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Screening a genomic library for genes involved in propionate tolerance in *Yarrowia lipolytica*

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Abstract

Microbial oils are regarded as promising alternatives to fossil fuels. For bio-oil production to be sustainable over the long term, utilizing low-cost substrates like volatile fatty acids (VFAs) is crucial. Increasing attention is being paid to one of the most common VFAs: propionate, a substrate that could be used to produce the odd-chain FAs of industrial interest. However, little is known about microbial responses to propionate-induced stress and the genes involved. Using genomic library screening, we identified two genes involved in propionate tolerance in *Yarrowia lipolytica*—*MFS1* and *RTS1*. Strains containing each of the genes displayed enhanced tolerance to propionate even when the genes were expressed in truncated form via a replicative plasmid. Compared with the control strain, the strain overexpressing *MFS1* under a constitutive promoter displayed greater tolerance to propionate: It had a shorter lag phase and higher growth rate in propionate medium (0.047 hr^{-1} versus 0.030 hr^{-1} for the control in 40 g/L propionate); it also accumulated more total lipids and more odd-chain lipids (10% and 3.3%, respectively) than the control. The strain overexpressing *RTS1* showed less tolerance for propionate than the strains harboring the truncated form (0.057 hr^{-1} versus 0.065 hr^{-1} in 40 g/L propionate medium) but still had higher tolerance than the control strain. Furthermore, the overexpression of *RTS1* seemed to confer tolerance to other weak acids such as lactate, formic acid, malic acid, and succinic acid. This work provides a basis for better understanding the response to propionate-induced stress in *Y. lipolytica*.

KEYWORDS

genomic library, propionate, stress response, tolerance, volatile fatty acids, *Yarrowia lipolytica*

1 | INTRODUCTION

Microbial oils (lipids and fatty acid (FA)-derived products) are viewed as promising alternatives to fossil fuels in the face of growing concerns over environmental issues and energy production. To ensure the long-term sustainability of bio-oils, much research has been dedicated to enhancing lipid production from microorganisms and minimizing the cost of substrates. As a result, there is a growing interest in using volatile FAs (VFAs) as carbon sources for lipid production (Beopoulos et al., 2008; Fei et al., 2011; Qiao et al., 2015). VFAs can be obtained from agro-industrial waste and several types of biodegradable organic waste (Papanikolaou, Galiotou-Panayotou, Fakas, Komaitis, & Aggelis, 2008); in theory, they should have higher conversion efficiencies than sugar-based carbon sources because of the shorter metabolic pathways involved (Gao, Li, Zhou, Cheng, & Zheng, 2017).

Abbreviations: DCW, dry cell weight; FA, fatty acid; FAME, fatty acid methyl ester; GC, gas chromatography; gDNA, genomic DNA; MFS, major facilitator superfamily; ORF, open reading frame; PCR, polymerase chain reaction; TFAs, total fatty acids; VFA, volatile fatty acid; WT, wild type

Propionate, one of the most common VFAs, can be used by *Yarrowia lipolytica* as its sole carbon source in lipid production (Fontanille, Kumar, Christophe, Nouaille, & Larroche, 2012; Kolouchová, Schreiberová, Sigler, Masák, & Řezanka, 2015; Gao et al., 2017). Recently, *Y. lipolytica* was metabolically engineered to produce unusual lipids, namely, odd-chain FAs, using propionate (Park, Dulermo, Ledesma-Amaro, & Nicaud, 2018). In the wild-type (WT) strain, lipid content ranged from 7.39% to 8.14% (w/w dry cell weight [DCW]), depending on the composition of the carbon source, and odd-chain FAs represented 22.9% to 36.5% of total lipids, corresponding to a concentration of 0.01–0.12 g/L. In the strain in which *PHD1* had been deleted (the gene codes for 2-methylcitrate dehydratase), lipid content was 8.1% (w/w DCW) higher than in the WT strain in minimal glucose and propionate media; odd-chain FAs also made up a greater percentage of total lipids (Δ*phd1* strain: 46.8% versus WT strain: 28.3%; corresponding to a concentration of 0.17 g/L in the former). Under the same conditions, in the obese Δ*phd1* strain, accumulated lipid content was 24.8% (w/w DCW) much higher than in the WT strain; the representation of odd-chain FAs among total lipids was slightly lower (41.9%) and corresponded to a concentration of 0.57 g/L.

Propionate and other weak acids have been used as food preservatives due to their potent inhibitory effects on microbial growth (Abbott et al., 2007). These effects have been observed in several studies of lipid production employing *Y. lipolytica* (Fontanille et al., 2012; Kolouchova et al., 2015; Park et al., 2018). Our previous study showed that propionate inhibited the growth of a WT strain: The growth rate was 0.236 hr^{-1} in 2 g/L propionate medium and dropped to 0.029 hr^{-1} in 100 g/L propionate medium (Park et al., 2018). For this reason, it will be necessary to improve microbial resistance to propionate and other weak acids if they are to be used as substrates in lipid production. Very few studies have looked at the tolerance and utilization of propionate by oleaginous yeast for lipid production. Consequently, it is crucial to have a better understanding of the molecular and regulatory responses of yeast to propionate if we wish to use the VFA as a carbon source.

Several research has investigated at stress response mechanisms in yeast, and efforts have been made to engineer strains with enhanced tolerance. Most studies aiming to improve tolerance to weak acids utilized acetic acid. For example, acetic acid tolerance in *Saccharomyces cerevisiae* was improved when the FA composition of the yeast's cell membrane was modified by overexpressing *ELO1* (Zheng et al., 2013). It was also improved by blocking aquaglyceroporin channels (Zhang et al., 2010) and by introducing the acetate consumption pathway (Wei, Quarterman, Kim, Cate, & Jin, 2013). In addition to traditional metabolic engineering approaches, such as overexpressing or knocking out one or more genes, genomic or global approaches are also proving increasingly successful in developing tolerant phenotypes (Santos & Stephanopoulos, 2008; Nicolaou, Gaida, & Papoutsakis, 2010). Borden and Papoutsakis (2007) screened for butanol-tolerant *Clostridium acetobutylicum* strains using genomic library enrichment. In *S. cerevisiae*, key genes involved in acetic acid tolerance were identified by screening deletion or overexpression libraries (Ding et al., 2013; Peña, Glasker, & Srienc, 2013). When researchers used transcript analysis to study the stress response of *S. cerevisiae* to a variety of weak organic acids (propionate, benzoate, sorbate, and acetate), it was revealed that acetate and propionate had a stronger impact on membrane-associated transport processes (Abbott et al., 2007). However, the mechanisms underlying propionate tolerance have remained elusive, regardless of the species examined (Guo & Olsson, 2014). As a result, it is necessary to carry out further research into the regulatory responses of yeast to propionate to develop more robust strains capable of employing propionate to produce odd-chain FAs.

In this study, a genomic library was constructed that contained the native promoters of *Y. lipolytica*. It was used to screen for propionate-tolerant strains, and two genes were identified. We also observed that the overexpression of the identified genes under the constitutive promoter enhanced propionate tolerance. Furthermore, tolerance to other organic acids was explored using the overexpression strains. This work gives insight into propionate stress responses and can help to develop more robust *Y. lipolytica* strains that exploit a wider range of substrates.

2 | MATERIALS AND METHODS

2.1 | Strains and media

The *Y. lipolytica* strains used in this study were derived from JMY7228 (Po1d *phd1 mfe1 tgl4* + pTEF-*DGA2* pTEF-*GPD1* hp4d-*LDP1*-*URA3*ex), which was derived from JMY3776 (Park et al., 2018). All the *Escherichia coli* and *Y. lipolytica* strains used in this study are listed in Table 1.

Media and growth conditions for *E. coli* were as described by Sambrook and Green (2012), and those for *Y. lipolytica* have been described by Barth and Gaillardin (1996). Rich medium (YPD) and minimal glucose medium (YNB) were prepared as described previously (Park et al., 2018). Minimal medium (YNB) contained 0.17% (w/v) yeast nitrogen base (without amino acids and ammonium sulfate, YNBww, Difco), 0.5% (w/v) NH_4Cl, and 50 mM KH_2PO_4-Na_2HPO_4 buffer (pH 6.8). The following carbon sources were added: 0.5% (w/v) glucose and 1–5% (w/v) propionate. Leucine was supplemented at 0.1 g/L when necessary. Solid media were prepared by adding 1.5% (w/v) agar.

2.2 | Construction of plasmids and strains (*E. coli* and *Y. lipolytica*)

Standard molecular genetic techniques were used in this study (Sambrook & Green, 2012). Restriction enzymes were obtained from New England Biolabs (MA, USA). The polymerase chain reactions (PCRs) were performed using an Applied Biosystems 2720 Thermal Cycler and employing GoTaq DNA Polymerase (Promega, WI, USA) and Q5 High-Fidelity DNA Polymerase (New England Biolabs).

The overexpression plasmids were constructed by ligating JMP62-LEU2ex-pTEF and the PCR fragments. The gene expression

TABLE 1 Strains used in this study

Strain name	Plasmid and genotype	Reference
Escherichia coli		
MGM collection	pINA240	(Barth & Gaillardin, 1996)
JME4010	pINA240-Pool 1 from MGM1221021	Fournier et al., unpublished
JME4014	pINA240-Pool 2 from MGM1221022	Fournier et al., unpublished
JME4018	pINA240-Pool 3 from MGM1221023	Fournier et al., unpublished
JME4022	pINA240-Pool 4 from MGM1221024	Fournier et al., unpublished
JME4026	pINA240-Pool 5 from MGM1221025	Fournier et al., unpublished
JME4030	pINA240-Pool 6 from MGM1221026	Fournier et al., unpublished
JME4034	pINA240-Pool 7 from MGM1221027	Fournier et al., unpublished
JME4038	pINA240-Pool 8 from MGM1221028	Fournier et al., unpublished
JME4042	pINA240-Pool 9 from MGM1221029	Fournier et al., unpublished
JME4046	pINA240-Pool 10 from MGM1221030	Fournier et al., unpublished
JME2563	JMP62-*LEU2*ex-pTEF	(Dulermo et al., 2017)
JME4569	JMP62-*LEU2*ex-pTEF-*MFS1i*	This study
JME4596	JMP62-*LEU2*ex-pTEF-*RTS1i*	This study
Yarrowia lipolytica		
JMY2900	Po1d *URA3 LEU2*	(Dulermo, Gamboa-Meléndez, Dulermo, Thevenieau, & Nicaud, 2014)
JMY3776	Po1d *phd1 mfe1 tgl4* + pTEF-*DGA2*-*LEU2*ex + pTEF-*GPD1*-*URA3*ex	Park et al., 2018
JMY7228	Po1d *phd1 mfe1 tgl4* + pTEF-*DGA2* pTEF-*GPD1* + hp4d-*LDP1*-*URA3*ex	This study
JMY7264	Po1d *phd1 mfe1 tgl4* + pTEF-*DGA2* pTEF-*GPD1* + hp4d-*LDP1*-*URA3*ex + *LEU2*	This study
JMY7588	JMY7228 + pINA240-*RTS1r*	This study
JMY7589	JMY7228 + pINA240-*MFS1r*	This study
JMY7567	JMY7228 + pTEF-*RTS1i*-*LEU2*ex	This study
JMY7569	JMY7228 + pTEF-*MFS1i*-*LEU2*ex	This study

cassettes to be transformed were prepared via the *Not*I digestion of the expression plasmids. The transformation of *Y. lipolytica* was carried out using a Frozen-EZ Yeast Transformation Kit (Zymo Research, CA, USA). Transformants were selected on YNB medium and verified by colony PCR. The expression cassettes were integrated randomly in the genome as described previously (Bordes, Fudalej, Dossat, Nicaud, & Marty, 2007).

2.3 | Genomic library construction

Y. lipolytica W29 genomic DNA (gDNA) was partially digested using the *Sau3*A restriction enzyme, and the resulting fragments (of up to 5 kb) were cloned into a pINA240 plasmid at the *Bam*H1 site (Figure 1). The recombinant plasmid conferred resistance to ampicillin, but the gene for tetracycline resistance was lost. The efficiency of gDNA cloning was verified by measuring the percentage of clones that were only resistant to ampicillin. The ampicillin-resistant *E. coli* colonies were mixed to form 10 independent pools (Fournier et al., unpublished, Table 1). They were then stored in the Microbiology and Molecular Genetics Collection (*Microbiologie et Génétique Moléculaire*, MGM) under the reference codes MGM1221021–MGM1221030 at the INRA Centre of Thiverval-Grignon (France). Transformant pools from the MGM collection were grown in 200 ml of LB ampicillin for 24 hr; 1 ml of culture was stored in 20% glycerol and kept in our laboratory collection at −80°C under the reference codes JME4010 to JME4046 (Table 1). The rest of the cultures were used for DNA plasmid preparation, which was carried out using the QIAGEN Plasmid Midi Kit (Hilden, Germany).

To verify the quality of the DNA plasmid pools, *E. coli* was transformed using 2 µl of the DNA preps from reference pool MGM1221021. Twenty random transformants were selected for plasmid extraction. Plasmids from the different clones presented different profiles upon digestion with restriction enzymes (i.e., *Eco*RI and *Bgl*II; Figure S1), indicating that each clone contained a different genomic insert.

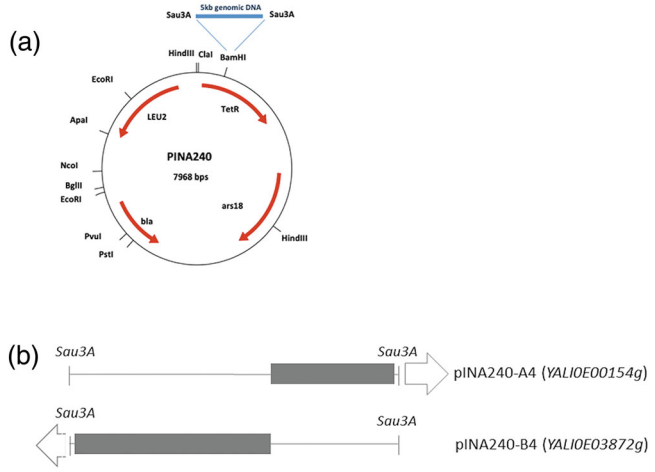

FIGURE 1 (a) Schematic map of the replicative plasmid pINA240 used to construct the gDNA library. The partial *Sau3*A genomic fragments were cloned at the *Bam*H1 dephosphorylated site. (b) Schematic map of the gDNA fragments inserted into JMY7588 (pINA240-A4) and JMY7589 (pINA240-B4). The grey bars indicate the genes' open reading frames (ORFs), and the white arrows show the ORFs of each gene that were not included in the genomic fragment [Colour figure can be viewed at wileyonlinelibrary.com]

2.4 | *In silico* sequence analysis

Gene and protein sequences were obtained from National Center for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov), UniproKB (the UniProt Knowledgebase) (http://www.uniprot.org/help/uniprotkb), and the yeast genomic database Génolevures (http://gryc.inra.fr/). The alignment of peptide sequences was performed using multiple alignment program MultAlin (multiple alignment program) (Institute of Biology and Protein Chemistry website: https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_multalin.html). Blast searches were carried out on the NCBI website (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Benchling software was employed for the gene sequence analysis and in silico plasmid construction (https://benchling.com/). Transmembrane domains were predicted using TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/).

2.5 | Screening for propionate-resistant transformants

Two plasmid library pools (MGM1221021 and MGM1221030) were used to transform *Y. lipolytica* JMY7228; the Frozen-EZ Yeast Transformation Kit was employed. The transformation mixture was incubated in liquid selection media (YNBD) overnight for the first screening corresponding to the selection of Leu+ transformants, then after the diluted cells (OD 0.005) were plated onto YNBD0.5P4 plate for the second screening (clones growing onto propionate media corresponding to a propionate stress). From the YNBD0.5P4 plates, we selected only big colonies, showing more tolerance to propionate. Around 150 colonies from each library pool (MGM1221021 and MGM1221030) were then transferred again onto propionate-containing medium (YNBD0.5P4) and grown for 3–5 days. The colonies showing better growth on propionate medium were subsequently transferred to YNBD medium and grown for 1–2 days to allow them to recover from propionate stress. The candidates were then assessed via colony PCR and propionate tolerance assays.

2.6 | Propionate tolerance assays

The tolerance of control and transformants strains was compared using spot assays or growth curves. In the spot assays, the cells were inoculated in 3 ml of YNBD1 and grown overnight. After their optical density (OD_{600}) values were adjusted to 1.0, these cell suspensions and three sequential dilutions (1:10, $1:10^2$, and $1:10^3$) were applied (3 µl) to the surface of YNB solid medium. The cultures were supplemented with adequate propionate or other organic acids and were incubated at 28°C for 3–5 days.

To test growth in liquid culture, precultures were inoculated in YNBD1 medium and grown overnight (28°C, 180 rpm). After their OD_{600} values were adjusted to 0.1, the precultures were transferred to 96-well plates containing fresh YNB medium with different concentrations of carbon sources. The strains were cultivated at 28°C with constant shaking for 120 hr. Growth was monitored by measuring the OD_{600} values every 30 min using a microtiter plate reader (Biotek Synergy MX, Biotek Instruments, Colmar, France). For each strain and set of conditions, we used two or three biological replicates.

2.7 | Quantifying lipid production

Lipid biosynthesis was performed in flasks using YNB medium containing 0.15% (w/v) NH_4Cl and 50 mM phosphate buffer (pH 6.8) with 0.5% (w/v) glucose and 4% (w/v) propionate. Lipids were extracted from 10 to 20 mg of freeze-dried cells and converted into their FA methyl esters (FAMEs) as per Browse, McCourt, and Somerville (1986). The FAMEs were analysed using gas chromatography (GC). More specifically, the GC analysis was carried out using a Varian 3900 GC equipped with a flame ionization detector and a Varian FactorFour VF-23ms column, where the bleed specification at 260°C was 3 pA (30 m, 0.25 mm, 0.25 µm). FAs were identified via comparison with commercial FAME standards (FAME32, Supelco). Their levels were quantified using the internal standard method, which involves adding 100 µg of commercial dodecanoic acid (Sigma-Aldrich). To determine DCW in a given flask, 10 ml of the culture was washed and lyophilized in a preweighed tube. The difference in mass was defined as the DCW, in milligram, of the cells found in 10 ml of culture. We used at least two biological replicates and calculated the mean and standard deviation.

3 | RESULTS

3.1 | Genomic library construction

The gDNA of *Y. lipolytica* was partially digested using the *Sau3*A restriction enzyme, and fragments of up to 5 kb in size were selected to construct the library. These fragments were cloned at the *Bam*HI position of the replicative plasmid (pINA240, [Barth & Gaillardin, 1996]) and used to form the library's 10 reference pools (Figure 1a). As a control, we used *Y. lipolytica* strain JMY7228 (*phd1*Δ). This strain is unable to use propionate as its sole carbon source; it is also more sensitive to propionate and thus reveals the compound's effects more distinctly (Park et al., 2018). The absence of the *PHD1* expression cassette in the genomic library was verified by PCR to avoid screening for positive clones arising from *PHD1* complementation (Figure S2).

3.2 | Screening for propionate-tolerant strains

A recent study showed that the deletion of *PHD1* in the methylcitrate pathway caused a severe growth defect on propionate: The engineered *phd1*Δ strain accumulated large amounts of odd-chain lipids (Park et al., 2018). To further increase lipid accumulation, we overexpressed the lipid droplet protein (*LDP1*), which enhances the storage of large amounts of triacylglycerol in intracellular lipid droplets. The *LDP1* gene was expressed under the control of the strong hp4d promoter (Madzak, Tréton, & Blanchin-Roland, 2000). The resulting obese *phd1*Δ *hp4-LDP1 (Leu-)* strain JMY7228 (Table 1) was complemented for leucine auxotrophy via transformation with the *LEU2* genomic fragment, giving rise to the prototrophic strain JMY7264.

At 120 hr of culture, compared with the control strain, the strain overexpressing *LDP1* had 9.7% higher biomass production, 1.83-fold greater total lipid content, and two-fold greater total lipid production (equivalent to a concentration 6.51 g/L; growth in YNBD6 medium; Table S2). These results were consistent with those from the study by Bhutada et al. (2018). We used the JMY7228 strain (Po1d *phd1 mfe1 tgl4* + pTEF-*DGA2* pTEF-*GPD1* hp4d-*LDP1*-*URA3*ex) as the starting strain in the library screening process since the strain had displayed much higher propionate sensitivity than other strains. To test propionate tolerance, glucose should always be supplemented together with propionate because the propionate-consumption pathway is blocked, as described above.

To find the optimal medium to use as we screened for propionate tolerance, growth of the control strain JMY7264 was monitored under different conditions, which varied based on (a) the cell amounts plated as determined via colony-forming units (OD_{600} of cell suspension from 0.002 to 0.1), (b) the glucose concentration (0.5–2% glucose combined with 0.4% propionate), and (c) the propionate concentration (1–4% propionate combined with 0.5% glucose; Figure S3). The results revealed that YNBD0.5P4 (0.5% [w/v] glucose and 4.0% [w/v] propionate) was the optimal screening medium because the control strain showed significantly less growth in this medium.

Because the transformation efficiency of the transformation kit was higher than that of the traditional LiAc method under our experimental conditions and because the same competent cells could be used later to transform other reference pools, we transformed two reference pools—MGM1221021 and MGM1221030—with the Frozen-EZ Yeast Transformation Kit. After transforming the same amount of DNA (308 ng) from each reference pool, Leu+ transformants on YNBD were obtained with transformation efficiency being 43,500 transformants/μg from MGM1221021 and 21,800 transformants/μg from MGM1221030, respectively. Propionate tolerant clones were selected as described in materials and methods. Among them, 150 colonies of transformants from each reference pool were transferred again onto propionate-containing plates (YNBD0.5P4) to screen for candidates displaying higher propionate tolerance. After 3–5 days of cultivation, 15 clones (seven from MGM1221021 and eight from MGM1221030) displayed better growth on propionate. These candidates were then transferred onto YNBD solid medium to allow them to recover from propionate stress; gDNA inserts were identified via colony PCR and sequence analysis. The inserts in the pINA240 plasmid were amplified with the primer pair P240-F1/P240-R1 or P240-F2/P240-R2 (Table S1) in the colony PCR.

Clearly defined PCR bands were amplified for clones A3 and A4 from among the seven MGM1221021 candidates and for clone B4 from among the eight MGM1221030 candidates (Figure S4). The PCR fragments were sequenced using primers P240-F2 and P240-R2 to determine the gDNA regions (Table S3). The sequence analysis showed that clone A3 contained 1-kb gDNA, which did not present any identifiable open reading frames (ORFs). The sequences of the gDNA in clones A4 (JMY7588) and B4 (JMY7589) contained partial sequences from the genes *YALI0E00154g* and *YALI0E03872g*, respectively (Figure 1b). In JMY7588, the plasmid contained a 2,610-BP genomic fragment that harboured 1,584 BP of the promoter region and 1,026 BP of *YALI0E00154g* (NCBI XP_503361.1), which codes for a 793-residue protein (UniProtKB/TrEMBL:Q6C7K1). In JMY7589, the plasmid contained a 2,135-BP genomic fragment that harboured 1,353 BP of the promoter region and 782 BP of *YALI0E03872g* (NCBI XP_503517.1), which codes for a 448-residue protein (UniProtKB/TrEMBL:Q6C745).

YALI0E00154g is homologous to *S. cerevisiae RTS1* (YOR014W), which encodes the regulatory subunit of protein phosphatase 2A (PP2A); the latter is involved in cell growth control, cell division control, and the stress response in this yeast (Evangelista, Rodriguez Torres, Limbach, & Zitomer, 1996; Ronne, Carlberg, Hu, & Nehlin, 2015). It has been reported that the deletion of *RTS1* caused sensitivity to temperature, ethanol, sorbate, and osmotic pressure and increased accumulation of CYC7 RNA, which is involved in the global stress response in *S. cerevisiae* (Evangelista et al., 1996; Shu, Yang, Hallberg, & Hallberg, 1997; Mollapour et al., 2004). In *Y. lipolytica*, PP2A was found to act as a regulator of glycogen metabolism (Queiroz-Claret, Jolivet, Chardot, Bergeron, & Meunier, 2002). However, neither PP2A nor its RTS1 subunit have been observed to play a role in the stress response to weak acids (e.g., propionate) in *Y. lipolytica*.

YALI0E03872g is similar to YJR124C in *S. cerevisiae*, but gene function remains unknown in both yeasts. From a BLAST performed on amino acid sequences (Zhang & Madden, 1997), *YALI0E03872g* was similar (~48%) to a major facilitator superfamily (MFS, pfam07690) transporter found in several fungi (e.g., *Nadsonia fulvescens* and *Metarhizium album*). The MFS transporter facilitates the transport of a variety of substrates, including ions, sugar phosphates, drugs, amino acids, and peptides, across cytoplasmic, or internal membranes. In addition, it has recently been shown that the MFS transporter regulates the stress response machinery and controls membrane potential and/or internal pH (Dos Santos, Teixeira, Dias, & Sá-Correia, 2014). In *Y. lipolytica*, using MultAlin (Combet, Blanchet, Geourjon, & Deléage, 2000), we found three genes coding for a putative MFS transporter: *YALI0E03872g*, *YALI0C08228g*, and *YALI0A15774g*. They were named *MFS1*, *MFS2*, and *MFS3*, respectively. The three proteins contain the characteristic cd06174 conserved motif of MFS secondary transporters and present nine putative transmembrane domains (Figures S5 and S6).

3.3 | Overexpression of *RTS1r* and *MFS1r* improved propionate tolerance

For simplicity's sake, we gave the name *RTS1r* to strain JMY7588 and *MFS1r* to strain JMY7589, in reference to the replicative plasmids that

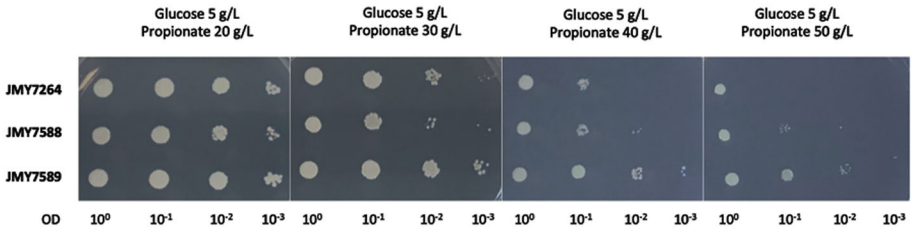

FIGURE 2 Results of the spot assay for the control strain (JMY7264), the *RTS1r* strain (JMY7588), and the *MFS1r* strain (JMY7589). Minimum media (YNBD0.5) containing different propionate concentrations were used. Pictures were taken after 3 days of growth at 28°C [Colour figure can be viewed at wileyonlinelibrary.com]

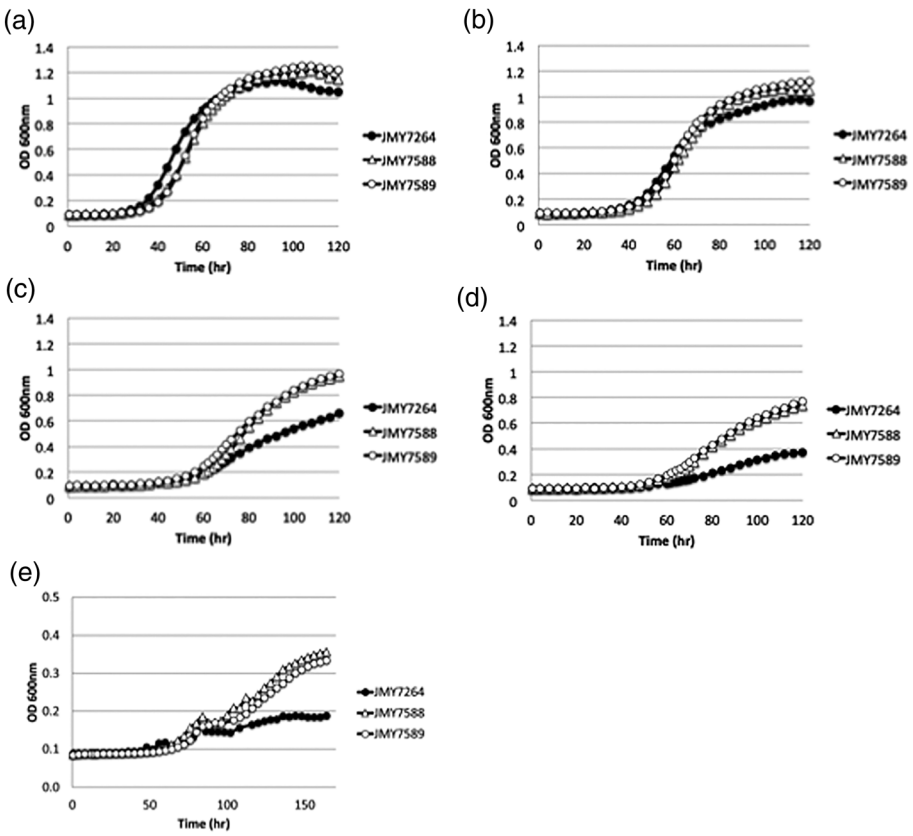

FIGURE 3 Growth curves of the control strain (JMY7264,), the *RTS1r* strain (JMY7588, Δ), and the *MFS1r* strain (JMY7589, ○) in YNBD0.5 liquid medium containing (a) 10 g/L of propionate, (b) 20 g/L of propionate, (c) 30 g/L of propionate, (d) 40 g/L of propionate, and (e) 50 g/L of propionate

expressed the truncated forms of the genes. The strains containing the full-length genomic genes were named *RTS1i* and *MFS1i* (i.e., they contained the integrated expression cassettes for the full-length genes).

To assess strain phenotype and propionate tolerance, the growth of the *RTS1r* and *MFS1r* strains on propionate media was compared with that of the control strain (JMY7264, derived from strain JMY7228 used in the library transformation process) by spot assays on agar plate and in liquid cultivation (Figures 2 and 3). As described above, the control—a leucine prototroph derivative—was not able to use propionate as its sole carbon source; we therefore added 5 g/L of glucose to the propionate media.

Compared with the control, both *RTS1r* and *MFS1r* displayed greater tolerance of propionate. However, *MFS1r* had higher tolerance than *RTS1r*, and it remained tolerant even at propionate levels of 50 g/L (Figure 2). In the liquid media, the growth curves of the two strains were similar, and their final OD_{600} values were slightly higher than that of the control strain in media containing 10 and 20 g/L of propionate (Figure 3). When the propionate concentration was more than 40 g/L, the difference in growth between the control and the two transformants was substantially greater. The final OD_{600} values of the two strains were almost twice of that of the control strain in YNBD0.5P4. Both *RTS1r* and *MFS1r* displayed improved growth rates under all the experimental conditions (Table S4). When the propionate concentration was 40 g/L, the maximal growth rates of *RTS1r* and *MFS1r* were 0.065 hr^{-1} and 0.054 hr^{-1} (increase of 2.17 and 1.8 fold over control), respectively. These results confirmed that the two strains displayed higher propionate tolerance than the control even though they were expressing truncated genes.

3.4 | Overexpression of *RTS1i* and *MFS1i* improved propionate tolerance

The library screening process identified two candidate strains that were found to display propionate tolerance even though they expressed truncated genes. To determine whether overexpression of the complete ORF under the control of a strong promoter could further increase propionate tolerance, the *RTS1* and *MFS1* genes were each cloned into the expression vector JME2563 under the pTEF1 constitutive promoter (JME2563; Figure S7). The gene expression cassettes were transformed into JMY7228, and gene integration was verified by colony PCR using the primer pairs pTEF-internal-Fw/RTS1-noBamHI-Rev and pTEF-internal-Fw/AvrII-E03872g-Rev for *RTS1* and *MFS1*, respectively.

To determine the effects of overexpression on propionate tolerance, two strains—JMY7567 and JMY7569—were constructed and evaluated under same conditions as described above (see Section 3.3.). As mentioned above, the strains were named *RTS1i* and *MFS1i*. The propionate tolerance of *MFS1i* was similar to that of *MFS1r*, as estimated from their growth rates, which means that *MFS1i* had

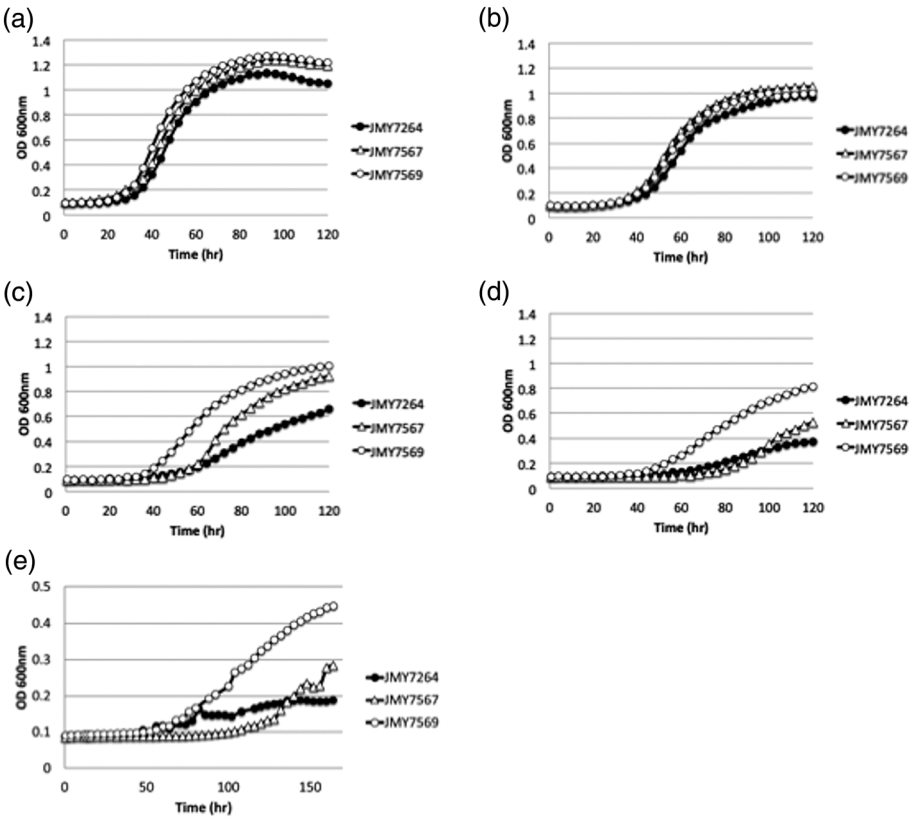

FIGURE 4 Growth curves of the control strain (JMY7264,), the *RTS1i* strain (JMY7567, Δ), and the *MFS1i* strain (JMY7569, ○) in YNBD0.5 liquid medium containing (a) 10 g/L of propionate, (b) 20 g/L of propionate, (c) 30 g/L of propionate, (d) 40 g/L of propionate, and (e) 50 g/L of propionate

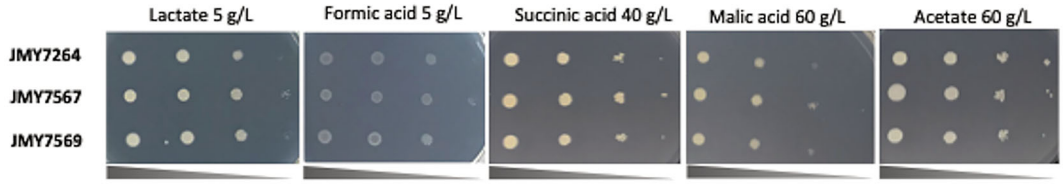

FIGURE 5 Results of the spot assay for the control strain (JMY7264), the *RTS1i* strain (JMY7567), and the *MFS1i* strain (JMY7569) with different weak acids. Pictures were taken after 5 days of growth at 28°C [Colour figure can be viewed at wileyonlinelibrary.com]

greater propionate tolerance than control (Table S4). When the propionate concentration was 30 g/L, *MFS1i* had a shorter lag phase and a higher growth rate than the control (Figure 4). These results indicate that *MFS1* is involved in propionate tolerance in *Y. lipolytica*. The propionate tolerance of *RTS1i* was similar to that of *MFS1i* at propionate concentrations of up to 20 g/L. However, at 30 g/L, *RTS1i* had a longer lag phase than *MFS1i*. At 40 and 50 g/L, its lag phase was even longer than that of the control strain, although it displayed a higher growth rate after the lag phase. When we compared the growth rates of *RTS1r* and *RTS1i* at 40 g/L, *RTS1r* appeared to display greater propionate tolerance than *RTS1i* (0.065 hr^{-1} versus 0.057 hr^{-1}). This difference between the two strains could be attributable to differences in gene length, promoter type, or plasmid type, as shown in another study (Nicaud, Fournier, La Bonnardière, Chasles, & Gaillardin, 1991).

We performed a spot assay to assess the general stress tolerance of *RTS1i* and *MFS1i*; we employed other weak organic acids, namely, acetate, lactate, formic acid, succinic acid, and malic acid, to determine whether overexpression increased tolerance more broadly (Figure 5). We observed the same differences in tolerance between *RTS1i* and *MFS1i* that we had already seen for propionate. JMY7567 (*RTS1i*) was more tolerant to lactate, formic acid, malic acid, and succinic acid than the control strain. In contrast, JMY7569 (*MFS1i*) did not show big difference on growth with organic acids in this condition. In the case of acetate, there were no differences in growth among strains, even at high concentrations (60 g/L). These findings suggest that *MFS1* is involved in a propionate-specific stress response. It also seems that, when *MFS1* is overexpressed, the general tolerance of weak acids is somehow sacrificed for increased propionate tolerance. In comparison, the overexpression of full-length *RTS1* increased tolerance not only to propionate but also to other weak acids. It has been found that the deletion or overexpression of *RTS1* resulted in different levels of tolerance, depending on parental strain and stressor type (Shu & Hallberg, 1995, Evangelista et al., 1996, Shu et al., 1997). Taken together, our results and those of previous studies suggest that PP2A probably has a functional role in more than one cell pathway.

3.5 | Overexpression of *RTS1* and *MFS1* improve odd-chain FA production

As shown in a previous study (Park et al., 2018), propionate is an important substrate for lipid synthesis in *Y. lipolytica*, especially when it comes to the production of odd-chain FAs. To determine whether increased propionate tolerance could improve total lipid accumulation and the production of odd-chain FAs, lipid synthesis by the *RTS1*- and *MFS1*-expressing strains was evaluated. After 120 hr of cultivation in a minimal glucose medium (YNBD0.5P4) containing a high concentration of propionate (40 g/L), the experimental strains had produced less biomass than the control strain (by 6.4–11.5%). Total lipid content was lower in the *RTS1*-expressing strains (by 4.5% and 23.1% for *RTS1r* and *RTS1i*, respectively), whereas it was higher in the *MFS1*-expressing strains (by 7.1% and 10.4% for *MFS1r* and *MFS1i*, respectively). Despite these low biomass and similar lipid content, the ratio of odd-chain lipids to total lipids was higher for all the experimental strains (Table S5).

4 | CONCLUSION

The objective of this study was to identify genes potentially involved in propionate tolerance in *Y. lipolytica*. To this end, we screened a plasmid-based genomic library harboring native promoters for propionate tolerance allowing identifying two genes of potential interest: *RTS1 (YALI0E00154g)* and *MFS1 (YALI0E03872g)*. We discovered that the initial transformants were expressing truncated genes. As a result, we then compared the phenotypes associated with the expression of the partial and full-length genes.

Two strains expressed *RTS1*, which encodes a regulatory subunit of the PP2A. They had different growth patterns on propionate that depended on gene length and promoter type. Growth was stronger, and lipid accumulation was greater for the strain expressing the truncated gene under a native promoter (*RTS1r*) than for the strain expressing the whole gene (including the ORF) under a strong promoter (*RTS1i*). Both strains (*RTS1r* and *RTS1i*) had higher levels of odd-chain lipid production than did the control strain. Further research should focus on whether these phenotype differences stem from differences in expression levels or differences in sequence conservation between the partial and full-length genes. Interestingly, the overexpression of *RTS1* seems to enhance tolerance to other weak acids, such as lactate, formic acid, malic acid, and succinic acid. Given that *RTS1* encodes a single subunit of PP2A—and that there is another regulatory subunit (encoded by *CDC55*) and a catalytic subunit (encoded by four genes)—the relationship among these subunits and its contribution to the tolerance of weak acids must be explored further to better understand the mechanisms at hand.

On propionate media, the *MFS1*-expressing strains (*MFS1r* and *MFS1i*) showed greater propionate tolerance, shorter lag phases, and higher growth rates than the control strain. They also accumulated

more lipids and more odd-chain lipids. On the basis of the sequence alignment results, we identified two more genes coding for a putative MFS transporter (named *MFS2* and *MFS3*). Further, characterization of the proteins encoding these genes would be helpful in clarifying the mechanisms underlying propionate tolerance in *Y. lipolytica*.

The role of these genes in the stress response to propionate and other weak acids remains unclear. Therefore, we need more studies that carry out expression analysis at the transcriptional level or metabolic flux analysis under conditions of propionate-induced stress to gain insight into the regulatory mechanisms. These are also crucial steps to engineer strains with improved tolerance for use in industry. Library enrichment and evolutionary engineering are promising strategies that employ pre-existing libraries or strains (Borden & Papoutsakis, 2007; Wright et al., 2011). In addition, combining computational and experimental approaches may also help to improve tolerance. The effects of overexpressing or disrupting multiple genes at the same time can be predicted using a computational model that has incorporated the experimentally determined effects of overexpressing or disrupting individual genes (Goodarzi et al., 2010).

Our results have laid the foundation for future research aimed at further improving propionate tolerance, which is crucial to use propionate as a substrate in the industrial production of valuable biochemicals such as odd-chain lipids. Furthermore, studies on propionate tolerance should augment *Y. lipolytica*'s ability to employ a wider range of substrates, including waste products and inexpensive materials, which will help make microbial production more competitive than petroleum-based production.

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CONFLICT OF INTEREST

None declared.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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