

# Improving Production of Malonyl Coenzyme A-Derived Metabolites by Abolishing Snf1-Dependent Regulation of Acc1

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**ABSTRACT** Acetyl coenzyme A (acetyl-CoA) carboxylase (ACCase) plays a central role in carbon metabolism and has been the site of action for the development of therapeutics or herbicides, as its product, malonyl-CoA, is a precursor for production of fatty acids and other compounds. Control of Acc1 activity in the yeast *Saccharomyces cerevisiae* occurs mainly at two levels, i.e., regulation of transcription and repression by Snf1 protein kinase at the protein level. Here, we demonstrate a strategy for improving the activity of ACCase in *S. cerevisiae* by abolishing posttranslational regulation of Acc1 via site-directed mutagenesis. It was found that introduction of two site mutations in Acc1, Ser659 and Ser1157, resulted in an enhanced activity of Acc1 and increased total fatty acid content. As Snf1 regulation of Acc1 is particularly active under glucose-limited conditions, we evaluated the effect of the two site mutations in chemostat cultures. Finally, we showed that our modifications of Acc1 could enhance the supply of malonyl-CoA and therefore successfully increase the production of two industrially important products derived from malonyl-CoA, fatty acid ethyl esters and 3-hydroxypropionic acid.

**IMPORTANCE** ACCase is responsible for carboxylation of acetyl-CoA to produce malonyl-CoA, which is a crucial step in the control of fatty acid metabolism. ACCase opened the door for pharmaceutical treatments of obesity and diabetes as well as the development of new herbicides. ACCase is also recognized as a promising target for developing cell factories, as its malonyl-CoA product serves as a universal precursor for a variety of high-value compounds in white biotechnology. Yeast ACCase is a good model in understanding the enzyme's catalysis, regulation, and inhibition. The present study describes the importance of protein phosphorylation in regulation of yeast ACCase and identifies potential regulation sites. This study led to the generation of a more efficient ACCase, which was applied in the production of two high-value compounds derived from malonyl-CoA, i.e., fatty acid ethyl esters that can be used as biodiesel and 3-hydroxypropionic acid that is considered an important platform chemical.

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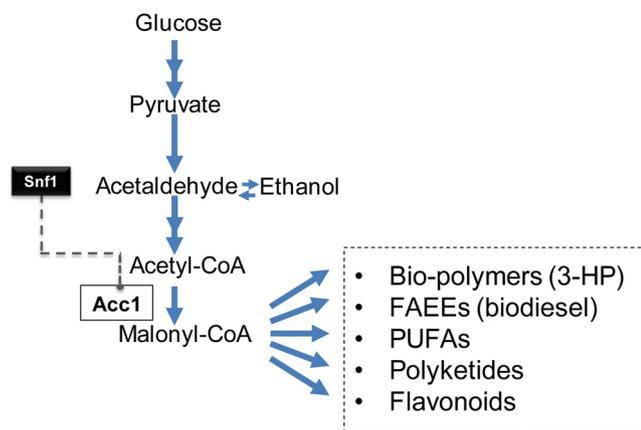
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Acetyl coenzyme A (acetyl-CoA) carboxylase (ACCase; EC 6.4.1.2), recognized as an essential enzyme in most living organisms, is responsible for the carboxylation of acetyl-CoA to form malonyl-CoA. ACCase is a multisubunit enzyme in prokaryotes, whereas it is a single, multidomain enzyme in eukaryotes. Although the structures are different between pro- and eukaryotic species, the reaction mechanisms are essentially the same in all types of cells (1).

ACCase plays a critical role in regulating fatty acid metabolism, as the enzyme catalyzes the first committed and rate-limiting step in fatty acid biosynthesis, representing an attractive target for therapeutic intervention in the control of obesity and the treatment of metabolic syndrome (2), in addition to being the site for development of new herbicides (1). Besides being an essential component for *de novo* fatty acid synthesis required for cell growth (1, 3, 4), malonyl-CoA serves as a major building block for a variety of relevant compounds in industrial biotechnology (Fig. 1),

such as polyketides and flavonoids (5, 6) or fatty acid-derived products (7, 8). However, it has been shown that the intracellular malonyl-CoA concentration is tightly regulated to be very low, resulting in limited production of malonyl-CoA-derived compounds (5–7, 9).

The transfer of malonyl-CoA-dependent pathways into heterologous hosts or their overexpression in native hosts for various applications relies on the availability of malonyl-CoA. Metabolic engineering applications of ACCase have been focused on the manipulation of enzyme levels through gene amplification. The first example was overexpression of a set of four *Escherichia coli* subunit proteins of ACCase in a coordinated manner, which resulted in a nearly 50-fold increased activity of the enzyme and a 6-fold increase in the rate of free fatty acid synthesis in *E. coli* (10). This was proven to be an effective approach for fatty acid production in additional studies, where a set of *E. coli* ACCase genes was (over) expressed in cyanobacteria (11) and *E. coli* (7, 9). This strategy also



**FIG 1** Malonyl-CoA works as a major building block. The primary fate of malonyl-CoA is to serve as a precursor for lipids. However, a wide range of industrially interesting fuels and chemicals are derived from malonyl-CoA. A cell factory with enhanced supply of malonyl-CoA could serve as a platform for malonyl-CoA-derived products. Acc1 is a critical enzyme for malonyl-CoA synthesis, and the global regulator Snf1 is involved in regulation of Acc1 at the posttranslational level.

allows the development of engineered strains for enhanced production of various fatty acid-derived products (12). In another study, introduction of *Corynebacterium glutamicum* ACCase, consisting of only two subunits, into *E. coli* led to an increase of the cellular malonyl-CoA concentration by 3-fold and improved malonyl-CoA-derived phloroglucinol production (5). Similarly, related to an increased availability of malonyl-CoA, it was observed that expression of the ACCase genes from *C. glutamicum* or *Photobacterium luminescens* in *E. coli* could lead to increased production of flavonones, which are direct precursors of flavonoids (13, 14). It was also reported that overexpression of ACCase in *Streptomyces coelicolor* resulted in an improved polyketide production (15). Changing the promoter of *ACC1* to the strong constitutive *TEF1* promoter in *Saccharomyces cerevisiae* has been seen to increase the production of 6-methylsalicylic acid by about 60% (6). Although many examples of ACCase overexpression were shown to be suitable for production of a variety of malonyl-CoA-derived products, these are still limited by the inherent low capacity of the enzyme itself and in particular its tight posttranslational regulation. Several other attempts to utilize overexpression of ACCase in various systems were not satisfactory (5, 8, 16), and there remains a need to increase ACCase enzyme activity by modifying its native regulation.

Microbial systems are increasingly being developed as production platforms for a wide variety of chemicals and biofuels to decrease the dependency on fossil resources. These target chemicals and biofuels are frequently produced by incorporating heterologous or modifying endogenous metabolic pathways in well-characterized host microorganisms, such as *E. coli* or *S. cerevisiae*. Nowadays, the yeast *S. cerevisiae* is often preferred as the host for the development of a new process due to its robustness and tolerance toward different environmental conditions, an extensive technology platform available for this organism, well-studied genetics, and a long history of industrial use with a well-established reputation (17, 18). There are therefore many examples that illustrate the suitability of this cell factory for the production of a range of fuels (16–18) and chemicals (17–19). In addition, to reduce the

production costs, many efforts have been reported to make *S. cerevisiae* efficiently grow on and metabolize pentoses that are present in hemicelluloses (20–23). Following decades of continuous efforts, *S. cerevisiae* is likely to become a key cell factory platform in the future (17, 18).

In *S. cerevisiae*, cytosolic ACCase is encoded by *ACC1* and mitochondrial ACCase by *HFA1*. To eliminate issues with transport across the mitochondrial membranes, it is desirable to assemble heterologous biosynthetic pathways in the cytosol for most biotechnological projects. Therefore, it is of great interest to understand the regulation of Acc1 activity. *ACC1* is transcriptionally regulated in coordination with phospholipid biosynthesis by positive (Ino2p and Ino4p) and negative (Opi1p) regulatory factors (4). Since it belongs to a family of biotin-dependent carboxylases, the efficiency of Acc1 biotinylation controls its activity at the protein level, and it has been reported that reduced levels of this vitamin affect the synthesis of fatty acids (24). Protein phosphorylation is one of the most important posttranslational modifications and plays a key role in signal transduction and gene regulation. It has been suggested that phosphorylation by Snf1 inactivates purified Acc1 (25, 26) (Fig. 1), and mutants lacking Snf1 display increased Acc1 activity (3, 25). Except for the regulation of Acc1, Snf1 is also responsible for the induction of genes involved in gluconeogenesis, glyoxylate cycle, and  $\beta$ -oxidation of fatty acids as well as involved in the general stress response, pseudohyphal growth, aging, and ion homeostasis (27). Therefore, Snf1 could not be simply deleted for enhancing Acc1 activity. Concerning phosphorylation of Acc1, one site at Ser1157 was verified by phosphoproteome analysis (28). However, it remains to be shown whether this serine (Ser) residue is the actual target site of Snf1-mediated phosphorylation. According to the phosphorylation recognition motif (Hyd-X-Arg-XX-Ser-XXX-Hyd) for Snf1 (29), besides Ser1157, an additional putative phosphorylation site was found at Ser659 of Acc1. There are no experimental data on phosphorylation of Ser659 reported so far. In this work, we intended to characterize these proposed phosphorylation sites of Acc1 via site-directed mutagenesis and evaluate the resultant mutant enzyme activities.

Modulation of Acc1 activity would directly affect the cellular concentration of the product malonyl-CoA, and, here, we demonstrate that engineering the protein at the posttranslational level can increase the enzyme activity of Acc1 and thereby enable improved flux through malonyl-CoA-dependent metabolic pathways for the production of chemicals and biofuels, e.g., (i) 3-hydroxypropionic acid (3-HP), a platform chemical that can be used in synthesis of a variety of chemical derivatives, such as acrylates and malonic acid (30), and (ii) fatty acid ethyl esters (FAEEs) that can be used as a main constituent of biodiesel used in trucks, buses, and trains (31).

## RESULTS

**Site-directed mutagenesis of Acc1 increases enzyme activity and total fatty acid content.** Ser1157 of Acc1 has been reported to be a site of phosphorylation (28), and using the phosphorylation recognition motif (Hyd-X-Arg-XX-Ser-XXX-Hyd) for Snf1 to search for additional possible phosphorylation sites, we identified Ser659 as a putative site besides Ser1157. These two possible phosphorylation sites of Acc1 were mutated from Ser to Ala (see details in Fig. S1 in the supplemental material). Thus, three versions of *ACC1* were constructed by PCR: (i) wild-type *ACC1* (*ACC1*<sup>wt</sup>),

TABLE 1 List of strains used in this study

Strain	Genotype or relevant characteristics	Gene overexpressed by plasmid		
		pSP-GM2	pIYC04	Source or reference
CEN.PK 113-5 D	MATa MAL2-8 <sup>c</sup> SUC2 ura3-52	NA <sup>a</sup>	NA	P. Kötter <sup>c</sup>
CEN.PK 506-1 C	MATa URA3 HIS3 TRP1 LEU2 SUC2 MAL2-8 <sup>c</sup> <i>snf1</i> (4-1899)::loxP-kanMX-loxP	NA	NA	P. Kötter
SC00	MATa MAL2-8 <sup>c</sup> SUC2 ura3-52 <i>snf1</i> Δ::loxP-kanMX-loxP	NA	NA	This study
SC01	SC00	Empty <sup>b</sup>	NA	This study
SC02	SC00	ACC1 <sup>wt</sup>	NA	This study
SC03	SC00	ACC1 <sup>ser1157ala</sup>	NA	This study
SC04	SC00	ACC1 <sup>ser659ala,ser1157ala</sup>	NA	This study
CB0	CEN.PK 113-5D	Empty	NA	16
CAW	CEN.PK 113-5D	ACC1 <sup>wt</sup>	NA	This study
CAS	CEN.PK 113-5D	ACC1 <sup>ser1157ala</sup>	NA	This study
CAD	CEN.PK 113-5D	ACC1 <sup>ser659ala,ser1157ala</sup>	NA	This study
CEN.PK 113-11 C	MATa <i>his3</i> -Δ1 <i>ura3</i> -52 MAL2-8 <sup>c</sup> SUC2	NA	NA	P. Kötter
CB2H1	CEN.PK 113-11 C	Empty	<i>ws2</i>	This study
CB2H2	CEN.PK 113-11 C	ACC1 <sup>wt</sup>	<i>ws2</i>	This study
CB2H3	CEN.PK 113-11 C	ACC1 <sup>ser1157ala</sup>	<i>ws2</i>	This study
CB2H4	CEN.PK 113-11 C	ACC1 <sup>ser659ala,ser1157ala</sup>	<i>ws2</i>	This study
HPY15	CEN.PK 113-11 C	Empty	<i>mcr<sub>Ca</sub></i>	This study
HPY16	CEN.PK 113-11 C	ACC1 <sup>wt</sup>	<i>mcr<sub>Ca</sub></i>	This study
HPY17	CEN.PK 113-11 C	ACC1 <sup>ser1157ala</sup>	<i>mcr<sub>Ca</sub></i>	This study
HPY18	CEN.PK 113-11 C	ACC1 <sup>ser659ala,ser1157ala</sup>	<i>mcr<sub>Ca</sub></i>	This study

<sup>a</sup> NA, strain was not transformed with any plasmid.

<sup>b</sup> Empty, strain was transformed with empty plasmid.

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(ii) *ACC1* with one site mutation at Ser1157 (*ACC1*<sup>ser1157ala</sup>), and (iii) *ACC1* with two site mutations at Ser1157 and Ser659 (*ACC1*<sup>ser659ala,ser1157ala</sup>).

To evaluate the effect of the point mutations on the ACCase activity, *ACC1*<sup>wt</sup>, *ACC1*<sup>ser1157ala</sup>, and *ACC1*<sup>ser659ala,ser1157ala</sup> were cloned into multicopy plasmid pSP-GM2 (32) and then transformed into *S. cerevisiae* CEN.PK 113-5 D to construct the strains CAW, CAS, and CAD (Table 1), respectively. CB0 holds the empty plasmid pSP-GM2 and was used as a control. ACCase activities in all strains were determined in stationary phase (Table 2). Overexpression of both wild-type and point-mutated *ACC1* enhanced the ACCase activity compared to that of control strain CB0. In strain CAW, which harbors *ACC1*<sup>wt</sup> on plasmid, ACCase activity was increased by 20%. While the enzyme activity was 80% higher in the single-mutated version of *ACC1* (*ACC1*<sup>ser1157ala</sup>) in strain CAS than for the control strain, overexpression of *ACC1*<sup>ser659ala,ser1157ala</sup> resulted in a 3-fold increase of ACCase activity, which was the highest increase.

Overexpression of wild-type or point-mutated Acc1 clearly led to an increase in the enzyme activity and thereby supposedly increased the formation of malonyl-CoA, a crucial precursor for fatty acid biosynthesis. Therefore, we also measured the concen-

tration of total fatty acids in strains CB0, CAW, CAS, and CAD (Table 2). We found no significant increase in the total fatty acid content when overexpressing *ACC1*<sup>wt</sup> compared with that of the control strain. Overexpression of *ACC1*<sup>ser1157ala</sup>, however, resulted in a 14 ± 0.2% improvement in the total fatty acid content, and overexpression of *ACC1*<sup>ser659ala,ser1157ala</sup> resulted in a 65 ± 0.3% increase in the total fatty acid content (strain CAD).

**Characterization of different Acc1 versions in the absence of SNF1.** The effect of increased activity of Acc1 shown in Table 2 could be explained by the introduced site mutations, Ser659 and Ser1157, which are suggested to function as phosphorylation sites recognized by Snf1 protein kinase (29). To evaluate this further, we expressed the two mutated enzymes as well as the wild-type form of the enzyme in an *snf1* strain.

*SNF1* was deleted in strain CEN.PK 113-5 D, resulting in strain SC00. Empty plasmid and plasmids holding three different versions of *ACC1* were introduced into SC00, resulting in strains SC01, SC02, SC03, and SC04 (Table 1). These strains were cultured in glucose-limited chemostat cultures (growth rate at 0.1 h<sup>-1</sup>), where Snf1 is normally active, and analyzed for their ACCase activity. No apparent physiological difference was found in chemostat cultures when different versions of Acc1 were over-

TABLE 2 Activities of acetyl coenzyme A carboxylase and total fatty acid content in strains holding different versions of *ACC1*<sup>a</sup>

Strain	Total fatty acid content (% of CDW)	ACCase activity (pmol/min/mg of cell-free protein extract)
CB0	7.1 ± 1.5	24 ± 3
CAW	7.4 ± 1.5	29 ± 3
CAS	8.1 ± 1.5	43 ± 4
CAD	11.7 ± 2.0	74 ± 9

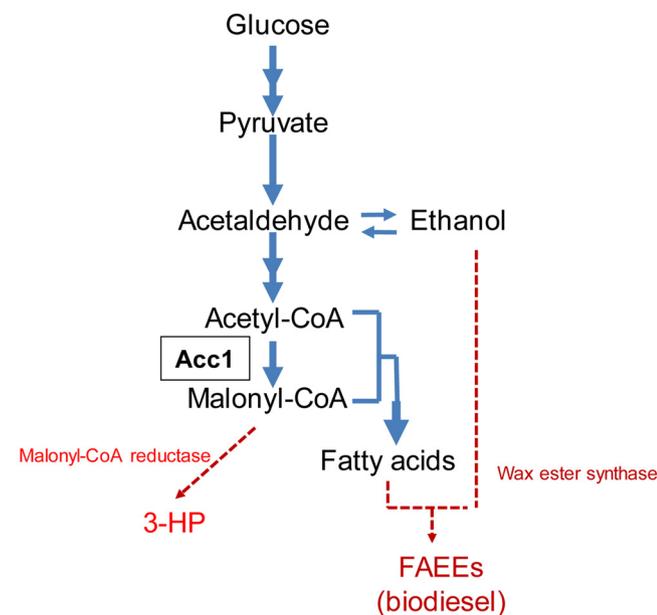
<sup>a</sup> CB0, control strain with empty plasmid; CAW, strain with overexpressed *ACC1*<sup>wt</sup>; CAS, strain with overexpressed *ACC1*<sup>ser1157ala</sup>; CAD, strain with overexpressed *ACC1*<sup>ser659ala,ser1157ala</sup>. The total fatty acid contents are expressed as percentages of dry-weight biomass. Strains were sampled at stationary phase. The reported results are the averages from three replicate experiments ± standard deviations.

**TABLE 3** Activities of acetyl coenzyme A carboxylase in *snf1* deletion strains holding different versions of *ACC1*<sup>a</sup>

Strain	Enzyme activity (pmol/min/mg of cell-free protein extract)
SC01	33 ± 8
SC02	57 ± 5
SC03	62 ± 9
SC04	63 ± 6

<sup>a</sup> SC01, control *snf1* deletion strain with empty plasmid; SC02, *snf1* deletion strain with overexpressed *ACC1*<sup>wt</sup>; SC03, *snf1* deletion strain with overexpressed *ACC1*<sup>ser1157ala</sup>; SC04, *snf1* deletion strain with overexpressed *ACC1*<sup>ser659ala,ser1157ala</sup>. Samples were taken from chemostat cultures after a steady state was maintained for about 50 h. The reported results are the averages from duplicate experiments ± standard deviations.

expressed. Compared to the higher enzyme activity for the mutated versions of *Acc1* in the presence of *SNF1* (Table 2), all the strains with overexpression of different versions of *ACC1*, i.e., strains SC02, SC03, and SC04, had similar ACCase activities in the absence of *SNF1* (Table 3). These results clearly indicate that Ser1157 and Ser659 play a role in regulation of *Acc1* activity, most likely as phosphorylation target sites of *Snf1*.



**FIG 2** Reconstruction of FAEEs and 3-HP biosynthesis in *Saccharomyces cerevisiae*. Single and double arrows represent single and multiple enzymatic steps; dashed arrows represent heterologous pathways. FAEEs, fatty acid ethyl esters; 3-HP, 3-hydroxypropionic acid.

**Using mutated *Acc1* for production of malonyl-CoA-derived products.** From the above-given information, it is clear that site-directed mutagenesis of *ACC1* results in increased activity, leading to higher total lipid content (Table 2), indicating increased supply of malonyl-CoA. We therefore wanted to evaluate if this phenomenon could be applied for improving the production of malonyl-CoA-derived products, of which FAEEs and 3-HP were taken as examples (see Fig. 2).

FAEEs can be produced by yeast following the introduction of a heterologous wax ester synthase gene (*ws2*), and the supply of malonyl-CoA has been found to be limiting the production (31). Therefore, as expected, overexpression of the different mutant *ACC1* genes increased FAEE production (Table 4). Compared to the FAEE-producing reference strain CB2H1 that holds only the *ws2* gene with an FAEE production of  $4.8 \pm 0.7$  mg/liter, strain CB2H2 that overexpresses *ACC1*<sup>wt</sup> has a slightly increased FAEE production (around 20%), while strain CB2H3 that overexpresses *ACC1*<sup>ser659ala</sup> showed a much higher increase (around 2-fold). Furthermore, overexpression of *ACC1*<sup>ser659ala,ser1157ala</sup> in strain CB2H4 resulted in the highest FAEE production, corresponding to a 3-fold improvement. Some physiological parameters of the different strains are listed in Table S1 in the supplemental material. The strains with higher activity of *Acc1* showed a lower growth rate, and their final biomass concentration was also reduced.

A second case study was the production of 3-HP. As shown in Fig. 2, biosynthesis of 3-HP can be realized directly from malonyl-CoA in two reaction steps catalyzed by the multifunctional malonyl-CoA reductase encoded by the *mcr<sub>Ca</sub>* gene from *Chloroflexus aurantiacus*, as reported before (33). The production of 3-HP (Table 4) in the different strains showed a trend similar to FAEE production. In HPY16, where *ACC1*<sup>wt</sup> was overexpressed, 3-HP production was improved by 60% compared to HPY15 that carries only *mcr<sub>Ca</sub>*. Interestingly, when *ACC1*<sup>ser659ala</sup> was overexpressed instead of the wild-type gene in HPY17, there was a more than 2.5-fold improvement in 3-HP production over the reference strain HPY15, and by overexpressing *ACC1*<sup>ser659ala,ser1157ala</sup> in HPY18, an improvement of more than 3.5-fold over HPY15 was obtained. Some physiological parameters of the different strains are listed in Table S2 in the supplemental material. A reduction in growth rate and biomass yield was found when the different versions of *ACC1* were overexpressed. With regard to the accumulation of 3-HP in glucose and ethanol phases (see Table S2), the similar increasing trend was observed when *ACC1*<sup>wt</sup>, *ACC1*<sup>ser659ala</sup>, or *ACC1*<sup>ser659ala,ser1157ala</sup> were overexpressed. More interestingly, it was found that the highest improvements, up to

**TABLE 4** Production of FAEEs and 3-HP in different strains holding different versions of *ACC1*

Strain	(Over)expressed genes ( <i>HIS3</i> and <i>URA3</i> ) <sup>a</sup>	Total FAEE production (mg/liter)	Total 3-HP production (mg/liter)
CB2H1	<i>ws2</i> and — <sup>b</sup>	4.7 ± 0.7	
CB2H2	<i>ws2</i> and <i>ACC1</i> <sup>wt</sup>	5.6 ± 0.8	
CB2H3	<i>ws2</i> and <i>ACC1</i> <sup>ser1157ala</sup>	9.5 ± 0.9	
CB2H4	<i>ws2</i> and <i>ACC1</i> <sup>ser659ala,ser1157ala</sup>	15.8 ± 2.5	
HPY15	<i>mcr<sub>Ca</sub></i> and —		82.2 ± 2.2
HPY16	<i>mcr<sub>Ca</sub></i> and <i>ACC1</i> <sup>wt</sup>		126.8 ± 5.9
HPY17	<i>mcr<sub>Ca</sub></i> and <i>ACC1</i> <sup>ser1157ala</sup>		210.9 ± 9.9
HPY18	<i>mcr<sub>Ca</sub></i> and <i>ACC1</i> <sup>ser659ala,ser1157ala</sup>		279 ± 19.8

<sup>a</sup> Gene (over)expressed in *HIS3* and *URA3* based plasmid.

<sup>b</sup> No gene was inserted for (over)expression.

6-fold (with  $ACC1^{ser659ala,ser1157ala}$ ) compared to the control, were in the ethanol phase, where Snf1 is most active.

## DISCUSSION

In *S. cerevisiae*, as well as in other higher eukaryotes, the activity of ACCase rather than the fatty acid synthetase complex determines the regulation of fatty acid synthesis (1). Therefore, ACCase is assumed to be a promising target for the development of therapeutics and herbicides and for biotechnology approaches.

Currently, a major challenge in understanding regulation of yeast Acc1 (encoding cytosolic ACCase) is to define its characteristics of posttranslational modification through protein phosphorylation. We here show that introduction of site mutation at Ser1157 and/or Ser659 of Acc1 resulted in an increase in Acc1 activity (Table 2), indicating that these two assumed phosphorylation sites are highly related to determining the enzyme activity *in vivo*. We further demonstrated that there is no difference in activity of the different mutant enzymes in an *SNF1* deletion strain. Phosphorylation at Ser1157 has been indicated in a previous study (28). Although no phosphorylation has been seen for Ser659 in any study so far, it may still occur. Our data strongly indicate that the introduced mutations were able to relieve Snf1-regulated repression of Acc1. Whether this regulation, presumably through protein phosphorylation, is directly or indirectly mediated by Snf1 remains to be seen. We also found that there is a 40% increased activity of ACCase in an *snf1* deletion strain compared to that of the reference strain (Tables 2 and 3), consistent with an earlier study that suggested a key role of phosphorylation on Acc1 activity with the evidence that *ACC1* expression is reduced in *snf1* mutants but still displays higher activity of the enzyme (25). It is also interesting that the deletion of *SNF1* in SC01 gives a higher ACCase activity than elevation of its transcriptional level by overexpression of *ACC1*<sup>wt</sup> on a plasmid in CAW (Tables 2 and 3). From these results, it is clear that activity of Acc1 is controlled primarily at the posttranscriptional level. Although there have been a variety of projects targeted to modulate Acc1 activity, this study is the first describing modulation at the posttranslational level. It is observed that the Acc1 activity measured in this study is much lower than in previous reports (34, 35). One possible explanation could be biotin deficiency. However, this would be reflected in a decrease in C<sub>18</sub> fatty acids (24), which is not the case in our strains. The reason for this difference could also be due to the fact that cell extracts were used directly for enzyme analysis in this study, and previous reports had purified the enzyme before enzyme analysis (34, 35). Differences in media and sampling times may also explain the differences in enzyme activity.

It is known that fatty acid biosynthesis highly depends on the activity of Acc1 (1, 3, 7), and here we also found a positive effect of the enhanced Acc1 activity on the total fatty acid content (Table 2), indicating limitations for fatty acid biosynthesis associated with Acc1 activity. Limitations in malonyl-CoA availability also impede biotechnological synthesis of many industrially relevant products.

Here, we demonstrated that expression of our identified more efficient Acc1 mutants could improve the production of two malonyl-CoA-derived products, i.e., FAEEs and 3-HP (Table 4). In both cases, the observed highest improvement was caused by overexpression of  $ACC1^{ser659ala,ser1157ala}$ , and the fold changes were similar to the improvement in ACCase activity by overexpression of this mutant enzyme (Table 2). This points to a signif-

icant flux control at Acc1 for production of both products. An impaired growth was observed in both cases when strains had an enhanced activity of Acc1 and their final biomass concentrations were also reduced, as shown in Tables S1 and S2 in the supplemental material. This is presumably due to an imbalanced synthesis of long-chain fatty acids or depletion of intermediates. A similar result has been reported in a previous review, which mentioned that overexpression of Acc1 together with Bp11 (encoding the enzyme biotin-apoprotein ligase) can even be lethal (4). A harmful effect on growth caused by overexpression of ACCase was also found in bacteria (10).

The highest production of FAEEs in CB2H4 reached 15.8 mg/liter, which is much lower than the value achieved using an engineered *E. coli* strain (31), but it is comparable with the highest FAEE production reported for *S. cerevisiae* from simple sugars, which is 17.2 mg/liter (36). In the latter study, fatty acid metabolism was heavily modified by eliminating nonessential fatty acid utilization pathways (36), and it is interesting that here almost the same titer was reached by simply overexpressing  $ACC1^{ser659ala,ser1157ala}$ . Samples for FAEE measurements were taken in stationary phase, as yeast cells can accumulate substantial amounts of lipids during the late exponential and stationary phases (37), which is also true for FAEE accumulation (see Table S1). During this phase, FAEEs were produced and ACCase would theoretically be inactivated by Snf1 (3). In the case of 3-HP, when overexpressing  $ACC1^{ser659ala,ser1157ala}$ , an approximately 6-fold increase was found during the ethanol phase, while only an approximately 2-fold increase was observed in the glucose phase compared to that in the control (see Table S2). This is probably due to the fact that the availability of cytosolic acetyl-CoA is higher during the ethanol phase than during the glucose phase (19). Still, the improvements when overexpressing the mutated Acc1 (5- to 6-fold) are greater than the improvements with the wild-type Acc1 (2-fold). This difference reflects the efficiency of converting acetyl-CoA into malonyl-CoA, indicating again a connection with Snf1-mediated phosphorylation, since the kinase is active mainly in this phase. These two cases suggest that regulation of this specific reaction was not only the result of gene expression but resides to a large extent in posttranslational modifications, which should be taken into account for future metabolic engineering strategies. There are several other examples of enzymes that are regulated posttranslationally rather than by expression level variation (38–40). A very good example is the control of yeast central metabolism by enzyme phosphorylation (40).

Yeast ACCase exhibits strong similarity to the respective enzyme sequences of mammals and plants (2). The AMP-activated protein kinase (AMPK) is responsible for phosphorylation and inhibition of ACCase in human; in yeast, Acc1 is phosphorylated by Snf1 (25, 26); Snf1-related protein kinases (SnRKs) play the regulatory role in higher plants. AMPK, Snf1, and SnRKs all belong to the Snf1 protein kinase family (41). The high conservation of ACCases from yeast to mammals and plants suggests that the yeast ACCase can be used as a model to study the molecular interactions in human physiology as well as for the development of targets in disease treatments (42), or it can be used as a guide to control lipid metabolism in plants for developing high-lipid-content plants as renewable and sustainable feedstocks for the production of liquid biofuels (43). Our findings represent a step forward in achieving these goals.

**Conclusion.** ACCase, which is crucial for the anabolism of malonyl-CoA and fatty acids (1), was also suggested to be the major rate-controlling step for production of a variety of high-value compounds derived from malonyl-CoA. Overexpression of ACCase has previously been applied in many studies for redirecting flux toward malonyl-CoA (1, 5–7, 9, 10). However, these approaches are still limited by the inherent low enzyme activity. In this study, it is interesting to note that a more efficient Acc1 was obtained by the introduction of two site mutations at Ser1157 and Ser659, which are suggested to be related to Snf1-mediated phosphorylation. The strains with higher activity of Acc1 showed a notable advantage for the production of malonyl-CoA-derived products, as demonstrated for the production of FAEEs and 3-HP. Our new finding is therefore also likely to be applicable for many other products that are derived from malonyl-CoA. The present study highlights the importance of engineering proteins along with metabolic engineering as a key strategy in achieving improved microbial cell factories.

## MATERIALS AND METHODS

**Strains and plasmids.** All DNA manipulations were carried out in *E. coli* DH5 $\alpha$  as described (44). All constructs have been checked by sequencing. The plasmids used are derived from pIYC04 (19) and pSP-GM2 (32).

The endogenous *ACC1* gene (wild type) encoding acetyl-CoA carboxylase was amplified from genomic DNA of CEN.PK 113-5D by PCR with Phusion high-fidelity polymerase (Thermo Scientific, Waltham, MA, USA). The primers are listed in Table S3 in the supplemental material. The *ACC1*<sup>ser1157ala</sup> single-point mutation and *ACC1*<sup>ser659ala,ser1157ala</sup> double-point mutation were introduced by oligonucleotide primers (details are shown in Fig. S1). Three versions of *ACC1* were digested with NotI and SacI and then ligated into the corresponding sites of pSP-GM2 and controlled by constitutive *PGK1* promoter.

For FAEE production in *S. cerevisiae*, the codon-optimized wax synthase gene (*ws2*) from *Marinobacter hydrocarbonoclasticus* DSM 8798 was adopted from a former study (16). The *ws2* gene was ligated into pIYC04 under control of the *TEF1* promoter. For 3-HP production in *S. cerevisiae*, the gene *mcr<sub>Ca</sub>* encoding malonyl-CoA reductase from *Chloroflexus aurantiacus* was codon optimized based on the codon preference of *S. cerevisiae* (GenScript, Piscataway, NJ, USA). The *mcr<sub>Ca</sub>* gene was ligated into pIYC04 downstream of the *TEF1* promoter.

*S. cerevisiae* strains used in this work are listed in Table 1. All experiments were performed in the background of CEN.PK (45, 46). Yeast transformation was conducted using the standard LiAc/SS carrier DNA/PEG method (47). *SNF1* was deleted in *S. cerevisiae* CEN.PK 113-5 D to construct strain SC00 using a fragment containing the *kanMX* marker flanked by *SNF1* up- and downstream regions amplified from genomic DNA of CEN.PK 506-1 C [MATa *URA3 HIS3 TRP1 LEU2 SUC2 MAL2-8<sup>C</sup> snf1* (4-1899)::loxP-*kanMX-loxP*] (48) by PCR, with the following oligonucleotides: *SNF1*-UP and *SNF1*-DOWN (see Table S3). The strains with *SNF1* deletion were selected from yeast extract-peptone-dextrose (YPD) plates containing G418 (0.2 mg/ml) and verified by PCR. Plasmids were transformed into the corresponding host strains for construction of new strains (Table 1). Successful mutants were picked from synthetic dextrose (SD) agar plates with the appropriate medium composition (36).

**Growth conditions.** Strains were grown on SD agar plates containing the appropriate medium composition for selection.

For enzyme activity and total fatty acid analysis, strains of *S. cerevisiae* were cultured in synthetic medium (19) with 20 g/liter glucose in shake flasks. The culture volume in the shake flasks was 20 ml with an initial optical density at 600 nm (OD<sub>600</sub>) of 0.02 after inoculation from precultures grown overnight in synthetic medium.

For FAEE and 3-HP production, strains of *S. cerevisiae* were cultured in synthetic medium (19) with 20 g/liter glucose in 1.0-liter Dasgip stirrer-

pro bioreactors (Dasgip, Jülich, Germany). The working volume was 0.6 liters, and the cultivation was carried out at an agitation of 600 rpm, with an aeration rate of 1 vvm (gas volume flow per unit of liquid volume per minute), a pH of 5, and using 2 M KOH and a controlled temperature of 30°C. The initial OD<sub>600</sub> of the cultures was around 0.02 after inoculation from precultures.

**Chemostat cultivation.** Chemostat cultures were carried out in 1.0-liter Dasgip stirrer-pro bioreactors with a 0.6-liter working volume under aerobic conditions using a dilution rate of 0.10 ( $\pm 0.005$ ) h<sup>-1</sup>. The pH was maintained at 5.0 with 2 M KOH, with a controlled temperature at 30°C, an airflow rate at 1 vvm, and agitation at 600 rpm. The emission of CO<sub>2</sub> and residual O<sub>2</sub> was monitored by Dasgip fedbatch pro gas analysis systems, equipped with off-gas analyzer GA4 based on zirconium dioxide and a two-beam infrared sensor (Dasgip). Strains were grown in synthetic medium containing (per liter) 10 g of glucose, 5 g of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 3 g of KH<sub>2</sub>PO<sub>4</sub>, 0.5 g of MgSO<sub>4</sub> · 7H<sub>2</sub>O, 1 ml of vitamin solution, 1 ml of trace metal solution, and 50 ml of Antiform 204 (27). Samples were taken from the cultures after a steady state was maintained for about 50 h. Steady state refers to cultures without detectable oscillations in values of pH, volume, and CO<sub>2</sub> and O<sub>2</sub> in the off-gas and biomass concentrations.

**Analytical methods.** The growth was measured by optical density at 600 nm and cell dry weight (CDW). CDW was determined by filtering 5 ml of the cell culture through a 0.45- $\mu$ m-pore-size nitrocellulose filter (Sartorius Stedim, Göttingen, Germany) and measuring the increased weight of the dry filter. The concentrations of residual glucose and external metabolites were analyzed using a Dionex Ultimate 3000 high-performance liquid chromatography (HPLC) system (Dionex Softron GmbH, Germering, Germany) with an Aminex HPX-87H column (Bio-Rad, California) using 5 mM H<sub>2</sub>SO<sub>4</sub> as the mobile phase (16).

**Lipid extraction and analysis.** Cells were harvested from a volume of 50 ml, washed twice with distilled water, and centrifuged at 3,000  $\times$  g for 5 min at 4°C. These samples were then frozen at -80°C and freeze-dried for about 3 days or until they appeared dry. Dried cells were weighed before extraction of lipids and FAEEs. The method for lipid extraction and separation was described previously (16). The analysis was performed in an ISQ single quadrupole GC-MS (Thermo, Fisher Scientific, Austin, TX) adopting the previously reported method for fatty acid methyl ester (FAME) or FAEE analysis (16, 49). FAME or FAEE peaks were identified using their spectrum patterns compared to their corresponding standard mix. The standard FAME mix consisted of methyl esters of myristic, palmitic, palmitoleic, heptadecanoic (internal standard), stearic, oleic, and arachidic acids (Sigma-Aldrich, St. Louis, LA, USA), and the standard FAEE mix consisted of ethyl esters of lauric, myristic, palmitic, palmitoleic, heptadecanoic (internal standard), stearic, and oleic acids (Cayman Chemical, Ann Arbor, MI, USA). The serial dilutions in hexane of FAME or FAEE mix standards were injected to generate standard curves for their quantification performed with the help of the QuanBrowser function in the Xcalibur software version 2.0 (Thermo Scientific).

**Determination of 3-HP.** The concentrations of 3-HP were determined by HPLC using the previously described method (33). Culture samples were centrifuged at 12,000  $\times$  g for 10 min, and the supernatants were filtered through a 0.20- $\mu$ m-pore-size nitrocellulose filter (Sartorius Stedim) and analyzed by a Summit HPLC (Dionex, Sunnyvale, CA, USA) with an Aminex HPX-87H column (Bio-Rad, Hercules) at 65°C using 0.5 mM H<sub>2</sub>SO<sub>4</sub> as the mobile phase at a flow rate of 0.5 ml min<sup>-1</sup>.

**Enzyme activity assay.** Cell-free extracts were prepared using a previously reported fast preparation method for enzyme analysis (16). Protein concentrations were measured according to the Bradford method. Acetyl-CoA carboxylase activities in the extracts were measured *in vitro* by monitoring the incorporation of labeled carbon from NaH<sup>14</sup>CO<sub>3</sub> into acid-stable reaction material as reported previously (50). ACCase activity was expressed as pmol of <sup>14</sup>C incorporated into acid-stable products per minute per milligram of cell-free protein extract.

## SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.01130-14/-DCSupplemental>.

- Figure S1, DOCX file, 0.1 MB.
- Table S1, DOCX file, 0.1 MB.
- Table S2, DOCX file, 0.1 MB.
- Table S3, DOCX file, 0.1 MB.

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