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Expression of Yarrowia lipolytica acetyl-CoA carboxylase in Saccharomyces cerevisiae and its effect on in-vivo accumulation of Malonyl-CoA

Humberto Pereira [a], Flávio Azevedo [a], Lucília Domingues [b], Björn Johansson [a,*]

[a] CBMA - Center of Molecular and Environmental Biology Engineering
[b] CEB - Centre of Biological Engineering, University of Minho, Campus de Gualtar, Braga, 4710-057, Portugal

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ABSTRACT

Malonyl-CoA is an energy-rich molecule formed by the ATP-dependent carboxylation of acetyl coenzyme A catalyzed by acetyl-CoA carboxylase. This molecule is an important precursor for many biotechnologically interesting compounds such as flavonoids, polyketides, and fatty acids. The yeast *Saccharomyces cerevisiae* remains one of the preferred cell factories, but has a limited capacity to produce malonyl-CoA compared to oleaginous organisms. We developed a new *S. cerevisiae* strain with a conditional allele of *ACC1*, the essential acetyl-CoA carboxylase (ACC) gene, as a tool to test heterologous genes for complementation. *Yarrowia lipolytica* is an oleaginous yeast with a higher capacity for lipid production than *S. cerevisiae*, possibly due to a higher capacity to produce malonyl-CoA. Measuring relative intracellular malonyl-CoA levels with an *in-vivo* biosensor confirmed that expression of *Y. lipolytica* ACC in *S. cerevisiae* leads to a higher accumulation of malonyl-CoA compared with overexpression of the native gene from an otherwise identical vector. The higher accumulation was generally accompanied by a decreased growth rate. Concomitant expression of both the homologous and heterologous *ACC1* genes eliminated the growth defect, with a marginal reduction of malonyl-CoA accumulation.

1. Introduction

Malonyl-CoA is an important precursor for a range of metabolites of biotechnological interest, including flavonoids, stilbenoids, and polyketides [1,2], as well as fatty acids, fatty alcohols, 3-hydroxypropionic acid, and biodiesel [3–5]. The enzyme malonyl-CoA synthetase binds free malonate to CoA in a plant malonate detoxification pathway [1], but malonyl-CoA is more commonly formed through the ATP-dependent carboxylation of acetyl-CoA by acetyl-CoA carboxylase (ACC). The highest expenditure of ATP in fatty acid synthesis can be accounted for by this metabolic step [6]. There are two main types of ACC, heteromeric and homomeric. The former is usually made up of four different monomers: biotin carboxylase, biotin carboxyl carrier protein, and carboxyl transferase α and β [7–9] and is present in bacteria and in the plastids of most plants. The latter combines all functional components into a single polypeptide chain and is found in the cytosol and mitochondria of eukaryotes [10,11].

The preferred industrial organism *Saccharomyces cerevisiae* is robust and tolerant to environmental stresses [12–15] and amenable to genetic manipulation [4,16–18]. The *S. cerevisiae* genome encodes a cytosolic ACC (*ScACC1*/YNR016C) [19] and a mitochondrial ACC (*ScHFA1*/YMR207C) [20]. The *ScACC1* gene is essential and necessary for the *de-novo* synthesis of lipids [19], while *ScHFA1* is necessary for respiration, probably due to a role in the synthesis of lipoic acid [20]. However, the *S. cerevisiae* metabolism is channeled towards glycolysis and fermentation rather than the production of compounds derived from acetyl- or malonyl-CoA. *S. cerevisiae* ACC is strictly regulated at both transcriptional and post-translational levels and has been identified as an important control point for the synthesis of fatty acids [21]. Attempts at increasing *S. cerevisiae* malonyl-CoA producing capacity have mainly focused on engineering the native gene *ScACC1* [22]. Overexpression of *ScACC1* was reported to increase production of stilbenoids [23], polyketides [2,23,24], fatty alcohols [3], 3-hydroxypropionic acid [4,5,25], biodiesel [4], and fatty acids [4,26–28]. However, apart from [27], none reported more than a 2-fold increase of their respective product. Furthermore, there is at least one instance where *ScACC1* overexpression reportedly

* Corresponding author.
E-mail address: bjorn_johansson@bio.uminho.pt (B. Johansson).

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had no effect on fatty acid production [29]. The *ScACC1* gene product, ScAcc1p is negatively regulated via phosphorylation mediated by AMP-activated protein kinase Snf1p [30] which could explain the relatively low increase in production of target compounds resulting from overexpression. However, *SNF1* deletion with or without *ScACC1* overexpression failed to increase fatty alcohol production [3]. The deletion of *SNF1* has many pleiotropic effects on metabolism [31] which may perhaps negate or obscure the effects of increased ScAcc1p activity. ScAcc1p mutants where the amino acid residues targeted by Snf1p were replaced seem to be more active, presumably due to reduced Snf1p mediated inhibition [2,4,5]. There are few examples of heterologous ACC expression in *S. cerevisiae:* wheat cytosolic ACC, as well as human ACC genes, were able to complement native *ScACC1* function [32,33]; expression of a bacterial heteromeric ACC from *Corynebacterium glutamicum* improved fatty acid production by 1.6-fold [34]; expression of *ACC1* from *Lipomyces starkeyi* improved total lipid accumulation, although not more than overexpression of the native *ScACC1* [28]. Most studies of the effects of ACC engineering ascertained the effect by an indirect variable such as the production of a certain target compound. *In-vitro e*nzymatic assays allow ACC activity to be measured by coupling the malonyl-CoA generating reaction to the production of a fluorescent product [2] or to the consumption of NADPH [35]. These assays rely on reagents such as acetyl-CoA which remain expensive even when produced in-house [36]. *In-vivo* biosensors based on the expression of green fluorescent protein (GFP) regulated by the intracellular concentration of malonyl-CoA have been developed for *S. cerevisiae* [5,37]. *In-vivo* measurements have the added advantage of gauging the effects of the enzyme in its native environment as opposed to *in-vitro* assays with cell extracts. These biosensors are based on the FapR transcription factor and respective operator (*fapO)* of *Bacillus subtilis* that control the expression of genes related to lipid metabolism. The binding of malonyl-CoA to FapR inactivates the repressor and allows the regulated genes to be expressed [38]. By expressing FapR and inserting *fapO* near the transcription start site of a GFP expression cassette, a malonyl-CoA sensor can be created. GFP fluorescence can then be measured *in-vivo* by either fluorescence spectroscopy [5] or flow cytometry [37]. *Yarrowia lipolytica* is perhaps the most studied oleaginous yeast from which several genes have been sourced for heterologous expression that enhances free fatty acid accumulation [39]. However, the heterologous expression of *Yarrowia lipolytica* ACC (*YlACC1*) has not been reported in *S. cerevisiae*. The objective of this work was to compare the malonyl-CoA production capacities of *ScACC1* and *YlACC1* by direct *in-vivo* measurement of malonyl-CoA concentration as well as assessing the physiological effects of expression of each gene alone or combinations of both genes.

2. Materials & Methods

2.1. Strains and media

YPD medium (1 % (w/v) Bacto yeast extract, 1 % (w/v) Bacto peptone, 2 % (w/v) glucose) was used for *S. cerevisiae* cultivation when selection for auxotrophic markers was not needed. Recombinant *S. cerevisiae* strains were cultivated on Synthetic Defined (SD) lacking components to select for auxotrophic markers. This medium contained 6.7 g/L Difco yeast nitrogen base without amino acids, 20 g/L of glucose, and drop-out amino acid mixture (Appendix I). Amino acid drop-out mixtures without uracil (SC Ura-), leucine (SC Leu-), or both (SC Ura- Leu-) were used as required for auxotrophic selection. Routine cloning procedures were performed using *Escherichia coli* XL1-Blue (Stratagene) which was maintained on Lysogeny Broth (LB) (0.5 % (w/v) Bacto yeast extract, 1 % (w/v) Bacto tryptone, 1 % (w/v) NaCl) supplemented with 100 μg/L ampicillin (LB Amp) when needed. Tetracycline (Tet) was added at a concentration of 111 mg/L when required. The *ScACC1* promoter in *S. cerevisiae* CEN.PK2-1C (*MATa ura3-52 his3-Δ1 leu2-3,112 trp1-289, MAL2-8c SUC2*) was replaced with the loxP-kanMX4-loxP-TDH3p-tc3 (TetON/OFF) cassette as described before [40] and the resulting strain was designated AccTet.

2.2. Vector construction

All plasmids and primers used in this study are listed in Tables 1 and 2, respectively. The LiAc method [18] was used for *S. cerevisiae* transformation. The genes *ScACC1* (Genbank JRIV01000180.1 22406.0.29107) and *YlACC1* (Genbank CP061014.1 1598078.0.1605344) were amplified from total DNA extracted from *S. cerevisiae* CEN.PK102-3A and *Y. lipolytica* PYCC 3347 as detailed by Philippsen et al. [41].

The *YlACC1* gene contains two short introns (106 and 360 bp) in the N-terminal part. Since both yeasts possess similar gene-splicing mechanisms [44], the introns could be expected to be processed. Subsequent experiments confirmed active expression (Fig. 1). The *ScACC1* and *YlACC1* PCR products were used to construct the four single gene expression vectors pYPK0_TEF1_ScACC1_TDH3, pYPK0_TEF1_YlACC1_TDH3, pYPK0_TDH3_ScACC1_PGI1, and pYPK0_TDH3_YlACC1_PGI1 by using the Yeast Pathway Kit cloning strategy as described before [43]. The expression cassettes in these plasmids were subsequently amplified and used to create plasmids

Table 1
Plasmids used in this work.

Plasmids	Description	Reference
pTDH3-tc3-3xHA	Plasmid containing the loxP-kanMX4-loxP-TDH3p-tc3 (TetON/OFF) cassette; amp^r; kanMX4	[40]
pLBL3	Plasmid used to confer leucine prototrophy; amp^r; LEU2	[42]
pYPKpw	Shuttle vector used for constructions; amp^r; URA3	[43]
pYPKa_Z_TEF1	Plasmids with promoters cloned in the *Zra*I restriction site; amp^r	[43]
pYPKa_Z_TDH3		
pYPKa_E_TDH3	Plasmids with terminators cloned in the *Eco*RV restriction site; amp^r	[43]
pYPKa_E_PGI1		
pJfapOfapR	Biosensor plasmid; amp^r; LEU2	[5]
pYPK0_TEF1_ScACC1_TDH3	pYPKpw derivative expressing *ScACC1* under control of the P_{TEF1} promoter; amp^r; URA3	This work
pYPK0_TDH3_ScACC1_PGI1	pYPKpw derivative expressing *ScACC1* under control of the P_{TDH3} promoter; amp^r; URA3	This work
pYPK0_TEF1_YlACC1_TDH3	pYPKpw derivative expressing *YlACC1* under control of the P_{TEF1} promoter; amp^r; URA3	This work
pYPK0_TDH3_YlACC1_PGI1	pYPKpw derivative expressing *YlACC1* under control of the P_{TDH3} promoter; amp^r; URA3	This work
pYPK0_TEF1_ScACC1_TDH3 _YlACC1_PGI1	pYPKpw derivative expressing *ScACC1* under control of the P_{TEF1} promoter and *YlACC1* under control of the P_{TDH3} promoter; amp^r; URA3	This work
pYPK0_TEF1_YlACC1_TDH3 _ScACC1_PGI1	pYPKpw derivative expressing *YlACC1* under control of the P_{TEF1} promoter and *ScACC1* under control of the P_{TDH3} promoter; amp^r; URA3	This work

Table 2
Primers used in this work.

Primer name	Primer sequence (5′->3′)
415_ScTDH3tpf	TTAAATAATAAAAAACACGCTTTTTCAGT
467_pCAPs_release_re	ATTTAAATCCTGATGCGTTTGTCTGCACAGA
468_pCAPs_release_fw	GTCGAGGAACGCCAGGTTGCCCACT
504_YlACC1f_SgsI	CCAAATGCGACTGCAATTGAGGACACT
505_YlACC1r_CpoI	TCCGTCACAACCCCTTGAGCAGCTCA
564_YlACC1_628_R	TCGTCCACTCCGGTTCCAGACCACG
567_pCAPsAjiIF	GTCGGCTGCAGGTCACTAGTGAG
568_pCAPsAjiIR	GTGCCATCTGTGCAGACAAACG
577_crp585-557	GTTCTGATCCTCGAGCATCTTAAGAATTC
578_crp42-70	GTTCTTGTCTCATTGCCACATTCATAAGT
586_YlACC1_6264_F	CTCCTCTCTCAAGAAGCAGC
622_ScPGI1tpr_PacI	TAATTAATTTTAGGCTGGTATCTTGATT
623_ScTDH3tpr_PacI	TAATTAATTTGTTTGTTTATGTGTGTTTATTCG
670_sc_acc1-Tc1B:	CCATCTTCTGTGGAGAAGACTCGAATAAGCTTTCTTCGCTCATATGTTCTCGAGGCCTAGG
671_sc_acc1-Tc2:	CGATACGATACGACACGATACGATACGACACGCTACTATAGCATAGGCCACTAGTGGATCTG
698_sc_acc1-B1:	ACCTGGCACTTCAATGTATTG
779_YlACC1_3445_rv	ACAAAGCAGACGACATGGTAGGCAG
780_YlACC1_3305_fwd	TCTTTGCCCACGATGATCCCTGGAT
781_YlACC1f_YPK	GCCAGGTTGCCCACTTTCTCACTAGTGACCTGCAGCCCACATGCGACTGCAATTGAGGACACT
782_YlACC1r_YPK	TAAATCCGGATATCCTGATGCGTTTGTCTGCACAGATGACTCACAACCCCTTGAGCAGCTCA
1123_New775	GTGCAATGCGGCCGCTGAC
1257_ScACC1_rv	AAATCCTGATGCGTTTGTCTGCACAGATGGCACTTATTTCAAAGTCTTCAACAAT
1258_ScACC1_fw	CCCACTTTCTCACTAGTGACCTGCAGCCGACAAATGAGCGAAGAAAGCT
1259_ScACC1middleRV	CCTTCGTGAACTCTAATATCTCC
1260_ScACC1middleFW	GCTCAAGTCTATATTCGTCG
1282_sc_acc1-T	GCGACCATGACAATGCTATTGATGG

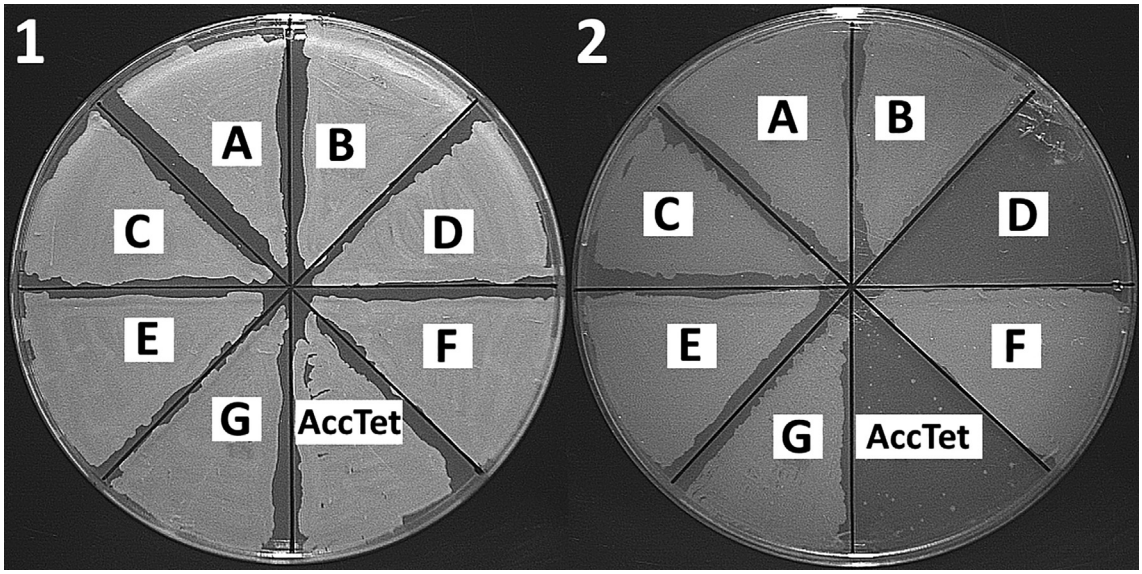


Fig. 1. Cultivation of AccTet and derived strains on solid YPD medium for 48 h without (1) or with (2) 111 mg/L tetracycline. *ACC1* genes from S. cerevisiae or Y. lipolytica or both are expressed using either the P_{TDH3} or P_{TEF1} promoters. (A-F) designates plasmids (Table 1) in the AccTet strain. (A) pYPK0_TEF1_ScACC1_TDH3. (B) pYPK0_TEF1_YlACC1_TDH3. (C) pYPK0_TDH3_ScACC1_PGI1. (D) pYPK0_TDH3_YlACC1_PGI1. (E) pYPK0_TEF1_ScACC1_TDH3_YlACC1_PGI1. (F) pYPK0_TEF1_YlACC1_TDH3_ScACC1_PGI1. (G) CEN.PK2-1C.

expressing both *ScACC1* and *YlACC1* simultaneously: pYPK0_TEF1_ScACC1_TDH3_YlACC1_PGI1 and pYPK0_TEF1_YlACC1_TDH3_ScACC1_PGI1.

The two cassettes were joined by recombination between the P_{TDH3} that is present in both cassettes. Primer pairs used for all PCR amplifications are listed in Table 3. All gene expression constructs were created using the pYPKpw plasmid as the backbone with the URA3 selection marker [43]. All the plasmid constructs were verified by colony PCR (Table 4) and Sanger sequencing. Sequencing results showed that the amplified *YlACC1* fragment had three single-nucleotide polymorphisms at positions 5532 (thymine to cytosine), 6349 (thymine to cytosine), and 6444 (guanine to adenine) when compared with the database sequence. This might be due to differences in the *Y. lipolytica* strain used (See Sequence alignments 1, 2, 25, 26, 31, and 32 in Supplementary materials). The cloning strategy for each construct was coded in python using pydna [45] and is available from a Git repository (https://github.com/MetabolicEngineeringGroupCBMA/Pereira_et_al_2022) and from a zenodo repository [46].

2.3. Physiological characterization and flow cytometry

The ACC expression vectors along with the malonyl-CoA biosensor plasmid pJfapOfapR [5] were used to create the strains listed in

Table 3
Templates and primers used for genetic construction.

Template	PCR product	Forward primer	Reverse primer
pTDH3-tc3-3xHA	loxP-kanMX4-loxP-TDH3p-tc3	670_sc_acc1-Tc1B	670_sc_acc1-Tc1B
pYPK0_TEF1_ScACC1_TDH3	TEF1_ScACC1α	577_crp585-557	1259_ScACC1middleRV
	ScACC1β_TDH3	1260_ScACC1middleFW	623_ScTDH3tpr_PacI
pYPK0_TDH3_ScACC1_PGI1	TDH3_ScACC1α	1123_New775	1259_ScACC1middleRV
	ScACC1β_PGI1	1260_ScACC1middleFW	578_crp42-70
pYPK0_TEF1_YlACC1_TDH3	TEF1_YlACC1α	577_crp585-557	779_YlACC1_3445_rv
	YlACC1β_TDH3	780_YlACC1_3305_fwd	623_ScTDH3tpr_PacI
pYPK0_TDH3_YlACC1_PGI1	TDH3_YlACC1α	1123_New775	779_YlACC1_3445_rv
	YlACC1β_PGI1	780_YlACC1_3305_fwd	578_crp42-70
pYPKa_E_TDH3	E_TDH3	568_pCAPsAjiIR	578_crp42-70
pYPKa_E_PGI1	E_PGI1		
pYPKa_Z_TDH3	Z_TDH3	577_crp585-557	567_pCAPsAjiIF
pYPKa_Z_TEF1	Z_TEF1		
***S. cerevisiae* genomic DNA (CEN.PK102-3A)**	*ScACC1*	1258_ScACC1_fw	1257_ScACC1_rv
pYPK0_TEF1_YlACC1_TDH3	*ScACC1α*	468_pCAPs_release_fw	1259_ScACC1middleRV
	ScACC1β	1260_ScACC1middleFW	467_pCAPs_release_re
***Y. lipolytica* genomic DNA (PYCC 3347)**	*YlACC1a*	781_YlACC1f_YPK	505_YlACC1r_CpoI
	YlACC1b	504_YlACC1f_SgsI	782_YlACC1r_YPK
pYPK0_TEF1_YlACC1_TDH3	*YlACC1α*	468_pCAPs_release_fw	779_YlACC1_3445_rv
	YlACC1β	780_YlACC1_3305_fwd	467_pCAPs_release_re

Table 4
Templates and primers used for diagnostic PCR.

pYPK0 constructs verified	Portions of plasmid amplified by PCR	Forward primer	Reverse primer
pYPK0_TEF1_ScACC1_TDH3	pYPKpw + P_{TEF1} + *ScACC1*	577_crp585-557	698_sc_acc1-B1
	ScACC1 + P_{TDH3} + pYPKpw	1282_sc_acc1-T	578_crp42-70
pYPK0_TDH3_ScACC1_PGI	pYPKpw + P_{TDH3} + *ScACC1*	577_crp585-557	698_sc_acc1-B1
	ScACC1 + P_{PGI1} + pYPKpw	1282_sc_acc1-T	578_crp42-70
pYPK0_TEF1_YlACC1_TDH3	pYPKpw + P_{TEF1} + *YlACC1*	577_crp585-557	564_YlACC1_628_R
	YlACC1 + P_{TDH3}	586_YlACC1_6264_F	622_ScPGI1tpr_PacI
pYPK0_TDH3_YlACC1_PGI1	pYPKpw + P_{TDH3} + *YlACC1*	577_crp585-557	564_YlACC1_628_R
	YlACC1 + P_{PGI1} + pYPKpw	586_YlACC1_6264_F	578_crp42-70
pYPK0_TEF1_ScACC1_TDH3 _YlACC1_PGI1	pYPKpw + P_{TEF1} + *ScACC1*	577_crp585-557	698_sc_acc1-B1
	ScACC1 + P_{TDH3} + *YlACC1*	1282_sc_acc1-T	564_YlACC1_628_R
	YlACC1 + P_{PGI1} + pYPKpw	586_YlACC1_6264_F	578_crp42-70
pYPK0_TEF1_YlACC1_TDH3 _ScACC1_PGI1	pYPKpw + P_{TEF1} + *YlACC1*	577_crp585-557	564_YlACC1_628_R
	YlACC1 + P_{TDH3} + *ScACC1*	586_YlACC1_6264_F	698_sc_acc1-B1
	ScACC1 + P_{PGI1} + pYPKpw	1282_sc_acc1-T	578_crp42-70

Table 5
Created strains that carry two plasmids with URA3 or LEU2 selection markers.

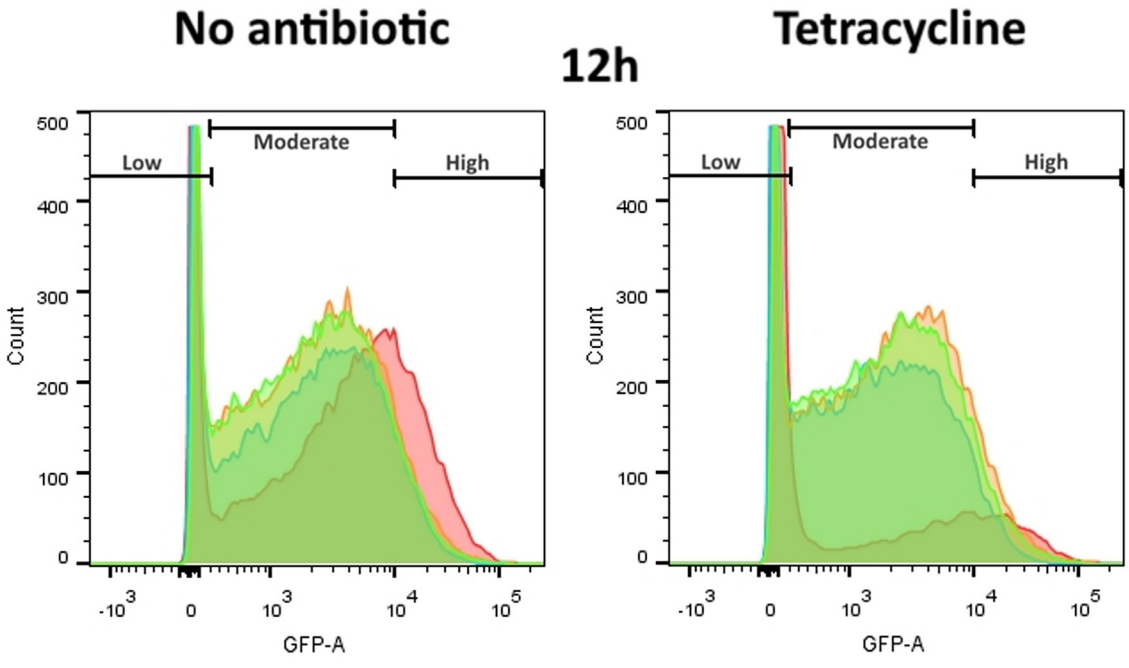
Strain name	Plasmid (URA3)	Plasmid (LEU2)	Original strain
pf	pYPKpw	pLBL3	AccTet
Sf1	pYPK0_TEF1_ScACC1_TDH3	pJfapOfapR	AccTet
Sf2	pYPK0_TDH3_ScACC1_PGI1	pJfapOfapR	AccTet
Yf1	pYPK0_TEF1_YlACC1_TDH3	pJfapOfapR	AccTet
Yf2	pYPK0_TDH3_YlACC1_PGI1	pJfapOfapR	AccTet
YSf	pYPK0_TEF1_YlACC1_TDH3_ScACC1_PGI1	pJfapOfapR	AccTet
SYf	pYPK0_TEF1_ScACC1_TDH3_YlACC1_PGI1	pJfapOfapR	AccTet
cfp	pYPKpw	pJfapOfapR	CEN. PK2-1C
cpp	pYPKpw	pLBL3	CEN. PK2-1C

Table 5. The resulting strains were cultured overnight in 5 mL of SC Ura- Leu- medium at 30 °C. These cultures were used to inoculate 7 mL of medium (SC Ura- Leu- or YPD) supplemented with tetracycline to an initial OD_{640nm} of 0.05 in a 50 mL culture tube and incubated at 30 °C with shaking at 200 rpm. Starting at 2 h to 6 h after inoculation, OD_{640nm} was measured every 2 h and the maximum specific growth rate was calculated from data during the exponential growth phase (Figures S1 and S2). At 12 h of cultivation, 1 mL of culture was collected for flow cytometry analysis. When needed, the samples were diluted so as to keep cell density at around 1×10^7 cells/mL. Cells were centrifuged at 16,000 g for 10 s and incubated for 30 min in a 100 µL phosphate-buffered saline (PBS) solution containing 4 % of formaldehyde. After a washing step with PBS, the cells were resuspended in 1 mL of PBS. Flow cytometry was performed using a BD™ LSR II flow cytometer. Cells were excited by a blue laser at 488 nm (50 mW), and green fluorescence signals were measured through a 525/50 bandpass filter for 30,000 cells per sample.

3. Results

3.1. ScACC1 and YlACC1 effects on maximal growth rates

The *ACC1* promoter in *S. cerevisiae* CEN.PK2-1C was replaced with a TetON/OFF promoter cassette as described by Kötter et al. [40], and the resulting strain was named "AccTet" (see Materials & Methods subsection 2.1. and Jupyter notebook "AccTet.ipynb"). This strain grows normally on YPD medium (Fig. 1.1 sector AccTet) but is unable to grow in medium supplemented with tetracycline (Fig. 1.2 sector AccTet). The loss of growth was a relatively stable phenotype and only sporadic residual growth could be detected after 48 h. The AccTet strain was transformed with multicopy plasmids carrying a URA3 marker and the *ScACC1* gene under the control of the P_{TDH3} promoter or the somewhat weaker P_{TEF1} promoter [47] (Table 1, pYPK0_TEF1_ScACC1_TDH3 and pYPK0_TDH3_ScACC1_PGI). The P_{TDH3} is induced by fermentable carbon sources, while the P_{TEF1} is active on both fermentable and non-



	Name	No antibiotic Low	No antibiotic Moderate	No antibiotic High	Tetracycline Low	Tetracycline Moderate	Tetracycline High
	CEN.PK2-1C	27	65	8	27	65	6
	AccTet	27	50	23	80	13	7
	P_{TEF1}-ScACC1	11	78	11	11	79	10
	P_{TDH3}-ScACC1	13	78	9	13	78	9

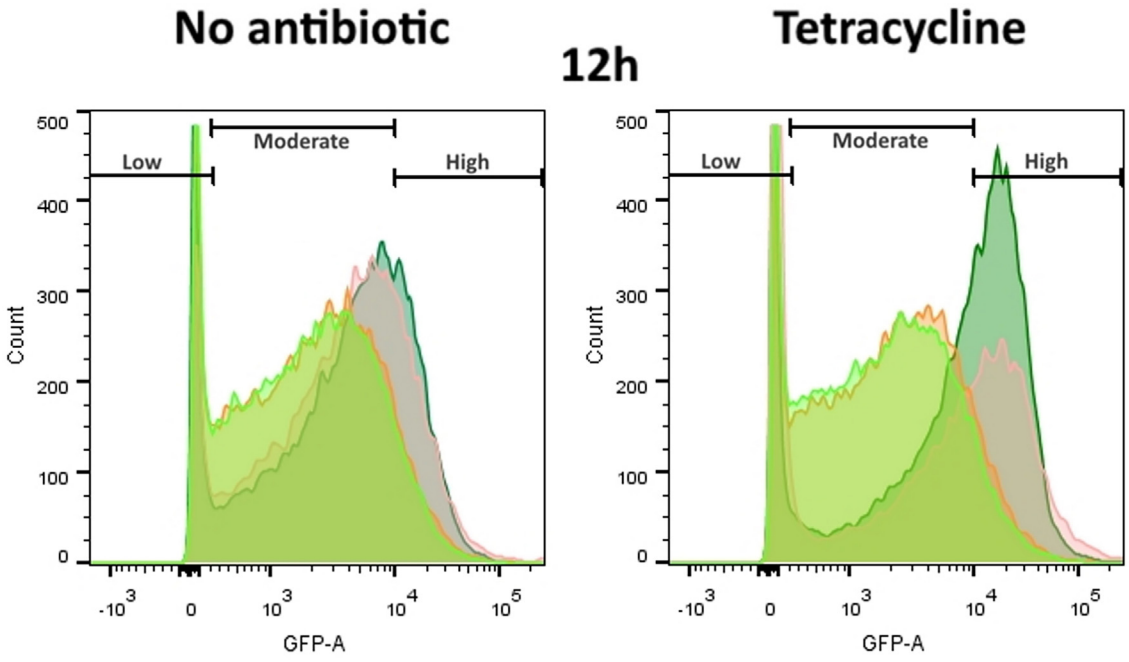
Fig. 2. Flow cytometry measurements with excitation at 488 nm of cells cultivated in liquid SC Ura- Leu- medium for 12 h without and with tetracycline. All strains carry the pJfapOfapR biosensor plasmid. The CEN.PK2-1C and AccTet strains carry the empty pYPKpw plasmid. P_{TEF1}-ScACC1 and P_{TDH3}-ScACC1 indicate the AccTet strain carrying pYPK0_TEF1_ScACC1_TDH3 and pYPK0_TDH3_ScACC1_PGI1, respectively.

fermentable carbon sources. The *ScACC1* gene controlled by the P_{TEF1} (Fig. 1.2 sector A) or P_{TDH3} (Fig. 1.2 sector C) restored growth on medium with tetracycline indicating that growth can be restored by the *ScACC1* gene carried on a plasmid. The *Y. lipolytica* ACC protein (UniProt A0A371C979) is quite similar to the *S. cerevisiae* protein (UniProt N1P4Q3), showing 64.1 % identity and 77.4 % similarity (See Sequence alignment 3 in Supplementary materials). It contains the domains with the functions of biotin carboxylation, ATP-grasp, CoA carboxyltransferase N-terminal, and CoA carboxyltransferase C-terminal in the same order as in the *S. cerevisiae* protein [48,49]. These can be visualized using the UniProt database web interface [50] (Fig. S3). The ACC gene from *Y. lipolytica* (*YlACC1*) was subcloned into expression vectors identical to the two used for the *S. cerevisiae* (Table 1, pYPK0_TEF1_YlACC1_TDH3 and pYPK0_TDH3_YlACC1_PGI1). These vectors were used to transform AccTet and the resulting transformants were subsequently plated on medium with and without tetracycline. Growth was observed on medium containing tetracycline with the gene under P_{TEF1}, but not under P_{TDH3} regulation (Figs. 1.1 and 1.2 sectors B and D). Our initial hypothesis was that the *YlACC* gene was inactive due to sequence errors introduced during the PCR process. However, the gene was verified by DNA sequencing confirming the identity of the cloned fragment. Interestingly, three single-nucleotide polymorphisms at positions 5532 (thymine to cytosine), 6349 (thymine to cytosine), and 6444 (guanine to adenine) were discovered, with the first changing the amino acid residue from phenylalanine to serine and the latter changing it from glycine to aspartic acid. This is possibly a difference between the *Y. lipolytica* strain used for DNA extraction (PYCC 3347) and the strain used as reference for the DNA sequence (DSM 3286). (See Sequence alignments 1 and 2 in Supplementary materials). The two strains that failed to grow on solid medium (Fig. 1.2 sectors D and AccTet) could grow in liquid YPD medium with tetracycline from an initial cell count of 1.5×10^6 for about two generations after which growth ceased (results not shown). Strains expressing both the *S. cerevisiae* and *Y. lipolytica* ACC genes simultaneously were also constructed. In each of the two plasmids, one gene was put under the control of P_{TEF1} and the other under P_{TDH3} (Table 1, pYPK0_TEF1_ScACC1_TDH3_YlACC1_PGI1 and pYPK0_TEF1_YlACC1_TDH3_ScACC1_PGI1). Surprisingly, AccTet strains carrying either of these constructs grew in the presence of tetracycline (Fig. 1.2 sectors E and F). The change of phenotype suggests an interaction between the genes that is not simply additive, as the presence of the *S. cerevisiae* allele restored growth.

3.2. ScACC1 and YlACC1 effects on malonyl-CoA accumulation

An *in-vivo* malonyl-CoA biosensor [5] was established by the transformation of CEN.PK2-1C and AccTet strains with the biosensor plasmid pJfapOfapR (LEU2) and the empty pYPKpw plasmid (URA3). This biosensor causes a malonyl-CoA inducible expression of green fluorescent protein, allowing the *in-vivo* measurement of malonyl-CoA concentration as fluorescence. Cells were analyzed by flow cytometry and the population was divided into three groups displaying "Low", "Moderate", and "High" fluorescence (Figs. 2, 3, and 4) to facilitate interpretation of the results.

In the absence of tetracycline, the CEN.PK2-1C strain and the AccTet strain displayed similar fluorescence accumulation (Fig. 2,

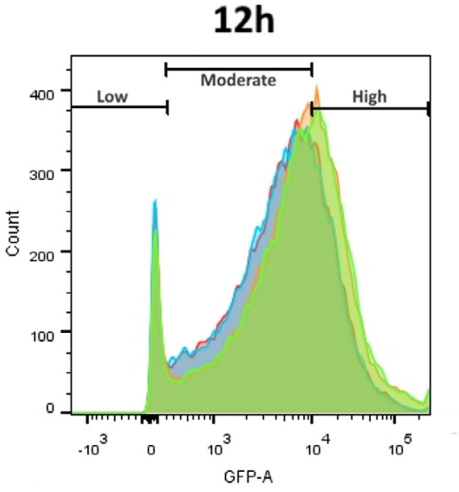


	Name	No antibiotic Low	Moderate	High	Tetracycline Low	Moderate	High
	P_{TEF1}-ScACC1	10	79	11	11	78	11
	P_{TDH3}-ScACC1	13	78	9	13	78	9
	P_{TEF1}-YlACC1	11	63	26	15	36	49
	P_{TDH3}-YlACC1	8	68	24	39	28	33

Fig. 3. Flow cytometry fluorescence measurements with excitation at 488 nm of cells cultivated in liquid SC Ura- Leu- medium for 12 h without and with tetracycline. All strains carry the pJfapOfapR biosensor plasmid. P_{TEF1}-ScACC1 and P_{TDH3}-ScACC1 indicate the AccTet strain carrying pYPK0_TEF1_ScACC1_TDH3 and pYPK0_TDH3_ScACC1_PGI1, respectively. P_{TEF1}-YlACC1 and P_{TDH3}-YlACC1 indicate the AccTet strain carrying pYPK0_TEF1_YlACC1_TDH3 and pYPK0_TDH3_YlACC1_PGI1, respectively.

"No antibiotic"). The AccTet strain had a higher fraction of cells in the "High" fluorescence group compared to CEN.PK2-1C, possibly because the promoter used to control its expression is stronger when turned on compared to the native promoter. Expression of plasmid-borne *ScACC1* genes controlled by either P_{TEF1} or P_{TDH3} promoters mostly increased the population of cells with intermediate fluorescence levels ("Moderate" fluorescence) and diminished the population with "Low" fluorescence. The expression of *ScACC1* on a plasmid increased the population with "Moderate" fluorescence from 50 to 65 % to 78–79 %. In the presence of tetracycline, the AccTet strain displayed markedly diminished fluorescence while the other strains produced a signal unaffected by tetracycline (Fig. 2, "Tetracycline"). Cells without the biosensor displayed only "Low" fluorescence (Fig. S4) as expected.

The expression of *YlACC1* had a dramatic effect on the fluorescence population distribution. Strains expressing *YlACC1* controlled by either of the two promoters had a higher fraction of cells with high fluorescence compared to strains expressing *ScACC1* (Fig. 3). The strain expressing *YlACC1* controlled by P_{TEF} showed strong fluorescence for 49 % of the population in the absence of tetracycline compared to 11 % for the *S. cerevisiae* gene controlled by the same promoter. As mentioned before, the strain expressing *YlACC1* controlled by the P_{TDH3} promoter has a strong growth defect (Fig. 1.2) in the presence of tetracycline. This defect seems to accompany a large population of cells with a low fluorescence signal (39 % Fig. 3, "Tetracycline"). These effects were present, but not as pronounced in the absence of tetracycline (Fig. 3, "No antibiotic").



	Name	Low	Moderate	High
	P_{TEF1}-ScACC1 P_{TDH3}-YlACC1	6	66	28
	P_{TEF1}-ScACC1 P_{TDH3}-YlACC1 w/ Tetracycline	6	65	29
	P_{TEF1}-YlACC1 P_{TDH3}-ScACC1	5	54	41
	P_{TEF1}-YlACC1 P_{TDH3}-ScACC1 w/ Tetracycline	6	53	41

Fig. 4. Flow cytometry fluorescence measurements with excitation at 488 nm of cells cultivated in liquid SC Ura- Leu- medium for 12 h without and with tetracycline. All strains carry the pJfapOfapR biosensor plasmid. P_{TEF1}-ScACC1 P_{TDH3}-YlACC1 and P_{TEF1}-YlACC1 P_{TDH3}-ScACC1 indicate the AccTet strain carrying pYPK0_TEF1_ScACC1_TDH3_YlACC1 and pYPK0_TEF1_YlACC1_TDH3_ScACC1, respectively.

There is an apparent moderating effect observed upon the removal of tetracycline. Fluorescence levels in strains expressing both ACC genes were not affected by the presence of tetracycline (Fig. 4). Since either the activation of the conditional allele or the presence of the *ScACC1* gene on a plasmid both relieve the growth defect (Fig. 1), the relief is probably linked to the ScAcc1p gene product. The extreme population shift seen for cells only expressing the *YlACC1* was also not observed, while the population of cells with very low fluorescence (Fig. 4, "Low") was reduced to 5–6 %. We measured the growth rates of strains used in the flow cytometry experiments (Fig. 5) in the presence and absence of tetracycline and in defined or rich media. Most strains grew at around 0.3 h^{-1} regardless of the medium used. With tetracycline, the AccTet derived strain containing only a *ScACC1* controlled by the TetON/OFF promoter grew at slower rates (0.09 ± 0.01 h^{-1} in SC Ura- Leu- Tet and 0.19 ± 0.06 h^{-1} in YPD Tet) until it ceased growing at an OD_{640nm} of around 0.1 (Figs. 5, S1, and S2). The strain expressing *YlACC1* under P_{TDH3} promoter control behaved in a similar fashion, though it grew slightly faster (0.11 ± 0.02 h^{-1} in SC Ura- Leu- Tet and 0.25 ± 0.08 h^{-1} in YPD Tet), and ceased growing at a higher OD_{640nm} of around 0.2 (Figs. 5, S1, and S2, D). The strain expressing *YlACC1* under P_{TEF1} promoter control grew at similar rates to *YlACC1* under P_{TDH3} promoter control (0.12 ± 0.02 h^{-1} in SC Ura- Leu- Tet and 0.28 ± 0.01 h^{-1} in YPD Tet) until the 10 h mark where growth deaccelerated (Figs. 5, S1, and S2, B). This is consistent with the results obtained on solid media for the corresponding strain without the malonyl-CoA biosensor (Fig. 1.2 sector "AccTet"). These growth defects were rescued by simultaneous expression of the *ScACC1* gene (Fig. 5 E and F). The biosensor plasmid did not add any significant burden as replacing it with an empty plasmid did not affect the growth rate much (Fig. 5 G and H).

The data displayed in Figs. 2 and 3 were compiled into a mean fluorescence reading by summing the fluorescence reading for each of the 30,000 events. These values are displayed in Fig. 6. This compilation makes it possible to compare the data obtained by flow cytometry with those reported using the same biosensor [5], but with average fluorescence readings from a fluorimeter. A 5.8-fold increase in fluorescence intensity is observed when using CEN.PK102-5B carrying a plasmid-borne copy of a *ScACC1* double mutant (S659A, S1157A) controlled by the P_{TEF1} when compared with the same strain carrying an identical construct but with the native *ScACC1* gene [5]. The AccTet expressing the *ScACC1* from the P_{TEF1} promoter (Fig. 6, A) is the strain most comparable to the wild-type reference strain used by Chen and coworkers [5]. The highest total fluorescence obtained was for the strain expressing *YlACC1* from a P_{TEF1} promoter and *ScACC1* from a P_{TDH3} promoter (Fig. 6, F). The ratio between the highest and lowest fluorescent counts was 3.2, somewhat lower than the one calculated for the *ScACC1* double mutant. However, background fluorescence is usually subtracted from data obtained with a fluorometer, while no such subtraction was made from the values in Fig. 6 contributing to a low ratio for the data obtained here. While other technical details such as media composition and cultivation strategy might also affect the comparability of results, the results show that heterologous expression of ACC genes is a viable alternative strategy for improving malonyl-CoA production capacity in *S. cerevisiae*.

4. Discussion

Enhancing malonyl-CoA production in *S. cerevisiae* by overexpression of the endogenous wild-type *ScACC1* gene has met with varied success [2,4,5,26–29,34]. So far, the most effective strategies

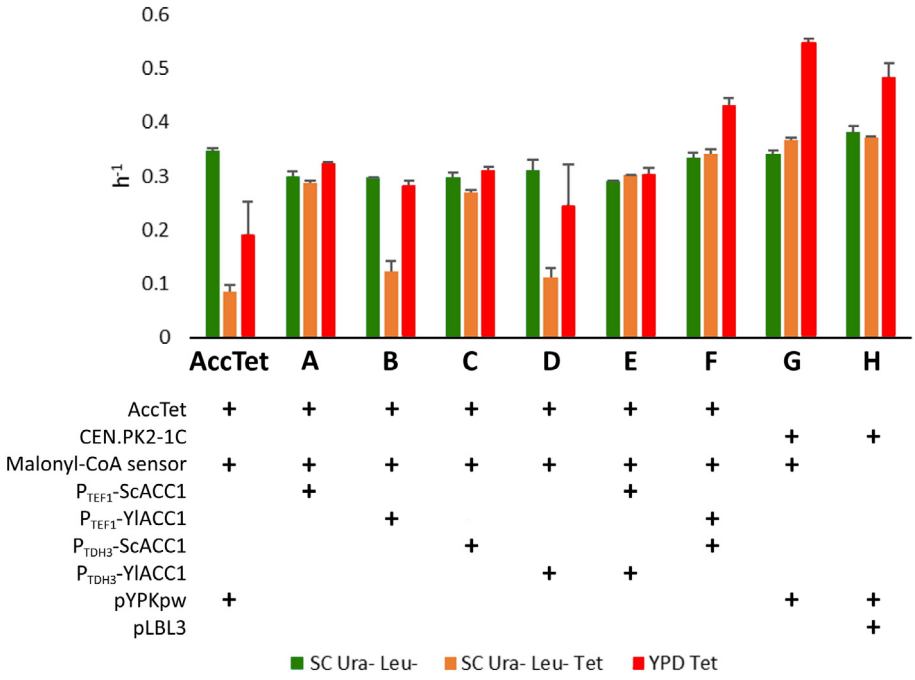


Fig. 5. Maximum specific growth rates for indicated strains in three different media. Rates were calculated for each of three replicas, error bars represent standard deviation. Media were SC Ura- Leu- (green), SC Ura- Leu- with tetracycline (orange) and YPD with tetracycline (red). Vectors pYPKpw and pLBL3 carry the URA3 and LEU2 markers, respectively. The former was used for ACC expression vector construction. pLBL3 is a similar vector but with LEU2 marker. (A-F) designates plasmids (Table 1) in the AccTet strain. (A) pYPK0_TEF1_ScACC1_TDH3 and pJfapOfapR. (B) pYPK0_TEF1_YlACC1_TDH3 and pJfapOfapR. (C) pYPK0_TDH3_ScACC1_PGI1 and pJfapOfapR. (D) pYPK0_TDH3_YlACC1_PGI1 and pJfapOfapR. (E) pYPK0_TEF1_ScACC1_TDH3_YlACC1_PGI1 and pJfapOfapR. (F) pYPK0_TEF1_YlACC1_TDH3_ScACC1_PGI1 and pJfapOfapR. (G) CEN.PK2-1C with pYPKpw and pJfapOfapR. (H) CEN.PK2-1C with pYPKpw and pLBL3. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

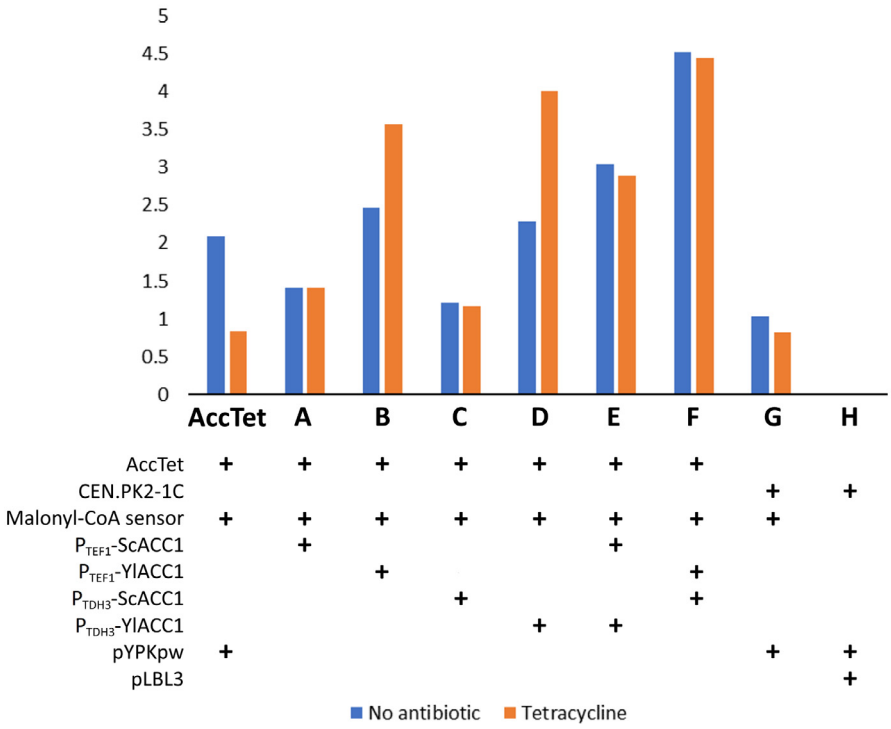


Fig. 6. Total fluorescence measured by flow cytometry. Strain designations are the same as in Fig. 5.

for increased malonyl-CoA production have focused on relieving negative regulation of the native ScAcc1p by removing target sites for kinases such as Snf1p. Expression of heterologous ACC genes remains an underexplored strategy for improving malonyl-CoA availability in *S. cerevisiae*. Previous attempts at expressing heterologous ACC for the improvement of malonyl-CoA production have been done while maintaining the expression of the native *S. cerevisiae* gene. Our results show that ACC gene products may interact in unexpected ways. The reduction in growth rate observed in strains with a high accumulation of malonyl-CoA agrees with previous findings of a negative correlation between activity and growth rate [4]. Since growth defects have been reported for different genes, it seems more likely that the effect is due to depletion of intermediates or the accumulation of malonyl-CoA rather than toxic effects of the accumulated protein. Expression of *ScACC1* and overexpression of the native ACC in *E. coli* both resulted in increased fatty acid production indicating increased malonyl-CoA production but with associated toxic effects [51]. It is not clear how the *ScACC1* and *YlACC1* genes interact. The gene products belong to phylogenetically close species [52] and naturally form homodimers. It is tempting to speculate that the proteins might form heterodimers that are less active or subject to the in-vivo regulation of the native ScAcc1p protein.

Engineering the supply of malonyl-CoA precursor acetyl-CoA has previously proved successful at increasing the production of malonyl-CoA-derived products. Engineering a pyruvate dehydrogenase bypass resulted in 7-fold increased flavonoid titer [53]. Heterologous expression of ATP citrate lyase has similarly proved successful at improving the production of 1-hexadecanol [3]. The addition of improved Coenzyme A generation via overexpression of a pantothenate kinase gene led to a 2-fold increase in product titer [53]. Combining these approaches with *YlACC1* expression has the potential of further improving malonyl-CoA titers. Moreover, it could help alleviate or elucidate the origin of the toxicity that we observe with increased ACC activity.

5. Conclusions

S. cerevisiae is a robust microorganism well suited for use in industrial applications. However, this yeast is very limited when it comes to generating malonyl-CoA-derived products. To the best of our knowledge, our work represents the first report of the heterologous expression of an ACC resulting in higher malonyl-CoA accumulation than overexpression of the native gene in *S. cerevisiae*. This result should spur the investigation of other heterologous genes in order to assess the full potential of the metabolic capacity in nature. However, the interaction between homologous and heterologous ACC genes underlines the need to study the expression of heterologous genes alone in absence of the homologous gene. These interactions could also be used in new strategies where temporal control of one or the other could be used to fine-tune malonyl-CoA production capacity for an optimal process.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.csbj.2022.01.020.

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