this gene showed normal growth on glucose and ethanol, but not on acetate (DeVirgilio et al., 1992). As discussed above, acetyl-coenzyme A synthetase seemed to play a crucial role in the appearance of both the short- and long-term Crabtree effect.

Outline of this thesis

The aim of the project was to investigate the function and regulation of acetyl-coenzyme A
synthetase in *S. cerevisiae* and more specifically, its role in the appearance of the Crabtree effect. To this end we cloned *ACS2*, the second gene encoding an acetyl-coenzyme A synthetase in *S. cerevisiae* and studied the effects of null mutations in two acetyl-coenzyme A synthetase genes, *ACS1* and *ACS2*. This work is described in chapter two of this thesis. In chapter three the kinetics of both proteins were compared with the available data on the 'aerobic' and 'anaerobic' isoforms as described in earlier literature. Carbon pulses were used to elucidate the transcriptional regulation of both genes during transient stages. The regulation of enzyme activity of both isoforms during glucose pulses to glucose-limited chemostat cultures, the short-term Crabtree effect, is the subject of chapter four. In chapter five we compared the regulation of the acetyl-coenzyme A synthetase genes during the short-term Crabtree effect with the transcriptional regulation of several genes encoding enzymes involved in acetyl-coenzyme A metabolism, i.e. those involved in fermentation, the Pdh bypass, ethanol- and acetate-consumption. Finally, these results are discussed in chapter six and a model is presented.
ACS2, a *Saccharomyces cerevisiae* gene encoding acetyl-coenzyme A synthetase, essential for growth on glucose.

Marco A. van den Berg and H. Yde Steensma
Summary

In *Saccharomyces cerevisiae* the conversion of pyruvate into acetyl-coenzyme A may proceed directly via the pyruvate dehydrogenase complex (Pdh) or indirectly via the so-called 'Pdh bypass', which requires the sequential action of pyruvate decarboxylase, acetaldehyde dehydrogenase and acetyl-coenzyme A synthetase. The relative contribution of both pathways to the rate of acetyl-coenzyme A synthesis varies in an unknown way with cultural conditions. To determine the possible role of acetyl-coenzyme A synthetase in this central part of metabolism, we have analysed the genes encoding this enzyme. Disruption of the recently cloned *ACS1* gene (DeVirgilio *et al.*, 1992) did not cause an apparent phenotype, except for a prolonged lag-phase during growth on glucose or C2-compounds like acetate and ethanol. In fact, a product from a different gene is responsible for acetyl-coenzyme A formation in the *acs1* mutant. We cloned a second gene encoding acetyl-coenzyme A synthetase, which we called *ACS2*. Inactivation of this gene caused an inability to grow on media containing glucose, but not on media with acetate or ethanol as the sole carbon source. This indicates that *ACS2* is essential for growth on glucose in batch cultures. The *acs1 acs2* double mutant was not viable. The role of both genes in glucose and acetate or ethanol metabolism is discussed.
Introduction

The yeast *Saccharomyces cerevisiae* is capable of producing ethanol while growing on glucose under fully aerobic circumstances. This phenomenon, generally known as the Crabtree effect, has been attributed to a limited respiratory capacity (Petrik et al., 1983). For a strictly respiratory metabolism, *S. cerevisiae* needs to coordinate the activities of glycolysis and citric acid cycle tightly. At this point in metabolism, pyruvate is the key intermediate. It can be converted directly to acetyl-coenzyme A, the fuel of the citric acid cycle, by the pyruvate dehydrogenase complex (Pdh), or indirectly via the so-called Pdh bypass. This bypass comprises three enzymes: pyruvate decarboxylase, Pdc; acetaldehyde dehydrogenase, Ald; acetyl-coenzyme A synthetase, Acs (Holzer and Goedde, 1957). Studies with aerobic, glucose-limited chemostat cultures of wild-type and isogenic pyruvate dehydrogenase-negative *S. cerevisiae* strains have shown that at low glycolytic fluxes, when metabolism is strictly respiratory, the pyruvate dehydrogenase complex is predominantly responsible for conversion of pyruvate into acetyl-coenzyme A (Pronk et al., 1994b). At higher glycolytic fluxes, caused by increasing the concentration of glucose in the medium or increasing the growth rate, the intracellular pyruvate concentration exceeds the low $K_{p}$ of the pyruvate dehydrogenase complex. The high $K_{p}$ of pyruvate decarboxylase (Holzer and Goedde, 1957) than results in an increased involvement of the Pdh bypass in pyruvate metabolism. However, Postma et al. (1989) observed that the capacity of pyruvate decarboxylase exceeds that of acetyl-coenzyme A synthetase. As a result acetate, acetaldehyde and ethanol are produced. This has led to the proposition that acetyl-coenzyme A synthetase may be an important factor in the appearance of the Crabtree effect.

Acetyl-coenzyme A synthetase is not exclusively involved in the Pdh bypass. It is also essential for growth on C2-compounds, like acetate or ethanol. The acetyl-coenzyme A formed from these carbon sources can be metabolised via the glyoxylate and citric acid cycles. It is not clear however, whether the same enzyme is involved in the Pdh bypass and growth on C2-compounds.

Satyanarayana and Klein (1974) described two forms of acetyl-coenzyme A synthetase: an 'aerobic' and an 'anaerobic' form. The enzymes differ in kinetics, appear under different physiological conditions, are located in different subcellular compartments and are immunologically different (Satyanarayana and Klein 1974; Klein and Jahnke, 1968, 1971, 1979; Satyanarayana et al., 1974). Despite these differences it was not clear whether the two proteins were encoded by one or two genes.

Recently, a *S. cerevisiae* gene encoding acetyl-coenzyme A synthetase was cloned by DeVirgilio et al. (1992). This gene, *ACSI*, showed 60-80% homology with acetyl-coenzyme A synthetase genes from other organisms. The open reading frame of *ACSI* has two ATG's in frame, separated by only 23 codons. Although only the second ATG is in agreement with the consensus sequence for translational start, it is still conceivable that two proteins with different N-termini are produced. Different start sites may yield enzymes with two subcellular locations (Wu and Tzagoloff, 1987; Slusher et al., 1991; Harington et al., 1994). A LEU2 insertion in *ACSI* resulted in an unexpected phenotype. The mutants were not able to grow on acetate and lacked the increase of ACS activity at the end of the exponential phase that is seen in wild type strains, but grew on ethanol (DeVirgilio et al., 1992). However, it could not be excluded that some active product was still produced. In the present report we describe the complete inactivation of the *ACSI* gene. The mutant still grows on all carbon sources tested.
A second acetyl-coenzyme A synthetase, of which the kinetics resemble that of the 'anaerobic' form described by Satyanarayana and Klein (1974), is demonstrated to be active in the mutant (Kortland et al., 1994). We also describe the cloning and characterisation of the gene responsible for the active protein in the *acs1* mutant. Disruption of this gene, which we called *ACS2*, resulted in mutants which are unable to grow on glucose, but do grow on ethanol. A model explaining the role of the two Acs genes in the metabolism of *S. cerevisiae* is discussed.

**Materials and methods**

**Strains**—The *S. cerevisiae* strains used in this study are listed in Table 1. T2-3D was chosen as host for the construction of Acs mutants for detailed physiological studies with chemostat cultures. Strains for such studies need to be isogenic with the wild type, prototrophic and homozygous diploids. Strain M5 was used as a backup strain since it is easy to transform and heterothallic.

The *Escherichia coli* strains XL1 blue (Bullock et al., 1987) and GM2 (Marinus, 1973) were used for plasmid amplifications.

**Plasmids**—The plasmids used in this study are summarised in Table 2. We recloned the 3.7 kb EcoRI-KpnI fragment of YCplac111::*ACS1* (kindly given to us by G. Barth and C. DeVirgilio), containing the *ACS1* gene, into pRS314 (Sikorski and Hieter, 1989). In the resulting plasmid, pRUL72, 90% of the *ACS1* open reading frame was replaced by the *APT1* expression cassette from pBEJ24 (Hadfield et al., 1990).

*ACS2* was disrupted *in vitro* after recloning the *XbaI-SalI* fragment of the YCp50 library (Rose et al., 1987) clone pRUL76 (this study) in YEp112 (Gietz and Sugino, 1988).

### Table 1. *Saccharomyces cerevisiae* strains.

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<th>Strain</th>
<th>Relevant genotype</th>
<th>Reference/Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>T2-3D</td>
<td>HO/HO ACS1/ACS1 ACS2/ACS2</td>
<td>Wenzel et al. (1992b)</td>
</tr>
<tr>
<td>GG620</td>
<td>HO/HO ACS1::APT1 ACS2/ACS2</td>
<td>This study</td>
</tr>
<tr>
<td>GG621</td>
<td>HO/HO acs1::APT1/acs1::APT1 ACS2/ACS2</td>
<td>A spore of GG620</td>
</tr>
<tr>
<td>GG624</td>
<td>HO/HO ACS1/ACS1 ACS2/acs2::Tn5ble</td>
<td>This study</td>
</tr>
<tr>
<td>GG625</td>
<td>HO/HO ACS1/ACS1 acs2::Tn5ble/acs2::Tn5ble</td>
<td>A spore of GG624</td>
</tr>
<tr>
<td>GG626</td>
<td>HO/HO ACS1/acs1::APT1 ACS2/acs2::Tn5ble</td>
<td>GG620 X GG624</td>
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<tr>
<td>M5</td>
<td>MATα/MATα leu2,3-112/leu2,3-112 ura3-52/ura3-52</td>
<td>Schaff et al. (1989)</td>
</tr>
<tr>
<td></td>
<td>trp1-92/trp1-92 HIS4/his4 ACS1/ACS1 ACS2/ACS2</td>
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<tr>
<td>GG627</td>
<td>MATα/MATα leu2,3-112/leu2,3-112 ura3-52/ura3-52</td>
<td>This study</td>
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<tr>
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<td>trp1-92/trp1-92 HIS4/his4 ACS1/acs1::APT1 ACS2/ACS2</td>
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Table 2. Plasmids.

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<td><em>ACS</em>1 as a 3.7 kb EcoRI-KpnI fragment</td>
<td>DeVirgilio et al. (1992)</td>
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<td>pRS314 with 3.7 kb EcoRI-KpnI fragment (<em>ACS</em>1)</td>
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<td><em>AptI</em> expression cassette</td>
<td>Hadfield et al. (1990)</td>
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<td><em>acs2::AptI</em> disruption plasmid</td>
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<td>YEplac112 with 3.7 kb EcoRI-KpnI of pRUL72 (<em>ACS</em>1)</td>
<td>This study</td>
</tr>
<tr>
<td>pRUL76</td>
<td>YCp50 clone containing <em>ACS</em>2</td>
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</tr>
<tr>
<td>YEplac112</td>
<td>multicopy plasmid</td>
<td>Gietz and Sugino (1988)</td>
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<td>pRUL77</td>
<td>YEplac112 with 5.5 kb <em>XbaI-Sall</em> of pRUL76 (<em>ACS</em>2)</td>
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<td>pUC21 with <em>Tn5ble</em> marker</td>
<td>A.L. van der Zanden</td>
</tr>
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<td>pRUL78</td>
<td><em>acs2::Tn5ble</em> disruption plasmid</td>
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</tr>
<tr>
<td>pRUL100</td>
<td>YEplac112 with 5.2 kb <em>CiaI-Sall</em> of pRUL76 (<em>ACS</em>2)</td>
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<td>YCplac33</td>
<td>single-copy plasmid</td>
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yielding pRUL77. The promoter and the complete open reading frame of *ACS*2 were replaced by the *Tn5ble* expression cassette, resulting in the final plasmid pRUL78. The *Tn5ble* cassette was first cloned from pUT332 (Gatignol et al., 1987; kindly provided by Cayla, Toulouse) to pUC21 (Vieira and Messing, 1991).

To study overexpression, *ACS*1 and *ACS*2 were cloned, using the fragments described above, in the multicopy plasmid YEplac112, yielding pRUL74 and pRUL77, respectively. pRUL100 is a derivative of pRUL77. It was constructed with the aid of a *XbaI-ClaI* linker (oligonucleotides used: 5'-CTAGGAAAGGAAAGGAAG-3' and 5'-CGCTTTCCCTTCC-TTTC-3') and thereby removing 294 nucleotides between the *XbaI* and *ClaI* sites. This plasmid was used to complement the *acs2* mutation.

*Media-S. cerevisiae* was grown either in complex medium (YEP, Sherman et al., 1983) or in minimal yeast medium (MY, Zonneveld, 1986). Glucose, raffinose, ethanol or acetate were used as carbon sources in final concentration of 2%, 0.67%, 3% and 0.5% respectively. For solid media 1.5% Difco agar was added. Final concentrations of supplements when required were: leucine (30 µg·mL⁻¹), tryptophan (20 µg·mL⁻¹), uracil (20 µg·mL⁻¹), histidine (20 µg·mL⁻¹), phleomycin (20 µg·mL⁻¹) and G418 (150 µg·mL⁻¹). Sporulation was induced by incubating cells two to seven days on sporulation medium (2% sodium acetate, 0.05% glucose, 0.8% Difco yeast extract and 2% Difco agar).

*E. coli* was either grown in Luria-Bertani medium (LB) or Terrific-Broth (TB; Sambrook et al., 1989). For solid media 1.5% Difco agar was added. Final concentrations of supplements when required were: ampicillin (60 µg·mL⁻¹), 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal; 20 µg·mL⁻¹) and isopropyl-β-D-thiogalactopyranoside (IPTG; 10 µg·mL⁻¹).

Recombinant DNA techniques—Standard protocols were followed for plasmid isolations, restriction enzyme analyses, PCR amplifications, ligations, gel electrophoresis and hybridisations (Sambrook et al., 1989). Yeast chromosomal DNA was isolated according to
Holm et al. (1986). For pulsed-field gel electrophoresis in a contour clamped homogeneous electric field apparatus (CHEF), the chromosomal DNA was prepared as described by Teunissen et al. (1993). Southern blotting was done by vacuum blotting onto nylon filters. DNA probes were labelled with \([\alpha^32P]dCTP\) according to the instructions of the Oligolabelling Kit (Pharmacia, Sollentuna).

ACS2 was cloned from the YCp50 library (average insert 10 kb) as follows. Five thousand colonies were grown overnight on nitrocellulose filters (Schleicher and Schuell, Dassel) on LB plates. The filters were treated as described by Woods (1984) and hybridised overnight at 48°C with a PCR amplified fragment of ACS1 (oligonucleotides used: 5'-TATGGGCCGTTCCGCAAGGGCG-3' and 5'-CGACAACACAGCACACTCCG-3'), followed by two washes of 20 minutes with 5x SSC (20x SSC is 3.0 M NaCl, 0.3 M sodium citrate, pH 7.0) plus 0.5% SDS at 48°C, to reduce a-specific hybridisation. Positive colonies were isolated and colony-purified. Plasmids from these clones were isolated and analysed.

Sequencing-Parts of the ACS2 clone were subcloned in pBluescript II KS+ (Stratagene Cloning Systems, La Jolla) and amplified in E. coli in 200 ml selective TB medium. Plasmid DNA was isolated using the Nucleobond AX kit (Macherey-Nagel, Düren). DNA sequences were determined on the A.L.F. DNA sequencer (Pharmacia, Roosendaal) using either the standard primers or oligos purchased from Pharmacia.

Sequence data were analysed using either the GeneSkipper software (EMBL, Heidelberg) or the GCG software (Devereux et al., 1984). The GenEMBL (release 86.0) and SwissProt (release 30.0) databases were screened to identify DNA and protein homologies, respectively.

Transformation-S. cerevisiae and E. coli were transformed by electroporation with a Bio-Rad Gene Pulser. Preparation of the yeast cells was according to Becker and Guarente (1991). E. coli competent cells were prepared as described by Dower et al. (1988). Selection of the phleomycin or G418 resistant yeast transformants was done as described by Wenzel et al. (1992a). However, sorbitol was not added to the selective plates.

Stability tests-Transformants were grown for more than ten generations in non-selective medium and plated onto the same medium. When the colonies were 4 mm in size, they were replica-plated onto selective and non-selective media. Stability was determined as the ratio between the number of colonies growing on selective and non-selective media.

Growth rates-Growth rates were determined as generation times in liquid medium. The cells were pregrown in mineral medium to late exponential phase and diluted to an \(A_{600}\) of 0.1 in 200 ml medium in 1 l flasks, and shaken with 250 rpm at 30°C. Growth was monitored by measuring the cell-density at hourly intervals. The exponential part of the growth curve was used to determine generation times and growth rates.

Preparation of cell-free extracts-Cell-free extracts were prepared from 50 ml cultures which were harvested at an \(A_{600}\) of 0.8. The cells were washed twice with ice-cold water and once with 100 mM potassium phosphate buffer (pH 7.5). Finally, the pellet was resuspended in 0.5 ml of phosphate buffer and a 0.25 volume of glassbeads was added (diameter 45 μm). Cells were disrupted by vortexing 10 minutes at 4°C. Cell debris was spun down for 30 minutes at 20,000x g. The supernatants were immediately used to assay protein concentrations and Acs
activities.

Protein determinations-The protein concentrations in cell-free extracts were estimated by the Bradford method (1976). Samples were adjusted to 100 µl by adding water and subsequently mixed with 1 ml of Bradford Reagent. The assay was calibrated using bovine serum albumin.

ACS assay-The specific activity of ACS was determined at 30°C as described by Postma et al. (1989). The reactions were done with 40 µg of protein and started by the addition of 100 mM potassium acetate. One unit (U) is defined as the amount of enzyme catalysing the conversion of 1 µmol of acetate per minute. Specific activities are expressed as U/(mg protein)-1.

RNA techniques-Total RNA was isolated according to Schmitt et al. (1990). Concentration and purity of the RNA were checked at 260 and 280 nm. From each isolate 15 µg were separated by electrophoresis in formaldehyde/1xTris-Acetate-EDTA agarose gels and blotted onto nylon membranes (essentially as described by Krockzec and Siebert, 1990). All probes used were derived from PCR-amplified fragments. The oligonucleotides used were : ACS1 (see Recombinant DNA techniques), ACS2 (5'-ACGCTTAGCTGTGTTGCCTTC-3' and 5'-CGAATGACGTATGCTGC-3') and PDA1 (5'-AGATCAATCAAATGCAT-3' and 5'-GGTACATGTTCGTACC-3'). We used PDA1 as an internal control, because it is more stable than the often used ACT1 (Wenzel et al., 1995). Hybridisation and detection were essentially the same as with DNA.

All solutions were made RNAse free by autoclaving for 40 minutes at 120°C. The gel trays were washed overnight with a 10% Glorix solution and were flushed immediately before use with RNAse free water.

Dissection-Sporulated cells were treated for 15 minutes with lyticase (5 mg/ml). The asci were dissected using the Singer MSM system.

Random spore analysis-Sporulated cells were resuspended in 1 ml water to an A600 of 0.8 and heated for 15 minutes at 56°C. This will kill all the vegetative cells and 99% of the spores. Colonies surviving the treatment will therefore be derived from a single spore.

Results

Disruption of the ACS1 gene-In order to completely inactivate the ACS1 gene, strains T2-3D and M5 were transformed with the linear 4.5 kb EcoRI-KpnI fragment of pRUL73 (Fig. 1A), yielding GG620 and GG627, respectively. Thereby 90% of the ACS1 open reading frame was replaced by the APT1 marker, as described in Materials and methods. Integration was confirmed with stability tests and by dissection of eighteen asci from both strains. In all tetrads G418 resistance segregated 2:2. Southern analysis of genomic DNA digested with various restriction enzymes (Fig. 1B and 1C) and of pulsed-field gel electrophoresis (CHEF) separated chromosomes (data not shown) confirmed proper integration at chromosome I. ACS1 was previously mapped at chromosome I (Steensma et al., 1993).

Northern analysis showed that disruption of ACS1 completely abolished transcription.
Total RNA was isolated from T2-3D and GG621 (acs1::APT1/acs1::APT1) cultures grown in YEPE to late exponential phase. ACS1 mRNA was only detected in ACS1/ACS1 strains (Fig. 6, lanes 1 and 2). Upon reintroduction of an intact copy of ACS1 either in single- or in multicopy vectors to the acs1 mutants the presence of ACS1 mRNA was restored (data not shown). Similar results were obtained with GG627 (data not shown).

Figure 1. Disruption of ACS1 by APT1. (A) ACS1 was disrupted in vitro by the APT1 expression cassette. In the resulting plasmid 90% of the gene is replaced. Yeast was transformed with the linear 4.5 kb EcoRI-KpnI acs1::APT1 fragment. (B) Expected fragment sizes. ACS1 is located at a 5.4 kb EcoRI-BglII fragment of chromosome I. Disruption by APT1 introduces a XhoI site, resulting in an EcoRI-XhoI and a XhoI-BglII fragment, both of 2.95 kb. (C) Southern blot of acs1::APT1 integrants. Chromosomal DNA was isolated and approximately 5 µg was digested with XhoI, EcoRI and BglII. The DNA was separated on a 0.7% agarose gel, blotted onto a nylon membrane and hybridised to the [α-32P]dCTP labelled 3.7 kb EcoRI-KpnI ACS1 fragment. Relevant genotypes are marked above each lane. Lane 1, T2-3D; lane 2, GG620-11A (=GG621); lane 3, GG620-11B; lane 4, GG620-11C; lane 5, GG620-11D.
Characterisation of acs1 mutants-To determine whether the disruption of ACSI disturbed yeast metabolism, we first analysed growth in batch cultures. The acs1 mutants showed an extended lag-phase in minimal medium with either glucose, ethanol or acetate as carbon source. This lag-phase varied between 10 and 40 hours. Once the yeasts entered exponential phase they showed the same maximum growth rate as the wild types (data not shown). Additionally, Acs activity determined in cell-free extracts prepared from batch cultures was equal in all strains (0.2 U·mg protein⁻¹). The possibility of contaminations with wild-type yeast was excluded, since the cells were checked for G418 resistance.

Comparison of cell-free extracts from acs1 mutant and wild-type revealed that the Acs activities in both strains exhibited different Kₘ values for acetate and ATP (Kortland et al., 1994). These results support the observations of Satyanarayana and Klein (1974) that at least two proteins in S. cerevisiae are capable of activating acetate to acetyl-coenzyme A. Moreover, the possibility of both proteins being transcribed from one gene could now be discarded. Hence, a second Acs gene must be present.

Cloning and sequence analysis of an ACSI homologue-In order to clone the gene responsible for Acs activity in the mutant, a YCp5 genomic library, with an average insert size of 10 kb (Rose et al., 1987), was screened under low stringency conditions with a PCR-amplified fragment of ACSI (see Materials and methods). Of the six clones isolated during this screen, two were identified as ACSI. The other four showed an approximately eightfold less intense signal with the probe as compared to the ACSI clones. All four had an identical insert of 7.4 kb. One of these, pRUL76, was selected for further analysis. First, it was shown by Southern blot analysis of pulse-field gel electrophoresis (CHEF) separated chromosomal DNA molecules that its yeast DNA insert originated from chromosome XII, using rDNA and the TRP1 gene as markers to discriminate between chromosomes XII and IV (data not shown). Second, several parts of the insert were subcloned in pBlueScript KS+ and sequenced. In total, 3844 nucleotides of the original insert were sequenced (Fig. 2).

An open reading frame (ORF) of 2049 nucleotides was found with 61.2% similarity to ACSI (2139 nucleotides). This ORF, named Acs2, encodes a putative protein of 683 amino acids with a calculated molecular mass of 75.4 kDa (Acs1p has a putative molecular mass of 79 kDa). At the protein level there is 73.6% similarity and 57.0% identity between the two putative Acs proteins (Fig. 3). The similarity is not conserved to specific parts of the proteins. High homologies were also found with genes encoding acetyl-coenzyme A synthetases from several organisms, including Aspergillus nidulans (62.4%; Connerton et al., 1990), Penicillium chrysogenum (61.2%; Martinez-Blanco et al., 1993), Neurospora crassa (59.8%; Connerton et al., 1990), Escherichia coli (48.7%; Blattner et al., 1993), Alcaligenes eutrophus (45.4%; Priefert and Steinbuchel, 1992) and Methanothrix soehngenii (44.6%; Eggen et al., 1991).

The codon bias index (CBI) calculated according to Sharp and Cowe (1991) is 0.451; indicating a moderately expressed gene (CBI of ACSI is 0.294). The gene was transcribed in both wild type S. cerevisiae and acs1 mutants (Fig. 6, lanes 1 and 3, where total RNA isolated from the wild type, acs1 and acs2 mutants is hybridised to the ACSI probe).

The putative promoter region of this gene contains several possible TATA elements, of which the first starting at -404 (TATAAA) is exactly according to the consensus (Struhl, 1987). The others are closer to the ATG, they begin at: -368 (TATA), -126 (TATATATA), -104 (TATATATATATATA) and -47 (TATA), respectively. Furthermore, at position -248
Figure 2. Sequence of the ACS2 gene. Nucleotide and derived amino acid sequence of the ACS2 gene. Possible initiator sequences are in italic and underlined. A putative AAS3 element in the promoter region of ACS2 is in bold and underlined. The trRNA\(^{Glu}\) is double underlined. The small unworn ORF is in bold.

an AAS3 box is present (Verd, 1990), which is involved in activation of several amino acid biosynthetic genes.

Upstream of the ACS2 gene, from positions -739 to -406, a small ORF of 330 nucleotides is present (Figs. 2 and 4). This ORF shows no homology with any of the sequences in the data bases. Furthermore, no putative TATA box is detectable upstream of this ORF. The CBI of this ORF is 0.108. So far, it is not known if it has a functional role in yeast.
### Figure 3. Alignment of Acs1p and Acs2p. The deduced amino acid sequences of ACS1 and ACS2 have been aligned using the GCG software (Deverue et al., 1982). Gaps have been inserted to optimise the alignment. The consensus is shown below the alignment. Identical amino acids are shown in capitals, conserved changes are shown with a "=".

<table>
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<tr>
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<th>Acs1p</th>
<th>Acs2p</th>
<th>Consensus</th>
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<td>1</td>
<td>MSPS</td>
<td>AVGSS</td>
<td>ERQSEEI</td>
</tr>
<tr>
<td>2</td>
<td>K3</td>
<td>I5</td>
<td>K7</td>
</tr>
<tr>
<td>51</td>
<td>QRP</td>
<td>IS</td>
<td>D6</td>
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<tr>
<td>23</td>
<td>HTY</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>64</td>
<td>S =</td>
<td>Q =</td>
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**Consensus**

- Acs1p: MSPS
- Acs2p: AVGSS
- Consensus: ERQSEEI

**Consensus**

- Acs1p: K3
- Acs2p: I5
- Consensus: K7

**Consensus**

- Acs1p: QRP
- Acs2p: IS
- Consensus: D6

**Consensus**

- Acs1p: HTY
- Acs2p: ...
- Consensus: ...

**Consensus**

- Acs1p: S
- Acs2p: Q
- Consensus: S1 = P

**Consensus**

- Acs1p: AM
- Acs2p: NV
- Consensus: CDR

**Consensus**

- Acs1p: PS
- Acs2p: FK
- Consensus: FDP

**Consensus**

- Acs1p: W
- Acs2p: N
- Consensus: A

**Consensus**

- Acs1p: IYP
- Acs2p: D
- Consensus: E7

**Consensus**

- Acs1p: NIL
- Acs2p: YDA
- Consensus: H7

**Consensus**

- Acs1p: EAI
- Acs2p: I8
- Consensus: A9

**Consensus**

- Acs1p: EAV
- Acs2p: I9
- Consensus: A9

**Consensus**

- Acs1p: KI
- Acs2p: E10
- Consensus: N10

**Consensus**

- Acs1p: K
- Acs2p: I11
- Consensus: N10

**Consensus**

- Acs1p: K
- Acs2p: I12
- Consensus: N10

**Consensus**

- Acs1p: R
- Acs2p: I13
- Consensus: N10

**Consensus**

- Acs1p: H
- Acs2p: E15
- Consensus: T10

**Consensus**

- Acs1p: I
- Acs2p: D16
- Consensus: T10

**Consensus**

- Acs1p: I
- Acs2p: D17
- Consensus: T10

**Consensus**

- Acs1p: V
- Acs2p: D18
- Consensus: T10

**Consensus**

- Acs1p: N
- Acs2p: E19
- Consensus: T10

**Consensus**

- Acs1p: D
- Acs2p: E20
- Consensus: T10

**Consensus**

- Acs1p: I
- Acs2p: D21
- Consensus: T10

**Consensus**

- Acs1p: V
- Acs2p: D22
- Consensus: T10

**Consensus**

- Acs1p: I
- Acs2p: D23
- Consensus: T10

**Consensus**

- Acs1p: V
- Acs2p: D24
- Consensus: T10

**Consensus**

- Acs1p: V
- Acs2p: D25
- Consensus: T10

**Consensus**

- Acs1p: V
- Acs2p: D26
- Consensus: T10

**Consensus**

- Acs1p: V
- Acs2p: D27
- Consensus: T10

**Consensus**

- Acs1p: V
- Acs2p: I
- Consensus: V

**Consensus**

- Acs1p: V
- Acs2p: I
- Consensus: V
Further upstream (positions -1076 to -1146) a glutamine tRNA gene was found in the complementary strand of the sequence. This was completely identical to the sequence of tRNA$_{\text{Gln}}$ published by Tschumper and Carbon (1982).

Overproduction of AcS-To confirm that both genes now available ($ACS1$ and $ACS2$) encode a functional AcS, we cloned both genes in a yeast multicopy vector (see Materials and methods) and introduced these into $S$. $cerevisiae$ M5. Two independent, colony-purified transformants of each transformation were inoculated in mineral medium with 3% ethanol prepared to determine AcS activity. Single-copy vectors containing the genes and empty vectors were

Figure 4. Inactivation of $ACS2$ by $Tn5ble$. (A) $ACS2$ was disrupted in vitro with the $Tn5ble$ marker. In the resulting plasmid 500 base pairs of the promoter and the complete open reading frame were replaced. The linear 2.8 kb $XbaI-PvuI$ $acs2::Tn5ble$ fragment was used to transform yeast. (B) Expected fragment sizes. $ACS2$ is partly located at a 2.8 kb $BglII$ fragment of chromosome XII. Disruption by $Tn5ble$ reduces the $BglII$ fragment to 1.9 kb. (C) Southern blot of $acs2::Tn5ble$ integrants. Chromosomal DNA was isolated and approximately 5 µg was digested with $BglII$. The DNA was separated on a 0.7% agarose gel, blotted onto a nylon membrane and hybridised to the [α-32P]dCTP labelled 2.8 kb $BglII$ $ACS2$ fragment as a probe. Relevant genotypes are marked above each lane. Lane 1, T2-3D; lane 2, GG624; lane 3, GG624-2A; lane 4, GG624-2B; lane 5, GG624-2C (=GG625); lane 6, GG624-2D.
included as controls. Yeast strains with either ACS1 or ACS2 on a multicycop vector showed an increased Acs activity of 0.66±0.16 U·mg protein⁻¹ and 4.7±0.5 U·mg protein⁻¹, respectively, as compared to yeasts with only one or no additional copy of both genes. In these strains, the activity varied over 0.22-0.35 U·mg protein⁻¹.

From these results we concluded that both the ACS1 gene cloned by DeVirgilio et al. (1992) and the ACS2 gene, encode functional ACS proteins.

Inactivation of ACS2-The ACS2 gene was disrupted by the Tn5ble expression cassette, which encodes for resistance against the antibiotic phleomycin. The whole ORF and 540 nucleotides upstream of ACS2 were replaced (Fig. 4). After confirming correct integration at the ACS2 locus with three different restriction enzymes, two strains were selected for further analysis; GG624 was derived from T2-3D and GG629 was derived from M5.

Eighteen asci of GG624 were dissected on YEPE. After three days a very clear 2:2 segregation was visible. In every tetrad two spores gave visible colonies and two spores germinated, but stopped growing after a few divisions (Fig. 5A). After transfer of these 'colonies' to plates with ethanol as the sole carbon source, they resumed growth. The same was seen after the dissection of GG629 asci. A second dissection of 36 asci on YEPE gave also a 2:2 segregation, but now all four colonies were visible. However, two remained about four times smaller than the other two (Fig. 5B). When dissection was performed on YEPE medium with 0.1% of glucose added, the pattern was the same as on YEPE (Fig. 5C). Since this suggested that ACS1 was glucose-repressible, we dissected the same strain on YEPE plus raffinose, a non-repressing carbon source. This resulted in exactly the same picture as seen during dissection on YEPE; four viable spores, however in every case two remained smaller than the other two (data not shown).

Testing the four spores of a typical tetrad from YEPE plates (Fig. 5) revealed that the phleomycin resistance segregated 2:2 (sensitive:resistant) in all tetrads. Phleomycin resistance was in every case linked to the two smallest colonies after dissection (Fig. 5D-F). Again the same pattern was seen for GG629.

Southern analysis showed that the 2:2 segregation of phleomycin resistance could be linked to the disruption of ACS2. In the heterozygous transformant (Fig. 4), Tn5ble was integrated in one of the two chromosomal copies of ACS2 and gave rise to an additional band hybridising with the probe as compared to wild type (lanes 2 and 1, respectively). The phleomycin resistant spores (lanes 5 and 6) were homozygous acs2::Tn5ble, whereas the two sensitive spores, remained homozygous ACS2 (lanes 3 and 4). Finally, Northern blot analysis of total RNA samples showed the complete inactivation of ACS2 (Fig. 6).

Complementation with ACS2-Disruption of ACS2 also removed the C-terminal part of the small unknown ORF upstream of ACS2. To prove that this ORF played no part in the drastic phenotype of the disruption, we complemented acs2 mutants with plasmids, containing ACS2 but not the small ORF. After dissection of the heterozygous GG629 transformed with plasmid pRUL77 we were able to isolate phleomycin-resistant colonies growing on glucose, thereby showing complementation. Since in pRUL77 only the first 20 bases of the small ORF were absent, plasmid pRUL100, containing only the last 26 nucleotides of the small ORF, were introduced in the haploid strain GG378 (ACS1 acs2::Tn5ble). The plasmid could complement the glucose-negative phenotype. After losing the plasmid during growth on non-selective
media, the yeast could only resume growth on media containing ethanol as the sole carbon source. These results show that *ACS2* is essential for growth on glucose and that the disruption of the small ORF is not responsible for the observed phenotype.

**acs1** *acs2* double mutant-To isolate a heterozygous *acs1* *acs2* mutant, we made a spore-to-spore cross of strains GG621 and GG624. The heterozygous double mutant (GG626) was isolated via restreaking on YEPD plus phleomycin and G418. Thirty-six asci of GG626 were dissected on YEPE plates. About 25% of the spores did not give colonies. Moreover, after testing the viable spores on phleomycin and/or G418 plates, no colonies resistant to both G418 and phleomycin were found. The same numbers were found after dissection on YEP plus raffinose, a non-repressing carbon source. These results suggested that the double mutant was not viable.

The same results were obtained with GG630, the heterozygous *acs1* *acs2* mutant which resulted from a cross between GG376 and a spore of GG629. However, in this background
we should be able to rescue the double mutant with the same plasmid as used in the rescue of the *acs2* mutant (pRUL77). After dissecting 96 ascii of transformed GG630, we were not able to isolate phleomycin and G418 resistant colonies, which grew on glucose. In spite of the low number of viable colonies (sporulation was induced after growth on selective MY and dissection was done on selective MY) we expected to find approximately five colonies. It was obvious that we had to screen a large number of spores to isolate a double mutant complemented by the plasmid. Therefore, we used random spore analysis to screen a high number of spores directly for growth on glucose, G418- and phleomycin resistance. We selected two candidates and checked with Southern analysis the chromosomal disruption of *ACS1* and *ACS2*, together with the presence of the *ACS2* multicopy plasmid. The results proved that the *acs1* *acs2* double mutant could be rescued with *ACS2* on a plasmid (data not shown).

**Discussion**

The data in this paper clearly indicates that *S. cerevisiae* contains two structural genes that each encode an active acetyl-coenzyme A synthetase. This is not unique in the central metabolism of *S. cerevisiae*. Enzymes such as phosphofructokinase, pyruvate decarboxylase, pyruvate carboxylase, malate synthase and citrate synthase have two or more isoenzymes with the same catalytic activity encoded by different genes (Heinisch, 1986; Hohmann, 1991; Brewster et al., 1994; Hartig et al., 1992; Rosenkrantz et al., 1986). In some cases the role of the different isoenzymes is not clear. In our case, disruption mutants, in which either *ACS1* or *ACS2* was disrupted, showed that the gene products are differentially regulated and have different physiological functions. After dissection on ethanol or raffinose the *acs2* mutant could form colonies. Hence, the Acs activity was produced from the *ACS1* gene. During repressing conditions i.e. glucose, with or without ethanol, however, the *acs2* mutant was not viable. It germinated, probably due to the presence of remaining Acs enzyme induced on sporulation medium, but stopped dividing after two or three divisions. Therefore, we think that *ACS1* is glucose-repressible and can possibly be induced by ethanol, acetaldehyde or acetate. An RC2 box in the promoter of *ACS1*, which is involved in (de)repression of *CYC1* (Guarente et al., 1984; Pfeifer et al., 1987), might play a role in this regulation. Other indications for Acs (de)repression were reported by Postma et al. (1989) and DeVirgilio et al. (1992). It thus appears that Acs1p is required for the assimilation of ethanol and acetate. *ACS2*, in contrast, is essential for growth on glucose and its product seems to be involved in the PDH bypass. We think that *ACS2* is a constitutive gene.

Why is *ACS2* essential for growth on glucose? Apparently, the Pdh complex, although it is active in batch cultures of wild-type yeast (Wenzel et al., 1993a), cannot meet the requirement for acetyl-coenzyme A in the *acs2* mutant. Three explanations are conceivable: (a) inhibition of Pdh, (b) toxic accumulation of acetate or, (c) a critical role of the subcellular compartmentation of acetyl-coenzyme A metabolism. Given the constant Pdh activity under a variety of conditions (Wenzel et al., 1993a), the first possibility seems unlikely. As yeasts are able to excrete large quantities of acetate, toxic accumulation of acetate also appears unlikely, leaving compartmentation as an attractive hypothesis.

Pdh produces acetyl-coenzyme A inside the mitochondria. However, it is not known if the mitochondrial carnitine acetyltransferase is able to transfer the acetyl moiety out of the
mitochondria (Kispal et al., 1993; Schmalix & Bandlow, 1993). At present, there are conflicting reports in the literature on the subcellular localisation of Acs. Different authors have reported mitochondrial, microsomal (Klein and Jahnke, 1971) and cytosolic (Kispal et al., 1991) localisation of the Acs activity. However, in view of its role in growth on carbon sources like acetate or ethanol and its carboxyterminal sequence, Acs1p may well be located in the peroxisomes. In many organisms the glyoxylate cycle is essential for growth on C2-compounds. This cycle consists of five enzymes: citrate synthase, Cit;aconitase, Aco; isocitrate lyase, Icl; malate synthase, Ms; malate dehydrogenase, Mdh. In S. cerevisiae Cit, Ms and Mdh have been located in the peroxisomes by different authors (McCammon et al., 1990; Lewin et al., 1990; Steffan and McAllister-Henn, 1992). The five genes encoding the yeast glyoxylate enzymes are glucose repressed and are induced during growth on non-fermentable carbon sources (Duntze et al., 1969; Hartig et al., 1992; Schöler and Schüller, 1993). ACS1 seems to share the same regulation pattern as these genes and is used for growth on C2-compounds. In addition, Acs1p has a C-terminus of SVKL, which resembles the SKL targeting sequence for peroxisomal enzymes (Gould et al., 1989). Peroxisomal enzymes with C-termini which diverge from the consensus sequence of this peroxisomal targeting signal (PTS1), like ARF, NKL and SKM are still essential and sufficient for peroxisomal targeting (De Hoop & AB, 1992). In addition to that, the Acs1p carboxy terminus VKL fits perfectly to the more general targeting consensus: a small amino acid at the first, a basic amino acid at the penultimate and a large non-polar amino acid at the C-terminal position (De Hoop & AB, 1992). If Acs1p is targeted to the peroxisomes it will be in one compartment with the rest of the glyoxylate cycle, which has then two direct supplies of acetyl-coenzyme A, the β-oxidation pathway and acetyl-coenzyme A synthetase.

The prolonged lag phase of the acls1 mutants in batch cultures might be explained by the kinetics of both Acs proteins. Preliminary results indicate that Acs2p has a 20-fold lower affinity for acetate than Acs1p (Kortland et al., 1994). Therefore, it seems plausible that acetate has to accumulate higher than normal concentrations to sustain growth in the acls1 mutant. The same is reported for cit1 mutants (Kispal et al., 1988).

Acs2p, on the other hand, might be cytosolic, due to a lack of any obvious targeting sequence. If compartmentation is the critical factor, the aces2 mutant is not able to provide cytosolic acetyl-coenzyme A while growing on glucose. This might be crucial in lipid biosynthesis. Furthermore, Acs2p seems to be a part of the Pdh bypass and therefore may be decisive in the appearance of the Crabtree effect. The other two enzymes of the Pdh bypass have been reported to be localised in the mitochondria (NAD+-dependent acetaldehyde dehydrogenase, Ald) and in the cytosol (NADP+-dependent Ald and Pdc) by Llorente and Nuñez de Castro (1977) and Van Urk et al. (1989), respectively. Work to identify the subcellular localisation of Acs1p and Acs2p, and the effect of overproducing Acs is currently in progress.

Acknowledgements

We wish to thank Claudio DeVirgilio and Gerold Barth for their kind gift of plasmid Ycp1ac111::ACS1, our colleagues Quirine van der Aart and Linda van der Zanden for their help with sequencing, Ophir Oppenheim for isolating the plasmid pRUL76, Jack Pronk and Hans van Dijken for advice, helpful discussions and critical reading of the manuscript.
The two acetyl-coenzyme A synthetases of *Saccharomyces cerevisiae* differ with respect to kinetic properties and transcriptional regulation

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Summary

*Saccharomyces cerevisiae* contains two structural genes, *ACS1* and *ACS2*, each encoding an active acetyl-coenzyme A synthetase. Characterisation of enzyme activities in cell-free extracts from strains expressing either of the two genes revealed differences in the catalytic properties of the two enzymes. The $K_M$ for acetate of Acs1p was about thirty-fold lower than that of Acs2p and Acs1p, but not Acs2p, could use propionate as a substrate. Enzyme activity measurements and mRNA analyses showed that *ACS1* and *ACS2* were both expressed during carbon-limited growth on glucose, ethanol and acetate in aerobic chemostat cultures. In anaerobic glucose-limited cultures, only the *ACS2* gene was expressed. Based on these facts, the products of the *ACS1* and *ACS2* genes were identified as the previously described 'aerobic' and 'anaerobic' forms of acetyl-coenzyme A synthetase, respectively. Batch and glucose-pulse experiments revealed that transcription of *ACS1*, is subject to glucose repression. A mutant strain lacking Acs2p was unable to grow on glucose in batch cultures, but grew readily in aerobic glucose-limited chemostat cultures, in which the low residual glucose concentration alleviated glucose repression. Experiments in which ethanol was pulsed to aerobic ethanol-limited chemostat cultures indicated that, in addition to glucose, also ethanol repressed *ACS1* transcription, although to a lesser extent. In contrast, transcription of *ACS2* was slightly induced by ethanol and glucose. Absence of *ACS2* prevented complete glucose repression of *ACS1*, indicating that *ACS2* (in)directly was involved in the transcriptional regulation of *ACS1*. 
Introduction

When *Saccharomyces cerevisiae* grows on acetate or ethanol, ATP-dependent activation of acetate to acetyl-coenzyme A is catalysed by acetyl-coenzyme A synthetase (EC 6.2.1.1). In addition to serving as the fuel for the citric acid cycle, acetyl-coenzyme A is an essential building block for the synthesis of lipids and some amino acids. During growth on glucose, direct formation of acetyl-coenzyme A from pyruvate is catalysed by the mitochondrial pyruvate dehydrogenase complex. Alternatively, conversion of pyruvate into acetyl-coenzyme A can be accomplished by the concerted action of the enzymes of the pyruvate dehydrogenase bypass: pyruvate decarboxylase, acetaldehyde dehydrogenase and acetyl-coenzyme A synthetase (Holzer and Goedde, 1957; Pronk et al., 1994b). Recent work has indicated that the latter pathway is essential for growth, probably for the provision of cytosolic acetyl-coenzyme A required for lipid synthesis (Van den Berg and Steensma, 1995; Flikweert et al., 1996).

In *S. cerevisiae*, a completely respiratory sugar metabolism is only observed at relatively low growth rates in aerobic, sugar-limited cultures (e.g. chemostat cultures). Upon exposure of such cultures to high sugar concentrations, metabolism becomes respirofermentative and pyruvate metabolism occurs predominantly via pyruvate decarboxylase (Petrik et al., 1983; Van Urk et al., 1988). Under such conditions acetate is formed as a byproduct, indicating that the *in vivo* activity of acetaldehyde dehydrogenase exceeds that of acetyl-coenzyme A synthetase. Acetate production can be a major problem in industrial high-biomass-density cultures of *S. cerevisiae* (e.g. for heterologous protein production), because this weak organic acid dissipates the pH gradient across the plasma membrane, leading to a reduction of the biomass yield (Verduyn, 1991).

In view of the central role of acetyl-coenzyme A synthetase in the carbon metabolism of *S. cerevisiae*, it is not surprising that the biochemistry of this enzyme has been studied in detail. Two immunologically distinct forms of the enzyme have been described (Satyanarayana et al., 1974). The two forms differed with respect to kinetic properties, substrate specificity and cellular localisation (DeVicenzi and Klein, 1970; Satyanarayana and Klein, 1973; Klein and Jahnke, 1979) and, based on their levels in shake-flask cultures, were called ‘aerobic’ and ‘anaerobic’ acetyl-coenzyme A synthetase (Satyanarayana and Klein, 1974). The differences in regulation, localisation and kinetic aspects suggest different roles in metabolism for both enzymes. Only the ‘aerobic’ form of the enzyme has been purified to homogeneity (Satyanarayana and Klein, 1976; Frenkel and Kitchens, 1977) and it has remained unclear whether the two forms are encoded by different genes.

Recently two structural genes, ACSI (DeVirgilio et al., 1992) and ACS2 (Van den Berg and Steensma, 1995), each encoding acetyl-coenzyme A synthetase, have been cloned from *S. cerevisiae*. Disruption of both genes was lethal (Van den Berg and Steensma, 1995), indicating that acetyl-coenzyme A synthetase is an essential enzyme in *S. cerevisiae*. Strains in which only ACS2 was disrupted grew normally on ethanol or acetate, but were unable to grow on glucose in batch cultures (Van den Berg and Steensma, 1995). This may be related to the observation that ACSI is subject to glucose repression (Kratzer and Schüller, 1995). In contrast, acsl mutants grew well on glucose (Van den Berg and Steensma, 1995).

The aim of the present study was to investigate the physiological function and regulation of both Acs proteins, and to determine whether they correspond to the ‘aerobic’ and ‘anaerobic’ forms of acetyl-coenzyme A synthetase. To this end, kinetic properties of acetyl-
coenzyme A synthetase were compared in cell-free extracts of isogenic wild-type, acs1 and acs2 strains S. cerevisiae. Furthermore, the transcriptional regulation of both genes was studied in batch and chemostat cultures.

Materials and methods

*Yeast strains*- Yeast strains used for detailed physiological studies should preferably lack auxotrophic markers and, whenever mutants are used, these should be isogenic to the wild-type. These criteria were met by using the set of isogenic strains constructed in a previous study (Van den Berg and Steensma, 1995); *Saccharomyces cerevisiae* T2-3D (HO/HO ACS1/ACS1 ACS2/ACS2), GG621 (HO/HO acs1::APT1/acs1::APT1 ACS2/ACS2) and GG625 (HO/HO ACS1/ACS1 acs2::Tn5ble/acs2::Tn5ble).

*Growth conditions and media*- For small-scale pilot experiments, cells were grown at 30°C in 750 ml shake-flasks (250 rpm) containing 100 ml of mineral medium (Zonneveld, 1986) supplemented with glucose (10 g l⁻¹), ethanol (30 g l⁻¹) or acetate (5 g l⁻¹).

Chemostat cultivation was performed in 2-liter laboratory fermenters (Applikon, Schiedam, The Netherlands), at a dilution rate of 0.10 h⁻¹. The working volume of the culture was kept at 1.0 liter via an electrical level sensor. Removal of the effluent from the centre of the culture ensured that biomass concentrations in the effluent line differed by less than 1% from those in the culture. The temperature was kept at 30°C and the pH was maintained at 5.0 by automatic addition of 2.0 M KOH. To maintain aerobic conditions, an air flow of 0.3 l·min⁻¹ through the culture was maintained using a Brooks 5876 mass-flow controller and the culture was stirred at 800 rpm. The dissolved oxygen concentration, continuously measured with an Ingold polarographic oxygen electrode, remained above 25% of air saturation in all aerobic experiments. For anaerobic growth experiments, the culture and the reservoir medium were sparged with nitrogen. To minimise oxygen diffusion, anaerobic cultures were equipped with Noprprene tubing. Chemostat data refer to steady state cultures without detectable metabolic oscillations. Culture purity was evaluated by phase-contrast microscopy at 1000x magnification and plating on selective media.

The mineral medium used in the chemostat experiments has been described (Verduyn et al., 1992). Carbon sources were added at a concentration of: glucose, 10 g l⁻¹; ethanol, 7.5 g l⁻¹ or acetic acid, 10 g l⁻¹. For anaerobic cultivation, the medium was supplemented with the anaerobic growth factors Tween80 and ergosterol as described (Verduyn et al., 1990) and the glucose concentration was 25 g l⁻¹.

*Pulse experiments*- After glucose-limited steady state cultures had been obtained, glucose was added by means of a syringe to give an initial concentration of 75 mM. Ethanol (40 mM) and combined ethanol/acetic acid (20 mM of each) pulses were added to ethanol-limited steady state cultures. Throughout the pulse experiments the medium flow into the fermenter and the removal of effluent were continued. The effluent was cooled on ice immediately after leaving the fermenter. At appropriate intervals samples were collected from the effluent line and used for metabolite determination and mRNA analysis.

*Determination of dry weight*- Dry weights of culture samples (10 ml) were determined using
nitrocellulose filters (pore size 0.45 μm; Gelman Sciences, U.S.A.). After removal of the medium by filtration, the filters were washed with demineralised water and dried in a Sharp R-7400 microwave oven for 20 min. Parallel samples varied by less than 1%.

Substrate and metabolite analysis-Glucose was determined with the Merck glucose-oxidase kit (no.14143). Ethanol was assayed with a colorimetric assay kit (EK 003, Leeds Biochemicals Ltd., Leeds, U.K.). Acetate was assayed using the Boehringer acetic acid kit (no. 148261).

Preparation of cell-free extracts-Cells (approximately 100 mg dry weight) were harvested by centrifugation at 5,000x g for 10 min, washed once with 100 mM potassium-phosphate buffer (pH 7.5, 4°C) and resuspended in 100 mM potassium-phosphate buffer pH 7.5, 2 mM MgCl₂ and 1 mM dithiothreitol. Cells were disrupted immediately by sonication with 0.7-mm diameter glass-beads at 0°C for 4x 30 s using an MSE sonicator (150 W output, 8 μm peak-to-peak amplitude). Whole cells and debris were removed by centrifugation at 20,000x g (10 min at 4°C). The clear supernatant, typically containing 2-4 (mg protein)·ml⁻¹, was used as cell-free extract. Protein concentrations were measured by the Lowry method.

Enzyme assays-The specific activity of acetyl-coenzyme A synthetase was determined at 30°C in a Hitachi spectrophotometer at 340 nm. The standard reaction mixture (1 ml) contained Tris-HCl (pH 7.7), 100 μmol; L-malate (pH 7.7), 10 μmol; coenzyme A, 0.2 μmol; ATP (pH 7.5), 8 μmol; NAD⁺, 1 μmol; MgCl₂, 10 μmol; malate dehydrogenase (3 U); citrate synthase (0.4 U), and cell-free extract. The reaction was started with 100 μmol potassium-acetate. Enzyme activities were calculated assuming an extinction coefficient of NADH of 6.3 mM⁻¹·cm⁻¹. One unit was defined as the amount of enzyme catalysing the acetate-dependent formation of 1 μmol NADH·min⁻¹ in the coupled assay. For determination of the Kₘ, the acetate or ATP concentration in the assay was varied, leaving the other components constant. Kinetic parameters were estimated by non-linear regression, assuming Michaelis-Menten kinetics, using the program Fig.P (Fig.P software).

This assay would also measure the combined activity of acetate kinase and phosphotransacetylase, acting together in hydrolysing acetyl-coenzyme A to generate ATP during anaerobic growth in bacteria (Grundy et al., 1993). However, they can also perform the reverse reaction, which generates acetyl-coenzyme A. To rule out the possibility that this two-step reaction is present in yeast (e.g. either ACS1 or ACS2 encodes an acetate kinase), we tested cell-free extracts from wild-type and mutant strains for the presence of phosphotransacetylase by omitting ATP from the reaction mixture and starting the reaction with 10 μmol acetyl-phosphate. Escherichia coli extracts were used as a positive control. No activity was observed in any of the three S. cerevisiae strains (data not shown), indicating that both ACS1 and ACS2 encode genuine acetyl-coenzyme A synthetases.

Activity of acetyl-coenzyme A synthetase with propionate as a substrate was determined in a discontinuous assay, measuring the propionate-dependent consumption of coenzyme A (Pronk et al., 1994a). Enzyme activity was calculated from the decrease in coenzyme A. One unit was defined as the amount of enzyme catalysing the propionate-dependent consumption of 1 (μmol coenzyme A)·min⁻¹.

Estimation of the capacities of Acs1p and Acs2p in wild-type cultures-Kinetic analysis of
acetate activation by cell-free extracts indicated that the acetyl-coenzyme A synthetases encoded by the ACS1 and ACS2 genes both obeyed Michaelis Menten-kinetics, but exhibited a substantially different $K_M$ for acetate. The ratio of the capacities of the two enzymes ($V_{max, ACS1}/V_{max, ACS2}$) was estimated by performing enzyme activity assays at two different acetate concentrations (1 and 100 mM, respectively). The ratio of the capacities was calculated from equation 1:

$$r_1 = \frac{V_{max, ACS1}}{V_{max, ACS2}}$$

$$r_{100} = \frac{V_{max, ACS1} \cdot 100}{V_{max, ACS2} \cdot 100}$$

$$1 = \frac{K_{S, ACS1} + 1}{K_{S, ACS1} + 100}$$

$$1 = \frac{K_{S, ACS2} + 1}{K_{S, ACS2} + 100}$$

In equation 1, $r_1$ and $r_{100}$ are the reaction rates observed with 1 and 100 mM acetate, respectively.

**RNA Techniques**—Total RNA was isolated from 2 ml samples as described (Schmitt et al., 1990), except that the final samples were stored in pure formamide. This reduced the total sample volume by a factor two, thus avoiding the need to concentrate samples with a low amount of RNA. For Northern blot analysis approximately 5 μg of each sample were mixed with an equal volume of sample buffer (2x MOPS, 2.88% formaldehyde and 0.1 mg.ml⁻¹ ethidium bromide) and separated on a 0.7% agarose gel containing 1.44% formaldehyde. The RNA molecules were transferred to a nylon membrane (Boehringer Mannheim) by vacuum blotting in 20x SSC (3.0 M NaCl, 0.3 M sodium-citrate). Hybridisation was carried out at 65°C for 18 hours in 5x SSC, 1% SDS, 20 mM sodium-pyrophosphate, 0.1% sodium-lauroylsarcosine and 1% blocking reagent (Boehringer Mannheim). Probes were prepared as described previously (Van den Berg and Steensma, 1995). The blots were washed twice for 10 min in 5x SSC, 1% SDS at 65°C. Quantification of the signals was done using the Phosphor Imager system (Molecular Dynamics, B&L Systems, Maarsse, The Netherlands). In all experiments, PDA1 mRNA was used as an internal loading standard. All solutions were made RNase free by autoclaving for 40 min at 120°C. Gel trays were washed overnight with a 10% Glorix solution.

**Results**

**Kinetic properties of the ACS1 and ACS2 gene products**—To investigate whether the gene products of the ACS genes correspond to the ‘aerobic’ and ‘anaerobic’ forms of acetyl-coenzyme A synthetase (Klein and Jahnke, 1971; Satyanarayana et al., 1974; Klein and Jahnke, 1979) the kinetic properties of the enzymes were compared. Ethanol-limited chemostat cultures of wild-type Saccharomyces cerevisiae and isogenic acs1 and acs2 mutants, grown at a dilution rate of 0.10 h⁻¹, were used as a reproducible source of biomass.

The ‘aerobic’ and ‘anaerobic’ forms of S. cerevisiae acetyl-coenzyme A synthetase have been reported to exhibit different affinities for acetate and ATP, with the ‘aerobic’ form exhibiting lower $K_M$ values for both substrates (DeVicenzi and Klein, 1970). The $K_M$ for acetate of the strain containing only ACS1 was about 30-fold lower than that of the strain
Table 1. Kinetic properties of acetyl-coenzyme A synthetase in cell-free extracts prepared from wild-type S. cerevisiae and in mutants in which either ACS1 or ACS2 was inactivated. Cells were grown in aerobic, ethanol-limited chemostat cultures (D = 0.10 h⁻¹).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Kinetic constants</th>
<th>Activity with propionate (U·mg protein⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$V_{max}$ (U·mg protein⁻¹)</td>
<td>$K_{M, \text{acetate}}$ (mM)</td>
</tr>
<tr>
<td>T2-3D</td>
<td>ACS1 ACS2</td>
<td>1.2 ± 0.1</td>
<td>0.60 ± 0.09</td>
</tr>
<tr>
<td>GG621</td>
<td>acs1 ACS2</td>
<td>0.34 ± 0.01</td>
<td>8.8 ± 0.5</td>
</tr>
<tr>
<td>GG625</td>
<td>ACS1 acs2</td>
<td>1.10 ± 0.01</td>
<td>0.32 ± 0.01</td>
</tr>
</tbody>
</table>

expressing only ACS2 (Table 1). In extracts from wild-type, the $K_{M}$ for acetate was close to that of GG625 extracts (Table 1). Extracts prepared from the three strains did not exhibit substantial differences with respect to their $K_{M}$ for ATP (Table 1).

A striking difference between the ‘aerobic’ and ‘anaerobic’ forms of acetyl-coenzyme A synthetase is the inability of the latter to activate propionate (DeVenicenzi and Klein, 1970). Extracts from wild-type and the isogenic mutant expressing only ACS1 were able to activate propionate, albeit at lower rates than acetate (Table 1). In contrast, activation of propionate was not observed with extracts from the strain expressing only ACS2 (Table 1). From these data we concluded that the ‘aerobic’ protein is encoded by the ACS1 gene and the ‘anaerobic’ protein by the ACS2 gene.

Growth of an acs2 mutant in glucose-limited chemostat cultures-S. cerevisiae mutants in which the ACS2 gene has been disrupted are unable to grow on glucose in batch cultures (Van den Berg and Steensma, 1995). This may be due to repression of ACS1 by glucose (Kratzer and Schüller, 1995). Alternatively, it may indicate that Acs2p has an indispensable function in glucose metabolism that cannot be met by Acs1p (e.g. due to different catalytic properties or subcellular compartmentation).

In a preliminary study, transcriptional regulation of both genes was studied in shake-flask cultures of wild-type yeast grown on glucose. ACS1 mRNA was not detectable during exponential growth, but appeared when the cells entered stationary phase (Fig. 1). Apparently, glucose repression of ACS1 was relieved when glucose was consumed and the culture switched to consuming ethanol and acetate produced during exponential growth. In contrast, ACS2 mRNA was observed throughout the growth curve (Fig. 1). Experiments in which glucose was added to acetate-grown batch cultures indicated that repression of ACS1 transcription started at glucose concentrations of 100 mg·l⁻¹ (data not shown).

The low residual substrate concentration in glucose-limited chemostat cultures is known to alleviate glucose repression (De Jong-Gubbels et al., 1995; Sierksstra et al., 1992a). We therefore tested whether a strain expressing only ACS1 was able to grow under these conditions by switching an ethanol-limited chemostat culture to medium with glucose as the sole carbon source. The strain rapidly adapted to glucose-limited growth. In the resulting glucose-limited steady state cultures its biomass yield was the same as that of the wild-type strain (Table 2). This indicated that glucose repression of ACS1 is the sole reason for the inability of acs2 mutants to grow on glucose in batch cultures.
**Figure 1. Growth and transcription of ACS1 and ACS2 in a glucose-grown shake-flask culture of wild-type S. cerevisiae T2-3D.** Total RNA was isolated at four time points (A through D) and used for Northern analysis. The abundance of ACS1 and ACS2 mRNA, normalised to the PDA1 signal, is represented in the bar diagram.

**Regulation of Acs genes in carbon-limited chemostat cultures**—To investigate effects of carbon source on the regulation of both genes, their expression was studied in carbon-limited chemostat cultures. In contrast to batch cultivation, chemostat cultivation can be carried out with a constant dissolved-oxygen concentration and growth rate, which is known to have substantial effect on acetyl-coenzyme A synthetase levels in *S. cerevisiae* (Postma et al., 1989). This enables studies on the regulation by carbon source without interference.

As the K_M values of the Acs gene products for acetate are different (Table 1), the ratio of their maximum activities in cell-free extracts (V_{max,ACS1}/V_{max,ACS2}) can be estimated by measuring acetyl-coenzyme A synthetase activities at two substrate concentrations (see materials and methods). These estimations indicated that in aerobic, carbon-limited chemostat cultures grown on glucose, ethanol or acetate, both genes were expressed simultaneously (Table 2). Under these conditions, ACS1 was responsible for most of the total capacity (Table 2). In the aerobic cultures grown on ethanol and glucose, expression levels of ACS2 were not substantially different, whereas the expression of ACS1 was higher in ethanol-limited cultures (Table 2).

In anaerobic glucose-limited chemostat cultures, Acs activity was lower than in aerobic cultures and appeared to be encoded exclusively by the ACS2 gene (Table 2). This is in line
Table 2. Acetyl-coenzyme A activities and biomass yields in cell-free extracts from carbon-limited chemostat cultures (D = 0.10 h$^{-1}$) of wild-type S. cerevisiae and mutants in which either ACS1 or ACS2 have been inactivated. Unless otherwise indicated, cultures were grown aerobically. The ratio of the maximum specific activities of the ACS1 and ACS2 gene products was calculated from enzyme activity assays at two different acetate concentrations (see Materials and methods).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Carbon source</th>
<th>Acs activity (U·mg protein$^{-1}$)</th>
<th>$V_{\text{max,ACS1}}/V_{\text{max,ACS2}}$</th>
<th>Biomass yield (g dry mass g substrate$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T2-3D</td>
<td>ACS1 ACS2</td>
<td>glucose</td>
<td>0.42</td>
<td>4.2</td>
<td>0.5</td>
</tr>
<tr>
<td>GG621</td>
<td>acs1 ACS2</td>
<td>glucose</td>
<td>0.24</td>
<td>-</td>
<td>0.5</td>
</tr>
<tr>
<td>GG625</td>
<td>ACS1 acs2</td>
<td>glucose</td>
<td>0.38</td>
<td>-</td>
<td>0.5</td>
</tr>
<tr>
<td>T2-3D</td>
<td>ACS1 ACS2</td>
<td>ethanol</td>
<td>1.26</td>
<td>26</td>
<td>0.6</td>
</tr>
<tr>
<td>GG621</td>
<td>acs1 ACS2</td>
<td>ethanol</td>
<td>0.30</td>
<td>-</td>
<td>0.6</td>
</tr>
<tr>
<td>GG625</td>
<td>ACS1 acs2</td>
<td>ethanol</td>
<td>1.04</td>
<td>-</td>
<td>0.6</td>
</tr>
<tr>
<td>T2-3D</td>
<td>ACS1 ACS2</td>
<td>glucose (anaerobic)</td>
<td>0.08</td>
<td>&lt;0.01</td>
<td>0.1</td>
</tr>
</tbody>
</table>

Table 3. Levels of ACS1 and ACS2 mRNA in steady state carbon-limited chemostat cultures (D = 0.10 h$^{-1}$) of wild-type S. cerevisiae T2-3D. The cultures were aerobic, unless stated otherwise. The levels of the two mRNAs have been normalised to the level of PDA1 mRNA. The level of each transcript during aerobic, glucose-limited growth was set at 1.

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Carbon source</th>
<th>Glucose</th>
<th>Ethanol</th>
<th>Acetate</th>
<th>Glucose (anaerobic)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACS1</td>
<td></td>
<td>1</td>
<td>1.4</td>
<td>1.8</td>
<td>0</td>
</tr>
<tr>
<td>ACS2</td>
<td></td>
<td>1</td>
<td>1.6</td>
<td>3.2</td>
<td>2.8</td>
</tr>
</tbody>
</table>

with the identification of ACS2 as the gene encoding the 'anaerobic' enzyme. Nevertheless, it should be noted that ACS2 expression is not limited to anaerobic or fermentative growth conditions (Table 2).

Northern analysis corroborated that in aerobic carbon-limited chemostat cultures grown on glucose, ethanol or acetate, both ACS1 and ACS2 were expressed, whereas only the ACS2 transcript was detected in anaerobic cultures (Table 3).

Transcription of ACS1 and ACS2 during transition from glucose-limited to glucose-excess conditions-In the experiments discussed above, growth was studied under carbon-limited conditions. Although industrial fed-batch cultures of S. cerevisiae are in principle sugar-limited, transient exposure to high sugar concentrations is inevitable due to imperfect mixing and the high sugar concentration in the feed of large-scale bioreactors. On lab-scale this situation can be simulated by glucose pulses to aerobic, glucose limited cultures. One of the consequences of such transient exposure of aerobic grown S. cerevisiae cultures to excess glucose is the accumulation of ethanol and acetate, the so-called short-term Crabtree effect (Van Urk et al., 1990). Since the capacity of acetyl-coenzyme A synthetase may be a relevant factor in the occurrence of acetate formation, transcriptional regulation of the Acs genes was
studied after addition of 75 mM glucose to aerobic, glucose-limited yeast cultures.

After applying the glucose pulse, three metabolic phases could be discerned (Fig. 2A). During the first phase (0-160 min), the glucose added to the culture was rapidly consumed. Glucose metabolism was accompanied by the production of ethanol and, to a lesser extent, acetate. In the second phase (170-380 min), ethanol was consumed, resulting in the formation of more acetate. This was consumed in the third phase (390-420 min). The levels of ACS1 and ACS2 mRNA during these phases were monitored by Northern blot analysis (Fig. 2B).

Transcription of ACS1 decreased below the detection limit within 10 minutes after addition of the glucose (Fig. 2B), confirming ACS1 glucose repression. As soon as glucose was exhausted and ethanol consumption started, ACS1 mRNA reappeared. During this phase ACS1 mRNA levels were two- to fourfold higher, compared to the steady state level (Fig. 2B). As the ethanol concentration in the culture decreased below 10 mM and acetate

![Graph A](image)

**Figure 2.** Transient responses of wild-type *S. cerevisiae* to a glucose pulse. At time zero 75 mmol of glucose was added to an aerobic, glucose-limited chemostat culture (D=0.10 h⁻¹) of *S. cerevisiae* T2-3D. A. Concentrations of glucose, ethanol and acetate. B. Relative abundances of ACS1 (•) and ACS2 (○) mRNA. *PDA1* mRNA was used as a loading standard; the steady state levels of both transcripts (t = 0) were set at 100 %. Note that the y-axis is exponential; points drawn on the x-axis were below the detection limit.
consumption started, a sharp increase of mRNA occurred (Fig. 2B). The maximum level, observed after 410 min, corresponded to approximately 40-fold the steady state value.

Throughout the pulse experiment, the levels of ACS2 mRNA varied between 60 and 180% of the steady state value, suggesting that transcription of ACS2 is relatively insensitive to the transient accumulation of metabolites. However, ACS2 completely disappeared at the end of the experiment (Fig. 2B). Only after several hours ACS2 mRNA returned to its steady state level (data not shown).

**Regulation of ACS1 expression by ethanol**—It is obvious that full transcription of ACS1 only occurred when ethanol is completely consumed (Fig. 2B). Either the gene is relieved from ethanol repression, or the increased level of acetate might induce ACS1 transcription. To further investigate the regulation by ethanol, C2-carbon pulses were given to ethanol-limited chemostat cultures. Since, full transcription of ACS1 occurred when the concentrations of ethanol and acetate were approximately 10-20 mM, we first added a mix of 20 mM ethanol and 20 mM acetate to an ethanol-limited culture. If the disappearance of any residual glucose was responsible for the increase of ACS1 mRNA levels (Fig. 2, sample 350 minutes and further), no increase of transcription is expected in this new experiment. On the other hand, repression by ethanol would lower the levels of ACS1 mRNA, whereas induction by acetate would increase it. As can be seen from Fig. 3A, the level of ACS1 mRNA decreased rapidly, although at a lower rate than after a glucose pulse (Fig. 2B), to approximately 20% of the steady state level. As soon as ethanol and acetate were consumed, ACS1 mRNA returned to the original level. The ACS2 mRNA level, on the other hand, increased to almost 200% before returning to the steady state level at the end of the pulse.

These data strongly suggest that ACS1 transcription is repressed by ethanol. This was confirmed in another pulse experiment, in which 40 mM ethanol was added to an ethanol-limited chemostat culture. Both genes responded exactly the same as during the ethanol/acetate pulse.

Apparently, ACS1 is repressed by ethanol and ACS2 is induced by ethanol.

**Transgene regulation of ACS1 transcription**—Since ACS1 and ACS2 catalyse the same reaction, it does not seem unlikely that interactions exist between the regulatory mechanisms affecting the transcription of both genes.

To test whether the transcription of ACS1 is influenced by the presence of an active ACS2 gene, glucose repression of ACS1 expression was compared in wild-type and GG625, which lacks an active ACS2 gene. When exponential-phase shake-flask cultures growing on acetate were pulsed with 1 g·l⁻¹ glucose, ACS1 expression in the wild-type showed exactly the same pattern as in the chemostat experiment shown in Figure 2B. Within 15 min after the pulse, ACS1 mRNA could no longer be detected (Fig. 4A). After 60 minutes the mRNA reappeared. In the acs2 mutant GG625, a decrease of ACS1 transcription was also observed. However, in this case significant levels of ACS1 mRNA remained present throughout the experiment (Fig. 4B). These results, showing that the regulation of ACS1 is influenced by the presence of an active ACS2 gene, were confirmed in glucose-pulse experiments with glucose-limited chemostat cultures of the acs2 mutant (data not shown).

In similar experiments, levels of the ACS2 transcript were monitored after glucose pulses to an acs1 mutant and the isogenic wild-type. ACS2 transcription did not differ in the two strains, neither in batch cultures nor in chemostat cultures (data not shown).
Figure 3. Regulation of ACS1 and ACS2 expression by ethanol. A. A mix of 20 mmol ethanol and 20 mmol acetate was added to an ethanol-limited chemostat culture. Concentrations of ethanol and acetate are shown in the upper panel. The middle panel shows the Northern blot hybridisations, with ACS1/PDA1 and ACS2/PDA1 mRNA, respectively. The relative abundance of ACS mRNAs are depicted in the lower panel. B. A 40 mmol ethanol pulse was given to an ethanol-limited chemostat culture. Concentrations of ethanol and acetate are shown in the upper panel. The middle panel shows the Northern blot hybridisations, with ACS1/PDA1 and ACS2/PDA1 mRNA, respectively. The relative abundances of ACS mRNAs are depicted in the lower panel.

Discussion

One of the goals of the present study was to determine whether the gene products of the ACS1 and ACS2 genes could be correlated with the ‘aerobic’ and ‘anaerobic’ forms of acetyl-coenzyme A synthetase described previously (DeVicenzi and Klein, 1970; Satyanarayana and Klein, 1973; Satyanarayana and Klein, 1974; Satyanarayana et al., 1974; Satyanarayana and Klein, 1976; Frenkel and Kitchens, 1977; Klein and Jahnke, 1979). Based on the difference in $K_M$ for acetate, substrate specificity and expression under anaerobic conditions, we concluded that ACS1 encodes the ‘aerobic’ form of the enzyme, whereas ACS2 encodes the ‘anaerobic’ form. Identity of the ACS1 gene product and the ‘aerobic’ isoenzyme is further supported by the correlation between the amino acid composition of purified ‘aerobic’ acetyl-coenzyme A synthetase (Satyanarayana and Klein, 1976) and the predicted protein sequence of the ACS1 product (DeVirgilio et al., 1992). So far, the ACS2 gene product (the ‘anaerobic form’) has not been purified to homogeneity. Attempts in our laboratory to achieve this goal were product (DeVirgilio et al., 1992). So far, the ACS2 gene product (the ‘anaerobic form’) has not been purified to homogeneity. Attempts in our laboratory to achieve this goal were

Figure 4. ACS1 transcription is influenced by the presence of ACS2. A glucose pulse (final concentration 5 mM) was given to an exponential-phase acetate-growing shake-flask culture of wild-type S. cerevisiae (A) and the acs2 mutant GG625 (B). The relative abundance of ACS1 mRNA, shown below each lane, is normalised to the signal of the PDA1 transcript. The ACS1 level at the start each experiment was set at 100%.
hindered by instability of partially purified preparations of the enzyme (Kortland et al., gene 1994). We propose that the terms 'aerobic' and 'anaerobic' acetyl-coenzyme A synthetase should no longer be used, because ACS2 is expressed not only during anaerobic growth, but also during aerobic growth on glucose, ethanol and acetate.

Palmitoyl-coenzyme A was reported to be a strong inhibitor of ACS1 encoded isoform (Satyanarayana and Klein, 1973). In the present study, palmitoyl-coenzyme A was found to be an equally effective inhibitor of acetyl-coenzyme A synthetase activity in cell-free extracts of all three strains (50% inhibition at 25 \( \mu \text{M} \) palmitoyl-coenzyme A; data not shown). Also in contrast to earlier reported (DeVicenzi and Klein, 1970) no difference was found in \( K_M \) for ATP of both gene products. However, it should be taken into account that both the cultivation conditions and assay procedures employed in the previous work (DeVicenzi and Klein, 1970; Satyanarayana and Klein, 1973) were different from those used in the present study.

Consistent with earlier reports (Kratzer and Schüller, 1995; Van den Berg and Steensma, 1995), ACS1 was found to be subject to glucose repression. Upon exposure of wild-type cells to glucose, the level of the ACS1 transcript decreased very rapidly: within 10 to 20 min after exposure to glucose concentrations as low as 0.1 \( \text{g} \cdot \text{l}^{-1} \), ACS1 was no longer detectable. A similar rapid decline of the transcript level was found for the tightly regulated genes FBP1, PCK1 and SDH1 and was attributed to glucose-accelerated mRNA turnover (Lombardo et al., 1992; Mercado et al., 1994). The possibility that this mechanism is also involved in the regulation of ACS1 transcription deserves further attention.

It has recently been proposed that, as part of the pyruvate dehydrogenase bypass, acetyl-coenzyme A synthetase is indispensable for the synthesis of lipids in the yeast cytosol (Flikweert et al., 1996). This implies that, in addition to its role in the dissimilation of ethanol and acetate, the enzyme also plays a vital role in assimilatory metabolism. The observation that both Acsl gene products are inhibited by a long-chain fatty acyl ester (e.g. palmitoyl-coenzyme A) is consistent with a role in lipid synthesis: inhibition by fatty-acyl coenzyme A esters is a logical feed-back mechanism to control this key assimilatory process.

The inability of acs2 mutants to grow on glucose in batch cultures, might lead to the conclusion that Acslp performs an essential role in glucose metabolism. However, the ability to grow in aerobic, glucose-limited chemostat cultures (no glucose repression of ACS1) showed that neither the kinetic properties nor the subcellular localisation of Acslp prevent it from operating as part of the Pdh bypass. A similar conclusion holds for the activity of acetyl-coenzyme A synthetase in gluconeogenic growth on ethanol and acetate: neither of the two acetyl-coenzyme A synthetases is indispensable for growth on these C2-compounds. Nevertheless, the regulation of transcription of both genes by these compounds was different. Kratzer and Schüller (1995) reported a strong derepression of ACS1 on ethanol. However, these experiments were done in uncontrolled shake-flask batch cultures. When the yeast was grown in an ethanol-limited chemostat culture and excess ethanol was added, it became clear that in fact ACS1 was repressed by ethanol (Fig. 3).

It is not clear whether ethanol itself caused repression of ACS1. Another possible candidate is acetaldehyde, which is formed after oxidation of ethanol. Unfortunately, due to the toxic and volatile nature of this compound it was not possible to pulse the cultures with acetaldehyde, or even measure its concentration during the pulse experiments.

The high expression of ACS1 during growth on acetate (Fig. 2) suggested that Acslp is primarily responsible for acetate activation during gluconeogenic growth. The relatively low \( K_M \) for acetate of Acslp enables it to gain enough energy to sustain growth, even at low
acetate concentrations. Acs2p, on the other hand, has a lower affinity for acetate, but was expressed during growth on glucose, when the main energy generating flux does not require Acs activity. Therefore, Acs2p is likely to be the major producer of cytosolic acetyl-coenzyme A, in total approximately 4% of the total amount of carbon (Flikweert et al., 1996), required for lipid and amino acid biosynthesis.

Acknowledgements

We thank R.A. Dingemans for technical assistance. This work was supported by the Dutch Ministry of Economic Affairs in the Framework of the ABON program 'Metabolic Fluxes in Yeasts and Fungi' and by the European Community in the framework of the research project 'From Gene to Product in Yeast: a Quantative Approach', which is part of the EC Framework IV Cell Factory Program.
The *Saccharomyces cerevisiae* acetyl-coenzyme A synthetase encoded by the *ACS1* gene, but not the *ACS2*-encoded enzyme, is subject to glucose catabolite inactivation

Patricia de Jong-Gubbels, Marco A. van den Berg, H.Y.de Steensma, Johannes P. van Dijken and Jack T. Pronk
Submitted for publication (1997)
Summary

Acetyl-coenzyme A synthetase (EC 6.2.1.1) catalyses the formation of acetyl-coenzyme A from acetate, coenzyme A and ATP. *Saccharomyces cerevisiae* contains two structural genes, *ACS1* and *ACS2*, that each encode an acetyl-coenzyme A synthetase isoenzyme. Transcription of both genes differs, but at present the physiological significance of the existence of two isoenzymes is not clear. In the present study, it was investigated whether, in addition to glucose repression of *ACS1* transcription, acetyl-coenzyme A synthetase levels are regulated by inactivation of the isoenzymes. To this end, regulation of the two *ACS* gene products was investigated after exposure of derepressed, ethanol-limited chemostat cultures of *S. cerevisiae* to excess glucose. After a pulse of 100 mM glucose to an ethanol-limited chemostat culture of wild-type *S. cerevisiae*, acetyl-coenzyme A synthetase activity decreased within 3 hours to 50% of its original activity. No such inactivation was observed in a strain in which *ACS1* was disrupted. On the other hand, acetyl-coenzyme A synthetase activity in a strain in which *ACS2* was disrupted decreased to 20% of its original activity within 3 hours after the pulse. Western blot analysis demonstrated that the *ACS1* product, but not the *ACS2* product was degraded after the glucose pulse. Inactivation kinetics of the acetyl-coenzyme A synthetase encoded by the *ACS1* gene strongly resembled those of isocitrate lyase. Control experiments with cycloheximide confirmed that the decrease of enzyme activity of the *ACS1* product was caused by glucose-induced inactivation, rather than by a combination of ceased synthesis and a high turnover of the protein.
Introduction

Depending on growth conditions, sugar metabolism in *Saccharomyces cerevisiae* (bakers’ yeast) can be either respiratory, fermentative or respirofermentative. Under anaerobic conditions fermentation is the only mode of ATP synthesis, resulting in low biomass yields (ca. 0.1 g biomass-g glucose⁻¹; Verduyn, 1991). A much higher biomass yield (ca. 0.5 g biomass-g glucose⁻¹) is reached during fully respiratory growth. In *S. cerevisiae*, this situation can only be achieved under fully aerobic, sugar-limited conditions and at relatively low growth rates (Käppeli, 1986; Postma *et al.*, 1989). Even under aerobic conditions however, alcoholic fermentation sets in immediately upon transition from glucose limitation to glucose excess (Fiechter *et al.*, 1981; Van Dijken and Scheffers, 1986). During this process, not only ethanol, but also acetate accumulates in the cultures (Van Urk *et al.*, 1988).

The branching point between respiratory and fermentative sugar metabolism is located at the level of pyruvate (Fig.1). Respiratory dissimilation of glucose requires that pyruvate, the product of glycolysis, is converted into acetyl-coenzyme A, the fuel of the TCA-cycle. Formation of acetyl-coenzyme A can occur via two pathways: directly via the pyruvate-dehydrogenase complex, or indirectly via the concerted action of the enzymes pyruvate decarboxylase, acetaldehyde dehydrogenase and acetyl-coenzyme A synthetase, a metabolic sequence known as the pyruvate dehydrogenase bypass (Pdh bypass; Holzer and Goedde, 1957; Pronk *et al.*, 1994b). This Pdh bypass partially overlaps with alcoholic fermentation: pyruvate decarboxylase is a key enzyme in both pathways (Fig.1). The regulation of the two pathways leading from pyruvate to acetyl-coenzyme A may have a significant effect on the distribution of pyruvate over fermentation and respiration (Holzer and Goedde, 1957).

In addition to its participation in the Pdh bypass, acetyl-coenzyme A synthetase plays a crucial role in the metabolism of ethanol and acetate (Fig. 1). Growth of *S. cerevisiae* on these two carbon sources requires the operation of the glyoxylate cycle and gluconeogenesis (Haarasila and Oura, 1975). In *S. cerevisiae*, key enzymes of these pathways are subject to

![Figure 1](Image)

Figure 1. Role of acetyl-CoA synthetase in sugar and ethanol metabolism by *Saccharomyces cerevisiae*. Numbered reactions are catalysed by the following enzymes: 1, pyruvate dehydrogenase complex (Pdh); 2, pyruvate decarboxylase (Pdc); 3, acetaldehyde dehydrogenase (Ald); 4, acetyl-coenzyme A synthetase (Acs); 5, alcohol dehydrogenase (Adh).
strict regulation and absent when fermentable carbon sources are available (Herrero et al., 1985; Gancedo, 1971).

The present study is focused on the regulation of acetyl-coenzyme A synthetase. S. cerevisiae contains two structural genes, ACS1 and ACS2, that each encode an active acetyl-coenzyme A synthetase (Van den Berg and Steensma, 1995). The enzyme encoded by the ACS1 gene appears to be predominantly responsible for the acetyl-coenzyme A synthetase activity found under respiratory growth conditions, whereas the ACS2-encoded isoenzyme is constitutively expressed at low levels (Van den Berg et al., 1996). Transcription of ACS1, but not of ACS2, is subject to rapid glucose repression (Kratzer and Schüller, 1995; Van den Berg et al., 1996). For some gluconeogenic and glyoxylate-cycle enzymes a similar rapid repression of transcription has been found to coincide with glucose-induced inactivation of the enzyme (López-Boado et al., 1987; Entian and Barnett, 1992; Minard and McAlister-Henn, 1992; Fernández et al., 1993).

The aim of the present study was to investigate whether and to what extent glucose-induced inactivation of Acs gene products occurs after exposure of S. cerevisiae to glucose excess. To this end, glucose was pulsed to ethanol-limited chemostat cultures of a wild-type S. cerevisiae strain and two isogenic deletion mutants. Acetyl-coenzyme A synthetase activity, as well as the amount of the proteins encoded by the two Acs genes, were determined in cell extracts.

Materials and methods

Yeast strains and maintenance-The Saccharomyces cerevisiae strains used in this study are derived from the homozygous, prototrophic wild-type strain T2-3D (HO/HO ACS1/ACS1 ACS2/ACS2) (Wenzel et al., 1992b). Construction of the isogenic mutant strains GG621 (HO/HO acs1::APT1/acs1::APT1 ACS2/ACS2) and GG625 (HO/HO ACS1/ACS1 acs2::Tn5ble/acs2::Tn5ble) has been described by Van den Berg and Steensma (1995). All strains were stored as frozen stock cultures at -80°C, containing 20% (v/v) glycerol. Subcultures of these frozen stocks were maintained on YEPE agar (Difco Yeast Extract 10 g·l⁻¹; Difco Peptone 20 g·l⁻¹; ethanol 10 g·l⁻¹ and agar 18 g·l⁻¹).

Chemostat cultivation-Chemostat cultivation was performed in 2-liter fermenters (Applikon, Schiedam, The Netherlands) at a dilution rate of 0.10 h⁻¹, a temperature of 30°C and at a stirrer speed of 800 rpm. The culture pH was maintained at 5.0 by automatic addition of 2.0 N KOH via an Applikon ADI-1020 controller. The working volume of the culture was kept at 1.0 liter by removal of effluent from the middle of the culture via an electrical level controller. This set-up ensured that biomass concentrations in the effluent differed by less than 1% from those in samples directly taken from the culture. An airflow of 0.3 l·min⁻¹ through all cultures was maintained using a Brooks 5876 gas flow controller (Brooks BV., Veenendaal, The Netherlands). The dissolved-oxygen concentration was measured with an Ingold polarographic O₂-electrode and remained above 30% of air saturation. All data presented refer to carbon-limited steady-state cultures without detectable oscillations (Sonlleitner, 1991). The mineral medium was prepared according to Verdun et al. (1992). Vitamins were filter-sterilised and added after heat sterilisation of the mineral medium. Pure ethanol was added without prior sterilisation to a final concentration in the medium of 7.5
g·l⁻¹ (equal to 333 mmol C·l⁻¹).

**Pulse experiments**—After ethanol-limited steady state cultures had been obtained at a dilution rate of 0.10 h⁻¹, a sterile glucose solution was pulsed into the fermenter by means of a syringe to give an initial concentration of 100 mM. Throughout the pulse experiments the medium flow into the fermenter and the removal of effluent were continued. The effluent was cooled on ice immediately after leaving the fermenter. At appropriate intervals, 10-15 ml samples were collected from the effluent line and used for metabolite determination and preparation of cell extracts. To investigate the steady state turnover of acetyl-coenzyme A synthetase, cycloheximide (10 mg·l⁻¹) was pulsed into ethanol-limited steady state cultures. During these control experiments, both the effluent and the medium pump were switched off and samples for preparation of cell-free extracts were taken every 15 minutes directly from the culture.

**Determination of culture dry weight**—Dry weights of culture samples (10.0 ml) were determined using nitrocellulose filters (pore size 0.45 μm; Gelman Sciences, USA). After removal of the medium by filtration, the filters were washed with demineralised water and dried in an R-7400 Magnetron Oven (Sharp Inc., Japan) for 20 minutes. Parallel samples varied by less than 1%.

**Substrate and metabolite analysis**—The glucose concentrations in the reservoir media and culture supernatants were determined with the Merck glucose-oxidase kit (no. 14143). Ethanol was assayed colorimetrically with an alcohol oxidase/peroxidase kit (BIRD Engineering, Schiedam, The Netherlands). Acetate was assayed by using the Boehringer acetic acid kit (no. 148261).

**Preparation of cell-free extracts and protein determination**—Cells from steady-state cultures or cells obtained during the pulse experiments (appr. 50 mg dry weight) were harvested by centrifugation at 5,000x g for 10 min, washed once with 100 mM potassium-phosphate buffer (pH 7.5, 4°C) and resuspended in 100 mM potassium-phosphate buffer, pH 7.5, containing 2 mM MgCl₂ and 1 mM dithiothreitol. The extracts were prepared immediately after washing by sonication of the cells with 0.7-mm diameter glass beads at 0°C for 2 min using an MSE sonicator (150 W output, 8 μm peak-to-peak amplitude). Whole cells and debris were removed by centrifugation at 20,000xg (20 min at 4°C). The clear supernatant was used for enzyme assays and Western blotting. Protein concentrations were determined by the Lowry method. Dried bovine serum albumin (fatty-acid free, Sigma) was used as a standard.

**Enzyme assays**—Enzyme assays were performed in a Hitachi model 100-60 spectrophotometer at 30°C with freshly prepared extracts. Reaction rates, corrected for endogenous rates, were proportional to the amount of extract. When necessary, extracts were diluted in sonication buffer. The assays were performed at 340 nm (E₃₄₀ nm of reduced pyridine-dinucleotide cofactors = 6.3 mM·cm⁻¹), except for isocitrate lyase. This enzyme was measured at a wavelength of 324 nm (E₃₂₄ nm of glyoxylate phenylhydrazone = 17.0 mM·cm⁻¹). All enzyme activities are expressed as μmol of substrate converted min⁻¹·(mg protein)⁻¹. The specific acetyl-coenzyme A synthetase activity was determined in a reaction mixture containing per ml: Tris-buffer (pH 7.7), 100 μmol; L-malate (pH 7.7), 10 μmol; coenzyme A, 0.2 μmol; ATP (pH 7.5), 8 μmol; NAD, 1 μmol; MgCl₂, 10 μmol; malate dehydrogenase (3 U); citrate
synthase (0.4 U), and cell-free extract. The reaction was started with 100 μmol potassium acetate. In order to discriminate between the activity of ACS1 and ACS2 gene products in the wild-type, some assays were performed with 1 μmol potassium acetate instead of 100 μmol. At this concentration Acs1p operates near saturation, whereas Acs2p operates far below its Vmax (Van den Berg et al., 1996). Fructose-1,6-bisphosphatase (EC 3.1.3.11) and isocitrate lyase (EC 4.1.3.1) were determined as described by De Jong-Gubbels et al. (1995).

SDS-PAGE and Western Blotting: Protein samples for SDS-PAGE and Western blot analysis were loaded on basis of equal protein concentration (20 μg). Samples were dissolved by boiling for 5 min in sample buffer (Sambrook et al., 1989). The proteins were separated on a 5% stacking and 10% running SDS-PAGE gel (Sambrook et al., 1989). Blotting was done for 1.5 h at 0.8 mA cm−² onto polyvinylidene difluoride membranes (Boehringer Mannheim) by a multiphor II Novabloot unit (Pharmacia), using standard buffers. Before use, the membranes were equilibrated for 15 s in methanol and flushed with water. Blocking was done overnight in TBST (50 mM Tris, pH 8.0; 0.3 M NaCl; 0.5% Tween20) with 1% blocking reagent (Boehringer Mannheim, Chemiluminescence Western Blotting Kit). After blocking, a 45 min incubation with 1:1000 diluted acetyl-coenzyme A synthetase rabbit antiserum in TBST with 0.5% blocking reagent followed. The antiserum, raised against purified Methanobatrachus soehngenii acetyl-coenzyme A synthetase (Jetten et al., 1989), was donated by W.M. de Vos (Agricultural University, Wageningen) and gave a single band on Western blots with a molecular weight as was expected for S. cerevisiae acetyl-coenzyme A synthetase. Subsequently, the membranes were washed three times for 15 min with TBST and incubated for 45 min with anti-mouse/anti-rabbit IgG-peroxidase (Boehringer Mannheim) in TBST plus 0.5% blocking reagent. Five final washes with TBST preceded the detection step, which was carried out according to the instructions of the manufacturer. Films (Kodak X-Omat AR) were exposed for approximately 2 min. All steps were performed at room temperature.

Results

Short term regulation of acetyl-coenzyme A synthetase in wild-type Saccharomyces cerevisiae: To investigate a possible role of glucose-induced inactivation in the regulation of acetyl-coenzyme A synthetase in S. cerevisiae, the wild-type strain T2-3D was grown in ethanol-limited, aerobic chemostat cultures (D = 0.10 h−¹). Under these conditions, both Acs genes are transcribed (Van de Berg et al., 1996). Exposure of these ethanol-limited cultures to excess glucose resulted in the onset of aerobic alcoholic fermentation, accompanied by the accumulation of acetate (Fig. 2A).

Figure 2. Effect of a 100 mM glucose pulse to an aerobic, ethanol-limited chemostat culture (D = 0.10 h−¹) of the S. cerevisiae wild-type strain T2-3D (A) Glucose, ethanol and acetate concentrations. (B) Activities of acetyl-coenzyme A synthetase, assayed with either 1 mM potassium acetate (○) or 100 mM potassium acetate (●), isocitrate lyase (□) and fructose-1,6-bisphosphatase (■). Initial activities were set at a 100% and were 0.76 U mg protein−¹ (1 mM, ○) and 1.05 U mg protein−¹ (100 mM, ○) for acetyl-coenzyme A synthetase, 0.14 U mg protein−¹ for isocitrate lyase (□) and 0.06 U mg protein−¹ for fructose-1,6-bisphosphatase (■). The dotted line represents the theoretically calculated dilution of the enzyme as a consequence of growth of the biomass.
Enzyme activity assays in cell extracts confirmed that isocitrate lyase and fructose-1,6-bisphosphatase (FBPase) were inactivated after glucose addition (Fig. 2B). Isocitrate lyase inactivation showed clear, linear inactivation kinetics, with approximately 20% of the original activity remaining 3 hours after the addition of glucose, consistent with earlier reports (Ordiz et al., 1995; 1996; López-Boado et al., 1987). Rapid, virtually complete inactivation of FBPase was achieved in the first 30 minutes after the pulse, again consistent with earlier reports (Gamo et al., 1994; Schork et al., 1994; 1995).

The acetyl-coenzyme A synthetase activity in the steady-state, ethanol-limited chemostat culture was 1.05 U·mg protein⁻¹. This activity declined linearly with time after exposure of the culture to excess glucose (Fig. 2B). Although the decline of the enzyme activity was less steep than in the case of isocitrate lyase, it could not be explained from dilution of the enzyme by growth of the biomass (dotted line, Fig. 2B). This suggested that either one or both Acs gene products are subject to glucose-induced inactivation.

As the two Acs proteins have different kinetic properties, it is possible to estimate the relative contribution of each enzyme by varying the acetate concentration in the enzyme assay (Van den Berg et al., 1996; see Materials and methods). Using 1 mM acetate as a substrate, thereby predominantly measuring the activity of Acs1p, the decline of the enzyme activity was steeper than when the standard assay was used (which contains 100 mM acetate and also measures the full activity of Acs2p). This preliminary experiment suggested that at least Acs1p is subject to glucose-induced inactivation. Confirmation of this conclusion was sought in experiments with isogenic deletion mutants in which only a single Acs gene was expressed.

Acs1p is subject to glucose-induced inactivation and degradation-To further investigate glucose-induced inactivation of Acs1p, the acs2 disruption mutant GG625 was grown in ethanol-limited continuous cultures at a dilution rate of 0.10 h⁻¹. In these cultures, expressing only Acs1p, the acetyl-coenzyme A synthetase activity was 1.10 U·mg protein⁻¹. Disruption of ACS2 did not significantly affect the pattern of metabolite formation after a glucose pulse. In particular, it is interesting to note that disruption of ACS2 did not result in an increased excretion of acetate (Fig. 3A).

A linear decrease of Acs1p activity was observed in cell extracts prepared from samples taken after the glucose pulse. After 180 min only 20% of the initial activity remained. The inactivation kinetics of Acs1p were similar to those of isocitrate lyase (Fig. 3B). Also in this strain, FBPase was rapidly inactivated after glucose addition. Control experiments in which 10 mg·l⁻¹ cycloheximide was added to steady-state cultures showed that inactivation of Acs1p activity after a glucose pulse was not due to an inherent instability of the protein or the presence of specific proteases at zero time (data not shown).

Figure 3. Effect of a 100 mM glucose pulse to S. cerevisiae GG625 (ACS1Δacs2). (A) Glucose, ethanol and acetate concentrations. (B) Activities of acetyl-coenzyme A synthetase (encoded by ACS1), assayed with 100 mM potassium acetate (○), isocitrate lyase (■) and fructose-1,6-bisphosphatase (□). Initial activities were set at a 100% and were 1.10 U·mg protein⁻¹ for acetyl-coenzyme A synthetase (○), 0.14 U·mg protein⁻¹ for isocitrate lyase (■) and 0.06 U·mg protein⁻¹ for fructose-1,6-bisphosphatase (□). The dotted line represents the theoretically calculated dilution of the enzyme as a consequence of growth of the biomass. (C) Western blots of cell-free extracts after the glucose pulse, using antisera raised against purified Methanotherix soehngenii acetyl-coenzyme A synthetase in rabbits (Jetten et al., 1989). Time intervals at which samples were taken indicated above each lane.
To investigate whether inactivation of Acslp was accompanied by degradation of the protein, its levels were monitored by immunoblotting of cell extracts prepared from the samples taken after the glucose pulse. Using an antiserum raised against acetyl-coenzyme A synthetase of the methanogenic bacterium *Methanothrix soehngenii* (Jetten *et al.*, 1989), which gave a specific reaction with the *S. cerevisiae* acetyl-coenzyme A synthetases, it became clear that the amount of Acslp decreased after the pulse (Fig. 3C).

**Acslp is not inactivated in the presence of glucose**—Previous experiments showed that acsl disruption strains, in which ACS2 is the only functional Ac gene, grow well on glucose, ethanol and acetate (Van den Berg and Steensma, 1995). Although this already suggests that glucose-induced inactivation of Acslp does not occur, we nevertheless investigated the influence of excess glucose on activity of Acslp. To this end, the acsl disruption strain GG621 was grown in ethanol-limited continuous cultures at a dilution rate of 0.10 h⁻¹. Although the acetyl-coenzyme A synthetase activity in this strain (0.30 U·mg protein⁻¹) was lower than in the wild-type (1.05 U·mg protein⁻¹) the pattern of metabolite formation was the same: approximately 100 mM ethanol and 1 mM acetate were produced within 180 min (Fig. 4A). Apparently, also disruption of ACS2 did not cause any significant effects on the accumulation of acetate in the cultures. Activity of Acslp only marginally decreased, to approximately 80% of its initial activity 3 hours after the addition of glucose (Fig. 4B). Immunoblotting confirmed that Acslp was not substantially degraded (Fig. 4C). The inactivation kinetics of isocitrate lyase and FBPase were similar to those in the wild-type. Inactivation of these C2-metabolising enzymes therefore seems not to be influenced by disruption of ACS1.

**Discussion**

Experiments with acsl disruption mutants of *Saccharomyces cerevisiae* have shown that the requirement for acetyl-coenzyme A synthetase during growth on glucose, ethanol and acetate can be met by each of the two structural genes ACS1 and ACS2 (Van den Berg and Steensma, 1995; Van den Berg *et al.*, 1996). Nevertheless, transcriptional regulation of these two structural genes differs strongly (Van den Berg *et al.*, 1996). Furthermore, as shown in this paper, the regulation of the two isoenzymes at the level of enzyme activity is strikingly different (Fig. 3B and 4B).

**ACS1** and its product exhibit a combination of strong glucose catabolite repression

**Figure 4.** Effect of a 100 mM glucose pulse to *S. cerevisiae* GG621 (Δacs1 ACS2). (A) Glucose, ethanol and acetate concentrations. (B) Activities of acetyl-coenzyme A synthetase (encoded by ACS2), assayed with 100 mM potassium acetate (O), isocitrate lyase (■) and fructose-1,6-bisphosphatase (□). Initial activities were set at a 100% and were 0.30 U·mg protein⁻¹ for acetyl-coenzyme A synthetase (O), 0.14 U·mg protein⁻¹ for isocitrate lyase (■) and 0.06 U·mg protein⁻¹ for fructose-1,6-bisphosphatase (□). The dotted line represents the theoretically calculated dilution of the enzyme as a consequence of growth of the biomass. (C) Western blots of cell-free extracts after the glucose pulse, using antisera raised against purified *Methanothrix soehngenii* acetyl-coenzyme A synthetase in rabbits (Jetten *et al.*, 1989). Time intervals at which samples are taken indicated above each lane.
(Kratzer and Schüller, 1995; Van den Berg et al., 1996) and glucose catabolite inactivation. Similar dual regulation systems have been found for a number of other S. cerevisiae enzymes, including key enzymes of gluconeogenesis, glyoxylate cycle and membrane transport of non-glucose sugars (Mercado et al., 1991; Fernández et al., 1993; Medintz et al., 1996). A differential regulation of isoenzymes is not unique for ACS1 and ACS2. For example, the malate dehydrogenase isoenzyme encoded by the MDH2 gene is subject to glucose-induced inactivation, whereas the MDH1 and MDH3 encoded isoenzymes are not (Minard and McAlister-Henn, 1992; McAlister-Henn et al., 1995).

In some cases, a strong down-regulation by glucose of enzyme activities that are not involved in glucose metabolism may prevent the occurrence of futile cycles or less efficient modes of metabolism. For example, the simultaneous expression of glycolytic enzymes and gluconeogenic enzymes can, under some growth conditions, lead to futile cycling of ATP (Navas et al., 1993; Wills, 1990). Such a teleological explanation is not readily available in the case of ACS1. First of all, acetyl-coenzyme A synthetase is an essential enzyme during growth on glucose (Flikweert et al., 1996) and secondly, the ACS2 gene continues to produce an active enzyme, even in the presence of glucose (Van den Berg and Steensma, 1995; Van den Berg et al., 1996). An alternative explanation for the tight regulation of ACS1 might be connected to a need to control the intracellular acetyl-coenzyme A/coenzyme A ratio in different subcellular compartments; it has been reported that disturbances in this parameter may negatively affect growth (Wenzel et al., 1992b).

At present, literature data on the localisation of acetyl-coenzyme A synthetase in S. cerevisiae are not consistent. Based on subcellular fractionation studies, different research groups have reported that the enzyme activity was either (partly) associated with mitochondria and microsomes (Klein and Jahnke, 1971; Klein and Jahnke, 1968) or that it was exclusively cytosolic (Kispal et al., 1991). The carboxyterminal VKL amino-acid sequence of Acslp has been identified as a potential microbody targeting sequence, whereas no clear targeting signals have been identified in the ACS2 sequence (de Hoop and AB, 1992; Van den Berg and Steensma, 1995). If the ACS1 product is indeed located in microbodies, this might offer an explanation for its inactivation: S. cerevisiae microbodies are known to be degraded in the presence of excess glucose (Chiang et al., 1996).

The inactivation pattern of Acslp and that of isocitrate lyase exhibit a striking similarity. Isocitrate lyase in S. cerevisiae has originally been reported in microbodies (Szabo and Avers, 1969), similar to the situation in many eukaryotic organisms (Levy, 1970). This would fit with the assumption that inactivation of Acslp is a direct consequence of its (putative) subcellular localisation in microbodies. However, other reports indicate that in S. cerevisiae, isocitrate lyase may be located in the cytosol, which sheds some doubt on this hypothesis (Duntze et al., 1969; McCammon et al., 1990; Taylor et al., 1996). Further work to establish the subcellular compartmentation of the acetyl-coenzyme A synthetase isoenzymes and isocitrate lyase in S. cerevisiae is clearly required.

In isocitrate lyase, an internal decapeptide between amino acids 37-46 has been implicated in glucose-induced inactivation (Ordiz et al., 1995). This sequence exhibits a weak (40 %) identity with the positions 22-31 in Acslp. Molecular analysis of the glucose-induced inactivation of Acslp is needed to find out if this weak homology is significant. Recently, cAMP-dependent phosphorylation of a specific sequence in the lcllp was also shown to be involved in glucose-induced inactivation (Ordiz et al., 1996). Several of such protein kinase recognition sites (Kemp and Pearson, 1990) can be detected in the deduced amino acid
sequence of Acs1p, however, it is unknown whether these sequences are functional.

At least theoretically, the kinetic and regulatory properties of acetyl-coenzyme A synthetase might have an important impact on the production of acetate that is invariably observed after exposure of aerobic, respiring *S. cerevisiae* cultures to excess glucose (Van Urk *et al.*, 1988; this study). However, the level of acetate accumulation of wild-type *S. cerevisiae* and the two *acs* disrupted strains did not differ significantly. Furthermore, in a strain expressing only *ACSI*, the rate of acetate production after a glucose pulse did not increase substantially as acetyl-coenzyme A synthetase became progressively inactivated. Similar results were obtained when glucose pulses were applied to glucose-limited chemostat cultures rather than ethanol-limited cultures (data not shown). These results indicate that inactivation of Acs1p is not a key mechanism responsible for the excretion of acetate during short-term exposure of *S. cerevisiae* to excess glucose.

**Acknowledgements**

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Northern blot analysis of 31 genes involved in acetyl-coenzyme A metabolism of *Saccharomyces cerevisiae* defines five putative regulatory elements

Marco A. van den Berg and H. Yde Steensma
Submitted for publication (1997)
Summary

Genes encoding proteins involved in the same metabolic pathway are often co-regulated, usually via common promoter sequences. Transcription of 31 genes of *Saccharomyces cerevisiae* was analysed during a transient response of a glucose pulse to a glucose-limited chemostat culture. In this way it was possible to determine the mRNA levels during the subsequent excess glucose-, ethanol- and acetate-phases, while external conditions as pH were kept constant. With this set-up we could distinguish between regulation by ethanol and regulation by acetate, something which is not possible using batch cultures. Here, we studied the regulation of genes involved in acetyl-coenzyme A metabolism.

To identify regulatory elements in promoters, genes with identical regulation patterns were grouped. They could be divided in four classes: no transcription under the conditions investigated, no regulation by glucose, induction by glucose and repression by glucose. Sequence alignment of genes with similar regulation patterns revealed five new, putative regulatory promoter elements. i. The glucose-inducible fermentation genes *PDC1* and *ADH1* share the sequence ATACCTTCSTT. ii. Acetate-repression might be mediated by the decamer CCCGAGRGGA, present in the promoters of *ACS2* and *ACR1*. iii. A specific element for the glyoxylate cycle was present in seven genes studied: *CIT2*, *ICL1*, *MLS1*, *MDH2*, *CAT2*, *ACR1* and *ACH1*. These genes were derepressed on ethanol or acetate. Their common promoter sequence, BCCWITSRNCCG, was also found in the promoters of *HAP1* and three ORFs with unknown function. iv. The sequence ACGTSCRGAATGA was found in the promoters of the partially ethanol-repressed genes *ACS1* and *YAT1*. v. Ethanol-induction, as seen for *ACS2* and *MDH1*, might be mediated via the sequence CGGSGCCGRAG.
Introduction

Carbon source dependent regulation is an important parameter in the transcription of genes. The best studied process in *Saccharomyces cerevisiae* is that of glucose repression. While this yeast is growing on excess glucose, genes involved in the assimilation of less-favoured carbon sources are repressed. These genes will be derepressed or even induced by the appropriate substrate when glucose is completely consumed (for reviews see Wills, 1990; Gancedo, 1992; Entian and Barnett, 1992; Ronne, 1995). Carbon source dependent regulation of several enzymes in the cells can redirect carbon fluxes and thereby influence metabolism. Our laboratory studies the interface between glycolysis and citric acid cycle, where respiratory and fermentative routes diverge. Acetyl-coenzyme A, the main fuel of the citric acid cycle, can be formed directly by the pyruvate dehydrogenase complex (Pdh) or indirectly by the so-called Pdh bypass (Fig. 1). The first step of this bypass, pyruvate decarboxylase

![Diagram of metabolic pathways](image)

**Figure 1.** Schematic overview of metabolism (pyruvate metabolism, glyoxylate cycle and C2-metabolism). Enzymes shown are pyruvate dehydrogenase, Pdh (1); pyruvate decarboxylase, Pdc (2); alcohol dehydrogenase, Adh (3); acetaldehyde dehydrogenase, Ald (4); acetyl-coenzyme A synthetase, AcS (5); citrate synthase, Cit (6); aconitase, Aco (7); isocitrate lyase, Icl (8); malate synthase, Mls (9); malate dehydrogenase, MdH (10). Included in this study, but not shown here, are acetyl carnitine transferase, Cat; acetyl-coenzyme A synthetase regulator, Acr; acetyl-coenzyme A hydrolase, Ach; a putative glyoxylate protein regulator, Fun34p.
(Pdc), is also part of alcoholic fermentation, which sets in under oxygen-limited conditions or during aerobic glucose excess (the Crabtree effect). A recent study showed that the direct route is not essential and can be completely replaced by the indirect three-step route (Pronk et al., 1994). However, such mutants are partially leucine-deficient, probably due to a change in the mitochondrial acetyl-coenzyme A:coenzyme A ratio (Wenzel et al., 1992). Blocking the Pdh bypass is thought to cause a shortage in cytosolic acetyl-coenzyme A, which is used for lipid synthesis (Flikweert et al., 1996). Disruption of acetyl-coenzyme A synthetase (Acs) is lethal (Van den Berg and Steensma, 1995). Their cellular toxicity further supports the importance of the internal (acetyl-) coenzyme A pools (Lee et al., 1990, 1996; Brass, 1994).

Recent studies showed that the two genes encoding acetyl-coenzyme A synthetase, ACS1 and ACS2, have distinct regulation patterns (Van den Berg et al., 1996). Many of the enzymes involved in the pathways shown in Fig. 1 are encoded by more than one gene. For some it is known that the isogenes are differentially regulated and/or encode proteins with a different cellular localisation (Rosenkrantz et al., 1986; Hartig et al., 1992; Steffan and McAllister-Henn, 1992). Detailed transcription studies are mostly done with one gene of interest (Wenzel et al., 1993a, 1994). Over the years many data have been generated from such studies (reviewed by Verdier, 1990; Svetlov and Cooper, 1995). Nevertheless, even when using these data, it is difficult to find common motifs (Fig. 2). For instance, included in this study are the genes encoding the glyoxylate cycle proteins. In batch cultures these genes are repressed by glucose and derepressed on non-fermentable carbon sources (Hartig et al., 1992; Fernández et al., 1993; Schöller and Schüller, 1994). Despite the vast amount of data, a common derepressing element, like Hap2/3/4 (Heme Activator Protein) or CSRE (Carbon Source Responsive Element), is not present in the promoters of the glyoxylate cycle genes (Fig. 2). Even the two genes which are specific for the cycle, ICL1 and MLS1, share no common regulatory promoter elements. So, they are either regulated via elements which have not been found yet, or, more likely, a high degree of freedom is allowed in the sequence of the elements involved.

Most studies have been performed in shake flask batch cultures. In such experiments varying values for pH and oxygen concentration may influence the transcription of genes. Recent work showed that carbon pulses to carbon-limited chemostat cultures are an excellent way to study carbon-source dependent transcription of genes (Sierksstra et al., 1992a; Van den Berg et al., 1996). Therefore, glucose was pulsed into a glucose-limited chemostat culture. With this set-up not only the effect of glucose on transcription could be studied, but also the effect of ethanol and acetate, both transiently formed during the consumption of glucose (Van Urk et al., 1988). By comparing promoters of genes with the same transcriptional pattern, we hoped to detect common sequences which might be new regulatory elements.

Materials and methods

Strain and cultivation-Saccharomyces cerevisiae wild-type T2-3D (HO/HO) was cultivated in carbon-limited chemostat cultures (D=0.1 h⁻¹, pH=5.0, 30°C), with 10 g l⁻¹ glucose as carbon source (Van den Berg et al., 1996). Glucose pulse experiments (adition of 75 mM of glucose) were performed as described previously (Van den Berg et al., 1996). Throughout the pulse experiments, the medium flow in to the fermenter and the removal of effluent was continued.
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Table 1. Genes and PCR oligonucleotides. The position of the 5' ends of the oligonucleotides are relatively to the ATG (+1). They are shown to left from 5' to 3'. The fragment of ADH2 was PCR-amplified, gel-purified and digested with Sau3AI. The digestion-mix was separated on gel and the 325 bp fragment was used as a 3' probe. Homologies shown are the highest among each set of isogenes. GPR1 is a gene from the yeast *Yarrowia lipolytica* (Barth et al., 1993).
Figure 2. Promoter analysis and codon bias index. Thousand basepairs upstream of each coding region were analysed (except for *ALD1*, see remark in Materials and methods). Analysis was done with the GCG-package (Deveraux, 1984). The following known promoter-elements are shown: O Hap2/3/4 (Heme Activator Protein); ▼ Abf1 (ARS Binding Factor); ▲ Rapl (Repressor Activator Protein); ● CSRE (Carbon Source Responsive Element); T, T-element; □ STRE (Stress Responsive Element); ▼ Adr1h (Alcohol Dehydrogenase Regulation, half site); ▼ Mig1 (Multicycopy Inhibitor of GAL gene expression); UAS (Upstream Activating Sequence); URS (Upstream Repressing Sequence) (Verdier, 1991; Svetlov and Cooper, 1995; Kellerman and Hollenberg, 1988; Butler and McConnel, 1988; Liao et al., 1991; Schöler and Schüller, 1994; Reardon et al., 1995). The codon bias index (CBI) is shown on the right of each gene (Sharp and Cowe, 1991).

Northern blot analysis—Total RNA was isolated and analysed by Northern blotting as described (Van den Berg et al., 1996). During the pulse experiments samples were collected, rapidly mixed with phenol and stored at -80 °C. When all samples were collected, the final steps of RNA isolation were performed. Northern blots were hybridised to Polymerase Chain Reaction (PCR) derived probes. The PCR reactions were done on total chromosomal yeast DNA with Goldstar DNA polymerase (Eurogentec) at the conditions suggested by the supplier. The products were purified using low-melting-point agarose (BRL, Bethesda), cut out and directly used for labelling (Oligolabelling Kit, Pharmacia). The PCR primers used are listed in Table 1. The fragment to be amplified was selected to avoid cross-hybridisation to the isolate(s). Special attention was given to *ADH1-ADH2* and *PDC1-PDC5*, both combinations are highly homologous. Therefore, fragments either 5' or 3' to the ORFs were amplified, so the probes would hybridise to the untranslated regions of the mRNA (Table 1). A larger fragment of *ADH2* was amplified and digested with *Sau3AI*. This enzyme cuts directly after the stop-codon and yields a 325 bp fragment 3' of the ORF, which was used as a probe.

Quantification of mRNA was done with a Shimadzu TLC Scanner CS-930 and a Phosphor Imager System (Molecular Dynamics, B&L Systems, Maarssen, The Netherlands). The levels of mRNA were calculated relative to the level of *PDA1* mRNA.

Data analysis—All sequence data used in this study were obtained from the *S. cerevisiae* genome sequence project (MIPS, http://speedy.mips.biochem.mpg.de), except for *ALD1* (Saigal et al., 1991), for which no match within the final genome sequence was possible (C. Ball, SGD, http://www.genome.stanford.edu/ Saccharomyces, personal communication). The ORFs YPL080c, YCR105w, YPR001w, YPR005c and YJL200c showed high homology with known genes, so they were included in this study and tentatively called *PDC7, ADH6, CIT3, ICL2, and ACO2*, respectively. The gene names *ICL2* and *ACO2* have recently been also used by other groups (Heinisch et al., 1996; Velot et al., 1996). One-thousand nucleotides upstream of each gene were screened, assuming that these contain all regulatory promoter elements, using the GCG-package (Deveraux, 1984). The list of regulatory elements used was a compilation of recent reviews (Verdier, 1990; Svetlov and Cooper, 1995) and reported elements (Kellerman and Hollenberg, 1988; Butler and McConnel, 1988; Liao et al., 1991; Schöler and Schüller, 1994; Reardon et al., 1995). The codon bias index was calculated according to Sharp and Cowe (1991), however both codons for asparagine were scored as optimal.
Results and Discussion

Studies on the transcriptional regulation of genes are mostly done with shake flask batch cultures (see for examples Denis et al., 1983; Hartig et al., 1992; Rosenkrantz et al., 1994; Schöller and Schüller, 1994). External conditions, as pH, might fluctuate in such cultures and therefore influence the transcription of genes. Recently, it was shown that carbon-limited chemostat cultures are an excellent tool in analysing gene transcription during steady-state and transient conditions at a constant pH (Sierkstra et al., 1992ab; Wenzel et al., 1993; Van den Berg et al., 1996). This is the first attempt to use this tool in analysing the transcriptional responses of a large set of genes. Although it was still a very complex situation (e.g. there was a continuous glucose feed during the pulse experiment) it was possible to group genes according to their transcriptional regulation pattern. By expanding this kind of analysis to more genes and more conditions it should be an excellent addition to the analysis of yeast genes (with unknown function).

Thirty-one genes encoding enzymes (in)directly involved in acetyl-coenzyme A metabolism of *Saccharomyces cerevisiae* (Table 1) were studied. They are part of several pathways: alcoholic fermentation, ethanol- and acetate-consumption, the Pdh bypass and the glyoxylate cycle (Fig. 1). Glucose was pulsed into a glucose-limited chemostat culture to a final concentration of 75 mM. During the transient response five phases could be distinguished according to the excess carbon source present: glucose, ethanol, ethanol/acetate, acetate and none (Fig. 3A). The yeasts consumed the excess glucose during the first 160

![Figure 3. Carbon source dependent regulation of gene transcription. A. A 75 mM glucose pulse was added to a glucose-limited steady state culture. Samples were taken for metabolite analysis. B. The pulse is divided in five stages, each with different excess carbon. Several samples were taken within each stage of the pulse for Northern analysis. Some typical transcriptional regulation patterns are presented. Not shown are the Northerns with PDC6, PDC7, ADH4, ADH6, ALD1, CIT3, ICL2 (all seven with no or very low transcription), DAL7, MDH3 (both more or less constant), ACS1 (comparable to YAT1), ICL1, MDH2, CAT2 (all as MLS1), PDC1, ADH1 (both glucose-induced), ADH3, CIT1, CIT2 and ACO1 (all four like PDC5).](image-url)
Table 2. mRNA levels during the transient response of a glucose pulse to a glucose-limited chemostat culture. The transient experiment, as shown in Fig. 3A, was analysed using five stages: Glucose-excess, 0-160 min; Ethanol-excess, 170-330 min; Ethanol/Acetate-mixture, 340-380; Acetate-excess, 390-420 min; no excess carbon, 480 and 720 min. All measurements have been normalised to the level of PDA1 mRNA. The transient response is not given in real values as this would make a good interpretation of the general patterns more difficult. ++, strong induction (8 to 40x); +, good induction (2 to 8x); +, small induction (appr. 2-fold); =, no change (steady state level); -, repression; --, strong repression (upto complete block); nq, not quantitated

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minutes, while producing ethanol and acetate. This was followed directly by the consumption of ethanol (170-300 min), which led to additional acetate production. Ethanol and acetate were consumed simultaneously, when the ethanol concentration was around 10 mM (330-370 min). Finally, acetate was consumed (380-420 min). At 480 and 720 minutes there was no excess carbon present. In the present set up we chose to continue the medium feed throughout the experiment, thereby keeping the volume constant.

Samples collected during the pulse were analysed for mRNA levels. Several transcriptional patterns could be distinguished. Some examples of Northern blots are depicted in Fig. 3B and the data are summarised in Table 2. One of the most stably expressed genes, as
compared to the level of 35S rRNA, was PDA1 (Fig. 3B). In several previous studies the PDA1 gene, encoding the Elα subunit of the pyruvate dehydrogenase complex, was also shown to be transcribed at a constant level (Sierkstra et al., 1992b; Wenzel et al., 1993, 1994, 1995). Seven other genes showed a comparable, relatively constant mRNA level: PDC5 (only a slight increase in mRNA during the pulse), ADH3, ADH5, (see remark for PDC5), ADH6, ALD1, DAL7 and MDH3. Five genes gave no visible transcript under the conditions employed: PDC6, PDC7, ADH4, ICL2 and CIT3 (Southern analysis was used as a control for the probes, data not shown). The 18 other genes were regulated by the three carbon sources tested. A small group of genes was induced on glucose: PDC1, ADH1 and ACO2. ACS2 and MDH1 were both induced on ethanol. Four genes were derepressed on acetate: ADH2, ACS1, YAT1 and FUN34. The remaining nine genes were all derepressed on both ethanol and acetate. These can be divided in two subgroups: complete glucose repression (ICL1, MLS1, MDH2, CAT2, ACR1 and ACH1) and no glucose repression (CIT1, CIT2 and ACO1).

Screening 1000 bp upstream of the ATG of each gene for known regulatory elements (Kellerman and Hollenberg, 1988; Butler and McConnel, 1988; Verdier, 1990; Liao et al., 1991; Schönler and Schüller, 1994; Reardon et al., 1995; Svetlov and Cooper, 1995) revealed several of these elements in each sequence (Fig. 2). However, there was no consistency within the groups with equal regulation as described above. Therefore, a sequence alignment was done within each group of equal regulation to identify common sequences. Five such sequences were found (Figs. 4-8), which will be discussed below.

**Alcoholic fermentation**—Pyruvate is converted into ethanol in two steps (Fig. 1). PDC1, the main producer of pyruvate decarboxylase in wild-type yeast (Hohmann, 1991), was induced on glucose (Table 2; Butler and McConnel, 1988). An Upstream Activating Sequence (UAS) at position -636/-535 relative to the ATG was reported to be necessary, but not sufficient for maximal glucose induction (Kellerman and Hollenberg, 1988; Butler and McConnel, 1988; Butler et al., 1990). In contrast to a recent report (Liesen et al., 1996) PDC1 was found not to be derepressed on ethanol. During the first 160 minutes of the experiment ethanol was present in considerable amounts, but PDC1 was not repressed (Table 2). Only when the excess glucose was consumed the mRNA level dropped, suggesting that the absence of inducing amounts of glucose was responsible for lower mRNA levels. Compared to 35S rRNA PDC5 was expressed at a more constant level, with a slight increase during the transient stages (Fig. 3 and Table 2). No transcript of PDC6 could be detected (Table 2), which was also reported by (Hohmann, 1991). However, in samples from acetate-limited steady state cultures a low level of mRNA was detected (data not shown). The putative PDC7 gene showed no transcription at all, which was not unexpected since its Codon Bias Index (CBI) is only 0.02, the lowest of all genes in this study.

The second step of fermentation is catalysed by alcohol dehydrogenase, an enzyme with a dual function. It is the final step in ethanol production during fermentative growth, but also the initial step in the utilisation of ethanol as a carbon source. Six Adh genes were analysed, ADH1 to 6. ADH1 was regulated in the same way as PDC1, induction by glucose, but the amount of mRNA dropped during the ethanol- and acetate-stages (Table 2; Denis et al., 1983). Again, this suggested lack of glucose induction rather than repression by ethanol. Ruohonen et al. (1995) suggested that a large region of 300 bp (-414/-700) was involved in glucose induction. Besides several elements (Fig. 2), this region contains an 11 bp sequence which is also present in the UAS of PDC1 (Fig. 4A). This element is found 73 times
A. Gene | Position | Sequence  
--- | --- | ---  
PDC1 | -628 | ATACCTTCCTT  
ADH1 | -639 | ATACCTTCCTT  
Consensus |  | ATACCTTCCTT  

B. Gene | Position | Sequence  
--- | --- | ---  
ACS2 | -497c | CCGGAGAGGA  
ACRI | -644 | CCGGAGGGGA  
Consensus |  | CCGGAGGGGA  

D. Gene | Position | Sequence  
--- | --- | ---  
ACS2 | -327 | CGGGGCCGGAG  
MDH1 | -330 | CGGGGCCGGAG  
Consensus |  | CGGGGCCGGAG

C. Gene | Position | Sequence  
--- | --- | ---  
ICL1 | -399 | TCCATTATCCCG  
MLS1 | -531c | TCCATTGAGCCG  
MDH2 | -501 | TCCATTGAGGCCG  
CAT2 | -240 | CCGGAGCCCAG  
ACRI | -811c | CCGGAGGGGA  
ACH1 | -332c | GGGGAGGGAGG  
CIT2 | -865 | TCCATTGATCCG  
Consensus |  | BCWTTGRRNCCG

B. Gene | Position | Sequence  
--- | --- | ---  
ACS1 | -491 | ACGTCCAGAATGA  
YAT1 | -366 | ACGGTGGAAATGA  
Consensus |  | ACGTSCRGAATGA

Figure 4. Sequence alignments of promoters with a common transcription pattern reveal putative regulatory elements. A. Glucose-induced promoters. Both PDC1 and ADH1 contain a common element in a region reported to be involved in glucose induction. B. Acetate-repressed promoters. Both ACS2 and ACRI show a gradual decrease in the amount of mRNA on acetate. Both promoters contain a CG-rich deamer sequence (c=complementary strand). C. C2-repressible promoters. The CSRE element of ICL1 (Schöler and Schüller, 1994) is found back in the promoters of MLS1, MDH2, CAT2, ACRI, ACH1 and CIT2, all repressed on glucose and derepressed on ethanol/acetate (c=complementary strand). D. Ethanol-inducible promoters. The promoters of ACS2 and MDH1 have a common sequence around position -330. E. Ethanol-repressed promoters. A sequence with high homology is found in the partially-ethanol repressed promoters of ACS1 and YAT1.

throughout the S. cerevisiae genome, while calculations predicted only 9 times, suggesting that the element has a specific function. The ADH3 gene might also be involved in ethanol production. It shows 73% homology with ADH1 and was expressed rather strongly (Fig. 3 and Table 2). Drewke et al. (1990) showed that a strain in which ADH1-4 were disrupted still produces ethanol, possibly via Adh5p.

In contradiction to earlier reports on experiments in shake flask batch cultures (e.g. Yu et al., 1989; Dombek et al., 1993), a faint amount of ADH2 mRNA was detected during glucose-limited and glucose-excess growth (Fig. 3B). Furthermore, no derepression or induction by ethanol was found. Ethanol even appeared to repress ADH2 transcription, since it had to disappear completely from the culture to give full transcription of ADH2 (Fig. 3B, t=370 min). Adr1p, a transcription factor which positively regulates ADH2 transcription (Yu et al., 1989, Verdone et al., 1996), is phosphorylated (and inactivated) on glucose and ethanol (Cherry et al., 1989; Vallari et al., 1992), thus corroborating our results for ADH2 transcription. Recently, Adr1p also has been implicated in the regulation of peroxisomes and peroxisomal proteins (Simon et al., 1991, 1995). Although there is no evidence for a peroxisomal function of Adh2p, it is curious that an ethanol-consuming enzyme is
derepressed on ethanol at the transcriptional level and derepressed on acetate. *ADH3*, encodes the mitochondrial alcohol dehydrogenase. The transcription was slightly elevated on non-fermentable carbon sources (Table 2; Young and Pilgrim, 1985). *ADH4* is rather distantly related to the genes discussed above. It has a high CBI, 0.63, but *ADH4* mRNA was not detected. This was also reported by Williamson and Paquin (1987). A T-element in close proximity to two Abf1 sites might mediate this silencing (Fig. 2). Drewke and Ciriacy (1988) reported transcription of *ADH4* in some brewery strains. *ADH6* is also rather distant: 44% homology to *ADH3*. A faint transcript, to low for quantification, could be detected in almost all samples tested (Table 2). It might function in the oxidation of ethanol or other alcohols as suggested by Ciriacy (1997).

**Ethanol consumption**-Ethanol is converted into acetyl-coenzyme A via three enzymes: alcohol dehydrogenase, acetaldehyde dehydrogenase and acetyl-coenzyme A synthetase (Fig. 1). Adh2p was believed to be the major ethanol consuming enzyme (Lutstorf and Megnet, 1968). However, as described above, ethanol repressed *ADH2* transcription. Another candidate for the first step in ethanol assimilation is the mitochondrial Adhp (Young and Pilgrim, 1985). The *ADH3* gene was slightly induced on ethanol (Table 2). The produced acetaldehyde can be consumed directly by the mitochondrial, NAD+-dependent acetaldehyde dehydrogenase, encoded by the *ALD1* gene (Saigal et al., 1991), or, alternatively, acetaldehyde could be transported to the cytoplasm where it will be oxidised by NADP+-dependent acetaldehyde dehydrogenase (Llorente and Nuñez de Castro, 1977). The *ALD1* gene was expressed at a constant, low level (Table 2). Acetate can be activated by acetyl-coenzyme A synthetase. *S. cerevisiae* contains two non-mitochondrial isoenzymes, Acs1p and Acs2p (Kispal et al., 1991; De Jong-Gubbels et al., 1996). *ACS2*, the gene encoding cytosolic Acs2p (De Jong-Gubbels et al., 1996), was induced on ethanol (Fig. 3).

**Acetate consumption**-Acetate is activated by acetyl-coenzyme A synthetase, which is encoded by two genes with different regulation; *ACS1* was repressed on glucose and ethanol, while *ACS2* was induced on ethanol (Fig. 3 and Table 2; Van den Berg and Steensma, 1995; Kratzer and Schüller, 1995; Van den Berg et al., 1996). *ACS1* transcription was maximal on acetate, when *ACS2* transcription was repressed (there is a slow decrease in *ACS2* transcript on acetate; Fig. 3B, t=390-420 min), therefore Acs1p most likely activates acetate, although Acs2p can also fulfill this function as was observed using acs1 mutants (Van den Berg and Steensma, 1995).

**The Pdh bypass**-The indirect route from pyruvate to acetyl-coenzyme A partially overlaps the three pathways described above. Two acetaldehyde dehydrogenases have been reported, one NAD+-dependent, mitochondrial and the other NADP+-dependent, cytosolic (Jacobsen and Bernofsky, 1974; Llorente and Nuñez de Castro, 1977). This is in contrast to Saigal et al. (1991) who found only one band reacting with antiserum using isoelectric focussing. It is not known if *ALD1* encodes either one or both of these proteins. Presumably, Ald1p acts in the mitochondrial consumption of ethanol and another protein is part of the cytosolic Pdh bypass. Since there are several ORFs of unknown function in the genome with homology to aldehyde dehydrogenases, one of these probably encodes the cytosolic acetaldehyde dehydrogenase. Remarkable is the downregulation of *ACS2* by acetate at the end of the experiment. This was also found for *ACRI* (Fig. 3), encoding an acetyl-coenzyme A synthetase regulator.
(Fernández et al., 1994). During this phase ACS1 was highly transcribed and might cause a feedback regulation on ACS2 and ACR1 transcription. Both genes share a decamer sequence in their promoter (Fig. 4B), and it will be interesting to see if this sequence mediates the negative response on acetate.

Glyoxylate cycle—This cycle incorporates the acetyl-coenzyme A generated from ethanol, acetate and β-oxidation into anabolism (Fig. 1; De Jong-Gubbels et al., 1995). The cycle was thought to be associated with peroxisomes, but only the citrate synthase and malate synthase reactions seem to be peroxisomal (Rosenkrantz et al., 1986; McCammon et al., 1990; Gangloff et al., 1990; Steffan and McAlister-Henn, 1992; Velot et al., 1996; Taylor et al., 1996). The initial step is the fusion of oxaloacetate with acetyl-coenzyme A to form citrate. Two genes have been described encoding citrate synthase: CIT1, encoding the mitochondrial isoform, and CIT2, encoding the peroxisomal isoform (Rosenkrantz et al., 1986; Lewin et al., 1990). During the ethanol and acetate stages of the pulse experiment the mRNA levels were slightly elevated (approximately 4-fold and 2-fold for CIT1 and CIT2, respectively; data not shown). This was also reported previously (Kim et al., 1986; Rosenkrantz et al., 1994). Rosenkrantz et al. (1994) identified a functional Hap2/3/4 element at -291/-273 in the CIT1 promoter, which accounted for derepression. An ORF with 54% homology to CIT1 and 52% homology to CIT2 was located at chromosome XI, which tentatively was named CIT3 (Table 1). The CBI of this gene is low, 0.08, indicating that it probably will not be transcribed in most standard laboratory experiments, which was indeed the case in this study (Table 2). It might be expressed in cit1 cit2 mutants, which still show some, although very low, citrate synthase activity (Kim et al., 1986).

The second step in the glyoxylate cycle is catalysed by aconitase. Two genes were analysed, ACO1 and ACO2 (Gangloff et al., 1990; Velot et al., 1996). In contrast to what was reported by Gangloff et al. (1990) no glucose repression of ACO1 was observed (Table 2). The Hap2/3/4 element at -290 could be involved in the elevated mRNA level during the ethanol and acetate stages (Table 2; Gangloff et al., 1990). The transcriptional regulation of ACO2 was very peculiar. First, there was a nine-fold induction by glucose (Fig. 3). During the transition from glucose to ethanol growth, ACO2 transcription rapidly declined to steady state level. Halfway through the acetate stage there was a second rapid decline in mRNA levels, which is unexplained (Fig. 3B).

Isocitrate is split into succinate and glyoxylate by isocitrate lyase. In accordance with previous studies the ICL1 gene was repressed on glucose and derepressed on ethanol and acetate (Table 2; Schöler and Schüller, 1993, 1994). Derepression is mediated via the CSRE (Carbon Source Responsive Element) at position -397/-389 (Schöler and Schüller, 1994). In contradiction to Heinisch et al. (1996) no transcription from ICL2 was detected (Table 2). However, they used a lacZ fusion to the promoter of ICL2 on a multi-copy plasmid.

Glyoxylate is fused with acetyl-coenzyme A to form malate. This step is catalysed by malate synthase, which participates in both carbon and nitrogen metabolism of S. cerevisiae. For this dual role of malate synthase the yeast has two genes, DAL7 and MLS1. DAL7 is the gene involved in nitrogen metabolism and therefore regulated by nitrogen and not carbon (Yoo and Cooper, 1989; Hartig et al., 1992; Table 2). MLS1 was repressed on glucose and derepressed on C2-compounds, exactly the same as ICL1 (Fig. 3).

The fifth and last step of the glyoxylate cycle is catalysed by malate dehydrogenase. Malate dehydrogenase was localised in three different compartments, each protein encoded
by a different gene, MDH1 (mitochondria), MDH2 (cytosol) and MDH3 (peroxisomes), respectively (Steffan and McAlister-Henn, 1992). Again a complex, but fine-tuned, system appears to be present. As is clear from the data presented in Fig. 3 and Table 2, all three genes were differentially regulated. MDH1 was repressed on glucose and induced on ethanol, but not acetate (Fig. 3B). The MDH1 promoter contains two glucose-repression mediating Mig1 sites (-666 and -505), which miss the essential upstream AT-rich element. The MDH2 promoter contains a Mig1 site at -297. MDH2 showed a regulation pattern typical of the glyoxylate cycle genes: glucose repression and C2-induction (Table 2). The third gene, MDH3, was not regulated by glucose, ethanol or acetate (Table 2). The protein is located in the peroxisomes, but was shown not to be a part of the glyoxylate cycle (Van Roermund et al., 1995). Northern blot analysis showed strong induction by oleate only (Van Roermund et al., 1995). This response is mediated via the so-called ORE (Oleate Responsive Element; Rottensteiner et al., 1996) at -341 in the promoter. Surprisingly, MDH1 and MDH2 also have such promoter elements.

Three of the glyoxylate cycle genes, ICL1, MLS1 and MDH2, showed exactly the same regulation pattern as CAT2, ACH1 and ACR1 (Fig. 3 and Table 2). Sequence alignment revealed an element present in all six promoters (Fig. 4C), which is based on the previously reported CSRE (Schöler and Schüller, 1994). CIT2, not repressed on glucose, but part of the glyoxylate cycle, contains also a CSRE, at -865 relative to the ATG (Fig. 4C). A CSRE is also present in the promoter of HAP1, a transcriptional activator of respiratory genes and transcribed on non-fermentable carbon sources. Furthermore, the promoters of three unknown ORFs YNL047c, YLL029w and YFL055w contain this element at positions -193, -505 and -417, respectively.

Acetyl-coenzyme A transport-Acetyl-coenzyme A is produced and consumed in all three compartments discussed above: mitochondria, cytosol and peroxisomes. Transport of the acetyl moiety over organellar membranes involves carnitine acetyltransferase (Atomi et al., 1993). Until now two genes have been cloned, CAT2 (Kispal et al., 1993) and YAT1 (Schmalix and Bandlow, 1993), both encoding carnitine acetyltransferase, although very distinct forms (Table 1). Both genes were regulated in a similar way: repression by glucose and derepression on C2-compounds (Fig. 3 and Table 2). However, YAT1, like ACS1, was also partially repressed on ethanol, since its mRNA level increased further when ethanol was consumed completely (Fig. 3). Sequence alignment of the ACS1 and YAT1 promoters revealed a common element (Fig. 4D). The regulation of CAT2 was similar to the glyoxylate cycle genes ICL1, MLS1 and MDH2.

Other genes-ACH1, ACR1 and FUN34, encode acetyl-coenzyme A hydrolase, acetyl-coenzyme A synthetase regulator and a putative glyoxylate protein regulator, respectively. The in vivo function of Ach1p is speculative. The gene was cloned via reversed genetics (Lee et al., 1989, 1990) and was highly homologous to the aacC gene of Acetobacter acetii (Fukaya et al., 1993) and the Neurospora crassa gene acu8 (Marathe et al., 1990; Connerton et al., 1992). In all three organisms disruption of these genes yields strains which poorly grow on acetate, but normally on ethanol (Connerton et al., 1992; Fukaya et al., 1993; Lee et al., 1996). Possibly, the acetyl-coenzyme A balance during growth on acetate is disturbed in such mutants.

The other two genes encode putative transcriptional regulators. Disruption of ACR1
decreases Acs1p activity, probably due to transcription inhibition (Fernández et al., 1994). FUN34 has 56% homology with the GPR1 gene (Glyoxylate Pathway Regulator) from Yarrowia lypolitica (Barth et al., 1993). Mutations in GPR1 abolished the activities of the glyoxylate cycle enzymes isocitrate lyase, malate synthase and acetyl-coenzyme A synthetase completely and the dominant allele GPR1-I inhibited growth on C2-compounds (Barth et al., 1993).

All three genes were subject to glucose repression. Like the glyoxylate cycle genes, ACH1 and ACR1 were derepressed on C2-compounds and contain both a CSRE in their respective promoters (Figs. 3B and 6). FUN34 was also repressed on ethanol (Fig. 3B).

General discussion- With respect to their regulation by glucose the genes can be divided into four groups: no transcription, not regulated by glucose, induced on glucose and repressed on glucose.

Five genes (PDC6, PDC7, ADH4, CIT3, ICL2) were not transcribed during the glucose pulse experiment. With the exception of ADH4 they have CBI's between 0.02 and 0.20 (Fig. 2) and therefore were not expected to give high expression. They might represent regulatory genes, pseudogenes or genes with a completely different function, since all, except PDC6, are only distantly related to their isogenes.

Twelve genes were not or only marginally influenced by glucose: PDA1, PDC5, ADH3, ADH5, ADH6, ALD1, ACS2, CIT1, CIT2, ACO1, DAL7 and MDH3. DAL7 and MDH3, are not part of the pathways in this study and are regulated by nitrogen and oleate, respectively.

The third group, induced on glucose, consists of three genes: PDC1, ADH1 and ACO2. PDC1 and ADH1 encode the two main enzymes of the fermentation pathway, which is active at high glucose concentrations. Both have Gcr1 elements around -600 in the promoter, which are found in all glycolytic promoters (Chambers et al., 1995), two in the UAS region of PDC1 (-608 and -587), and one near the Rap1 site in the ADH1 promoter (-649). An additional common sequence of 11 bp is found within the UAS of both genes (Fig. 4A). The function of ACO2 is not known yet, and therefore it is difficult to explain why it was induced on glucose.

The fourth group consists of glucose-repressed genes. Two of them, ACS1 and ADH2, were transcribed in aerobic glucose-limited steady state cultures. The other nine genes of this group were not transcribed under these conditions (Table 2). Seven out of the nine genes, which were repressed even during glucose-limited growth, have one or more Mig1 sites, although all, except FUN34, miss the AT-rich region in front of the element. The promoters of ADH2 and ACS1 lack Mig1 sites. The ACS1 promoter contains an RC2 binding site, which was implicated in the repression of CYC1 transcription (Pfeifer et al., 1987). This site might mediate the anaerobic repression of ACS1 (Van den Berg et al., 1996).

The glucose-repressed group can be divided in three subgroups. Six genes (ICL1, MLS1, MDH2, CAT2, ACR1 and ACH1) were derepressed on ethanol and acetate. MDH1, comprises the second subgroup of glucose-repressed genes, the ethanol inducible genes. There is a specific region of homology with ACS2 (Fig. 4D), which was also induced on ethanol, but not repressed on glucose. Like other positively acting elements as ORE, Gal4 and CSRE, this region contains a CGG triplet. The consensus sequence is also found at -316 in ORF YPL135w. It would be interesting to see if this ORF is also induced on ethanol.

Finally, four genes (ADH2, ACS1, YAT1 and FUN34) were not only repressed on glucose, but also (partially) by ethanol. They only reached full transcription when the yeasts were in
the acetate phase (Fig. 3 and Table 2). The partially ethanol repressed ACS1 and YAT1 genes share a similar promoter sequence (Fig. 4E), which is unique in the S. cerevisiae genome for these two genes. Acs1p, for acetate consumption, Fun34p, a glyoxylate cycle protein regulator, and Yat1p, a mitochondrial carnitine shuttle, are expected to be highly expressed on acetate. In contrast to the acetate-derepressed ADH2 gene there are no Adr1 sites present in the promoters of ACS1, YAT1 and FUN34. The FUN34 promoter, contains the sequence AATACAGGAAAGGT (-413), which is almost identical to the sequence, which mediates acetate induction of the Candida tropicalis ICL1 gene (Atomi et al., 1995).

Although the in vivo function of the five putative regulatory elements described in this paper (Fig. 4A) still must be analysed, the method applied was successful and when extended to more genes and conditions it might reveal other putative promoter elements.

The data obtained from Northern blots suggested the following model for carbon metabolism. When glucose is limited the main flux of carbon will go via the Pdh complex to the citric acid cycle (Pronk et al., 1994b), with the exception of a small amount of pyruvate that is converted via Ald and Acs2p to cytosolic acetyl-coenzyme A (Flikweert et al., 1996). Upon excess glucose metabolism shifts to fermentation and ethanol is produced. When ethanol is consumed it might enter the mitochondria, where it is converted to acetaldehyde by Adh3p and subsequently to acetate by mitochondrial Ald1p. Both ADH3 and ALD1 were expressed constitutively (Table 2). Alternatively, acetaldehyde is transported to the cytosol and oxidised to acetate by cytosolic Ald. Next, acetate is activated by the cytosolic acetyl-coenzyme A synthetase, Acs2p (De Jong-Gubbels et al., 1996b), which gene ACS2 is induced by excess ethanol. Why ADH2, encoding cytosolic alcohol dehydrogenase, is switched on when all the ethanol is consumed, is not known. When acetate is the carbon source, it is presumably metabolised by Acs1p, since ACS1 transcription is almost 40-fold increased on acetate (Van den Berg et al., 1996). ACS2 transcription decreases on acetate (Fig. 3B) and Acs1p has a much lower Km for acetate than Acs2p (Van den Berg et al., 1996). Acs1p is thought to be peroxisomal and therefore the acetyl-coenzyme A produced might be directly fused to oxaloacetate or glyoxylate, catalysed by the only two peroxisomally localised enzymes of the glyoxylate cycle: citrate synthase Cit2p and malate synthase Ms1p (Rosenkrantz et al., 1986; McCammon et al., 1990).

Acknowledgements

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Summary and general discussion
Acetyl-coenzyme A is an important intermediate in many metabolic pathways. Besides functioning as a building block for lipids and several amino acids, it is also an intermediate in energy generating pathways as β-oxidation and citric acid cycle. The yeast *Saccharomyces cerevisiae* can produce acetyl-coenzyme A, amongst others, via the β-oxidation, pyruvate dehydrogenase or acetyl-coenzyme A synthetase (Fig. 1). Work by Van Urk (1989) and Postma *et al.* (1989) suggested that limitations of the latter enzyme triggered the occurrence of the so-called Crabtree effect, e.g. aerobic alcoholic fermentation. The aim of this project was to study the regulation of acetyl-coenzyme A synthetase and to determine its role in the appearance of the Crabtree effect.

In *S. cerevisiae* acetyl-coenzyme A synthetase was shown to be encoded by two genes: *ACS1* and *ACS2* (Chapter 2). These genes were differentially regulated; *ACS1* was repressed by glucose and ethanol, while *ACS2* was induced by ethanol (Chapter 3). The gene products differed in $K_m$, cellular localisation and regulation (Table 1). Acs2p, the cytosolic enzyme (De Jong-Gubbels *et al.*, 1996b), is probably nesseacy to produce acetyl-coenzyme A for cytosolic lipid synthesis (Flikweert *et al.*, 1996), whereas the transcriptional regulation of *ACS1* suggested that Acs1p, the peroxisomal protein (De Jong-Gubbels *et al.*, 1996b), is mainly used for metabolising acetate, although both enzymes can fulfill either function (Chapter 3).

When yeast was cultivated in glucose-limited chemostat cultures, where glucose repression is absent, both genes were transcribed (Chapter 3). When the short-term Crabtree effect was stimulated by addition of excess glucose, the pool of acetyl-coenzyme A synthetase changed rapidly. Transcription from the gene *ACS1* was completely inhibited within 10-15 minutes and the protein encoded by this gene was inactivated (Chapters 3 and 4). Although, the amino acid sequence RHALKTPKK, which resembles the ubiquitine-destruction box (Glotzer *et al.*, 1991), was present in Acs1p and faint bands of 20-30 kDa larger than Acs1p were detected during some inactivation experiments, we were not able to detect ubiquitinated forms of Acs1p with immunoprecipitation (data not shown). *ACS2* transcription and the activity of Acs2p remained more or less constant (Chapters 3 and 4).

Immediately after the addition of glucose, ethanol and acetate were produced by the culture; i.e. the short-term Crabtree effect. What causes the Crabtree effect? Is it the observed decrease in Acs activity, in particular Acs1p activity, leading to acetate accumulation, which triggers, in turn, ethanol production, as suggested by Postma *et al.* (1989)? Aristidou *et al.* (1995) showed that a reduction in acetate production by *Escherichia coli* is highly advantageous for biomass and heterologous protein production. Therefore, one way to approach the question was to overproduce acetyl-coenzyme A synthetase. In experiments where either *ACS1* or *ACS2* on multi-copy plasmids increased the Acs activity during glucose-limited growth 3-7 fold, no decrease in the amount of byproducts formed after addition of glucose was seen (De Jong-Gubbels *et al.*, 1996a). However, when wild-type cells were precultivated in ethanol-limited chemostat cultures and then a glucose pulse was given, a significant decrease in the amount of the acetate produced was observed (De Jong-Gubbels *et al.*, 1997). Here, the total Acs activity was increased 3-fold, mainly due to increased Acs1p activity (Chapter 3). Hence, it is clear that Acs is not the only rate-limiting step in carbon metabolism triggering the appearance of the Crabtree effect. Additional steps, which seem to be repressed by glucose, are at least equally important. This was not surprising since other studies also showed that overproduction of a single enzyme did not increase the flux through a pathway (Niederberger *et al.*, 1992). Usually the sensitivity of the flux through a pathway

<table>
<thead>
<tr>
<th>Gene</th>
<th>ACS1</th>
<th>ACS2</th>
<th>'aerobic' ACS</th>
<th>'anaerobic' ACS</th>
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<tr>
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<td></td>
<td></td>
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<td>XII</td>
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<tr>
<td>Transcription</td>
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<td></td>
<td></td>
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</tr>
<tr>
<td>Glucose</td>
<td>complete repression</td>
<td>on</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethanol</td>
<td>partial repression</td>
<td>induction</td>
<td></td>
<td></td>
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<tr>
<td>Acetate</td>
<td>derepression</td>
<td>repression</td>
<td></td>
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<tr>
<td>Anaerobic</td>
<td>complete repression</td>
<td>on</td>
<td>lethal, during batch growth</td>
<td>on glucose</td>
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<tr>
<td>Disruption</td>
<td>no effect</td>
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### Protein

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<th>215 kDa</th>
<th>250 kDa, 151 kDa</th>
<th>347 kDa</th>
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<td></td>
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<td>Localisation</td>
<td></td>
<td></td>
<td></td>
<td>microsomal</td>
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<tr>
<td>K&lt;sub&gt;M&lt;/sub&gt; acetate</td>
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<td>8.8 mM</td>
<td>0.37 mM, 0.28 mM</td>
<td>5.9 mM</td>
</tr>
<tr>
<td>V&lt;sub&gt;max&lt;/sub&gt;</td>
<td>1.1 U·mg&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>0.34 U·mg&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>0.20 U·mg&lt;sup&gt;-1&lt;/sup&gt;</td>
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<tr>
<td>K&lt;sub&gt;M&lt;/sub&gt; ATP</td>
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<td>1.3 mM</td>
<td>1.2 mM, 1.0 mM</td>
<td>3-10 mM</td>
</tr>
<tr>
<td>Propionate</td>
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<td>-</td>
<td>+</td>
<td>-</td>
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<td>no</td>
<td>yes</td>
<td>no</td>
</tr>
<tr>
<td>Palmitoyl-CoA inhibition</td>
<td>yes</td>
<td>yes</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

is expressed as the 'Flux Control Coefficient' and each single enzyme has its own 'Control Coefficient' (the factor which determines the relative sensitivity of an enzyme). In most cases the 'Control Coefficients' of all the enzymes of a pathway are more or less equal (Niederberger et al., 1992; Cornish-Bowden et al., 1995). Therefore, only when all the enzymes involved were increased, the flux through the pathway will be increased (Schaaff et al., 1989; Niederberger et al., 1992).

Combining the results from this project with data from literature the following hypothesis of carbon metabolism is suggested (Fig. 1). When <i>S. cerevisiae</i> is grown in a glucose-limited chemostat culture the pyruvate produced by the cytosolic glycolysis is directed into the mitochondrial citric acid cycle via pyruvate dehydrogenase (Pdh; Pronk et al., 1994b). When there is excess glucose available, the flux from pyruvate is redirected via pyruvate decarboxylase (Pdc1p/Pdc5p) and alcohol dehydrogenase (Adh1p) to alcoholic fermentation (Fig. 1; Van Urk, 1989; Sierkstra et al., 1992a). The ethanol produced can subsequently be used as a carbon and energy source. As suggested in Chapter 5 of this thesis ethanol might be oxidised in the mitochondria. Peroxisomes are known to be repressed during growth on
Figure 1. Schematic representation of carbon metabolism. The enzyme abbreviations used are: Pdc1p/Pdc5p, pyruvate decarboxylase; Ald1p, acetaldehyde dehydrogenase; Adh1p/Adh2p/Adh3p, alcohol dehydrogenase; ACS1p/ACS2p, acetyl-coenzyme A synthetase; Pdh, pyruvate dehydrogenase; Pyc1p/Pyc2p, pyruvate carboxylase; Cit2p, citrate synthase; Aco, aconitase; Icl1p, isocitrate lyase; Mls1p, malate synthase; Mdh2p/Mdh3p, malate dehydrogenase.

ethanol (Tuttle and Dunn, 1995), suggesting a limited role in ethanol metabolism. The cytosolic route via Adh2p seems to play a limited role, since ADH2 transcription was shown to be repressed by ethanol (Chapter 5). Therefore, the mitochondrial alcohol dehydrogenase (Adh3p; Young and Pilgrim, 1985) might be the best candidate for the initial oxidation of ethanol, since ADH3 was shown to be slightly induced by ethanol (Chapter 5). Subsequently, either the mitochondrial acetaldehyde dehydrogenase Ald1p (Saigal et al., 1991) or the cytosolic isoenzyme, possibly encoded by ALD6 (Bussey, 1997), converts acetaldehyde into acetate, which can be activated to acetyl-coenzyme A by Acs2p. Transcription of ACS2 is also induced by ethanol ( Chapters 3 and 5).

During excess glucose S. cerevisiae also excretes acetate, which later can be used for growth. The 40-fold increased transcription of the ACS1 gene during excess acetate and the low $K_M$ of Acs1p for acetate suggests that acetate under these conditions is mainly activated by Acs1p (Chapter 3), the peroxisomal acetyl-coenzyme A synthetase (De Jong-Gubbels et al., 1996b). The produced acetyl-coenzyme A can be used directly by the two acetyl-coenzyme A consuming reactions of the glyoxylate cycle, catalysed by citrate synthase and malate synthase, which are both peroxisomal (Cit2p and Mls1p in Fig. 1; Rosenkrantz et al., 1986; McCammon et al., 1990). The glyoxylate cycle is completed in the cytosol. From citrate glyoxylate is produced by two reactions, catalysed by aconitase (Aco) and isocitrate lyase (Icl1p). Besides glyoxylate, what must transported back into the peroxisomes, the latter enzyme also produces succinate, that can be used by the citric acid cycle. Regeneration of oxaloacetate, to complete the cycle, also takes place in the cytosol via oxidation of malate by malate dehydrogenase (Mdh2p). All three enzymes were shown to be cytosolic (Gangloff et al., 1990; McCammon et al., 1990; Steffan and McAllister-Henn, 1992; Taylor et al., 1996). The peroxisomal malate dehydrogenase, Mdh3p, functions in the opposite way, to regenerate the NAD consumed during β-oxidation (Van Roermund et al., 1995).

In this thesis the regulation of acetyl-coenzyme A synthetase and its role in the appearance of the short-term Crabtree effect in the yeast S. cerevisiae has been studied. Although the enzymatic activity is decreased immediately in response to excess glucose and this might add to the excretion of acetate, it must be concluded that the enzyme is not the only bottle-neck in metabolism causing alcoholic fermentation. Further research must elucidate all the steps involved.
References


peroxisomes in *Saccharomyces cerevisiae* is impermeable to NAD(H) and acetyl-CoA under *in vivo* conditions. *EMBO J.* 14:3480-3486.


Samenvatting

Functie en regulatie van acetyl-coenzym A synthetase in de gist *Saccharomyces cerevisiae*.

De bakkersgist *Saccharomyces cerevisiae* wordt gekweekt op een mengsel van suikers. Deze suikers kan het organisme omzetten in energie en bouwstenen voor nieuwe cellen. In negen stappen wordt één molecuul glucose omgezet in twee moleculen pyruvaat, de zogenaamde glycolyse (Fig. 1). Het vervolg vanaf pyruvaat wordt bepaald door de condities waarin de cellen gekweekt worden. Is zuurstof afwezig (anaërobe omstandigheden), dan wordt pyruvaat in twee stappen omgezet in ethanol; de zogenaamde alcoholische fermentatie. Is er wel zuurstof aanwezig (aërobe omstandigheden) dan kan pyruvaat in de mitochondriën volledig worden verbrand tot kooldioxide. In vergelijking met de alcoholische fermentatie levert dit acht maal zoveel energie (=ATP) op. Er is echter nog een derde situatie. Als de gist teveel suikers krijgt gaat hij ethanol produceren onder aërobe omstandigheden. Dit wordt het Crabtree effect genoemd (Hoofdstuk 1). Waarom gaan cellen onder zulke gunstige omstandigheden (zuurstof en veel suiker aanwezig) minder energie uit het substraat halen?

Uit eerder werk was bekend dat de afbraak van glucose tot pyruvaat heel snel gaat, zodat de externe suikerconcentratie de pyruvaatconcentratie in de cel bepaalt. Het enzym pyruvataaldehydegensase, dat de koppeling verzorgt tussen de glycolyse en de citroenzuur cyclus (Fig. 1), heeft een lage affiniteitsconstante en een lage maximale verwerkingsnelheid voor pyruvaat. Dit betekent dat de capaciteit gelimiteerd is en alleen bij een lage concentratie pyruvaat alles via dit enzym, Pdh, wordt omgezet. Het enzym pyruvatdecarboxylase, wat de eerste stap vanaf pyruvaat richting ethanol katalyseert (Fig. 1), heeft een hoge affiniteitsconstante en een hoge maximale verwerkingsnelheid voor pyruvaat. Bij lage pyruvaat concentraties zal de koolstofflux daarom richting citroenzuur cyclus gaan en bij hoge pyruvaat concentraties richting alcoholische fermentatie.

Er is echter een theoretische mogelijkheid om ethanol productie bij hoge glucose concentraties te voorkomen. De enzymen aceetaldehyde dehydrogenase en acetyl-coenzym A synthetase kunnen tesamen uit aceetaldehyde acetyl-coenzym A vormen, dat vervolgens in de citroenzuur cyclus gebruikt kan worden. De activiteit van het enzym acetyl-coenzym A synthetase neemt echter af met toenemende groeisnelheid en hogere concentraties glucose in het medium, zodat al snel acetaat wordt uitgescheiden. Acetaat is een zwak zuur, dat de proton gradiënt over de membraan ontkoppelt. De gist moet extra glucose omzetten in ethanol om snel voldoende energie te krijgen om de proton gradiënt in stand te houden. Het enzym acetyl-coenzym A synthetase lijkt dus een belangrijke schakel in het optreden van het Crabtree effect. Het Crabtree effect zorgt voor een lagere opbrengst in de industriële bakkersgistproductie en is daarom ongewenst.

Het doel van dit proefschrift was inzicht te krijgen in de functie en regulatie van acetyl-coenzym A synthetase in bakkersgist. Daartoe zijn eerst de genen gekloneerd, welke coderen voor het enzym (Hoofdstuk 2). Dit blijken er twee te zijn: *ACS1* en *ACS2*. Beide genen blijken op verschillende wijze gereguleerd te worden: de transcriptie van *ACS1* wordt volledig geremd door glucose en partieel door ethanol, terwijl *ACS2* transcriptie wordt gestimuleerd door ethanol (Hoofdstuk 3). Ook verschillen de geproduceerde enzymen van elkaar. Acs1p heeft een lage affiniteitsconstante voor acetaat, bevindt zich in de peroxisomen en wordt geïnactiveerd door glucose. Acs2p heeft een hoge affiniteitsconstante voor pyruvaat, is gelokaliseerd in het cytosol en stabiel in de aanwezigheid van glucose (Hoofdstukken 3 en
4). Disruptiemutanten van *acs1* verschillen in groei niet wezenlijk van het wild-type, terwijl de *acs2* mutatie letaal blijkt tijdens batch kweek op glucose (Hoofdstuk 2). Uit ander werk is inmiddels gebleken dat de Pdh bypass route, zoals de gezamenlijke werking van pyruvaatdecarboxylase, acetaldehyde dehydrogenase en acetyl-coenzym A synthetase ook wel wordt genoemd (Fig. 1), essentieel is voor de productie van cytosolisch acetyl-coenzym A. Dit gebruikt het organisme waarschijnlijk voor het maken van vetten. Resumerend lijkt Acs2p voornamelijk nodig voor de productie van acetyl-coenzym A ten bate van cellmateriaal, en Acs1p vooral nodig bij groei op acetaat.

Wat heeft dit te betekenen voor het Crabtree effect? In wild-type cellen daalt de totale acetyl-coenzym A activiteit onmiddellijk na toevoeging van een overmaat glucose, doordat

![Diagram](image)

Figuur 1. Schematische weergave van het koolstofmetabolisme in *S. cerevisiae*. Verklaring van de genummerde enzymen: 1, pyruvaatdecarboxylase (Pdc); 2, alcoholdehydrogenase (Adh); 3, pyruvaatdehydrogenase (Pdh); 4, acetaldehyde dehydrogenase (Ald); 5, acetyl-coenzym A synthetase (Acs).
Acs1p wordt geïnactiveerd. Dit gaat samen met de productie van ethanol en acetaat (Hoofdstuk 4). Is deze bij-product vorming een gevolg van de lagere acetyl-coenzym A synthetase activiteit? Dit is getest door de activiteit in de cel te verhogen met behulp van extra genecopieën. In het geval van meer ACS2 copieën geeft dit 3 maal zoveel activiteit, terwijl met meer ACS1 copieën zelfs 7 maal zoveel activiteit wordt verkregen. Dit leidt echter niet tot een reductie in de hoeveelheid bijproduct vorming na additie van de overmaat glucose. Worden wild-type cellen echter voorgekweekt op ethanol en wordt dan een overmaat glucose toegevoegd, dan daalt de hoeveelheid bij-producten drastisch. In deze cellen is de hoeveelheid acetyl-coenzym A synthetase activiteit, voor het toedienen van de puls, 3 maal zo hoog als bij groei op glucose, zodat geconcludeerd kan worden dat acetyl-coenzym A synthetase in ieder geval niet de enige beperkende factor is in het metabolisme van de bakkersgist, waardoor deze onder volledig aërobe omstandigheden de energetisch onvoordelig route van alcoholische fermentatie gebruikt. In Hoofdstuk 5 is beschreven hoe verschillende genen betrokken bij het metabolisme van acetyl-coenzym A op identieke wijze gereguleerd blijken te zijn. Verder onderzoek zal echter moeten uitsluiten welke enzymen, naast acetyl-coenzym A synthetase, nog meer van belang zijn bij het optreden van het Crabtree effect.
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