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RESEARCH ARTICLE

Use of a wine yeast deletion collection reveals genes that influence fermentation performance under low-nitrogen conditions

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ABSTRACT

A deficiency of nitrogenous nutrients in grape juice can cause stuck and sluggish alcoholic fermentation, which has long been a problem in winemaking. Nitrogen requirements vary between wine yeast strains, and the ability of yeast to assimilate nitrogen depends on the nature and concentration of nitrogen present in the medium. In this study, a wine yeast gene deletion collection (1844 deletants in the haploid AWRI1631 background) was screened to identify genes whose deletion resulted in a reduction in the time taken to utilise all sugars when grown in a chemically defined grape juice medium supplemented with limited nitrogen (75 mg L^{-1} as a free amino acid mixture). Through micro-scale and laboratory-scale fermentations, 15 deletants were identified that completed fermentation in a shorter time than the wildtype (c.a. 15%–59% time reduction). This group of genes was annotated to biological processes including protein modification, transport, metabolism and ubiquitination (*UBC13, MMS2, UBP7, UBI4, BRO1, TPK2, EAR1, MRP17, MFA2* and *MVB12*), signalling (*MFA2*) and amino acid metabolism (*AAT2*). Deletion of *MFA2*, encoding mating factor-a, resulted in a 55% decrease in fermentation duration. *Mfa2*Δ was chosen for further investigation to understand how this gene deletion conferred fermentation efficiency in limited nitrogen conditions.

Keywords: *Saccharomyces cerevisiae*; yeast deletion library; genome wide screening; limited nitrogen; *MFA2*; mating a-factor

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INTRODUCTION

To complete fermentation successfully (i.e. utilisation of all fermentable sugars), yeasts require sufficient yeast assimilable nitrogen (YAN). The presence of sufficient YAN supports appropriate cell growth and metabolism (Bell and Henschke 2005; Ugliano *et al.* 2007). The rate at which a cell grows and maintains its metabolic activity can influence the regulation of other pathways (e.g. sugar metabolism), which in turn affects fermentation kinetics (Bely, Sablayrolles and Barre 1990; Sablayrolles *et al.* 1996; Alexandre and Charpentier 1998). YAN metabolism also leads to the production of various aroma and flavour compounds, which contribute to the organoleptic properties of the wine (Vilanova *et al.* 2007). Therefore, YAN availability is important in fermentation by significantly influencing cell growth, rate of sugar catabolism and wine flavour (Bell, Ough and Kliewer 1979; Henschke and Jiranek 1993; Ugliano *et al.* 2007).

Although YAN is one of the main nutrient groups needed during fermentation, it is often not present in sufficient amounts in grape must to enable yeast to utilise all sugar. The amount and composition of YAN present in the juice can vary depending on factors such as the grape variety, viticultural management practices, soil, climate and degree of ripeness (Henschke and Jiranek 1993; Alexandre and Charpentier 1998; Bell and Henschke 2005). For example, the YAN content in Australian grape juices varies between 50 and 350 mg L^{-1} (Ugliano *et al.* 2007). YAN concentrations below 150 mg L^{-1} increase the risk of stuck and sluggish fermentation as a consequence of yeast metabolism being affected (Jiranek, Langridge and Henschke 1995a; Alexandre and Charpentier 1998; Bell and Henschke 2005). Stuck and sluggish fermentations result in wine left with residual sugar, which is not desirable in certain wine styles (Bisson 1999). Moreover, unfermented sugars can support spoilage organisms (McClellan, Does and Bisson 1989) producing undesirable aromas and flavours, as well as volatile acidity (Alexandre and Charpentier 1998). The depletion of YAN in juices can also lead to the liberation of excessive H_2S in wine (Jiranek, Langridge and Henschke 1995b; Ugliano *et al.* 2009).

In order to minimise problematic fermentations due to YAN insufficiency, it is common practice for winemakers to supplement fermentations with inorganic ammonium salts. These supplementations are often carried out empirically without determining the nitrogen requirement of the inoculated strain or the nitrogen content of the grape juice (Jiranek, Langridge and Henschke 1995a; Torrea *et al.* 2011). Poorly considered supplementation could result in wine with increased levels of residual nitrogen that encourage microbial instability in the bottled wine, and thus reduce quality (Jiranek, Langridge and Henschke 1995a; Bell and Henschke 2005). Also, YAN supplementation plays an important role in modulating the production of both primary metabolites and volatile compounds, which can affect wine aroma and flavour (Torrea *et al.* 2011) positively or negatively if not balanced or over threshold (Ugliano *et al.* 2007).

An alternative paradigm to supplementation is to use yeast strains that are able to efficiently utilise the YAN available in juice in order to complete fermentation. Commercial wine yeasts have different requirements or demands for YAN (Jiranek, Langridge and Henschke 1995a; Gutiérrez *et al.* 2012; Gutierrez *et al.* 2013; Brice *et al.* 2014). Yeasts with lower YAN demands are less likely to undergo nitrogen (N) starvation, enabling successful completion of fermentation in YAN-deficient juices (Manginot, Roustan and Sablayrolles 1998; Gardner, Poole and Jiranek 2002). Therefore, identification of genetic determinants conferring N-efficient behaviour has great merit. Previous work by this group utilising a transposon mutant library in both a laboratory and wine yeast background identified genes impacting N-efficiency and fermentation efficiency (Gardner *et al.* 2005; Zhang *et al.* 2018). This study builds on that work by screening a library of open reading frame deletants constructed in a wine yeast background.

Accordingly, the fermentation capabilities of a partial wine yeast deletion collection were analysed in high-throughput micro-fermentations under limited-N conditions and mutants grouped according to their performance. Those with the shortest fermentation were tested in laboratory-scale cultures to confirm their ability to complete N-limited fermentation quickly. The deleted genes of these mutants are of interest as they represent potential targets for strain improvement. Among the genes highlighted was *MFA2*, encoding mating pheromone a-factor. This is the first report describing the dual function of *MFA2* in mating as well as fermentation.

MATERIAL AND METHODS

Strains and media

The parental prototrophic haploid strain AWRI1631 (*MATa*) was derived from a widely used commercial strain (N96; Borneman *et al.* 2008). A wine yeast deletion collection containing 1844 mutants was prepared in AWRI1631 by replacement of non-essential genes with the *KanMX* cassette (Wach *et al.* 1994) as described for a subset of these deletants (Varela *et al.* 2012). Strains were routinely grown on YPD medium (1% yeast extract, 2% bactopeptone, 2% D-glucose; Guthrie and Fink 2002) with 200 mg L^{-1} of G418 sulfate for deletant strains and with 2% agar when solid media were required for plating.

Chemically defined grape juice medium (CDGJM; McBryde *et al.* 2006) containing 200 g L^{-1} sugar as equimolar amounts of glucose and fructose with 75 mg L^{-1} of a mixture of amino acids and ammonium chloride (Henschke and Jiranek 1993) was used for micro-scale and laboratory-scale fermentations. To mimic red grape juice, 3 g L^{-1} polyphenol extract powder (Cat: Tppr, OenoProd, Beaune, France) was also added (Walker *et al.* 2014).

Micro-fermentation of the wine yeast deletion collection in CDGJM

Measurement of cell growth

The wine yeast deletion collection and the wildtype strain, AWRI1631, were inoculated from –80°C glycerol stocks (2 μL) into 96-well microtiter plates (Corning Costar®, Corning, New York, USA; Cat No. CLS3596) containing 200 μL of YPD and incubated statically for 48 h at 28°C. Cells were resuspended by agitation, and 2 μL of the culture were inoculated into 200 μL of CDGJM in 96-well microtiter plates and covered with clear breathable sealing film (Axygen Corning, NY, USA; AXY BF-400) to allow measurement of growth as optical density at 600 nm using a spectrophotometric plate reader (Tecan, Mannedorf, Switzerland; M200 Infinite). All liquid handling was performed using an automatic platform fitted with an 8-channel pipetting head (CAS-3800, Corbett Robotics, QLD, Australia).

Measurement of fermentation progress

Fermentation progress was measured using a sacrificial sampling approach as described in Liccioli *et al.* (2011b). In brief, four copies of the same plate were prepared by transferring 6 μL

of overnight YPD culture into 600 μL of CDGJM in deep 96-well plates (Axygen; Cat No. P-DW-20-C) and covered with a cloth membrane (Diversified Biotech, Dedham, MA; Cat No. BEM-1). One plate of the four copies was collected and stored at −20°C at each sampling point during fermentation to enable subsequent determination of residual sugar content. This approach was used to eliminate the potential influence of multiple samplings from the same small volume fermentations. Residual glucose and fructose were quantified enzymatically as described in Boehringer-Mannheim (1989) with final volumes adjusted to 200 μL for analysis in 96-well microtiter plates and with Megazyme (Bray, Ireland) reagents. Liquid handling during plate preparation and enzymatic analysis was performed robotically.

Evaluation of strains in laboratory-scale fermentations

Flask fermentations (100 mL) in this study were conducted in CDGJM using either an automated 96-flask fermentation platform or 250 mL Erlenmeyer flasks fitted with an airlock (Walker *et al.* 2003). The automated fermentation platform built on a Tecan Freedom EVO consisted of four 24-position water jacketed carriers, to maintain temperature with magnetic stirrers to continuously agitate the fermentations. Each flask was comprised of a customised 100 mL Schott bottle with a fermentation lock and septum-sealed sampling port. The liquid handling arm of the Freedom EVO collected samples (~1 mL) at regular intervals for off-line analysis. Two hundred microliter from each sample was used to measure growth (see above) and the remaining samples were stored at –20°C prior to the enzymatic determination of residual sugar.

Calculation of area under the curve and fermentation duration to determine fermentation performance

Fermentations were considered complete and total fermentation duration determined when residual sugar content was less than 2.0 g L^{-1}. The residual sugar values measured at each sampling point were used to plot fermentation curves for each mutant from the 100-mL fermentations. The area under the curve (AUC) of each profile was then calculated using the composite trapezoid rule using GraphPad Prism 6 (GraphPad software INC., La Jolla, CA, USA) as described by Liccioli, Chambers and Jiranek (2011a). Where the AUC values of the deletants were less than that of the wildtype, the deletants were considered to have an improved fermentation efficiency.

Statistical analysis

A two-way analysis of variance (ANOVA) (Dunnett's multiple comparison test) was performed to determine the statistical significance between the fermentation performance of the individual mutants and the wildtype during fermentation. A scatter plot representing correlation between residual sugar and growth was performed using Pearson r correlation. Observed differences in the expression level of gene(s) in the wildtype and deletants were statistically tested using a two-way ANOVA (Bonferroni's multiple comparisons test). All of these statistical analyses were performed using GraphPad Prism 6 (GraphPad software INC., La Jolla, CA, USA).

Primers used in this study

The primers and corresponding sequences used in this study are shown in Table 1. Primers were sourced from Sigma Aldrich (Australia).

Confirmation of identity of individual deletants

The identity of individual deletants was authenticated by amplification of the deletion cassette using gene-specific primers (Table 1) and comparison of the DNA sequence to the reference genome of S288c using WU-BLAST2 (http://www.yeastgenome.org/blast-sgd). For deletants incorrectly labelled, the barcode region of the *KanMX* deletion cassette was amplified using primers U1 and D1 (Table 1). The tool FASTA barcodes (http://www.ttuhsc.edu/som/cbb/FASTAbarcodes/) was used to identify the query sequence to the up and down tags of the deletion library (McMahon *et al.* 2011).

Construction of an *MFA1* deletion in AWRI1631

An *MFA1* deletion strain was constructed in the haploid wine strain AWRI1631 using a PCR-based deletion strategy (Baudin *et al.* 1993; Wach *et al.* 1994). The *mfa1*Δ::*KanMX* deletion cassette was sourced from the *MFA1* deletion clone from the prototrophic BY4741 collection (Mulleder *et al.* 2012). The cassette was PCR amplified using VELOCITY DNA polymerase (Bioline, London, UK; 21098) with gene-specific primers A and D (Table 1) according to the manufacturer's instructions. The identity of the PCR product was confirmed by sequencing and validated products were then transformed into AWRI1631 using the lithium acetate method (Gietz and Schiestl 2007). Transformants were selected on YPD plates containing G418 sulfate (200 mg L^{-1}), and the deletions were confirmed by PCR amplification and sequencing.

Quantitative PCR for gene expression studies

To study gene expression, a quantitative PCR (qPCR) experiment was conducted following the MIQE guidelines (Bustin *et al.* 2010; Taylor and Mrkusich 2014). Samples of the culture harvested during fermentation at similar residual sugar concentrations (approximately 128, 83, 35 and 10 g L^{-1}) were used to compare transcript abundance between strains. Cells were collected by centrifugation, and resuspended in TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA; 15596026) followed by snap freezing in liquid N and storage at –80°C. Total RNA was extracted using Zymo (Irvine, CA, USA) Research kits (Direct-zol RNA MiniPrep R2050) as per the manufacturer's instructions. RNA purity and integrity were estimated from OD_{260}/OD_{280} (Tecan M200 Infinite) and gel electrophoresis. cDNA was synthesised from the total RNA (BioRad, Hercules, CA, USA, iScript kit; 170-8897) and any contaminating DNA removed with an Ambion TURBO DNAfree kit (Thermofischer, Waltham, MA, USA; AM1907). Relative quantification of the transcripts was performed on a Biorad CFX-96 Real Time PCR system with Ssofast Evagreen Supermix (BioRad; 172-5203) with primers as listed in Table 1. The amplification cycle included an initial denaturation at 95°C for 30 s followed by 40 cycles at 95°C for 5 s and 60°C for 5 s. A sample maximisation strategy was used with all six biological replicates being amplified in duplicate. At the end of each run, a melt curve was prepared for all samples from 65°C to 95°C. Transcript abundance of the target genes was normalised with abundance of reference genes (*TAF10*, *ALG9*; Teste *et al.* 2009) in the same sample. The stability of the housekeeping genes was determined using qbase + software (Vandesompele *et al.* 2002) and primer efficiency was determined by running a standard curve using a serially diluted cDNA sample. The mean fold change of expression for given genes per sample for different time points was determined using a relative quantification method (Schmittgen

Table 1. Primers used in this study.

Gene name	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
TDA7[a]	TAATGCGATTATTTCGCTTGTAAAT	GTATGTTAAAATGCCTTCCAGATTG
UBP7[a]	GCATCCGGTAATACTAACAAGAGAA	AATAATAATGGTGGTAATGGCAATG
AIM26[a]	GCTGGTATTGACAGGTAACTATGCT	GAATCCAGTTCGTTCTCAAACTTAG
MFA2[a]	TGATAGTTTCCTTTTCCGTTAAGTG	TGGCTCAAAACTTTTTCACTTTTAC
UBI4[a]	AATAATCCTGGATAAACCAATTTCG	CTTTTCCCTTTTGGTAGTCACAATA
MKT1[a]	ATTAAGAACAAGACGAAAAATGGTG	AACTTCAATCTATCCAGTAACGGTG
MMS2[a]	CACCACTATTGCTCATTTTGTACTG	TATTTATTATTGGCTTGGACTGGAG
MRP17[a]	ATACCCACTCAAACAAACTCATTGT	CTTAGTCAGGATTGTCGTTTAGCAT
MVB12[a]	TGATTTCATGTATTTTTGGTTCAGA	GAAATTGACGATGGAGAAGAAGTAA
AAT2[a]	ATACACAATTACTCCAGTAGCTGCC	TATCCTTTATGTTCATGGGAGTTGT
BRO1[a]	GCCTTCCCTTTTCTTGATGTATATT	ACAGTCTAGCACGAAAAAGAAGAGA
TPK2[a]	TACAATTCTGGCCTTCTTACCTAAA	TAATTTTTGCACTGAGATCATGAGA
INA1[a]	CTCAGTACTCGTTTAGCTTGAATCC	TGGGCACTTACTCATACATCAAATA
EAR1[a]	TACAGGCTTTAAGAACCTTGCTCTA	TCATACGTTCTTCTTGTGACACATT
MFA1[a]	GCCCATACCTTTATTCTTTGTTCTT	AAGAAACAATGAAGCAAGATAAGGA
MFA1[c]	ATGCAACCATCTACCGCTAC	CTAAGCAATAACACATGCTGGG
MFA2[c]	GATCACCACTGCTTCCACAC	TTAAGCGATAACACAGGCGG
TAF10[d]	ATATTCCAGGATCAGGTCTTCCGTAGC	GTAGTCTTCTCATTCTGTTGATGTTGTTGTTG
ALG9[d]	CACGGATAGTGGCTTTGGTGAACAATTAC	TATGATTATCTGGCAGCAGGAAAGAACTTGGG
U1[b]	GATGTCCACGAGGTCTCT	
D1[b]		CGGTGTCGGTCTCGTAG
KanB[b]	CTGCAGCGAGGAGCCGTAAT	
KanC[b]		TGATTTTGATGACGAGCGTAAT

[a]Deletion cassette amplification.
[b]Identification of clones that were incorrectly labelled in the wine yeast library.
[a,b]Primer sequences were from Saccharomyces Genome Deletion Project (http://wwwsequence.stanford.edu/group/yeast_deletion_project/Deletion_primers_PCR_sizes.txt).
[c]Primers for qPCR target genes were designed using Primer3 software (http://simgene.com/Primer3).
[d]Primers for qPCR reference genes were adapted from (Teste *et al.* 2009).

and Livak 2008) using the qbase + software (Vandesompele *et al.* 2002), Excel (Microsoft Office 2013) and GraphPad Prism (GraphPad Prism 6—GraphPad software INC., La Jolla, CA, USA).

RESULTS

Construction of a wine yeast deletion library

To ensure that the most industrially relevant genetic background was used in this study, a wine yeast deletion library was constructed in the haploid wine yeast derivative AWRI1631 (Borneman *et al.* 2008). Using the approach reported previously (Varela *et al.* 2012), a total of 1844 mutants were constructed (Table 1, Supporting Information). All the deletants were checked by PCR amplification and sequencing of the deletion cassette.

High-throughput screening (micro-fermentation) of the wine yeast deletion collection

In order to identify genes that influence fermentation duration when only supplied with minimal N (75 mg L^{-1}), the sugar utilisation profile of clones from this wine yeast deletion collection was compared to that of the wildtype strain, AWRI1631, in a high-throughput micro-fermentation screening in CDGJM (Fig. 1). To ensure supplied nitrogen would protract fermentation, preliminary experiments were also undertaken with the addition of sufficient N (450 mg L^{-1}). The wildtype strain was able to utlise all available sugar in ~60 h in comparison to ~200 h when only minimal N is available (data not shown). Additional fermentation wells containing the wildtype were sampled at a higher frequency to accurately determine the most appropriate sampling points (A–D) for the deletants (Fig. 2A). Initially, the wildtype consumed sugars slowly (43 g L^{-1} by 96 h); however, fermentation became more vigorous after 96 h. Using this information, residual sugars were measured for the 1844 deletants at time points B (121 h) and C (146 h). The extent of sugar catabolism by the wine yeast deletants was highly variable (Fig. 2B), when the concentration of residual sugars for the wildtype was 42 ± 8 g L^{-1} (time point C). Given this range, the deletants were classified into three groups: 1040 with residual sugar around that of the wildtype (30–60 g L^{-1}), 282 with more residual sugar (>60 g L^{-1}) than the wildtype and 522 with markedly less residual sugar (<30 g L^{-1}) than the wildtype group (Table 2, Supporting Information). Growth was also monitored for the wildtype and the deletants during the early stages of micro-fermentation (Fig. 2A; Table 1, Supporting Information). The wildtype reached stationary phase at 113 h with an optical density of 1.77. Therefore, using optical density at 113 h and residual sugars at 146 h (time point C), a correlation was plotted to identify whether the fermentation abilities of the deletants were dependent on growth (Fig. 3). A weak negative correlation was observed, emphasising a small effect of growth on fermentation progression (i.e. increase in growth of the deletants leading to a decrease in residual sugar). Further analysis of the 522 deletants, which completed fermentation in a shorter time than the wildtype, identified that the majority (408 i.e. 78%) of the deletants had higher optical density than the wildtype.

Based on the results of this screening exercise, 92 candidates with the greatest reduction in fermentation duration and no growth defects were chosen for further evaluation (Screen II, Table 3, Supporting Information). These candidates were selected as they were observed to utilise significantly more sugar

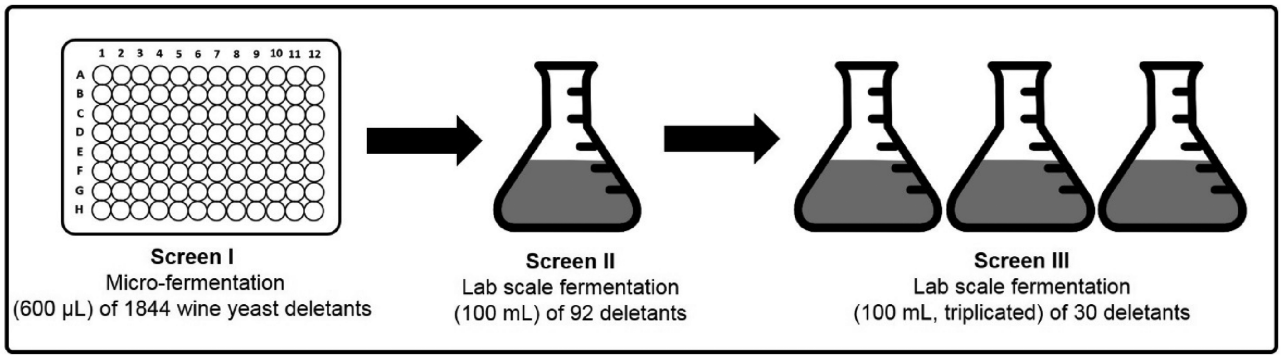
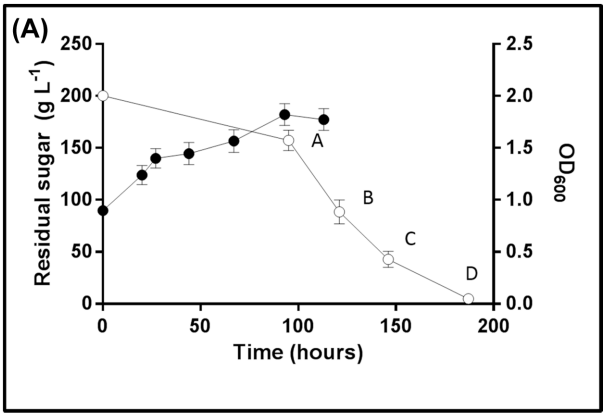


Figure 1. Schematic representation of the screening of the wine yeast deletion library for fermentation performance in a CDGJM with limited nitrogen (75 mg N L^{-1}).



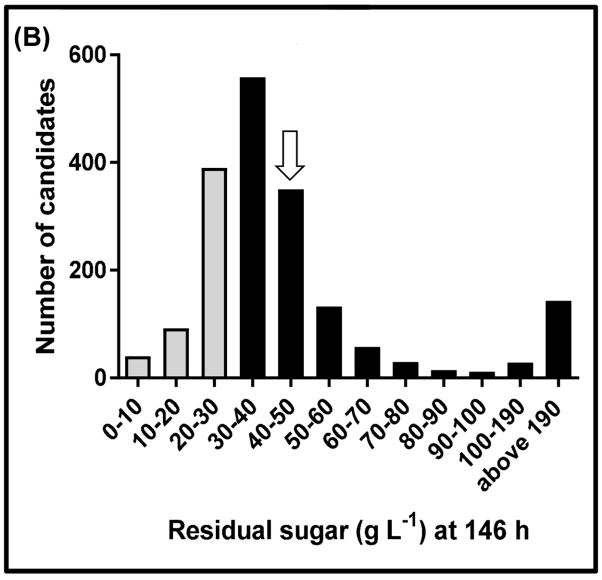


Figure 2. (A) Fermentation performance of AWRI1631 during micro-fermentation. Sugar utilisation (open symbols) at four time points: A (95 h), B (121 h), C (146 h) and D (187 h). Growth as estimated by optical density at 600 nm (solid symbols). Sixteen replicates were analysed. (B) Histogram representing the distribution of deletion mutants (number) based on the residual sugar content at time point C (146 h). The arrow indicates the residual sugar concentration range of the wildtype, AWRI1631 (42 ± 8 g L^{-1}) at time point C. Candidates for further analysis were chosen from the groups with grey shaded bars.

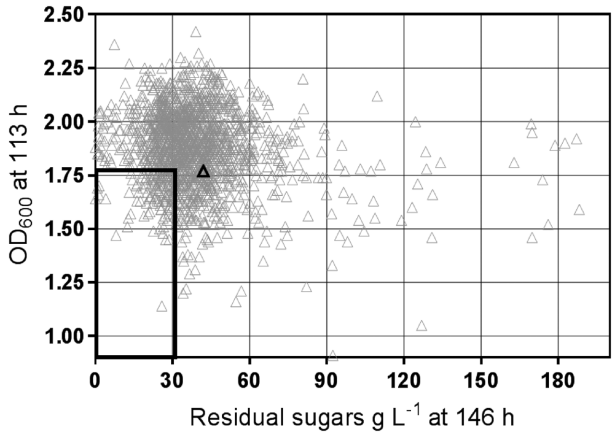


Figure 3. Scatter plot of the residual sugar (at time point C, 146 h) and growth (OD_{600} at 113 h) of the deletion mutants (grey) and the wildtype (black) during micro-fermentation. Deletants (114) exhibiting shorter fermentation duration and decreased growth in comparison to the wildtype are highlighted in a bold box. Correlation coefficient (r) = –0.1616.

than the wildtype, having less than 48 and 26 g L^{-1} of residual sugar at time points B and C, respectively—i.e. approximately half of that of the wildtype (88 and 42 g L^{-1} of residual sugar at time point B and C, respectively). Of the possible 522 candidates, an arbitrary total of 92 of the most fermentation efficient deletants was chosen since this number (plus controls) could be accommodated in the 96-position fermentation platform.

Confirmation of deletant fermentation performance in laboratory-scale fermentations

The fermentation performance of the 92 shortlisted deletants was confirmed in laboratory-scale (100 mL), non-replicated fermentations (screen II) since this format allowed greater control of experimental conditions and better representation of an anaerobic, wine-like fermentation (Walker *et al.* 2014). Results of this experiment highlighted 48 deletants that completed fermentation in less time than the wildtype (Fig. 4A), with 30 utilising all sugar within 131–205 h compared to 229 h for the wildtype. As well as total fermentation time, the AUC of fermentation curves of individual deletants was also used for a more holistic view of the pattern of sugar utilisation by which deletants were ranked (Liccioli, Chambers and Jiranek 2011a). AUC values calculated for the 30 strains were 7185–9516 (i.e. 72%–95% of the wildtype at 9999; Table 4, Supporting Information). These 30 candidates were further assessed in a third screen

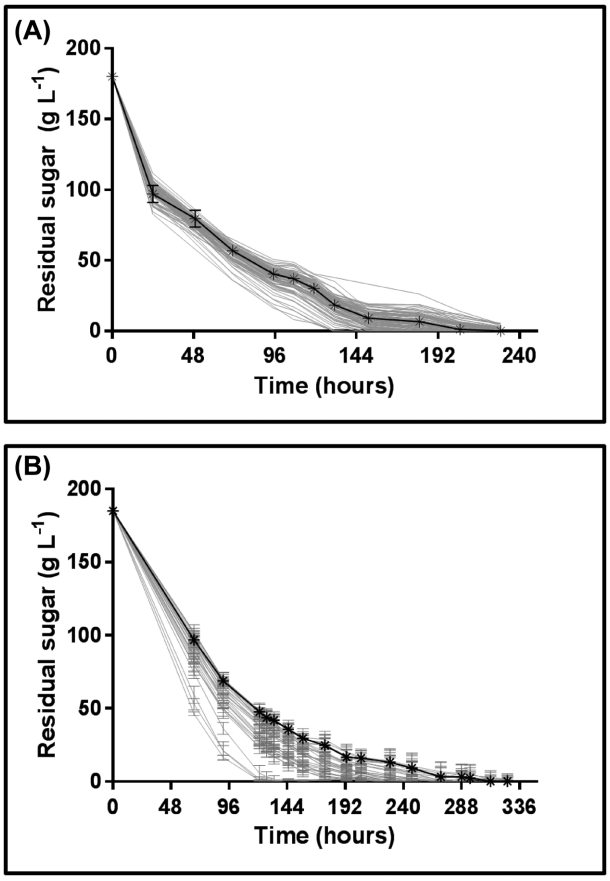


Figure 4. Sugar utilisation of the wildtype (AWRI1631, black line) compared to the shortlisted 92 deletants (grey lines) over time. Fermentation was conducted in 100 mL of CDGJM with 75 mg N L^{-1} at 28°C on a robotic sampling platform. **(A)** Screen II of the shortlisted 92 yeast deletants (single cultures) compared with the wildtype (four replicates). **(B)** Screen III of the selected 30 candidates in triplicate, along with the wildtype strain (six replicates). Standard deviation of the mean is represented as error bars.

conducted in triplicate (Fig. 4B). Fifteen of these deletants utilised all sugars in significantly less time than the wildtype (Table 2). *Tda7*Δ and *aim26*Δ were the most rapid deletants, completing fermentation in 121 h, which was 172 h ahead of the wildtype (293 h). Closely following were *ubp7*Δ and then *mfa2*Δ (Table 2). The remaining 11 deletants exhibited fermentation durations ranging from 63% to 85% of the wildtype. The AUC for individual deletants representing sugar utilisation over time was calculated as being 54%–85% of AWRI 1631, with *tda7*Δ (AUC = 8911) being the most efficient and *ear1*Δ (AUC = 14073) being the least efficient compared to the wildtype (AUC = 16399; Table 2).

Characterisation of the deletants mfa1Δ and mfa2Δ

To date, *MFA2* has only been annotated to mating and cell-type regulation (Michaelis and Herskowitz 1988; Johnson 1995; Galgoczy *et al.* 2004). Our results of improved fermentation performance under N-limited conditions for *mfa2***Δ** allude to a dual function of this gene. In order to explore the link between efficient fermentation and the yeast mating factor, the function of the two genes encoding mating a-factor, *MFA2* and *MFA1*, were further studied. The sugar utilisation and growth profile of *mfa1*Δ was similar to the wildtype (AWRI1631), whilst growth and fermentation duration of *mfa2*Δ were reduced (Fig. 5). The expression of *MFA1* and *MFA2* during fermentation was also examined. RNA was extracted from cultures of mutant (AWRI1631 *mfa2*Δ) and parental (AWRI1631) strains when the sugar content was approximately 128, 83, 35 and 10 g L^{-1} in each culture. Differential expression of *MFA1* and *MFA2* was observed in the wildtype, with *MFA2* being expressed to a greater extent (~1.93-fold NREL) compared to *MFA1* (~0.17-fold NREL) at the first time point (Fig. 6A and B). The expression of both genes markedly declined at 83 and 35 g L^{-1} of residual sugar (corresponding to 58.5% and 82.5% sugar consumption, respectively). In the case of *MFA2*, expression recovered in the wildtype in the final sample (95% of sugar consumed; Fig. 6B). The absence of *MFA2* transcript in the AWRI1631 *mfa2*Δ strain confirmed the deletion of the *MFA2* gene (Fig. 6B). Interestingly, expression of *MFA1* was upregulated by ~6- to 7-fold in the *MFA2* deletant at the initial sampling point (128 g L^{-1}). Although *MFA1* expression declined subsequently, it remained ~4-fold higher than in the wildtype (Fig. 6A).

DISCUSSION

Yeasts differ in fermentative capabilities in N-deficient must, which is dependent upon their demand for nitrogen (N) to maintain glycolytic flux—the genetic basis of which is the focus of recent research (Brice *et al.* 2013, 2014; Tesnière, Brice and Blondin 2015). In order to generate N-efficient strains, it is important to identify genes and processes that allow the yeast to perform well in low-N juices. Gene deletions provide a paradigm to better understand the mechanisms behind the improved fermentation phenotype exhibited by these strains. Although there are several strategies that are now available for strain improvement, whole genome investigations using collections of yeast mutants with single defined gene deletions (Winzeler *et al.* 1999; Giaever *et al.* 2002) provide opportunities to readily study gene functionality, and so identify targets for strain improvement (Donalies *et al.* 2008). Some studies have successfully used deletion collections in auxotrophic laboratory yeast backgrounds to investigate growth phenotypes in response to exposure to single stress factors associated with fermentation. Examples include the identification of genes essential for tolerance to ethanol and other alcohols (Fujita *et al.* 2006; van Voorst *et al.* 2006; Teixeira *et al.* 2009), high pressure and low temperature (Abe and Minegishi 2008), acetic acid (Mira *et al.* 2010), oxidative and chemical stress (Tucker and Fields 2004), high glucose (Teixeira *et al.* 2010), anaerobic growth (Reiner *et al.* 2006) and low temperature (Salvadó *et al.* 2016). Most recently, this approach has been used by this group to define the Fermentome, genes essential to allow the successful completion of fermentation under the multistress conditions of a high-sugar, juice-like medium (Walker *et al.* 2014). Alternatively, genes important to the response of yeast to stress have been identified through transcriptional analyses following exposure to stresses such as N limitation (Backhus *et al.* 2001; Rossignol *et al.* 2003; Mendes-Ferreira *et al.* 2007a,b; Contreras *et al.* 2012; Brice *et al.* 2013; Barbosa *et al.* 2015). Research in a wine yeast background to identify genes related to N-efficiency in wine-like fermentations has been limited to the screening of random collections of transposon mutants of non-essential genes (Gardner *et al.* 2005; Zhang *et al.* 2018). The present study sought to fill this knowledge gap.

The need to supply sufficient N (both in amount and specific amino acid composition) to meet the auxotrophic requirements of laboratory strains used in previous studies has meant

Table 2. Fermentation performance of fermentation efficient deletants.

Deleted gene	Gene function	FD (h) (FD ± SD)	FD % of wildtype	AUC (AUC ± SD)	AUC % of wildtype (AUC % ± SD)
TDA7[a]	Topoisomerase I Damage Affected	121 ± 0	41 ± 0	8911 ± 114	54 ± 0
UBP7[a]	UBiquitin-specific Protease	123 ± 3	42 ± 1	9332 ± 275	57 ± 1
AIM26[a]	Altered Inheritance rate of Mitochondria	121 ± 0	41 ± 0	9602 ± 482	58 ± 2
MFA2[a]	Mating Factor A	131 ± 12	45 ± 4	10 825 ± 431	66 ± 2
UBC13[a]	Ubiquitin-conjugating	185 ± 17	63 ± 6	12 205 ± 645	74 ± 3
UBI4[a]	Ubiquitin	193 ± 0	66 ± 0	12 225 ± 306	74 ± 1
MMS2[b]	Methyl MethaneSulfonate sensitivity	209 ± 18	71 ± 6	12 475 ± 720	76 ± 4
MRP17[a]	Mitochondrial Ribosomal Protein	201 ± 7	69 ± 2	12 643 ± 85	77 ± 1
MKT1[a]	Maintenance of K2 Killer Toxin	191 ± 15	65 ± 5	12 712 ± 360	77 ± 2
MVB12[a]	MVB sorting factor of 12 kilodaltons	193 ± 0	66 ± 0	13 239 ± 211	81 ± 1
AAT2[b]	Aspartate AminoTransferase	215 ± 28	73 ± 10	13 273 ± 986	81 ± 6
BRO1[b]	BCK1-like Resistance to Osmotic shock	227 ± 21	78 ± 7	13 387 ± 699	81 ± 4
INA1[c]	Indicator of Abscission	225 ± 48	77 ± 16	13 567 ± 1375	82 ± 8
TPK2[a]	Takashi's Protein Kinase	201 ± 7	69 ± 2	13 593 ± 184	83 ± 1
EAR1[d]	Endosomal Adaptor of Rsp5p	249 ± 38	85 ± 13	14 073 ± 269	85 ± 6
wildtype AWRI1631		293 ± 21	100	16 399 ± 752	100

Fermentation performance was calculated as AUC and FD (fermentation duration) of the 15 shortlisted gene deletants (from Screen III) and percentage difference in AUC and FD of the deletants in comparison to the parent, AWRI1631 (i.e. AUC % = (mutant AUC/parent AUC) × 100 and FD % = (mutant FD/parent FD) × 100). Fermentation duration corresponds to the time (h) by which residual sugar was <2 g L^{-1}. Values represent the average of FD/AUC ± SD. Deletants significantly different in fermentation completion when compared to the parent are represented by
[a]$P < 0.0001$; [b]$P < 0.001$; [c]$P < 0.01$; [d]$P < 0.1$ (two-way ANOVA).

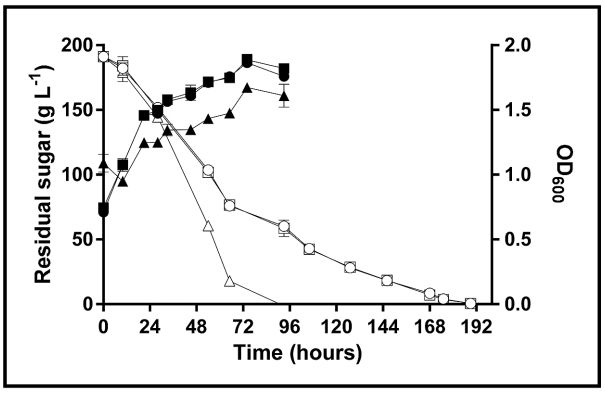


Figure 5. Fermentation performance of *mfa1*Δ, *mfa2*Δ and the wildtype AWRI1631. Fermentations (100 mL) were conducted in triplicate in CDGJM with 75 mg N L^{-1}. Data points represent the average value ± standard deviation. Sugar utilisation over time of the individuals (open symbols). Growth as estimated by optical density at 600 nm of the individuals (solid symbols). Circle; AWRI1631, square; AWRI1631 *mfa1*Δ and triangle; AWRI1631 *mfa2*Δ.

that investigations related to N efficiency were limited to high N content (Walker *et al.* 2014). Several studies have shown that supplementation can affect metabolism through the blockage of pathways, or by combinatorially influencing metabolic networks, thereby making quantitative comparisons of growth and physiological studies difficult (Pronk 2002; Mulleder *et al.* 2012). The recent availability of prototrophic libraries in laboratory yeast (Mulleder *et al.* 2012) and wine yeast (Varela *et al.* 2012) has made it possible to conduct studies under limited-N conditions, overcoming previous shortcomings.

In this study, a prototrophic wine yeast deletion library (Varela *et al.* 2012) was used to identify genes influencing fermentation in a wine-like limited N medium. Based on the sugar utilisation profile of the wildtype, preliminary screening via micro-fermentations (Table 1, Supporting Information) of the wine yeast deletion collection allowed mutants to be segregated into groups based on the extent of sugar utilisation. Fermentation by 282 of the mutants (15%, Fig. 2B; Table 2, Supporting Information) was protracted indicating that these genes were essential for the timely completion of fermentation. Walker *et al.* (2014) used the deletion library in the auxotrophic laboratory strain BY4743 to identify 93 genes (i.e. the fermentome) essential for fermentation in CDGJM under high N (450 mg N L^{-1}) and high-sugar (200 g L^{-1}) conditions. Interestingly, despite differences in the experimental conditions of these studies (i.e. N availability and strain background), our list of 282 protracted deletants included 15 of the fermentome genes (Table 3) identified by Walker *et al.* (2014), underscoring the universal importance of these. A further 14 deletants of fermentome genes, however, had no effect in the present study (i.e. in the AWRI1631 background), since they were not protracted compared to the wildtype. Further work to explore the influence of strain background and growth conditions will help resolve such apparent differences. More importantly, the wine yeast deletion library needs to be expanded to a complete collection from the current 1844 deletants or at least the remaining 64 fermentation essential genes identified by Walker *et al.* (2014) in order to assess their essentiality in the conditions of the present study.

Twenty eight percent of the wine yeast deletion library mutants (522 deletants) completed fermentation in a shorter time, alluding to the function of these genes negatively influencing fermentation progress. Whether these genes are directly related to nitrogen metabolism or instead are general regulators of fermentation is worthy of future study. Of these,114 deletants were capable of enabling shorter fermentation time in spite of exhibiting decreased growth. In general, fermentation rate has been reported to correlate with biomass (Varela, Pizarro and Agosin 2004), further evaluation of this group of genes may reveal exceptions. However, these 522 genes are potential targets for guided strain improvement and thus were assessed in greater detail. A shortlist of the best performing 92 strains (<48 and <26 g L^{-1} of sugar at time point B and C, respectively—i.e. ∼50% of that of the wildtype) were chosen for further evaluation in 100 mL fermentations. Fifteen strains completed

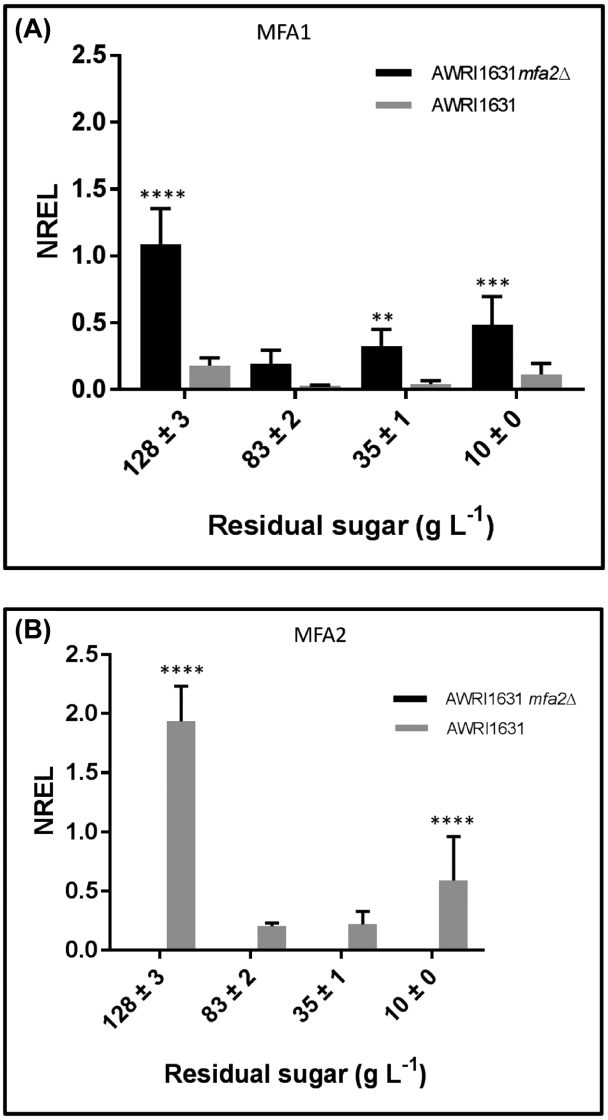


Figure 6. Expression of *MFA1* **(A)** and *MFA2* **(B)** in AWRI1631 and AWRI1631 *mfa2*Δ strains. Normalised relative expression level (NREL) is shown at four selected residual sugar levels (g L^{-1}). Two-way ANOVA was used to determine the significant differences in expression levels between *mfa2*Δ and the wildtype. Significant difference is indicated as $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$.

fermentation in between 41% and 69% of the time taken by the parent (Table 2). Gene Ontology (GO) analysis (Table 5, Supporting Information) revealed that 11 out of these 15 genes were over-represented for the GO terms related to different stages of ubiquitination and protein modification/turnover or metabolic processes: protein polyubiquitination (*UBI4*, *UBC13* and *MMS2*), protein deubiquitination (*UBP7*, *UBI4* and *BRO1*), protein modification by small protein conjugation (*UBP7*, *UBI4*, *BRO1*, *MMS2* and *UBC13*), post-translational protein modification (*UBP7*, *TPK2*, *UBI4*, *BRO1*, *MMS2* and *UBC13*) protein metabolic process (*UBP7*, *TPK2*, *UBI4*, *BRO1*, *MFA2*, *MMS2*, *MKT1*, *EAR1*, *UBC13* and *MRP17*) and endosomal transport (*MVB12*, *EAR1* and *BRO1*). The remaining four genes include *AAT2* (involved in aspartate aminotransferase in nitrogen metabolism) and three with unknown function, *INA1*, *TDA7* and *AIM26*.

Ubiquitination is a protein degradation process that is required in many cellular processes such as the cell cycle, endocytosis and stress response (Hershko 1997; Staub and Rotin 2006). In a study of the stress response of yeast under sake brewing conditions, 235 genes related to stress response or ethanol fermentation were deleted and investigated for their impact on ethanol production (Wu *et al.* 2009). Amongst the 235 genes were *UBI4*, *UBP7*, *UBC13* and *TPK2*, which have also been identified in our study. Wu and co-workers (2009) demonstrated that deletion of ubiquitin-related genes, such as *UBI4* and *UBC13*, increased fermentation abilities producing significantly more ethanol than the parental strain. Furthermore, enhanced fermentation abilities of *ubi4*Δ were confirmed in a number of laboratory yeast strain backgrounds (Wu *et al.* 2009).

An important group of proteins regulated by ubiquitination are plasma membrane proteins, in particular, amino acid permeases. Ubiquitination of these initiates endocytosis and the eventual degradation of the ubiquitin-bound protein in the vacuole/lysosome via the multivesicular body (MVB) pathway (Hicke 2001). Genes such as *BRO1* and *EAR1* (in combination with *SSH4*) play a role in the regulation of Gap1p (general amino acid permease) degradation by ubiquitination in the presence of ammonium (Nikko, Marini and Andre 2003; Leon, Erpapazoglou and Haguenauer-Tsapis 2008). However, deletion of *BRO1* (Nikko, Marini and Andre 2003), *MVB12* (Oestreich *et al.* 2007) or *EAR1* (in combination with *ssh4*Δ; Leon, Erpapazoglou and Haguenauer-Tsapis 2008) have been shown to disrupt this pathway thereby interfering with the degradation process. Interestingly, *ear1*Δ (in combination with *ssh4*Δ) results in the localisation of Gap1p to the vacuolar membrane rather than the vacuolar lumen, where it would otherwise be degraded (Leon, Erpapazoglou and Haguenauer-Tsapis 2008). Furthermore, *bro1*Δ results in the recycling of Gap1p to the cell membrane without ubiquitination in the presence of ammonium (Nikko, Marini and Andre 2003), essentially desensitising *GAP1* to nitrogen catabolite repression at the protein level. It is possible to consider that the lack of regulation of amino acid permeases by the MVB pathway may play a role in successful low-N fermentation. However, it is not known how the gene deletions in this study affect the degradation of other amino acid permeases or amino acid catabolic enzymes, which could also play a role in the observed enhanced fermentation performance.

MFA2 was further investigated to understand how deletion of this mating gene positively affected fermentation. To date, *MFA2* has only been annotated to mating and cell-type regulation (Michaelis and Herskowitz 1988; Johnson 1995; Galgoczy *et al.* 2004). *MFA2*, together with *MFA1*, considered functionally redundant, is responsible for the production of a signalling molecule (a-factor) in a *MATa* cell (Michaelis and Herskowitz 1988). Both *MFA1* and *MFA2* produce a precursor (36 and 38 amino acids, respectively), which undergoes several modification steps involving different genes before the mature a-factor molecule is transported outside the cell through the ABC transporter *STE6* (review by Michaelis and Barrowman 2012). We hypothesise that mating pheromone production and processing is an energy-consuming process. Therefore, in a pure culture of a haploid heterothallic strain, such as within this study where no mating or switching of mating types occurs, the ability to downregulate synthesis/processing of mating factor-a may lead to a metabolic efficiency that benefits fermentation. This finding is in keeping with the finding that deletants of *MFA2* are haploproficient in either carbon- or nitrogen-limited media (Delneri *et al.* 2008). Deletion of *MFA2* also results in lower biomass (Fig. 5) suggesting that fermentation capability on a per cell basis far exceeds that of the parent. This may be related to metabolic efficiencies due to modification of the mating pheromone a-factor pathway. Given

Table 3. List of genes essential in timely completion of fermentation.

ORF	Gene name	Gene function description
YEL051W	VMA8	Subunit D of the V1 peripheral membrane domain of V-ATPase
YHL020C	OPI1	Transcriptional regulator of a variety of genes
YKR007W	MEH1	Component of the EGO and GSE complexes
YFR053C	HXK1	Hexokinase isoenzyme 1
YDR247W	VHS1	Cytoplasmic serine/threonine protein kinase
YGR063C	SPT4	Spt4p/5p (DSIF) transcription elongation factor complex subunit
YJR033C	RAV1	Subunit of RAVE complex (Rav1p, Rav2p, Skp1p)
YLL007C	LMO1	Homolog of mammalian ELMO (Engulfment and celL MOtility)
YMR263W	SAP30	Component of Rpd3L histone deacetylase complex
YNL076W	MKS1	Pleiotropic negative transcriptional regulator
YOR209C	NPT1	Nicotinate phosphoribosyltransferase
YOR221C	MCT1	Predicted malonyl-CoA:ACP transferase
YOR265W	RBL2	Protein involved in microtubule morphogenesis
YPR036W	VMA13	Vacuolar Membrane Atpase
YPR074C	TKL1	TransKetoLase

ORF, open reading frame.

Table 4. List of genes interacting with *MFA2* involved in carbohydrate metabolism/transport or storage.

Type of interaction	Gene	Gene function
Negative	GAL10	GALactose metabolism
Negative	GID7	Glucose-induced degradation deficient
Negative	HXT5	HeXose Transporter
Negative	STL1	Sugar transporter-like protein
Negative	PCL6	Pho85 cycLin

Adapted from Costanzo *et al.* (2016).

that deletion of *MFA2* resulted in a culture with significantly increased (34%) sugar catabolism compared to the parent, we chose to also examine *MFA1*, a functionally redundant gene encoding a-factor. As the *mfa1*Δ deletant was absent in the deletion collection used in this work, the *MFA1* deletant (*mfa1*Δ::*KanMX*) was constructed in the same background as *mfa2*Δ, AWRI1631, to determine whether an *mfa1*Δ exhibited a similar fermentation phenotype. In fact, *mfa1*Δ deletant performed no differently to the wildtype in terms of fermentation and growth (Fig. 5), suggesting potentially different roles for the *MFA1* and *MFA2* gene products.

In the wildtype background, transcription of *MFA2* was dominant to that of *MFA1* (Fig. 6A and B). However, when *MFA2* was deleted, *MFA1* produced a pattern of expression through fermentation that implied a compensation for the *MFA2* gene product in *mfa2*Δ. Contrasting with these results, others have shown *MFA1* and *MFA2* to be transcribed at similar levels and to be functionally equivalent. Thus, deletion of one of the mating factor genes did not prevent the strain being mating proficient (Michaelis and Herskowitz 1988; Chen *et al.* 1997). However, here we show that these genes are not functionally equivalent, at least in terms of their impact on fermentation performance. Further work is needed to determine the basis for the link between these genes and fermentation, the extent to which they are functionally equivalent in mating type functions, interactions with related genes and the basis for their differential expression.

Novel interactions between genes have been identified when unexpected phenotypes result from mutations in two or more genes (Dixon *et al.* 2009; Costanzo *et al.* 2010). Nevertheless, gene interaction networks (adapted from Costanzo *et al.* 2016 and the Saccharomyces Genome Database) already indicate that *MFA1* and *MFA2* genes interact with different sets of genes, including genes involved in either carbohydrate metabolism, transport or storage (Table 4). Future studies should examine these genes for their potential role in fermentation efficiency in an *mfa2*Δ strain.

CONCLUSION

Nitrogen-efficient strains offer a means for avoiding or reducing the problems associated with the fermentation of grape juices low in N, but the basis for these efficiency differences is not known. Motivated by this and recognising the fact that wine and laboratory yeast strains can behave differently under wine-like conditions, we generated and exploited a partial wine yeast deletion collection to identify genes whose deletion improved fermentation performance in low-N media. Fifteen deletants were identified through a robust three-stage screening process. The unexpected link between one of these gene deletions, *mfa2*Δ, and fermentation remains to be explored at a more fundamental level. Further candidates would likely be found with completion and screening of the library with all non-essential genes, with all showing promise as the basis for construction of optimised strains for industry use, perhaps by genome editing (Bao *et al.* 2014). Their use would avoid the need for YAN supplementation or at least reduce the incidence of problem fermentations in unsupplemented juices.

SUPPLEMENTARY DATA

Supplementary data are available at *FEMSYR* online.

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Conflict of interest. None declared.

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