Identification of Yeast Genes Affecting Production of Hydrogen Sulfide and Volatile Thiols from Cysteine Treatment during Fermentation

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Abstract

Hydrogen sulfide (H_2S), well-known for its undesirable rotten-egg odour, is often produced during fermentation by the yeast *Saccharomyces cerevisiae* when nitrogen becomes depleted. However, an early burst of H_2S generated by yeast from cysteine could contribute to the formation of the fruity varietal thiols 3-mercaptohexan-1-ol (3MH) and 3-mercaptohexyl acetate (3MHA) through reaction with (*E*)-2-hexenal, which is otherwise rapidly metabolised. The goal of this project is to identify genes and pathways leading to H_2S generation from cysteine and thus enhance the tropical aromas in wine that appeal to many consumers.

Using a candidate gene approach, *TUM1* was for the first time identified to play a crucial role in the early production of H_2S from cysteine. Overexpressing *TUM1* elevated production of H_2S, while its deletion reduced the H_2S by half. Furthermore, deletion of either *MET17* or *MET2* led to an additional delayed burst of H_2S, suggesting that a portion of the H_2S generated from cysteine is fed directly into the sulfate assimilation pathway. Triple deletants of *STR2*, *STR3* and individual *MET* genes, were shown to require both *MET17* and *TUM1* to bypass the transsulfuration pathway and grow on high concentrations of cysteine as the sole sulfur source. These results illustrate that cysteine is not converted to sulfate or sulfite, but rather to sulfide via a novel pathway requiring the action of Tum1p.

The failure to identify a specific QTL associated with H_2S formation from cysteine using a set of 96 fully sequenced M2 x F15 progeny, suggests multiple genes affect the trait. To identify additional genes, a modified version of bismuth-containing indicator agar resembling grape juice was developed and used to screen both AWRI1631 wine yeast and BY4741 deletion collections. Both Δ*lst4* and Δ*lst7* strains were observed to form lighter coloured colonies and produce significantly less H_2S than the wild-type on high concentrations of cysteine. Further investigations revealed that deleting genes involved in cysteine transportation such as *AGP1*, *GNP1*, *MUP1*, *STP1* and *DAL81* all resulted in reduced production of H_2S from cysteine. These findings demonstrated, for the first time, that genes involved in regulating cysteine uptake could affect H_2S formation from cysteine and therefore selecting wine yeasts with ability to take up supplemented cysteine efficiently could maximise aromatic thiol production.

Preliminary results indicate that the higher levels of 3MH/A could be achieved by modulating *TUM1* and cysteine supplementation. In addition, polysulfides, that may affect the sensory

quality of wine, were detected for the first time in yeast undergoing fermentation on high concentrations of cysteine by the fluorescent probe SSP4. Finally, an up-to-date review of recent study on sulfur metabolism in *S. cerevisiae* is presented, which includes suggestions for future research in this field.

In conclusion, these findings not only have greatly advanced our current understanding of *S. cerevisiae* cysteine catabolism, but also could be applied to develop better yeast strains, as well as novel winemaking practices to enhance tropical aromas of wines.

Declaration

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Thesis Structure

This thesis consists of seven chapters presented as a combination of conventional (Chapter 1, 3, 5 and 7) and publication (Chapter 2, 4 and 6) formats. References and supplemental material are provided at the end of each chapter.

Chapter 1 Literature review and summary of research aims.

Chapter 2 Huang CW, Walker ME, Fedrizzi B, Roncoroni M, Gardner RC and Jiranek V. The yeast *TUM1* affects production of hydrogen sulfide from cysteine treatment during fermentation. *FEMS Yeast Research* 2016;**16**:fow100. https://doi.org/10.1093/femsyr/fow100

Chapter 3 Development of a screening assay for detecting H_2S production from cysteine.

Chapter 4 Huang CW, Walker ME, Fedrizzi B, Gardner RC and Jiranek V. Yeast genes involved in regulating cysteine uptake affect production of hydrogen sulfide from cysteine during fermentation. *FEMS Yeast Research* 2017;**17**:fox046. https://doi.org/10.1093/femsyr/fox046

Chapter 5 H_2S, varietal thiols and polysulfides.

Chapter 6 Huang CW, Walker ME, Fedrizzi B, Gardner RC and Jiranek V. Hydrogen sulfide and its roles in *Saccharomyces cerevisiae* in a winemaking context. *FEMS Yeast Research* 2017;**17**:fox058. https://doi.org/10.1093/femsyr/fox058

Chapter 7 Conclusions and future directions.

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Chapter 1

Literature review and summary of research aims

This literature review was mostly written within the first 6 months of candidature and only covers the literature up to August 2014. The purpose of this literature review was to provide the background information and establish a theoretical framework for this PhD project. For a more updated literature, please refer to the introduction sections in Chapter 2 to 5 and Chapter 6: Minireview.

1.1 Introduction

Humans have been making and enjoying wine for thousands of years (Pretorius 2000; McGovern *et al.* 2004). The earliest archaeological evidence of a winemaking facility dates back at least 6000 years, with the discovery of grape crushing basins, large storage jars and drinking cups in an Armenian cave (Barnard *et al.* 2011).

The "real winemakers", the living yeasts, which carry out alcoholic fermentation to convert grape juice into wine, were not identified until the 1860s by Louis Pasteur (see review by Barnett 2000). Nowadays, the significance of the yeast *Saccharomyces cerevisiae* to the final sensory quality of wine is well established and most large-scale wineries inoculate with selected *S. cerevisiae* wine yeast strains to produce specific, reproducible styles of wine of consistent quality (Pretorius 2000; Swiegers *et al.* 2009).

Volatile sulfur compounds produced by yeast are key contributors to wine aroma. For example, hydrogen sulfide (H_2S) is a volatile molecule, having the distinctive and undesirable aroma of rotten eggs. Much effort has been made to trying to understand its formation and how to remove it from finished wines (Swiegers and Pretorius 2007). However, it has recently been shown that early production of H_2S following the addition of yeast to grape juice, may contribute to the production of desirable varietal thiols 3-mercapto-hexanol (3MH) and 3-mercaptohexylacetate (3MHA) in Sauvignon Blanc (Harsch *et al.* 2013). Therefore complete removal may not be wanted in all circumstances, and a better understanding of the mechanisms of H_2S formation is required, especially early in fermentation.

According to Wine Australia annual reports 2013-2014, Sauvignon Blanc was the most popular varietal in Australia (up 10% to $640 million in wine sales), however most of the Sauvignon Blanc is imported from New Zealand. Studies have revealed that the unique, tropical, fruity style of New Zealand Sauvignon Blanc wines that many consumers prefer, can be attributed to the significantly higher concentrations of varietal thiols: 3MH and 3MHA found in these wines, when compared to Sauvignon Blanc wines from other countries e.g. Australia, France, South Africa and USA (Lund *et al.* 2009; Benkwitz *et al.* 2011).

This PhD project has two primary goals. Firstly, to identify the yeast genes responsible for H_2S formation from sulfur sources such as cysteine, to better understand the mechanisms behind H_2S production. The second goal is to determine whether modification of these genes can affect not only production of H_2S early in fermentation, but increase 3MH and 3MHA

production associated with enhanced tropical fruity aromas. The application of these findings has potential benefit to the Australian wine industry, in providing not only new wine styles, but allowing the industry to compete in the premium wine market.

This literature review will give an overview of the current mechanisms proposed for the production of unpleasant, rotten-egg aroma of H_2S and tropical, fruity aroma of varietal thiols by yeast during fermentation. The timing of hydrogen sulfide production and its role as a thiol precursor will also be highlighted. Finally, a theoretical framework for this PhD project will be described in Summary of Research Aims (Section 1.7).

1.2 Production of hydrogen sulfide by yeast during fermentation

The undesirable, rotten-egg odour of H_2S in wine has a very low perception threshold (1.1-1.6 μg L^{-1}) and is a very common problem for the global wine industry (Siebert *et al.* 2010). Most H_2S in wine is produced by yeast during fermentation and it can be generated by yeast via different mechanisms and sources, which include the inorganic sulfur compounds: sulfate, sulfite and elemental sulfur, as well as the organic sulfur compounds: glutathione and cysteine (Rauhut 2009; Ugliano and Henschke 2009).

Studies have shown that different amounts of H_2S (~0 to 300 μg L^{-1}) are generated by different yeast strains under the same fermentation conditions (Rauhut 1993; Spiropoulos *et al.* 2000; Kumar, Ramakrishnan and Bisson 2010), indicating that genetic differences among yeast strains play a significant role in influencing H_2S production. Other factors such as the juice nutrient content (e.g. nitrogen and vitamins), turbidity, soluble solids, titratable acidity, and fermentation temperature have all been reported to affect the level of H_2S produced during wine fermentation (Wainwright 1970, 1971; Eschenbruch and Bonish 1976; Vos and Gray 1979; Jiranek, Langridge and Henschke 1995a; Karagiannis and Lanaridis 1999; Spiropoulos *et al.* 2000; Bohlscheid *et al.* 2007).

1.2.1 H_2S production from the sulfate assimilation pathway

Most H_2S produced by yeast is from the sulfate assimilation pathway (SAP) (Fig. 1.1). The sulfur-containing amino acids, cysteine and methionine are generally present at very low

concentrations in grape juice (<20 mg L^{-1}), therefore yeast have to make their own for growth (Rauhut 2009; Ugliano and Henschke 2009). The main sulfur source for yeast to synthesise sulfur-containing amino acids is sulfate, which is usually abundant in grape juice (~160 to 700 mg L^{-1}) (Rauhut 2009; Ugliano and Henschke 2009). Extracellular sulfate (SO_4^{2-}) is transported into yeast cells by high affinity transporters, Sul1p and Sul2p (Cherest *et al.* 1997), wherein it is reduced to sulfite (SO_3^{2-}). Sulfite is further reduced to sulfide (S^{2-}) by sulfite reductase, which is encoded by the *MET10* (α subunit) and *MET5* (β subunit) genes (Thomas and Surdin-Kerjan 1997). The sulfide formed is integrated into the nitrogen-containing precursor, *O*-acetyl-L-homoserine (OAH), to form homocysteine, which is progressively converted into cysteine and methionine (Thomas and Surdin-Kerjan 1997).

Sulfite is a potential source of H_2S. It is commonly added as aqueous potassium metabisulfite (up to 100 mg L^{-1}), or gaseous sulfur dioxide (SO_2) to harvested grapes, juice and wine to prevent oxidation and inhibit unwanted microbes during winemaking (Stratford and Rose 1986; Ugliano and Henschke 2009). Sulfite in the juice can diffuse into yeast cells and be reduced to sulfide by sulfite reductase. Sulfite is considered the likely preferred sulfur source over sulfate, as less energy is required for yeast to synthesise sulfur-containing amino acids (Hallinan, Saul and Jiranek 1999; Ugliano and Henschke 2009).

The liberation of excessive H_2S usually arises when there is not enough OAH to combine with the sulfide generated by yeast. This condition is frequently encountered during wine fermentation when yeast assimilable nitrogen (YAN) is limited in grape juice (Jiranek, Langridge and Henschke 1995a), as it is rapidly consumed during fermentation.

This situation can be avoided by the addition of nitrogen supplements such as diammonium phosphate (DAP) to the juice during fermentation (Henschke and Jiranek 1993). Although this approach reduces most of the H_2S from the SAP, some yeast strains have been observed to continue producing H_2S even if sufficient DAP is supplemented (Ugliano, Kolouchova and Henschke 2011). Furthermore, DAP is required to be added prior to YAN being totally consumed and within the permissible limits (~1.7 g L^{-1}) (Australian and New Zealand Food Standard 4.5.1). Moreover, excessive addition of DAP does not only increase the risk of acetate ester e.g. ethyl acetate (volatile acidity) formation, which is known to mask other positive aromas (Ugliano *et al.* 2007), but also the production of ethyl carbamate, a known carcinogen (Adams and van Vuuren 2010).

Whilst reductive off-flavours are removable through treating the wine with copper sulfate ($CuSO_4$) – known as copper fining (~0-1.0 mg L^{-1}; Iland, Ewart and Sitters 1993); the concerns of this approach are that of copper toxicity. The legal limits vary between countries from no specific limit in Australia (Australian and New Zealand Food Standard 4.5.1) to 0.5 mg L^{-1} in the United States (Linderholm *et al.* 2010) and being not permitted in Japan (Japan External Trade Organization 2011). Whilst $CuSO_4$ does remove the negative smell of H_2S, a drawback is the unwanted removal of positive fruit aromas e.g. varietal thiols (Darriet *et al.* 2001; Swiegers and Pretorius 2007).

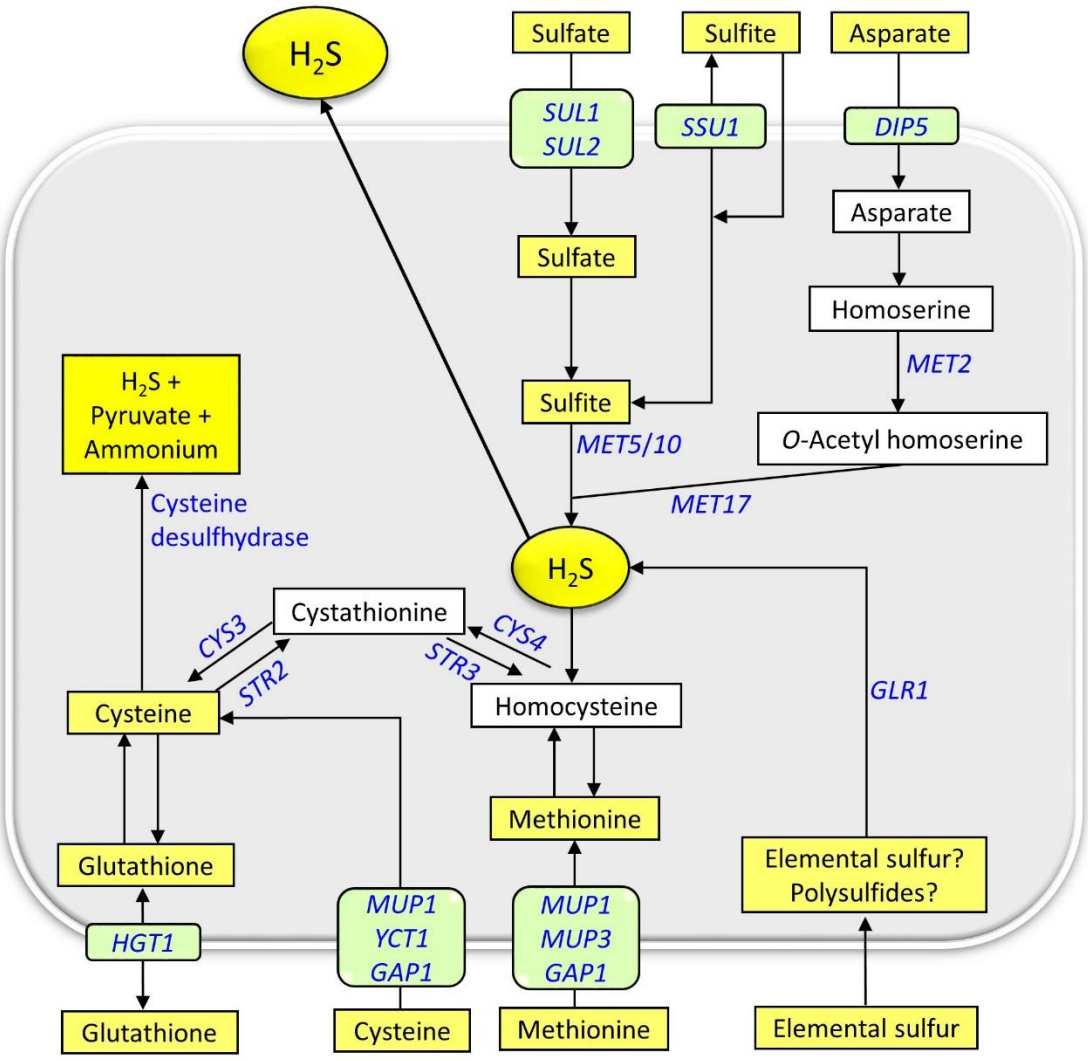

Figure 1.1. Pathways used by *Saccharomyces cerevisiae* to metabolise sulfur sources and H_2S production (according to Ugliano and Henschke 2009; Sato *et al.* 2011; Harsch and Gardner 2013).

1.2.2 Low H_2S-producing wine strains

DAP and $CuSO_4$ treatments require winemakers to spend extra money and time after yeast inoculation. A more efficient strategy to manage H_2S production during fermentation would be to prevent its formation through the use of low H_2S-producing yeast strains.

Yeasts differ in their ability to produce H_2S (Rankine 1963, 1964; Zambonelli, Soli and Guerra 1984) and scientists have identified the activity of the enzyme sulfite reductase within the SAP, as critical for the control of yeast H_2S production (Linderholm *et al.* 2006; Linderholm *et al*. 2008). The approach of targeting the yeast enzyme sulfite reductase in the SAP has successfully led to development of several commercial low-H_2S yeast strains such as Distinction® (Maurivin Yeast, Australia) and Vivace (Renaissance Yeast, Canada).

Researchers at the Australian Wine Research Institute (AWRI) used ethylmethane sulfonate (EMS) to induce random mutations in a commercial diploid wine yeast (PDM; Mauri Yeast, Australia). They found the low-H_2S mutants generated had non-synonymous mutations in either *MET5* or *MET10*, resulting in amino acid substitutions within the sulfite reductase protein, and either partial or complete inactivation of the enzyme (Cordente *et al.* 2009).

Linderholm and coworkers (2010) identified a genetic variant of *MET10* in UCD932, following a screen of several commercial and native yeast strains. The single mutational change $T_{662}K$ in *MET10* whilst not abolishing the activity of sulfite reductase, resulted in a reduction in H_2S formation. This trait was transferable into other yeast backgrounds (Linderholm *et al.* 2010).

Recently, a dominant $R_{301}G$ mutant allele of *MET2* in a few wine yeast strains including Zymaflore F15 (Laffort, France), was reported as being able to significantly reduce H_2S during wine fermentation (Huang, Roncoroni and Gardner 2014). The yeast *MET2* encodes homoserine *O*-acetyl transferase (HTA), which catalyses the acetylation of homoserine to *O*-acetyl homoserine, which is subsequently combined with sulfide to yield homocysteine (Fig. 1.1) (Baroni *et al.* 1986). Huang, Roncoroni and Gardner (2014) proposed the $R_{301}G$ mutation in *MET2* to increase the activity of HTA and thereby efficiency in converting homoserine into *O*-acetyl homoserine. The ready supply of *O*-acetyl homoserine available to bind to H_2S, prevented release of the odorous gas into wine (Huang, Roncoroni and Gardner 2014).

1.2.3 H_2S production from elemental sulfur, glutathione and cysteine

Elemental sulfur is another potential source of H_2S. Elemental sulfur is sprayed in the vineyard to fight grapevine powdery mildew (*Erysiphe necator*) and other pests. The residual sulfur in grape must may be spontaneously converted to sulfide under the anaerobic and low pH fermentation conditions established by yeast (Thomas *et al.* 1993; Linderholm *et al.* 2008). Yeast can also reduce cytotoxic elemental sulfur to glutathione and sulfide using NADPH-glutathione reductase, encoded by *GLR1* (Sato *et al.* 2011).

The tripeptide, glutathione (L-γ-glutamyl-L-cysteinyl-glycine) exists naturally in grape must (ranging from 1.3 to 102 mg L^{-1}), and can also be synthesised by yeast (Rauhut 2009). It is a vital antioxidant and storage molecule for sulfur and nitrogen in yeast; contributing to an estimated 0.5–1% of dry cell weight (Elskens, Jaspers and Penninckx 1991; Rauhut 2009). Glutathione protects various aromatic compounds in wine from oxidation (Ugliano *et al.* 2011; Kritzinger, Bauer and Du Toit 2012) but is a potential source of H_2S (Rauhut 2009). It has been shown that addition of glutathione (greater than 50 mg L^{-1}) to grape must (even when added as part of rehydration nutrients (~516 mg L^{-1} of glutathione)), results in higher H_2S production (Rauhut 2009; Winter *et al.* 2011a).

The genes and mechanisms by which glutathione leads to increased H_2S during fermentation are not fully understood. It has been proposed that glutathione is first hydrolysed to cysteine, which is subsequently degraded by cysteine desulfhydrase to release H_2S under nitrogen source limited conditions (Tokuyama *et al.* 1973; Rauhut 2009). Surprisingly, an estimated 40% of H_2S production is suggested to come from glutathione, at least in sulfate-containing and nitrogen-limiting media (Hallinan, Saul and Jiranek 1999).

Cysteine is another precursor, given that its addition into grape juice also leads to increased H_2S production (Giudici and Kunkee 1994; Jiranek, Langridge and Henschke 1995a). It has been suggested that a pathway other than the SAP is used by yeast to generate H_2S from cysteine (Winter and Curtin 2012). The enzyme, cysteine desulfhydrase is proposed to cleave cysteine into H_2S, pyruvate and ammonia when preferred nitrogen sources are limited (Tokuyama *et al.* 1973). However, the yeast gene encoding this enzyme activity has not been characterised.

Tryptophanase encoded by tnaA from *Escherichia coli* (Awano *et al.* 2003) and cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE) in mammals is able to degrade cysteine to

release H_2S (Singh *et al.* 2009). However, the deletion of equivalent yeast genes (e.g. *cys4*) did not result in a similar reduction in H_2S (Linderholm *et al.* 2008).

Cysteine, elemental sulfur and sulfite are not considered significant sources of H_2S under wine making conditions (Jiranek, Langridge and Henschke 1995a; Ugliano and Henschke 2009), as only small amounts of cysteine (<20 mg L^{-1}) are found in grape juice (Henschke and Jiranek 1993; Ugliano and Henschke 2009) and excessive use of elemental sulfur and sulfite can be avoided. Nonetheless, commercial yeast nutrient products containing cysteine e.g. Laffort FreshArom, may be added during fermentation to increase the concentration of antioxidant glutathione, to preserve thiols (O'Kennedy 2013). Supplementation of rehydration nutrients that are rich in glutathione can affect the timing of H_2S production and boost the concentrations of tropical fruit aroma of 3MH/A (Winter and Curtin 2012). On this basis, the mechanisms by which yeast generate H_2S from these sulfur sources are worth further investigation.

1.3 Methods for H_2S detection

1.3.1 Bismuth-containing media

Commercially available BiGGY (Bismuth Glucose Glycine Yeast) agar was originally used to isolate *Candida* from pathological samples (Nickerson 1953) but it has become a routine indicator medium to screen for the H_2S-forming potential of wine yeasts (Jiranek, Langridge and Henschke 1995b; Cordente *et al.* 2009; Linderholm *et al.* 2010). The sulfide produced reacts with bismuth in the medium to form a dark-brown precipitate of bismuth sulfide. However, others have demonstrated that colony colour on BiGGY agar does not always correlate to the amount of H_2S produced by yeast in grape juice (Kumar *et al.* 2010; Spiropoulos *et al.* 2000). Jiranek, Langridge and Henschke (1995b) suggested that colony colour on BiGGY agar only reflects the maximum sulfite reductase activity in yeast strains. The disagreement between BiGGY agar and fermentation can be attributed to the difference in the nutrient composition e.g. nitrogen and the physical fermentation conditions e.g. temperature (Linderholm *et al.* 2008; Ugliano, Kolouchova and Henschke 2011).

Bismuth citrate (11 g L^{-1}) and agar (20 g L^{-1}) have also been added to both Chemically Defined Grape Juice Medium and natural grape juice (Jiranek, Langridge and Henschke 1995b; Huang, Roncoroni and Gardner 2014). Whilst these indicator plates give similar results to those obtained from BiGGY agar, grape juice(s) are highly recommended when examining H_2S production in wine yeast (Kumar, Ramakrishnan and Bisson 2010).

1.3.2 Metal-impregnated paper methods

H_2S quantification is widely undertaken using paper strips that have been impregnated with metals such as silver nitrate or lead acetate, attached to the top of fermentation flasks (Natusch, Sewell and Tanner 1974; Cordente *et al.* 2009; Linderholm *et al.* 2010). The carbon dioxide (CO_2) produced during fermentation flushes H_2S up to the headspace where it reacts with the metals on the strips to form dark-coloured precipitates of silver or lead sulfide.

For screening purposes, a larger piece of paper treated with silver nitrate can be applied on top of the 96-well plate and sealed with Breathe-Easy sealing membranes (Sigma–Aldrich, St. Louis, USA) (Duan *et al.* 2004). However, this membrane screening method requires the addition of cysteine (~300 mg L^{-1}) to increase sensitivity for the formation of dark-coloured precipitates. Furthermore, multiple membranes for multiple time points are needed to study the kinetics of H_2S formation during fermentation.

1.3.3 Metal containing detector tubes

Commercial, pre-calibrated H_2S detector tubes (KITAGAWA, Japan or GASTEC, Japan) that are packed with silver nitrate or lead acetate can be inserted on top of the fermentation flasks and used to monitor H_2S production accurately and easily during fermentation (Ugliano and Henschke 2010). This method is based on a similar principle as the metal-impregnated paper; the CO_2 produced during fermentation flushes the H_2S into the detector tubes. H_2S then reacts with the metals in the tube and forms a dark-coloured precipitate. The length of the dark-coloured band formed is proportional to the total H_2S produced by yeast during fermentation (Park 2008).

1.3.4 Methylene blue

Methylene blue becomes colourless when it is reduced by hydrogen sulfide (Fischer 1883). The amount of hydrogen sulfide that leads to methylene blue decolourisation can be quantified by measuring the absorbance using a spectrophotometer (Acree *et al.* 1971). Winter and Curtin (2012) have further developed this method to monitor H_2S production in a 96-well plate (200 μL) format for high throughput screening. As methylene blue is added directly into the medium, this method could potentially be more sensitive in monitoring H_2S production during fermentation than methods that rely on CO_2 evolution to flush H_2S into the headspace. However, it has been observed that colourless methylene blue can revert back to its blue colour during fermentation. This may be due to evaporation of H_2S or oxidation of the dye (Winter and Curtin 2012). The other limitation of the method is the H_2S detection threshold and linearity (up to 50 μg), as the reduced colourless methylene blue does not change even when more H_2S is produced by yeast.

1.3.5 Gas Chromatography Mass Spectrometry

Gas Chromatography Mass Spectrometry (GC-MS) can be used to quantify H_2S and other sulfur compounds in wine (Nguyen *et al.* 2010). However, this method is not suitable to monitor H_2S production during fermentation or for high-throughput screening purposes due to the associated high cost and lengthy run time required.

1.4 Timing and retention of H_2S produced during fermentation

Hydrogen sulfide has been observed to be typically produced by yeast in two bursts as shown in Figure 1.2 (Thomas *et al.* 1993; Park 2008; Ugliano *et al.* 2009; Winter and Curtin 2012). The first peak is usually larger and occurs during the early stages of fermentation. It has been suggested that the first peak of H_2S comes primarily from the sulfate assimilation pathway, that is used by yeast to assimilate sulfate into the sulfur amino acids for growth and the amount of H_2S produced during this period is strain dependent (Thomas *et al.* 1993). The second peak of H_2S is relatively small and occurs towards the end of fermentation. It has been proposed that this peak could be related to the composition of the medium and the sulfur-containing compounds e.g. cysteine and glutathione may contribute to this peak through their degradation to H_2S by yeast under nitrogen-limiting conditions (Ugliano *et al.* 2009).

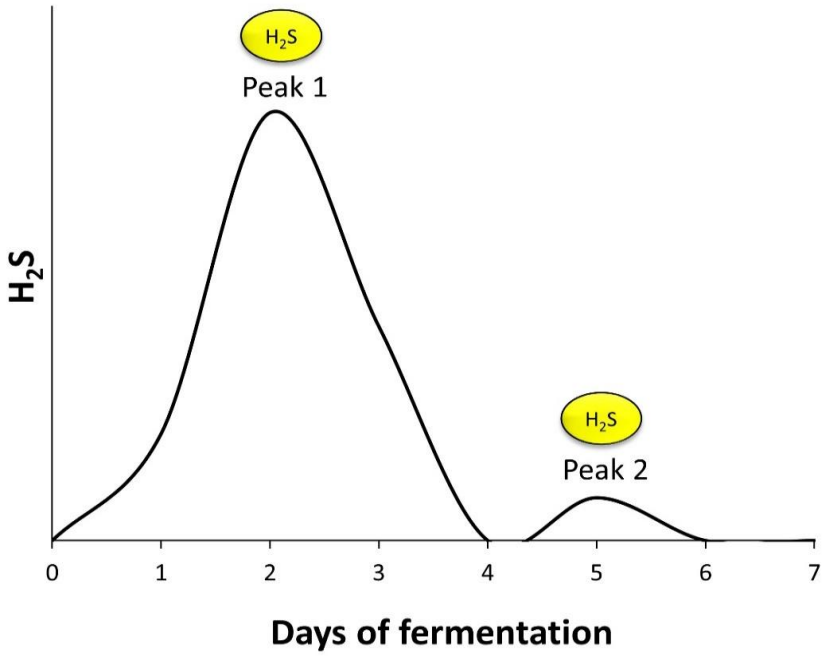

Figure 1.2. Schematic of the kinetics of H_2S produced by yeast during a typical fermentation (according to Thomas *et al.* 1993; Park 2008; Winter and Curtin 2012).

It has been assumed that if more H_2S is produced by yeast during fermentation, the finished wine will contain more residual H_2S. This speculation was confirmed by Park (2008), who used the lead acetate H_2S detector tubes to demonstrate that high levels of H_2S production during fermentation were correlated with higher concentrations of H_2S measurable in the finished wine. Moreover, Henschke and Jiranek (1991) and Park (2008) alluded to the second peak of H_2S being more likely to remain in the finished wine compared to the first peak of H_2S. An explanation for this is that in the later stages of fermentation, sugar consumption decreases, resulting in less CO_2 being evolved to push H_2S out of the wine.

These findings contrast that of Ugliano, Kolouchova and Henschke (2011), who used gas chromatography to measure H_2S in the finished wine and showed that the kinetics and concentrations of H_2S production during fermentation did not correlate with H_2S concentrations in the finished wine. The authors suggested the lack of correlation could be the result of H_2S reacting with compounds such as phenolics or quinones in the wine. Other factors, including the yeast strains used and the winemaking practices (e.g. punch-down or pump-over frequency) have all been suggested to affect the final wine H_2S content (Park 2008; Ugliano, Kolouchova and Henschke 2011).

Nevertheless, these studies show a complexity of H_2S formation and retention during fermentation. Further work is needed to understand the mechanisms of H_2S formation at different stages of fermentation and its impact on the final wine.

1.5 Volatile thiols

Three volatile thiols are particularly important in wine aroma: 4-mercapto-4-methylpentan-2-one (4MMP), which is responsible for a boxwood aroma; and 3-mercaptohexan-1-ol (3MH) and 3-mercaptohexyl acetate (3MHA) being reminiscent of passion fruit and grapefruit aromas, respectively (Tominaga *et al.* 1998).

These volatile thiols have very low detection thresholds of 0.8 ng L^{-1} (4MMP), 60 ng L^{-1} (3MH) and 4 ng L^{-1} (3MHA) and are key aroma compounds in many white and red wines such as Riesling, Cabernet Sauvignon, Merlot and especially in Sauvignon Blanc (Tominaga *et al.* 1998; Tominaga *et al.* 2000; Murat, Tominaga and Dubourdieu 2001; Dubourdieu *et al.* 2006). It has been shown that the distinctive, fruity style of New Zealand Sauvignon Blanc

can contain significantly higher levels of 3MH and 3MHA compared to wines from France, Australia, South Africa, Spain, and the United States (Lund *et al.* 2009).

The volatile thiols, 4MMP and 3MH, are usually not detected in unfermented grape juice thus it has been proposed that they are derived from odourless precursors in grape juice that are cleaved by yeast enzymes (carbon-sulfur lyases) during fermentation (Dubourdieu *et al.* 2006; Swiegers *et al.* 2007). The volatile thiol, 3MHA is also not present in grape juice and no precursor in grape has been found yet. It has been suggested that 3MHA is derived from 3MH by yeast alcohol acetyltransferase, which is encoded by *ATF1* (Swiegers *et al.* 2006a). Moreover, it has been shown that yeast strains vary in their ability to convert 3MH into 3MHA (Swiegers *et al.* 2009).

1.5.1 Precursors of volatile thiols

Three groups of precursors for volatile thiols have been proposed.

The first group are cysteinylated precursors, such as S-3-(4-mercapto-4-methylpentan-2-one)-cysteine (cys-4MMP) and 3-S-cysteinylhexan-1-ol (cys-3MH) (Darriet *et al.* 1995; Tominaga *et al.* 2000). Yeast *IRC7* encodes a ß-lyase that is responsible for releasing 4MMP from cysteinylated precursors (Thibon *et al.* 2008; Roncoroni *et al.* 2011).

The second group of precursors are glutathionylated precursors, such as *S*-3-(4-mercapto-4-methylpentan-2-one)-glutathione (GSH-4MMP) (Fedrizzi *et al.* 2009) and 3-*S*-glutathionylhexan-1-ol (GSH-3MH) (Roland *et al.* 2010a). The mechanisms of 4MMP release from GSH-4MMP and 3MH release from GSH-3MH are not well understood. GSH-3MH (Glu-Cys-3MH-Gly) is suggested to first be cleaved into single amino acids (glutamic acid, cys-3MH and glycine) by γ-glutamyltranspeptidase and carboxypeptidase. The cys-3MH formed is then cleaved to release free 3MH (Winter *et al.* 2011b).

The third group of precursors are mesityl oxide for 4MMP and (*E*)-2-hexenal for 3MH. This group of precursors requires the addition of sulfur compounds, such as H_2S produced by yeast during fermentation or cysteine and glutathione in grape musts in order to form thiols (Schneider *et al.* 2006). Even though mesityl oxide could be a possible precursor for 4MMP, it has not been detected in juice (Schneider *et al.* 2006). (*E*)-2-hexenal, also known as green leaf volatile (GLV), is produced by the enzymatic oxidation of unsaturated lipids when the

grapes are crushed during the pre-fermentation treatment (Drawert 1974). The concentrations of (*E*)-2-hexenal range from a few to hundreds of micrograms per litre, depending on the grape variety and pre-fermentation treatment (Subileau *et al*. 2008).

Several studies have reported that the conversion efficiency for 4MMP and 3MH from these three groups of proposed precursors is very low (from 0.1 to 12%; Dubourdieu *et al.* 2006; Subileau *et al.* 2008; Roland *et al.* 2010b). Furthermore, it has been shown that there is no correlation between the concentrations of the precursors in the juice and the final thiols in wine (Pinu *et al.* 2012). Therefore, alternative pathways and precursors for the formation of 4MMP and 3MH remain to be explored.

1.5.2 Modulation of thiols by yeast

Yeast species and strains differ in their ability to release 4MMP and 3MH and convert 3MH to 3MHA (Murat *et al.* 2001) and use of high potential thiol-producing yeast strains such as VIN7 (4MMP), VIN13 (3MH) and QA23 (3MH to 3MHA) could increase desirable fruity volatile thiols in Sauvignon Blanc wines (Swiegers *et al.* 2009).

The ability of yeast to produce fruity volatile thiols could be enhanced by overexpressing genes encoding ß-lyase. For instance, yeast overexpressing *Escherichia coli* tnaA, which encodes tryptophanase (cysteine-β-lyase), increased 4MMP and 3MH in model fermentations (Swiegers *et al.* 2007). In addition, overexpression of the full-length *S. cerevisiae* allele of *IRC7* in wine yeast, Zymaflore F15 (Laffort, France), enhanced 4MMP, 3MH and 3MHA production in Sauvignon Blanc wines (Roncoroni *et al.* 2011). Similarly, overexpression of yeast *STR3*, encoding cystathionine β-lyase, resulted in increased 3MH in Sauvignon Blanc wine (Holt *et al.* 2011). However, using these genetically modified yeasts to make wine is currently not allowed in most countries, including Australia.

Another approach to enhance thiols is co-inoculation of wine yeast strains. A study by King *et al.* (2008) has shown that Sauvignon Blanc wine made by inoculation with both commercial wine yeasts, VIN7 (Anchor Yeast) and QA23 (Lallemand), contained higher levels of 3MH and 3MHA compared to the wine made by VIN7 or QA23 alone. The increase in the production of 3MH/A could be due to VIN7 and QA23 complementing each other as VIN7 is a high 3MH releaser and QA23 is an efficient converter of 3MH to 3MHA (Swiegers

et al. 2006b).

Although studies have suggested that some *Saccharomyces bayanus* var. *uvarum* strains are capable of producing high levels of thiols, they also produce higher levels of ß-phenylethyl alcohol and its acetate, which could mask the thiol-related aromas of Sauvignon Blanc wines (Dubourdieu *et al.* 2006). Interestingly, interspecific hybrids of *S. cerevisiae* x *S. bayanus* were observed to release more 4MMP and 3MH without producing the undesirable ß-phenylethyl alcohol and its acetate, as compared to wines made by the *S. cerevisiae* strain VL3 (Dubourdieu *et al.* 2006). Furthermore, non-*Saccharomyces* yeasts such as *Pichia kluyveri* (Frootzen, Chr. Hansen, Denmark) have also been shown to increase volatile thiol production (Anfang, Brajkovich and Goddard 2008).

An alternative strategy to enhance concentrations of varietal thiols is by co-inoculation of specific *Saccharomyces* and non-*Saccharomyces* yeasts (Anfang, Brajkovich and Goddard 2008; Zott *et al.* 2011). It has been shown that wine yeast VL3 and an isolate of *Pichia kluyveri* from New Zealand, at a 1:9 starting ratio, significantly boosted the 3MHA levels in Sauvignon Blanc wines compared with single species ferments (Anfang, Brajkovich and Goddard 2008). These findings warrant further exploration of the mechanisms by which 3MHA production can be increased through manipulation of *Saccharomyces* and non-*Saccharomyce*s yeast.

1.6 H_2S and thiol production

It has been proposed that H_2S could potentially contribute to the formation of 3MH through reaction with (*E*)-2-hexenal. However, studies have shown that less than 1% of 3MH in wine is produced via this pathway (Schneider *et al*. 2006; Subileau *et al*. 2008).

Harsch and colleagues (2013) suggested that the low conversion efficiency is likely the result of insufficient early H_2S production during fermentation. The authors observed that (*E*)-2-hexenal is rapidly metabolised by yeast during the first 24 h after inoculation under commercial winemaking conditions. As such, little or no (*E*)-2-hexenal is present to react with the H_2S produced by yeast when assimilable nitrogen is depleted in the grape juice (Fig. 1.3). Nevertheless, Harsch *et al.* (2013) could demonstrate addition of H_2S (e.g. 100 mg L^{-1} of $NaSH{\cdot}xH_2O$) to grape juice prior to fermentation significantly increased concentrations of 3MH and 3MHA in the wine.

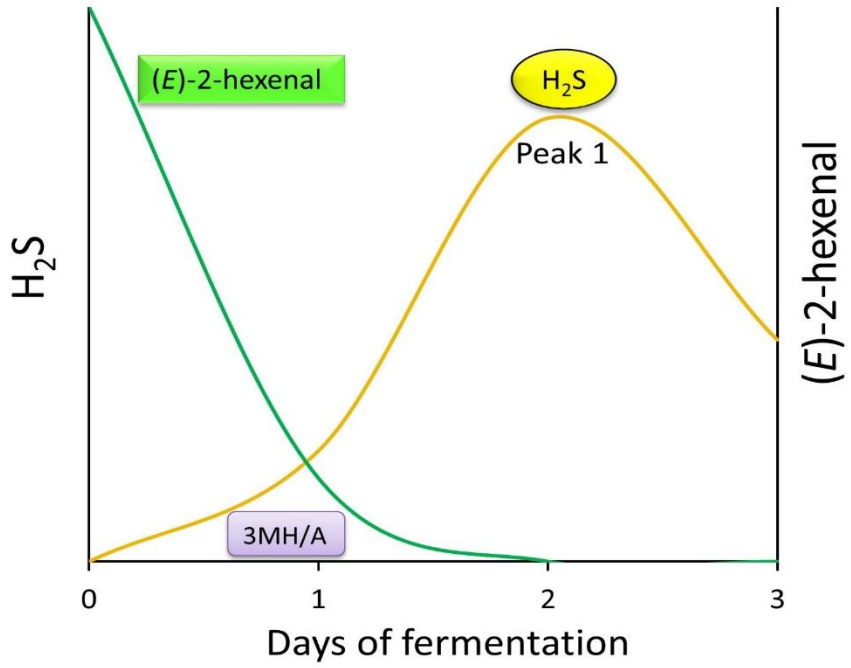

Figure 1.3. (*E*)-2-hexenal degradation and H_2S production during fermentation. A limited amount of early H_2S is produced by yeast to react with rapidly metabolised (*E*)-2-hexenal to form 3MH/A (adapted from Harsch *et al.* 2013).

Interestingly, two yeast single-gene deletants, Δ*met17* and Δ*cys4*, which are known as high H_2S producers (Linderholm *et al.* 2008), have been observed to increase 3MH and 3MHA production in Sauvignon Blanc wine (Harsch and Gardner 2013).

Winter *et al.* (2011a) observed that H_2S production was shifted to an earlier stage of fermentation by supplying rehydration nutrients, which could be responsible for the increased 3MH, 3MHA and decreased H_2S production. Furthermore, the authors used microarray analysis to show that this early H_2S was not due to changes in the expression of genes involved in the sulfate assimilation pathway, but was likely derived from glutathione − one of the ingredients of the rehydration nutrient, Laffort Dynastart® (Winter *et al.* 2011a).

These findings collectively suggest that H_2S could be a critical thiol precursor if yeast is able to produce significant amounts of H_2S in the early stages of fermentation before (*E*)-2-hexenal is metabolised by the yeast.

1.7 Summary of research aims

Studies have shown that H_2S can be generated by yeast through three characterised mechanisms: (1) from the sulfate assimilation pathway in which sulfate or sulfite are reduced to sulfide by sulfite reductase (Thomas and Surdin-Kerjan 1997), (2) via the reduction of inorganic sulfur by glutathione reductase (encoded by *GLR1*; Sato *et al.* 2011) and (3) the reduction of cysteine by a cysteine desulfhydrase (Tokuyama *et al.* 1973). In yeast, the cysteine desulfhydrase activity is thought to be encoded by *IRC7*, since the purified ß-lyase encoded by this gene acts directly on cysteine (Santiago and Gardner 2015).

These three pathways do not account for all H_2S produced during fermentation, since strains lacking functional Irc7p still produce H_2S when grown with cysteine or glutathione (Winter and Curtin 2012). There must be a fourth, novel pathway in yeast that leads to H_2S, involving genes not yet annotated for this function.

The major goal of this project is to determine the pathways and enzymes involved in the generation of H_2S from sulfur sources such as cysteine. One pathway proposed is via the formation of polysulfides, derived from elemental sulfur, cysteine or oxidised glutathione (GSSG). Thus, as part of this project, the formation of polysulfides in yeast from these substrates, and whether yeast enzymes are required for this pathway will be determined.

The experimental work planned for the project is as follows:

1) **Quantitative trait locus (QTL) mapping of genes linked to H_2S from cysteine**

   The $R_{301}G$ mutation in *MET2* that causes lower H_2S production, was recently identified by Huang, Roncoroni and Gardner (2014) through a quantitative trait locus (QTL) mapping approach. Preliminary trials indicated that Zymaflore F15 produced more H_2S than Oenoferm M2 from cysteine. Therefore, a similar strategy will be undertaken whereby the set of 96 fully sequenced F2 progenies from the M2 x F15 cross, will be used to conduct laboratory scale (100 mL) fermentations in the presence and absence of cysteine. The H_2S production will be measured by H_2S detector tubes (Komyo Kitagawa, Japan), and the H_2S data of the 96 sequenced progenies will be mapped to the corresponding individual genomes to identify potential QTLs associated with H_2S formation from cysteine.

2) **Testing a set of yeast candidate deletion mutants to identify genes required for H_2S production from cysteine.**

   A candidate gene approach will also be used to identify genes affecting H_2S production from cysteine. Several candidate genes deletants such as the *MET* gene deletants (e.g. Δ*met3/5/10*), the ß-lyase gene deletants (e.g. Δ*str3*, Δ*bna3* and Δ*cys3*) (Howell *et al.* 2005; Holt *et al*. 2011; Harsch and Gardner 2013) and the cysteine transporter deletants (e.g. Δ*gap1,* Δ*mup1* and Δ*yct1*) will be tested for their H_2S production from cysteine (During-Olsen *et al.* 1999; Kosugi *et al.* 2001; Kaur and Bachhawat 2007).

3) **Developing a screening assay for detecting H_2S production from cysteine**

   Another powerful strategy to identify yeast genes responsible for H_2S production is screening yeast single-gene deletion libraries (Linderholm *et al*. 2008; Yoshida *et al.* 2011). To accomplish this, a simple, reliable high-throughput assay for H_2S detection is necessary. As mentioned earlier, BiGGY agar plates (Nickerson 1953), metal (copper sulfate or lead nitrate) containing agar plates (Kim, Huh and Fay 2009; Yoshida *et al.* 2011), silver nitrate membranes (Duan *et al.* 2004) and the methylene blue method (Winter and Curtin 2012) have all been developed for high-throughput, H_2S detection screening purposes, however, these methods have their own limitations. This project will first test a few of the currently available screening methods and the

assay considered to be most suitable will be further developed, in order to screen H_2S production from cysteine.

4) Screening yeast deletion libraries for H_2S production from cysteine

The modified 96-well format, H_2S screening method will be used to screen the yeast deletion libraries. Currently, there are several yeast deletion libraries available including laboratory BY4743/BY4742/BY4741 deletion libraries and the partial AWRI1631 wine yeast deletion library (Varela *et al.* 2012). A few strains from these libraries will be initially tested to determine which yeast genetic background generates significant amounts of H_2S from cysteine, to be easily distinguished on the indicator medium.

5) Genetic manipulation and varietal thiol production

The candidate genes identified will be further investigated by construction of yeast knock-out/over-expression strains through PCR-based homologous recombination strategy (Guthrie and Fink 1991; Gietz *et al*. 1992). The yeast knock-out/over-expression strains will be used to conduct laboratory scale (100 mL) fermentations where in H_2S production will be measured. The concentrations of varietal thiols, 3MH and 3MHA, in wine will also be measured to examine the relationship between H_2S and thiol formation (Herbst-Johnstone *et al.* 2013).

6) Development of a detection method for polysulfides in yeast and wine

Berg *et al.* (2014) identified that the more soluble, linear form of sulfur: inorganic polysulfide (Sn^{2-}) is used as a sulfur storage molecule and can be oxidised to sulfate in bacteria (*Beggiatoa* spp.). It has also been proposed by Sato *et al.* (2011) that toxic, insoluble elemental sulfur may be converted into more soluble polysulfides first before entering yeast cells via a "polysulfide shuttle", where it is metabolised to sulfide.

Since the formation of polysulfides from elemental sulfur, cysteine or oxidised glutathione (GSSG) may be involved, a part of this project will specifically investigate polysulfide formation and degradation and any enzymes required for this process (Berg *et al.* 2014). To achieve this, an easy and reliable method for polysulfide detection is necessary. This work will investigate an HPLC-based method

for polysulfide detection in yeast (Rohwerder and Sand 2003; Kamyshny, Borkenstein and Ferdelman 2009).

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Chapter 2

The yeast *TUM1* affects production of hydrogen sulfide from cysteine treatment during fermentation

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Contribution to the Paper	Designed and performed experiments, interpreted data, wrote the manuscript and acted as corresponding author.		
Overall percentage (%)	70		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
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By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

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RESEARCH ARTICLE

The yeast TUM1 affects production of hydrogen sulfide from cysteine treatment during fermentation

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ABSTRACT

The undesirable rotten-egg odour of hydrogen sulfide (H_2S) produced by yeast shortly after yeast inoculation of grape musts might be an important source of desirable varietal thiols, which contribute to tropical aromas in varieties such as Sauvignon Blanc. In this study, we observed that *Saccharomyces cerevisiae* strains produce an early burst of H_2S from cysteine. Both Δ*met2* and Δ*met17* strains produce a larger burst, likely because they are unable to utilise the H_2S in the sulfate assimilation pathway. For the first time, we show that *TUM1* is partly responsible for the early production of H_2S from cysteine. Overexpressing *TUM1* elevated production of H_2S, whilst its deletion yields only half of the H_2S. We further confirmed that yeast convert cysteine to H_2S by analysing growth of mutants lacking components of the transsulfuration pathway. High concentrations of cysteine overcame this growth block, but required *TUM1*. Collectively, the data indicate that *S. cerevisiae* does not convert cysteine to sulfate or sulfite, but rather to sulfide via a novel pathway that requires the action of Tum1p. The findings of this study may allow the improvement of commercial yeasts through the manipulation of sulfur metabolism that are better suited towards production of fruit-driven styles.

Keywords: *Saccharomyces cerevisiae*; *TUM1*; hydrogen sulfide; varietal thiols; sulfate assimilation pathway; transsulfuration pathway

INTRODUCTION

Volatile sulfur compounds produced by yeast are important contributors to wine aroma. Some sulfur compounds such as hydrogen sulfide (H_2S) are responsible for an unpleasant, rotten-egg aroma (Rauhut 1993). On the other hand, the other class of sulfur compounds, such as 3-mercapto-hexanol (3MH) and 3-mercaptohexylacetate (3MHA), contribute positive tropical fruity aromas (Swiegers and Pretorius 2007).

(*E*)-2-Hexenal, also known as green leaf volatile, in grape musts is generated by the enzymatic oxidation of unsaturated lipids when the grapes are crushed during the prefermentation treatment (Drawert 1974). Depending on the grape variety and

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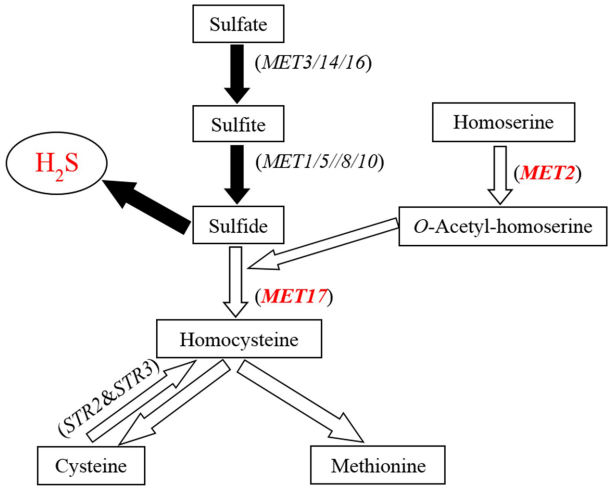

Figure 1. The SAP in *S. cerevisiae*. The black arrows indicate the major route for H_2S production during fermentation. Deletion of *MET2* or *MET17* increases H_2S production (adapted from Ugliano and Henschke 2009; Harsch and Gardner 2013).

prefermentation treatment, the concentrations of (*E*)-2-Hexenal can vary from a few to hundreds of micrograms per litre (Schneider *et al.* 2006).

Interestingly, (*E*)-2-Hexenal can react with H_2S to form 3MH. However, studies have indicated that <1% of 3MH in wine is produced via this H_2S–C6 pathway (Schneider *et al.* 2006; Subileau *et al.* 2008). Harsch *et al.* (2013) proposed that the lack of significant amounts of early H_2S produced by yeast to react with (*E*)-2-Hexenal (which is rapidly metabolised by yeast during the first 24 h post-inoculation) is responsible for the low conversion efficiency.

Most H_2S produced by yeast during fermentation is from the sulfate assimilation pathway (SAP) (Fig. 1) in which sulfate or sulfite is ultimately reduced to sulfide by sulfite reductase encoded by *MET10* (α subunit) and *MET5* (β subunit) (Thomas and Surdin-Kerjan 1997). H_2S is usually not released until yeast assimilable nitrogen becomes limited in grape juice during fermentation, as under replete nitrogen conditions, the sulfide is further metabolised to form methionine, cysteine and glutathione (Jiranek, Langridge and Henschke 1995).

Studies have shown that yeast can generate H_2S from cysteine (Tokuyama *et al.* 1973; Jiranek, Langridge and Henschke 1995). However, cysteine is not considered to be a significant source of H_2S under winemaking conditions given the small amounts of cysteine in grape must (<20 mg L^{-1}), e.g. Bordeaux musts (3 mg L^{-1}) (Pripis-Nicolau *et al.* 2001; Ugliano and Henschke 2009).

Winter and coworkers demonstrated that the supply of rehydration nutrients led to increased 3MH and 3MHA and decreased H_2S levels (Winter *et al.* 2011). They observed that H_2S production was shifted to an earlier stage of fermentation and proposed that cysteine, derived from glutathione—a component of the rehydration nutrient, is used by yeast for the early H_2S production. Therefore, a potential strategy to enhance thiol formation is to develop a yeast strain capable of generating significant amounts of early onset of H_2S from cysteine, which would be available to react with the transient (*E*)-2-Hexenal.

Santiago and Gardner (2015) established that the yeast *IRC7* gene encodes a cysteine desulfydrase which cleaves cysteine to generate H_2S. Interestingly, whilst most yeast strains possess a 38 bp deleted, non-functional as a ß-lyase *IRC7* variant (Roncoroni *et al.* 2011), they are still able to produce H_2S when grown with cysteine (Winter, Cordente and Curtin 2014), alluding to a novel pathway for H_2S formation, involving genes not yet annotated for this function.

In this study, we have confirmed that an early burst of H_2S production is induced by high concentrations of cysteine and show that there is a delayed burst of H_2S produced in strains deleted for either *MET17* or *MET2*. We then attempted to identify genes involved using both quantitative trait locus (QTL) and candidate gene approaches. Candidate genes tested were based on those previously identified in a genome-wide screen using the BY4742 deletion library as affecting H_2S production from cysteine (Winter, Cordente and Curtin 2014), but in several genetic backgrounds. This study represents the first report of the role of Tum1p in the production of H_2S from cysteine; the *TUM1* gene has not been previously annotated to this biological process.

METHODS

Yeast strains and culture

The yeast strains used for this study are listed in Table 1. YPD media (10 g L^{-1} yeast extract, 20 g L^{-1} peptone and 20 g L^{-1} glucose) was used for standard yeast propagation at 28°C. Synthetic dextrose minimal media (SD) (6.7 g L^{-1} yeast nitrogen base without amino acids and 20 g L^{-1} glucose) was used to select against uracil auxotrophic strains. Strains transformed with *KanMX*, *HphMX* or *NatMX* deletion cassettes were selected on Geneticin or G418 sulfate (200 mg L^{-1}; Astral, NSW, Australia), Hygromycin (300 mg L^{-1}; Astral, NSW, Australia) or Nourseothricin sulfate (ClonNAT; Bioscientific Pty. Ltd, Australia) (100 mg L^{-1}), respectively (Goldstein and McCusker 1999).

Yeast growth was measured using a Tecan Infinite M200 microplate reader, whereby the absorbance of 0.2 mL cultures was read every 24 h at 600 nm (OD 600 nm) with 1 min shaking prior to measurement. Yeast starter cultures (2% sugar, non-sulfate CDGJM plus 0.15 mM methionine) were centrifuged, washed twice and resuspended in sterile water. The starter cultures (~1.5×10^5 cells mL^{-1}) were inoculated in a 96-well plate (Costar 3596, Sigma-Aldrich, NSW, Australia) containing 150 μL Sulfur-Free Chemically Defined Grape Juice Medium (SFCDGJM; CDGJM lacking $MgSO_4.7H_2O$, methionine and cysteine) supplemented with 5 mM L-cysteine (168149, Sigma-Aldrich, NSW, Australia), and incubated for 72 h at 28°C in triplicate. The composition of CDGJM medium was identical to Henschke and Jiranek (1993) except amino acid and diammonium phosphate supplementation was altered to reflect Marlborough Sauvignon Blanc juice (Harsch *et al.* 2010; Santiago and Gardner 2015).

Genetic manipulation and strain construction

Polymerase chain reactions (PCR) were performed using Velocity DNA polymerase (Bioline, Australia). Yeast deletion strains were confirmed using Kan B or Kan C primers together with gene-specific primers as reported in Table S1 (Supporting Information). Yeast transformation was performed using the lithium acetate method (Gietz *et al.* 1992).

Plasmid pJC1, a 2-μm-based plasmid containing the *PGK1* promoter and *URA3* selectable marker (Crous, Pretorius and Van Zyl 1995; Martin *et al.* 2003), was used to overexpress the *TUM1* gene. *TUM1* was amplified from BY4743 genomic DNA using *TUM1-EcoRI*-F and *TUM1-XhoI*-R primers (Table S1). The PCR product was digested with restriction enzymes *Eco*RI and *Xho*I, purified (Wizard Plus SV Minipreps, Promega, USA)

Table 1. Yeast strains used in this study.

Strain	Genotype, phenotype and comments	Origin
Zymaflore F15	Wild-type diploid; a commercial wine yeast	Laffort, France
Oenoferm M2	Wild-type diploid; a commercial wine yeast	Lallemand, Australia
M2xF15 progeny (1 ~ 96)	Wild-type diploid progeny of M2xF15; 23 tetrads, plus 4 random spores, 96 individuals	Huang, Roncoroni and Gardner (2014)
BY4743	*MATa/α, his3-Δ1/his3-Δ1, leu2-Δ0/leu2-Δ0, LYS2/lys2-Δ0, met15-Δ0/MET15, ura3-Δ0/ura3-Δ0*	Euroscarf
BY4743 Δ*met3*	*met3::KanMX*	Euroscarf
BY4743 Δ*met5*	*met5::KanMX*	Euroscarf
BY4743 Δ*met10*	*met10::KanMX*	Euroscarf
BY4743 Δ*met17*	*met17::KanMX*	Euroscarf
BY4743 Δ*tum1*	*tum1::KanMX*	Euroscarf
BY4743 Δ*uba4*	*uba4::KanMX*	Euroscarf
BY4743 Δ*ncs2*	*ncs2::KanMX*	Euroscarf
BY4743 Δ*ncs6*	*ncs6::KanMX*	Euroscarf
BY4743 Δ*urm1*	*urm1::KanMX*	Euroscarf
BY4743 Δ*ahp1*	*ahp1::KanMX*	Euroscarf
BY4743 Δ*str2*	*str2::KanMX*	Euroscarf
BY4743 Δ*yhr112c*	*yhr112c::KanMX*	Euroscarf
BY4743 Δ*yll058w*	*yll058w::KanMX*	Euroscarf
BY4743 Δ*yml082w*	*yml082w::KanMX*	Euroscarf
BY4743 Δ*ygr012w*	*ygr012w::KanMX*	Euroscarf
BY4743 Δ*bna3*	*bna3::KanMX*	Euroscarf
BY4743 Δ*irc7*	*irc7::KanMX*	Euroscarf
BY4743 Δ*vam7*	*vam7::KanMX*	Euroscarf
BY4743 Δ*fra1*	*fra1::KanMX*	Euroscarf
BY4743 Δ*fra2*	*fra2::KanMX*	Euroscarf
BY4743 Δ*mrs3*	*mrs3::KanMX*	Euroscarf
BY4743 Δ*isu1*	*isu1::KanMX*	Euroscarf
BY4743 (pJC1)	BY4743 with (pJC1)	This study
BY4743 (*TUM1*ox)	BY4743 with (pJC1+*TUM1*)	This study
BY4742	*MATα, his3-Δ1,leu2-Δ0, ura3-Δ0, lys2-Δ0*	Euroscarf
BY4742 Δ*met2*	*met2::NatMX*	This study
BY4742 Δ*met3*/Δ*str2*/Δ*str3*	*met3::KanMX; str2::HphMX; str3::NatMX*	This study
BY4742 Δ*met14*/Δ*str2*/Δ*str3*	*met14::KanMX; str2::HphMX; str3::NatMX*	This study
BY4742 Δ*met16*/Δ*str2*/Δ*str3*	*met16::KanMX; str2::HphMX; str3::NatMX*	This study
BY4742 Δ*met5*/Δ*str2*/Δ*str3*	*met5::KanMX; str2::HphMX; str3::NatMX*	This study
BY4742 Δ*met10*/Δ*str2*/Δ*str3*	*met10::KanMX; str2::HphMX; str3::NatMX*	This study
BY4742 Δ*met1*/Δ*str2*/Δ*str3*	*met1::KanMX; str2::HphMX; str3::NatMX*	This study
BY4742 Δ*met8*/Δ*str2*/Δ*str3*	*met8::KanMX; str2::HphMX; str3::NatMX*	This study
BY4742 Δ*met17*/Δ*str2*/Δ*str3*	*met17::KanMX; str2::HphMX; str3::NatMX*	This study
BY4741	*MATa, his3-Δ1, leu2-Δ0, met15Δ0, ura3Δ0*	Euroscarf
BY4741Δ*met3*	*met3::KanMX*	Euroscarf
BY4741Δ*met5*	*met5::KanMX*	Euroscarf
BY4741Δ*met10*	*met10::KanMX*	Euroscarf
AWRI1631	Haploid wine strain	AWRI
AWRI1631 Δ*tum1*	*tum1::KanMX*	AWRI
AWRI1631 Δ*cys3*	*cys3::KanMX*	AWRI
AWRI1631 Δ*cys4*	*cys4::KanMX*	AWRI
AWRI1631 Δ*irc7*	*irc7::KanMX*	AWRI
AWRI1631 Δ*str3*	*str3::HphMX*	This study
AWRI1631 Δ*vps25*	*vps25::KanMX*	AWRI
AWRI1631 Δ*vps36*	*vps36::KanMX*	AWRI
AWRI1631 Δ*fra1*	*fra1::KanMX*	AWRI
AWRI1631 Δ*tum1*/Δ*str3*	*tum1::KanMX; str3::HphMX*	This study
Sigma 1278b (pJC1)	Lab strain, *MATa*, *ura3Δ0* (pJC1)	This study
Sigma 1278b (*TUM1*ox)	Lab strain, *MATa*, *ura3Δ0* (pJC1+*TUM1*)	This study
Oenoferm M2 *ura3Δ* (pJC1)	Wine strain, *MATa*, *ura3Δ0*, ho::*HphMX* (pJC1)	This study
Oenoferm M2 *ura3Δ* (*TUM1*ox)	Wine strain, *MATa*, *ura3Δ0*, ho::*HphMX* (pJC1+*TUM1*)	This study
AWRI796 *ura3Δ* (pJC1)	Wine strain, *ura3::kanMX* (pJC1)	This study
AWRI796 *ura3Δ* (*TUM1*ox)	Wine strain, *ura3::KanMX* (pJC1+*TUM1*)	This study
Zymaflore F15 *ura3Δ* (pJC1)	Wine strain, *MATa*, *ura3Δ0* (pJC1)	This study
Zymaflore F15 *ura3Δ* (*TUM1*ox)	Wine strain, *MATa*, *ura3Δ0* (pJC1+*TUM1*)	This study
Lalvin L2056 *ura3Δ* (pJC1)	Wine strain, *ura3Δ::KanMX* (pJC1)	This study
Lalvin L2056 *ura3Δ* (*TUM1*ox)	Wine strain, *ura3Δ::KanMX* (pJC1+*TUM1*)	This study
Maurivin B *ura3Δ* (pJC1)	Wine strain, *ura3Δ::KanMX* (pJC1)	This study
Maurivin B *ura3Δ* (*TUM1*ox)	Wine strain, *ura3Δ::KanMX* (pJC1+*TUM1*)	This study

ox = overexpression.

and ligated with pre-digested pJC1 plasmid (*Eco*RI and *Xho*I). Transformation, plasmid propagation and verification of the recombinant plasmid (pJC1+*TUM1*) by restriction digestion were performed according to Sambrook, Fritsch and Maniatis (1989). The plasmids pJC1 (as control) and the (pJC1+*TUM1*) were transformed into the uracil-minus yeast strains described in Table 1. Transformants were selected on synthetic dextrose (SD) uracil drop-out plates (Amberg, Burke and Strathern 2005).

Triple deletants of *STR2*, *STR3* and individual *MET* genes were constructed by sequential deletion of *STR2* followed by *STR3* in the individual *MET* gene deletants available from the haploid BY4742 deletion library (Table 1). The deletion of *STR2* was achieved by amplifying the *HphMX* cassette in plasmid pAG32 plus ∼100 bp of homologous untranslated sequence flanking *STR2* (using the primers pair Del-*str2*-F and Del-*str2*-R; Table S1). The PCR products were used for yeast transformation with selection of transformants on YPD agar plates containing hygromycin (300 mg L^{-1}). *Str2::HphMX* deletants were confirmed by PCR using primers *Hph*-I-F and RC*str2* (Table S1).

The *STR3* and *MET2* genes were deleted using a similar approach. The NatMX cassette in plasmid pAG25 was amplified together with ∼100 bp of homologous untranslated sequence flanking *STR3* or *MET2* using Del-*str3*-F and Del-*str3*-R or Del-*met2*-F and Del-*met2*-R primers, respectively (Table S1). Yeast transformants were selected on YPD agar plates containing nourseothricin (100 mg L^{-1}). *Str3::NatMX* and *Met2::NatMX* deletants were verified using primer pairs *Nat*-I-F and RC*str3* and *Nat*-I-F and RC*met2* (Table S1).

Fermentations and H_2S quantification

Yeast starter cultures were prepared by inoculating a single yeast colony into starter medium (2% sugar, non-sulfate CDGJM plus 0.15 mM methionine) for 24 h at 28°C. The starter culture was centrifuged, washed and resuspended in sterile water to inoculate 100 mL of medium (non-sulfate CDGJM plus 5 mM cysteine and 0.15 mM methionine) at 2.5×10^6 cells L^{-1}. The additional amounts of histidine (200 mg L^{-1}), leucine (300 mg L^{-1}), uracil (100 mg L^{-1}) and lysine (300 mg L^{-1}) were added for auxotrophies (Harsch *et al*. 2010). Fermentations were conducted in triplicate in 250 mL flasks at 28°C with shaking at 100 rpm. Fermentation progress was monitored daily as weight loss due to CO_2 evolution (Bely, Sablayrolles and Barre 1990). H_2S produced by yeast during fermentation was detected by either lead acetate (4H: 1–2000 ppm; GASTEC, Japan) or silver nitrate (120SF: 1–1000 ppm; KITAGAWA, Japan) H_2S detector tubes that tightly fitted into the glass airlock of the flask (Park 2008).

Data analysis

The mean, standard error of the mean (SEM) and *t* test (two samples assuming unequal variances) were performed using Microsoft Excel 2013 (Microsoft, Redmond, Washington, USA). Analysis of variance (ANOVA) and Tukey's honestly significant difference test were performed using JMP software (SAS Institute, Cary, NC, USA). Statistical significance was set at the confidence level of 5%.

QTL mapping

The R/QTL package (Broman *et al*. 2003) was used for mapping of QTLs. Single QTL analysis for quantitative traits was performed using the Haley–Knott regression. Significance thresholds were generated for every trait by 1000 permutations of the phenotype data. Data were normalised between runs, by making the centre of the distribution 0 and the standard deviation 1.

RESULTS

High cysteine causes an early burst of H_2S production and deletion of *MET17* or *MET2* leads to an additional delayed burst

The *MET* genes associated with the SAP are well known for their roles in affecting H_2S production during fermentation (Cordente *et al*. 2009; Linderholm *et al*. 2010; Huang, Roncoroni and Gardner 2014; Noble, Sanchez and Blondin 2015). However, their roles in H_2S production from cysteine are less well understood. Here, the *MET* gene deletants were fermented in sulfate-free CDGJM supplemented with 5 mM cysteine and 0.15 mM methionine to examine their roles in H_2S production from cysteine.

The concentration of 5 mM (605.8 mg L^{-1}) cysteine was chosen because a distinguishable amount of H_2S could be detected by H_2S detector tubes in initial trials (data not shown). In addition, a similar amount of cysteine (4.1 mM or 500 mg L^{-1}) was applied in a previous study, without biomass formation being affected (Winter, Cordente and Curtin 2014). A minimal concentration (0.15 mM) of methionine was supplemented to facilitate cell growth. No H_2S was detectable when yeast strains were fermented in non-sulfate CDGJM plus 0.15 mM methionine (Fig. 2A). Furthermore, no H_2S was detected in uninoculated, non-sulfate CDGJM plus 5 mM cysteine and 0.15 mM methionine (Fig. 2A). Therefore, this medium was considered ideal for studying H_2S production by yeast from cysteine.

BY4743 produced an early burst of H_2S when high concentrations of cysteine are added to the fermentation (Fig. 2A). Because this yeast strain lacks an active *IRC7* gene, this known cysteine desulfydrase activity cannot be responsible for the H_2S that is produced. All of the *MET* gene deletant strains also produced a similar burst of H_2S after 48 h of fermentation (Fig. 2A). However, the Δ*met17* strain produced a significant amount of additional H_2S in a more prolonged time. In addition, the *MET2* deletant of the related BY4742 strain also produced elevated H_2S compared to the corresponding wild type (Fig. 2B).

This early burst of H_2S production from cysteine must come from an as-yet-unidentified pathway, independent of both *IRC7* and most of the SAP, as strains lacking the individual *MET* genes did not affect H_2S production. The late H_2S observed for Δ*met17* and Δ*met2* strains could be derived from the SAP, as sulfate, sulfite or sulfide.

The addition of 5 mM cysteine did transiently slow the growth of BY4743 on the first day compared to BY4743 when fermented in non-cysteine containing CDGJM (Fig. S1, Supporting Information). However, BY4743 was able to overcome the toxic effect of cysteine by the second day and the overall fermentation kinetics of BY4743 grown on 0 or 5 mM cysteine were similar. The fermentation kinetics were also similar for the BY4743 *MET* gene deletants, suggesting that the additional H_2S observed for the Δ*met17* strains was not related to growth.

QTL mapping of genes linked to H_2S from cysteine

Zymaflore F15 was observed to produce more H_2S than Oenoferm M2 when fermented in non-sulfate CDGJM plus 5 mM cysteine and 0.15 mM methionine (Fig. 3A). Genome sequencing of the parental strains enabled single nucleotide polymorphisms at individual chromosomal loci to be identified. To map genes responsible for H_2S production from cysteine, a set of 96

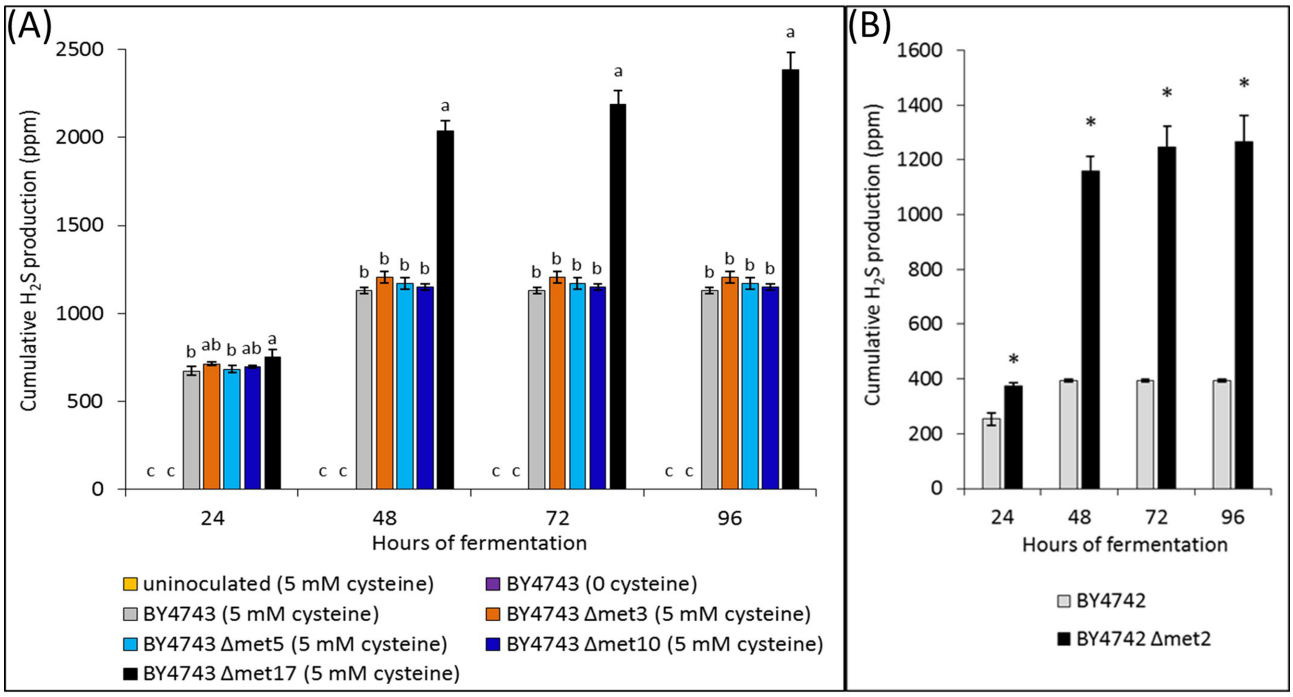

Figure 2. (A) Cumulative H_2S production from BY4743 and its *MET* gene deletants **(B)** BY4742 and BY4742 Δ*met2* during 24 h–96 h of fermentation. Fermentations were performed in 100 mL of non-sulfate CDGJM and 0.15 mM methionine plus or minus 5 mM cysteine at 28°C ($n = 3$) with shaking at 100 rpm. H_2S was measured with silver nitrate (120SF: 1–1000 ppm; KITAGAWA, Japan) H_2S detector tubes **(A)**, which were replaced at regular intervals. H_2S was measured by lead acetate (4H: 1–2000 ppm; GASTEC, Japan) H_2S detector tubes for **(B)** BY4742 and BY4742 Δ*met2* strains. Data represent mean values of triplicate fermentations ± standard error of the mean (SEM). Samples not connected by the same letter are significantly different (ANOVA, Tukey's HSD). Asterisks above bars represent significant differences compared to the wild types (*$P < 0.05$, two-tailed Student's *t* test).

fully sequenced M2xF15 progeny (Huang, Roncoroni and Gardner 2014) was fermented in triplicate in non-sulfate CDGJM supplemented with 5 mM cysteine and 0.15 mM methionine. H_2S production was measured over the course of the ferment using H_2S detector tubes. The final reading on the tubes (averages of replicates) was used as input for QTL analysis.

The logarithm of the odds scores for the individual chromosomes revealed that none of the peaks were significant even at the 5% level (Fig. 3C) despite variation for H_2S production among the 96 progenies (Fig. 3B). This indicates that there was no readily identifiable correlation between H_2S production from cysteine and a specific gene or DNA region.

Deletion of *TUM1* gene yields only half of the H_2S from cysteine

The inability to identify a specific QTL associated with H_2S production from cysteine may be due to insufficient sample sizes or indicate the presence of multiple genes affecting the trait (Bloom *et al.* 2013; Winter, Cordente and Curtin 2014). It was therefore decided to investigate this process by testing candidate gene deletants, based on results from previous studies (Winter, Cordente and Curtin 2014; Santiago and Gardner 2015), in order to decipher the genetic basis behind how H_2S is formed during fermentation on high cysteine. Deletants used in this study are in the homozygous diploid auxotrophic laboratory strain BY4743 background (Euroscarf) and the genes of interest identified were further validated in the haploid wine yeast strain AWRI1631 background (AWRI Wine Yeast Deletion Library collection) (Varela *et al.* 2012).

The ß-lyase gene, *IRC7*, has been previously identified as responsible for H_2S production from cysteine (Santiago and Gardner 2015). Therefore, several ß-lyase candidate gene deletants (Holt *et al.* 2011; Harsch and Gardner 2013) from two yeast gene deletion libraries were evaluated in this study for their roles on H_2S production from cysteine.

Deletion of *TUM1* gene in the laboratory strain BY4743 resulted in half of the H_2S production (Fig. 4A) and most importantly, deletion of *TUM1* did not affect the fermentation performance (Fig. S2, Supporting Information). The other ß-lyase candidate gene (YML082W) deletants did not have as big and impact on H_2S formation compared to Δ*tum1* and the lower H_2S production observed such as from BY4743 Δ*yml082w* may be related to its slower growth rate (data not shown). Deletion of *TUM1* in the AWRI1631 background also reduced H_2S production from cysteine significantly (Fig. 4B).

Overexpression of *TUM1* elevated production of H_2S from cysteine

The reduction in H_2S production to half in yeast strains lacking *TUM1* clearly demonstrated the importance of *TUM1* in H_2S production from cysteine. The impact of *TUM1* was also tested by overexpressing the *TUM1* gene originating from BY4743 in different genetic backgrounds. In all cases, overexpression of *TUM1* led to increased H_2S production at the early stage of fermentation (Fig. 5). Both BY4743 and Sigma 1278b are laboratory yeast strains whereas the others are *ura3* auxotrophic strains derived from commercial wine yeast strains. As no H_2S was detected in the normal sulfate-containing CDGJM (data not shown), it can be

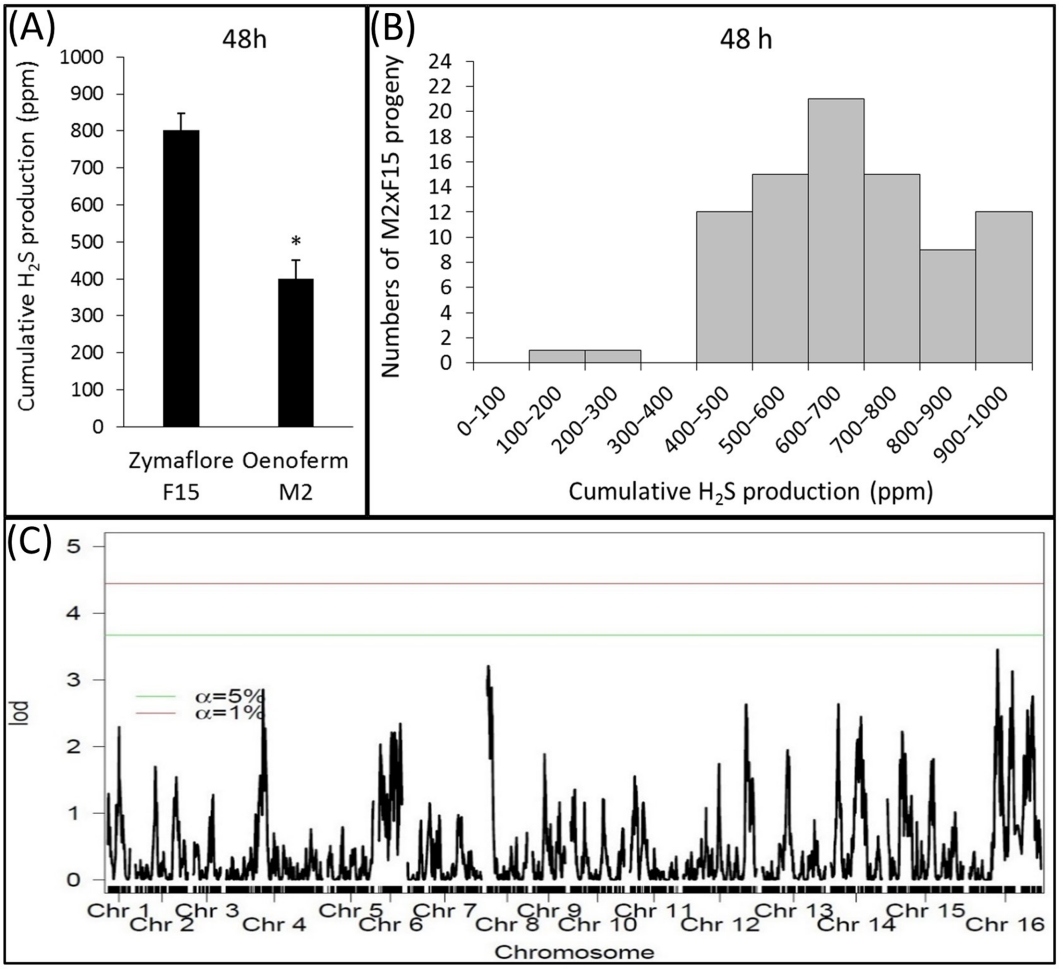

Figure 3. (A) Cumulative H_2S production from Zymaflore F15 and Oenoferm M2, **(B)** the 96 M2xF15 progenies, **(C)** LOD scores for H_2S production in 96 M2xF15 progenies plotted along the 16 yeast chromosomes. Green horizontal line indicates the 5% and red indicates the 1% significance cut-off. Fermentations were performed in 100 mL of non-sulfate CDGJM and 0.15 mM methionine plus or minus 5 mM cysteine at 28°C ($n = 3$) with shaking at 100 rpm. H_2S was measured with silver nitrate (120SF: 1–1000 ppm; KITAGAWA, Japan) H_2S detector tubes, which were replaced at regular intervals. The mean H_2S released is shown and error bars show SEM. Asterisks above bars represent significant differences compared to the wild types (*$P < 0.05$, two-tailed Student's *t* test).

concluded that the effect of *TUM1* is specific to production from cysteine.

Fate of cysteine

The observed increase in H_2S production in the BY4742 Δ*met2* and BY4743 Δ*met17* deletants, when grown in high cysteine (5 mM) medium, alluded to the formation of an unknown intermediate, derived from the degradation of cysteine, which is able to be metabolised through SAP. A series of experiments were carried out to determine the fate of cysteine.

The presence of sulfate and sulfite was measured by ion chromatography (OIV-MA-AS313-16), in high-cysteine medium prior to and after fermentation with BY4743 and BY4743 Δ*met17* strains. However, no sulfate or sulfite was detectable (data not shown). The aspiration method (Rankine and Pocock 1970; Fujita *et al.* 1979) was also used to determine if any SO_2 (detection limits ~0.5 mg L^{-1}) was present but again no sulfite could be detected (data not shown).

Hydrogen sulfide production from cysteine catabolism was further investigated in double deletants of *MET17* and the individual *MET* genes of the SAP. BY4741 was chosen because of the pre-existing Δ*met17* mutation. It was proposed that if cysteine is converted to sulfate then deletion of *MET3* would prevent the reduction of sulfate so that less H_2S would be expected than in BY4741 (Δ*met17* alone). If cysteine is converted to sulfite, then deletion of *MET3* would not affect H_2S production, but deletion of *MET1*, *MET5* or *MET10* would reduce H_2S, as sulfite could not be further assimilated. However, amounts of H_2S produced by these double deletants were similar to each other indicating that yeast does not catabolise cysteine to sulfate or sulfite (Fig. S3, Supporting Information).

Yeast can bypass the known *STR2/STR3* transsulfuration pathway and grow on high cysteine, but require both *MET17* and *TUM1*

It has been shown that Δ*str2* and Δ*str3* strains are unable to grow on a medium containing 0.2 mM cysteine or glutathione as sole sulfur source (Hansen and Johannesen 2000). However, overexpression of a full-length functional *IRC7* gene in a Δ*str3* deletant of the wine yeast F15 restored growth on 0.5 mM cysteine as the only sulfur source (Santiago and Gardner 2015). The authors proposed that extra H_2S produced by Irc7p action on cysteine could

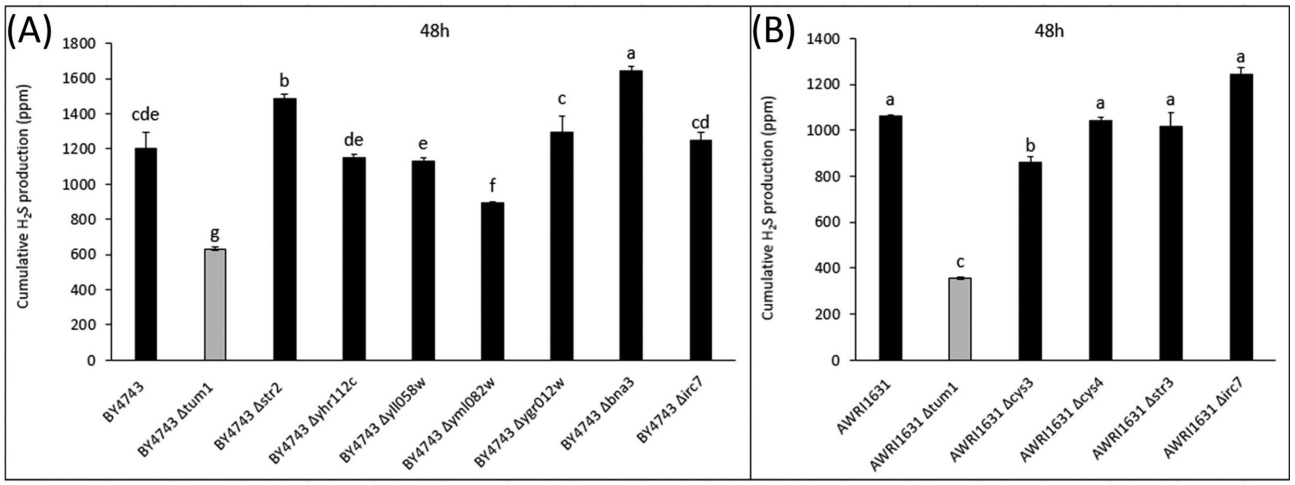

Figure 4. Cumulative H_2S production from the ß-lyase candidate gene deletants in **(A)** laboratory strain BY4743 **(B)** and wine yeast strain AWRI1631 backgrounds. Fermentations were performed in 100 mL of non-sulfate CDGJM and 0.15 mM methionine plus 5 mM cysteine at 28°C ($n = 3$) with shaking at 100 rpm. H_2S was measured with silver nitrate (120SF: 1–1000 ppm; KITAGAWA, Japan) H_2S detector tubes, which were replaced at regular intervals. The mean H_2S released is shown and error bars show SEM. Samples not connected by the same letter are significantly different (ANOVA, Tukey's HSD).

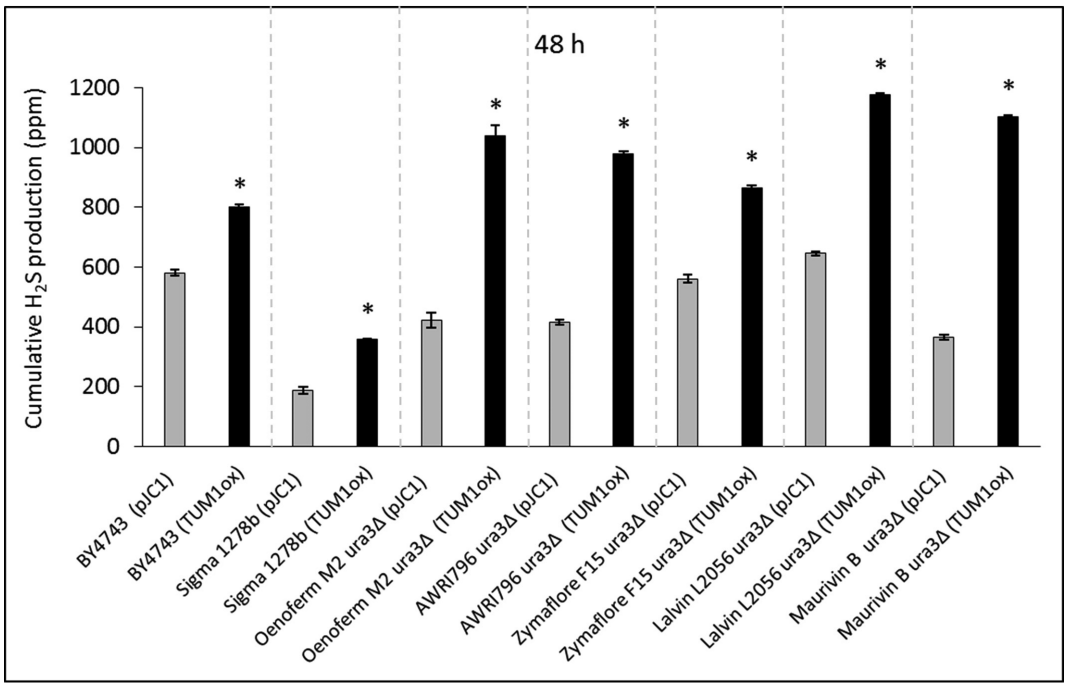

Figure 5. Cumulative H_2S production from the wild types and *TUM1* overexpression strains in different yeast genetic backgrounds (separated by dotted lines). Fermentation was carried in 100 mL of non-sulfate CDGJM plus 5 mM cysteine and 0.15 mM methionine at 28°C with shaking at 100 rpm. H_2S was measured by lead acetate (4H: 1–2000 ppm; GASTEC, Japan) H_2S detector tubes and the mean H_2S released is shown. Error bars indicate SEM. Fermentations by BY4743 and Oenoferm M2 strains were performed in triplicate, whilst only duplicate fermentations were done for the other strains. ox denotes overexpression. Asterisks above bars represent significant differences compared to the wild types (*$P < 0.05$, two-tailed Student's *t* test).

be metabolised via the SAP to produce methionine, enabling the bypass of the transsulfuration pathway and growth of the Δ*str3* strain on cysteine.

The growth of the Δ*str2* and Δ*str3* strains on high concentrations of cysteine as sole sulfur source media has not been previously investigated. The observation that yeasts are capable of generating additional H_2S from cysteine led to the speculation that Δ*str2* and Δ*str3* strains could also bypass the transsulfuration pathway, allowing growth under excess cysteine conditions. Furthermore, the ability of individual triple deletants of *STR2*, *STR3* and various *MET* genes to grow would reveal whether cysteine was converted to sulfate, sulfite or sulfide, prior to synthesis of methionine via SAP.

Triple deletants were constructed in the BY4742 genetic background (wild type for *MET17*), whereby individual *MET* gene deletants were further modified such that coding sequences of *STR2* and *STR3* were replaced with the *str2::HphMX* and *str3::NatMX* deletion cassettes (see the section 'Material and Methods'). The growth of the triple deletants was tested in Sulfur-Free Chemically-Defined Grape Juice Medium (SFCDGJM;

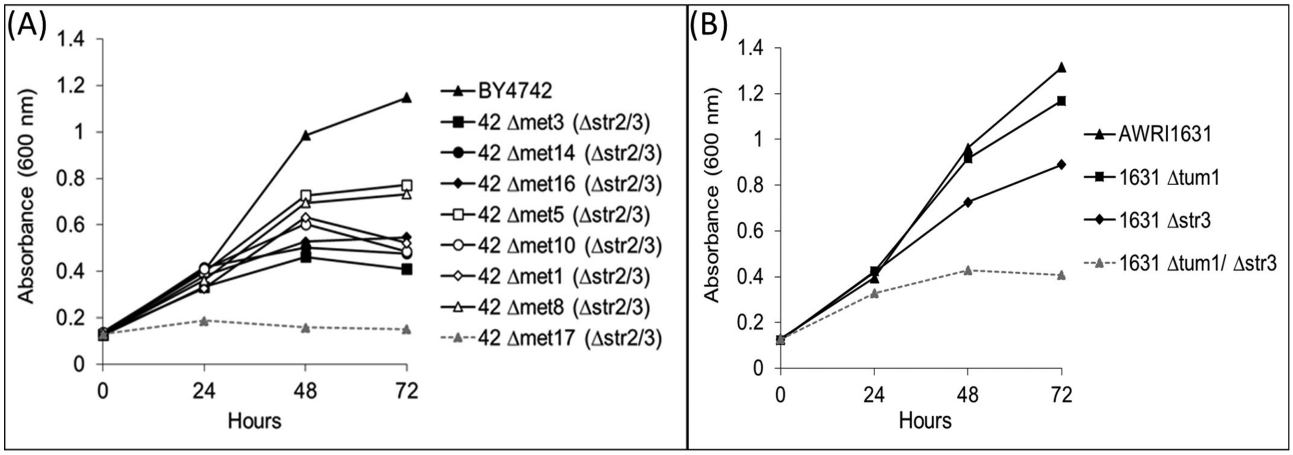

Figure 6. (A) Yeast *MET17* and **(B)** *TUM1* genes are necessary to bypass the *STR2/STR3* transsulfuration pathway and grow on high concentration of cysteine as sole sulfur source. Strains were grown at 28°C for 72 h in SFCDGJM plus 5 mM cysteine. The absorbance was measured by microplate reader every 24 h with 1 min shaking at OD600 nm. Values are means of triplicate wells.

CDGJM lacking $MgSO_4.7H_2O$, Met and Cys) supplemented with 5 mM cysteine (Santiago and Gardner 2015). Cysteine was essential for growth, as all strains showed only minimal growth in SFCDGJM, which lacked this amino acid (data not shown). The strains also failed to grow on low cysteine (0.1 mM), confirming that the deletion of *STR2* and *STR3* had been successful and that the transsulfuration pathway was inactive in these mutants (data not shown).

All of the triple deletants could grow with 5 mM cysteine as sole sulfur source, with the exception of Δ*str2*/Δ*str3*/Δ*met17* strain (Fig. 6A). These findings suggest that Δ*str2* and Δ*str3* strains can bypass the transsulfuration pathway when cultured in medium containing high cysteine. The data show that cysteine is not being converted to sulfate, as the deletion of *MET3*, *MET14* or *MET16*, which would block flow of sulfate through the SAP, did not affect growth. Sulfite was also not an intermediate, as the deletion of *MET1*, *MET5*, *MET8* or *MET10*, which block the reduction of sulfite to sulfide, did not prevent growth.

BY4742 Δ*met17*/Δ*str2*/Δ*str3* was the only strain that could not grow, consistent with the hypothesis that sulfur derived from supplementation with high cysteine most likely enters the SAP as sulfide.

We have already alluded to *TUM1* gene as playing a key role in H_2S production from cysteine and therefore the growth of Δ*tum1*/Δ*str3* double deletant was investigated. Deletion of *STR3* in wine strain AWRI 1631 was clearly able to bypass the transsulfuration pathway, as it could grow in a high cysteine medium (Fig. 6B). A slower growth rate was observed for the Δ*tum1*/Δ*str3* double deletant. The reduced ability to generate H_2S from cysteine in this strain therefore correlates with reduced growth on cysteine as sole sulfur source, consistent with the interpretation that cysteine enters the SAP as sulfide.

Genes involved in tRNA thiolation, vacuolar maintenance and iron–sulfur homeostasis have limited effect on H_2S production from cysteine

A number of other candidate genes were screened for their role in the conversion of cysteine to H_2S. In *Saccharomyces cerevisiae*, the gene *TUM1* together with *URM1*, *UBA4*, *NCS2* and *NCS6* has been identified as being involved in the wobble modification in tRNAs. Tum1p is a sulfur transferase that accepts persulfide sulfur from Nfs1p and transfers it to Uba4p (Noma, Sakaguchi and Suzuki 2009). The yeast deletants (BY4743 Δ*urm1*, Δ*uba4*, Δ*ncs2*, Δ*ncs6*, Δ*ahp1*), which represent the other genes involved in tRNA thiolation, were investigated to determine whether they also affected H_2S production from cysteine. Interestingly, none of the deletants tested showed a decrease in H_2S production (Fig. S4, Supporting Information).

Winter, Cordente and Curtin (2014) showed that the vacuole-related gene deletants in the BY4742 laboratory strain background reduced H_2S production from cysteine significantly, while mutants in iron–sulfur homeostasis increased production. However, BY4742 is auxotrophic for lysine and has been reported to ferment much more slowly in grape juice than BY4743 unless 10-fold lysine (300 mg L^{-1}) was supplemented (Harsch *et al.* 2010). Therefore, we decided to reexamine that some of the low H_2S producers (vacuole deletants) and high H_2S producers (iron–sulfur homeostasis deletants) in the auxotrophic laboratory strain BY4743 and the prototrophic wine yeast strain AWRI1631 backgrounds. Our results show that the vacuole mutant, BY4743 Δ*vam7*, did produce less H_2S than BY4743 (780 vs 1200 ppm), but it also fermented more slowly than the wild type. Surprisingly, Δ*vps25* and Δ*vps36* in the AWRI1631 background did not produce less H_2S, even they did ferment relatively slower than the wild type (Fig. S5A and B). None of the iron–sulfur homeostasis deletants (Δ*fra1*, Δ*fra2*, Δ*mrs3* and Δ*isu1*) in BY4743 background or AWRI1631 Δ*fra1* produced more H_2S from cysteine (Fig. S6, Supporting Information).

DISCUSSION

In this study, we have confirmed that *Saccharomyces cerevisiae* is capable of producing an early burst of H_2S from high concentrations of cysteine during fermentation and revealed that *TUM1* is a crucial gene affecting this H_2S production. Part of the H_2S produced is normally utilised by yeast cells as a sulfur source for growth.

High intracellular concentrations of cysteine are cytotoxic, and therefore there are mechanisms to prevent its accumulation (Stipanuk 2004). In human cells, this is achieved by converting cysteine to sulfite and sulfate using cysteine dioxygenase and sulfite oxidase (Sörbo and Ewetz 1965; Lombardini, Singer and Boyer 1969). In other species such as tobacco, cysteine is converted to sulfate (Smith 1975), whilst *Candida albicans*

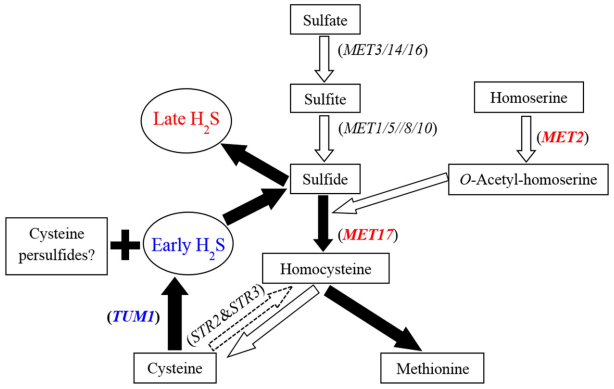

Figure 7. Model for cysteine catabolism in *S. cerevisiae*. *TUM1* (blue font) is proposed to be the principal gene responsible for the early burst of H_2S production, which is observed during fermentation. Cysteine persulfides may also be involved in this pathway. Part of the sulfide produced then enters the sulfur assimilation pathway, and this gives an additional burst of (late) H_2S if the *MET2* or *MET17* gene (red font) is deleted. The black arrows indicate the possible route used by the Δ*str2* and Δ*str3* deletants, when the *STR2/STR3* transsulfuration pathway is blocked (white dashed arrow), enabling growth on high concentrations of cysteine as the sole sulfur source (adapted from Harsch and Gardner 2013; Fräsdorf, Radon and Leimkühler 2014).

converts cysteine to sulfite through cysteine dioxygenase, encoded by the *CDG1* gene (Hennicke *et al.* 2013). This study confirms earlier findings that high cysteine is toxic to yeast (Kumar *et al.* 2006; Santiago and Gardner 2015) and that yeast responds to high cysteine in fermentation conditions by producing a burst of H_2S (Tokuyama *et al.* 1973; Jiranek, Langridge and Henschke 1995; Winter and Curtin 2012).

In this study, a larger delayed burst of H_2S was produced from cysteine in strains with deletions of either *MET17* or *MET2*. We propose that this elevated burst of H_2S occurs because these strains are unable to utilise H_2S in the SAP; hence, all of the H_2S produced from cysteine is diffused into the medium in these strains, rather than a proportion being reincorporated into the SAP. In support of this explanation, we present experimental evidence that deletants of *STR2* and *STR3* can bypass the transsulfuration pathway when grown in media containing high concentrations of cysteine as the sole sulfur source. Thus, yeast cells must possess an alternative pathway to obtain methionine in media with high concentrations of cysteine. Our genetic data using combinations of trans-sulfuration mutants with deletions of individual *MET* genes suggest that it is the H_2S generated from cysteine catabolism that is fed directly into the SAP and is utilised for the synthesis of methionine.

In addition, we identified a role for the *TUM1* gene in this novel yeast pathway of cysteine catabolism. Deletion of *TUM1* reduced H_2S production from cysteine, while overexpressing *TUM1* increased H_2S in different yeast genetic backgrounds. The requirement for *TUM1* for full growth of transsulfuration pathway mutants on high cysteine confirmed the role of this yeast gene in the production of H_2S from cysteine.

A new model (Fig. 7) for cysteine catabolism in *S. cerevisiae* is proposed in which the *TUM1* gene plays a key role. The precise role played by *TUM1* in yeast cysteine catabolism remains to be determined. However, because *TUM1* was the only gene involved in tRNA thiolation to effect H_2S production from cysteine, it seems unlikely that the cellular process of tRNA thiolation plays a role in cysteine catabolism in *S. cerevisiae*. Our data further suggested that neither sulfate nor sulfite is produced as part of this yeast pathway.

In humans, cysteine is first metabolised by cysteine aminotransferase to 3-mercaptopyruvate, which is then converted to pyruvate and protein-bound persulfide by TUM1 (sulfurtransferase). The H_2S is then released from protein-bound persulfide when reducing systems such as glutathione or thioredoxin are present (Shibuya *et al.* 2009; Mikami *et al.* 2011). The human TUM1 protein and yeast Tum1p are orthologues (Mathew, Schlipalius and Ebert 2011). In addition, alignment of the Tum1p sequences from several yeast strains, available from the *Saccharomyces* Genome Database (SGD), indicates that Tum1p are highly conserved among *S. cerevisiae* strains (http://www.yeastgenome.org/cgi-bin/FUNGI/alignment.pl?locus=tum1&submit=Submit&rm=display_result). Whilst the crystal structure of yeast Tum1p has been solved at 1.90 A resolution (Qiu *et al.* 2012), it remains to be confirmed as to whether the protein can act like a human sulfurtransferase.

In mammals, cystathionine β-synthase (CBS, EC 4.2.1.22) and cystathione γ-lyase (CSE aka CGL, EC 4.4.1.1) (Kashfi and Olson 2013) have also been found to degrade cysteine to release H_2S. However, the equivalent genes (*STR3* and *CYS3*) in yeast do not produce H_2S (Linderholm *et al.* 2008; Winter, Cordente and Curtin 2014), which was also confirmed in this study by demonstrating that deletion of *STR3* in the wine yeast AWRI1631 background did not affect H_2S production (Fig. 4B).

In humans, the biological roles of the TUM1 protein range from thiolation of cytosolic tRNAs to the generation of H_2S as a signaling molecule both in mitochondria and the cytosol (Fräsdorf, Radon and Leimkühler 2014). To our best knowledge, *S. cerevisiae* Tum1p has only been annotated to the thiolation of cytosolic tRNAs (Noma, Sakaguchi and Suzuki 2009). The biological role of the H_2S generated from cysteine by Tum1p in *S. cerevisiae* remains unknown but it is tempting to speculate that it may also have the similar function as 3MST in humans, in generating H_2S and cysteine persulfide and thus glutathione polysulfides, as signalling molecules (Ida *et al.* 2014; Santiago and Gardner 2015). This may explain why most yeast strains possess a 38 bp deleted, non-functional as a ß-lyase *IRC7* variant (Roncoroni *et al.* 2011) as an alternative pathway(s) exists for H_2S formation from cysteine. The Δ*tum1* deletant did not completely eliminate H_2S formation from cysteine therefore further studies are needed to investigate the involvement of other genes, pathways and polysulfides in the yeast cysteine catabolism process.

Winter, Cordente and Curtin (2014) proposed that vacuole plays a central role in H_2S production from cysteine, based on the phenotypes of several vacuole mutants such as BY4742 Δ*vam7*, which grew more slowly but produced less H_2S than wild type at the same growth stage. Surprisingly, our results show that the vacuole mutants, Δ*vps25* and Δ*vps36* in AWRI1631 backgrounds, did not reduce H_2S production. Our results do show that BY4743 Δ*vam7* produced less H_2S than wild type; however, we suspected that the poor growth of the strain is the major factor contributing to the lower H_2S production. This is because poor growth will result in less vigorous production of CO_2, which is required to sparge H_2S into the H_2S detector tube during fermentation (Park 2008). We also did not observe elevated H_2S production for the iron–sulfur homeostasis deletants (Δ*fra1*, Δ*fra2*, Δ*mrs3* and Δ*isu1*) in BY4743 and Δ*fra1* in AWRI1631 backgrounds. At this stage, we could not conclude whether the vacuole and iron–sulfur homeostasis related genes affect formation of H_2S from cysteine during fermentation and we agree with Winter and coworkers that further investigations using a more sensitive detection methods for H_2S inside the yeast cells are required to better understand the roles of these genes in yeast cysteine catabolism.

The findings of this study greatly facilitate our knowledge of the process of yeast cysteine catabolism. Polysulfides as possible signalling molecules have attracted much attention lately, but the majority of studies have been performed in mammalian and plant cells (Mishanina, Libiad and Banerjee 2015; Höfler *et al.* 2016). The identification of the new important role played by yeast Tum1p in the generation of H_2S (and possibly polysulfides) from cysteine suggests that yeast could also be a useful model organism for studying human diseases such as Parkinson and Alzheimer, which involve a defect in cysteine catabolism (Heafield *et al.* 1990). The yeast *TUM1* also allows the improvement of commercial yeast strains for wide range of applications in the food industry. *TUM1* overexpression strains that produce H_2S from cysteine more efficiently could potentially increase 3MH/A in wine via the H_2S–C6 pathway. This would be useful to boost thiol aromas in grape juice, characteristic of the fruit-driven wine styles such as Sauvignon Blanc (Harsch *et al.* 2013). Wort can contain high levels of cysteine (up to 35 mg L^{-1}); therefore, *TUM1* deletion strains, which produce less H_2S from cysteine, might be valuable to the brewing industry in managing H_2S, and creating the fruit-driven styles of beers such as American pale ale (Lawrence and Cole 1972; Priest and Stewart 2006).

SUPPLEMENTARY DATA

Supplementary data are available at FEMSYR online.

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Conflict of interest. None declared.

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Supplementary Material

Supplementary Table S1: PCR primers and sequences

Name	**Sequence (5'–3')**	**Use**
TUM1-EcoRI-F	GCATGAGAATTCATGCCATTATTTGATCTTATTTCTCCAAAAGC	Primer pair of *TUM-XhoI*-R: Overexpression of *TUM1* using the pJC1 plasmid
TUM-XhoI-R	GCATGACTCGAGTTAATCTCTGTTTTCAGCAATCCACTC	See above
Del-*str2*-F	CGATAGTAGAATGACGCTCATGTGCCGATTCGGGACCTACATAAACAAGAGAGAGAAGAGGAACAGAAGA**TTTAGCTTGCCTTGTCCCCGCCGGGT**	Primer pair of Del-*str2*-R: deletion of *STR2* using the pAG32 plasmid (hygromycin B resistance).
Del-*str2*-R	CGCTGTTTGTCGTGTCCGGACAATCAGCATTAAAATTTTCTACAGAGTTTTCTAATACATATAAACATTTTGGCT**TCGACACTGGATGGCGGCGTTAGTATCG**	See above
Del-*str3*-F	GCTCTGTGTGCTTACAGTTCATTAAAAACAAAAAATATCAAGCAAACAACAAAAGCATAGAAGCAAAAAG**TTTAGCTTGCCTTGTCCCCGCCGGGT**	Primer pair of Del-*str3*-R: deletion of *STR3* using the pAG25 plasmid (nourseothricin resistance).
Del-*str3*-R	ACTATTTAAAGTTACTATCTTTGGATTTGAACCTTATAAAAAAAAAAAAAAAGGTACATGCATATTTTCCC**TCGACACTGGATGGCGGCGTTAGTATCG**	See above
Del-*met2*-F	CAGGGTCCGTCTAAAGTTTCTCTTTATTTTGGAATAATAGAAAAGAAAGAAAAAAACGTAGTATAAAAGGA**TTTAGCTTGCCTTGTCCCCGCCGGGT**	Primer pair of Del-*met2*-R: deletion of *MET2* using the pAG25 plasmid (nourseothricin resistance).
Del-*met2*-R	GTCTATATATAAATATAGATATAGATATACATGTACTGGTTTATCTATGTTATGCCT	See above

	GAGGTATGTGTGGTAT**TCGACACTGG ATGGCGGCGTTAGTATCG**	
Hph-I-F	TGCTGATCCCCATGTGTATCACTGGCA AACTG	Confirmation of deletion using the *HphMX* cassette
Nat-I-F	TTAGGATTTGCCACTGAGGTTC	Confirmation of deletion using the *NatMX* cassette
kanB	CTGCAGCGAGGAGCCGTAAT	Confirmation primer: Confirmation of deletion using the *KanMX* cassette
kanC	TGATTTTGATGACGAGCGTAAT	Primer pair of any Reverse Confirmation primer: Confirmation of deletion using the *KanMX* cassette
FC*met1*	TTCTATTTTCGTTATTGGTTTCTCG	Primer pair of RC*met1*: Confirmation of deletion using the *KanMX* cassette
RC*met1*	AGCAATCTTTTTACTTGTTGTTTGG	See above
RC*met2*	AAATGTGGAAAGCTCTAAAGCACTA	Primer pair of *Nat*-I-F: Confirmation of deletion using the *NatMX* cassette
FC*met3*	GTAATTTTGTAACTCACCGCATTCT	Primer pair of RC*met3*: Confirmation of deletion using the *KanMX* cassette
RC*met3*	TTGAAATTAATGTCGACCAGTATGA	See above
FC*met5*	TTCATCACGTGCGTATTATCTCTTA	Primer pair of RC*met5*: Confirmation of deletion using the *KanMX* cassette
RC*met5*	TTTATTCTTCACCTCGTTTTCATTC	See above
FC*met8*	ATGCCATTTCAGTTACAACCTAGTC	Primer pair of RC*met8*:

		Confirmation of deletion using the *KanMX* cassette
RC*met8*	CATGTCTAGAAGTTGGAAGGTTGTT	See above
FC*met10*	AAAGAAAACACTATCAACATTCCCA	Primer pair of RC*met10*: Confirmation of deletion using the *KanMX* cassette
RC*met10*	ATCTCAGTTCATTGTAGTCTCGCTT	See above
FC*met17*	CATCCTCATGAAAACTGTGTAACAT	Primer pair of RC*met17*: Confirmation of deletion using the *KanMX* cassette
RC*met17*	CCTCTTTTGTAACTTGGTCCTACAA	See above
FC*tum1*	TGACCAGCTTTTCATACTTTCTACC	Primer pair of RC*tum1*: Confirmation of deletion using the *KanMX* cassette
RC*tum1*	ATCAGTTTTCCCTCTGCTTTAAGAT	See above
FC*uba4*	GCAGAATTCAGAAATCAAAGTGAAT	Primer pair of RC*uba4*: Confirmation of deletion using the *KanMX* cassette
RC*uba4*	TTTGTACACCAAAGAGCAGTGTAAA	See above
FC*ncs2*	TCTTTCTTTCCTTCCACTTAATCCT	Primer pair of RC*ncs2*: Confirmation of deletion using the *KanMX* cassette
RC*ncs2*	AACAGGTCTAGTGCAATAAATGAGC	See above
FC*ncs6*	CTGCAAACACAGAATGAGCTACTTA	Primer pair of RC*ncs6*: Confirmation of deletion using the *KanMX* cassette
RC*ncs6*	GATATCCACATCTAGCGCATCTATT	See above
FC*urm1*	GCGACAGAGTAGTGGATGTTTTTAT	Primer pair of RC*urm1*: Confirmation of deletion using the *KanMX* cassette

RC*urm1*	AAACACAAGAAACATCTAAAGTCCG	See above
FC*ahp1*	TTTCTGATTTGTAATTATACGGGGA	Primer pair of RC*ahp1*: Confirmation of deletion using the *KanMX* cassette
RC*ahp1*	ATTTTGTTCGAAACGCATATAATGT	See above
FC*str2*	AATAATTGCTCTTCCACAGAAAATG	Primer pair of RC*str2*: Confirmation of deletion using the *KanMX* cassette
RC*str2*	AAGTTCCATCTCACAAACAAATCTC	See above
RC*str3*	ATTTCTATATTCCTTTTACGACCGC	Primer pair of *Nat*-I-F: Confirmation of deletion using the *NatMX* cassette
FC*yhr112c*	TTATCACGTGTGATTATGTGGTCTC	Primer pair of RC*yhr112c*: Confirmation of deletion using the *KanMX* cassette
RC*yhr112c*	CAAGAAAAACTTCCCAGTGTAGAAA	See above
FC*yll058w*	CTCTCAAGAAAGGATGTTGAAGAAG	Primer pair of RC*yll058w*: Confirmation of deletion using the *KanMX* cassette
RC*yll058w*	TGTGTATAAACCTTAAAATAGCGGC	See above
FC*bna3*	CGCACAAGCTAACCTTTATGTAGTT	Primer pair of RC*bna3*: Confirmation of deletion using the *KanMX* cassette
RC*bna3*	CTAGAGATTTAGGCAAGGTAGGACC	See above
FC*irc7*	AAATTGATAACGATTTTATTGTCGC	Primer pair of RC*irc7*: Confirmation of deletion using the *KanMX* cassette
RC*irc7*	TGGCTACTAGATGGTTGTCTACTCC	See above
FC*vam7*	AGTAACCGTCACCTGAACAACTTAC	Primer pair of RC*vam7*: Confirmation of deletion using the *KanMX*

		cassette
RC*vam7*	AAGTTTAGACAGGTTTTTGGGTCTT	See above
FC*fra1*	ATTTTGTTTTGTTTTTGACGACTTC	Primer pair of RC*fra1*: Confirmation of deletion using the *KanMX* cassette
RC*fra1*	CAGTATAAATCACTAGGGGGCTGTA	See above
FC*fra2*	GTTTGTATGTGGGCTTAATCTGTTC	Primer pair of RC*fra2*: Confirmation of deletion using the *KanMX* cassette
RC*fra2*	TTTTACTTCATAGGTTTGAACTCGC	See above
FC*mrs3*	GCTTCTTAGGGTTTTGTTTTAGGTC	Primer pair of RC*mrs3*: Confirmation of deletion using the *KanMX* cassette
RC*mrs3*	TTCTAACTAACTAACCAAGGAGGGC	See above
FC*isu1*	AAAAATGACAAGCTATTTCCGTGTA	Primer pair of RC*isu1*: Confirmation of deletion using the *KanMX* cassette
RC*isu1*	AAACAAATATATAAGGGGGAAGTGG	See above
FC*cys3*	ACCCCATACCACTTCTTTTTGTTAT	Primer pair of RC*cys3*: Confirmation of deletion using the *KanMX* cassette
RC*cys3*	CCTTCTTGATCTCGTTCTAGTTCTG	See above
FC*cys4*	ACAACTTCAACTTCACCCAAGTAAG	Primer pair of RC*cys4*: Confirmation of deletion using the *KanMX* cassette
RC*cys4*	TTGACAGTGACGTTTACAGATAGGA	See above
FC*vps25*	TCTACTGAAGAGTCTGCATTTTGTG	Primer pair of RC*vps25*: Confirmation of deletion using the *KanMX* cassette
RC*vps25*	AGTTGAATGGTAAGGTTCAAGACTG	See above
FC*vps36*	ATGTCTGGTGCAGTGTATGTAAGAA	Primer pair of RC*vps36*:

		Confirmation of deletion using the *KanMX* cassette
RC*vps36*	GGTCGAACTTGACAAAAATAAAAGA	See above

[a] PCR amplicons were confirmed with primer pairs (gene specific primer (forward) and Kan B, or gene specific primer (reverse) and KanC).

[b] KanB and KanC primers designed from Yeast Deletion Project (www-sequence.stanford.edu/group/yeast_deletion_project/deletions3.html)

[c] Bold font denotes sequence (positions 77 to 92 of *TEF1* promoter and 1699 to 1727 of terminator in plasmids pAG 25 and 1245 to 1271 in pAG 32 (Goldstein and McCusker 1999).

[d] *EcoRI* and *XhoI* restriction sites are underlined.

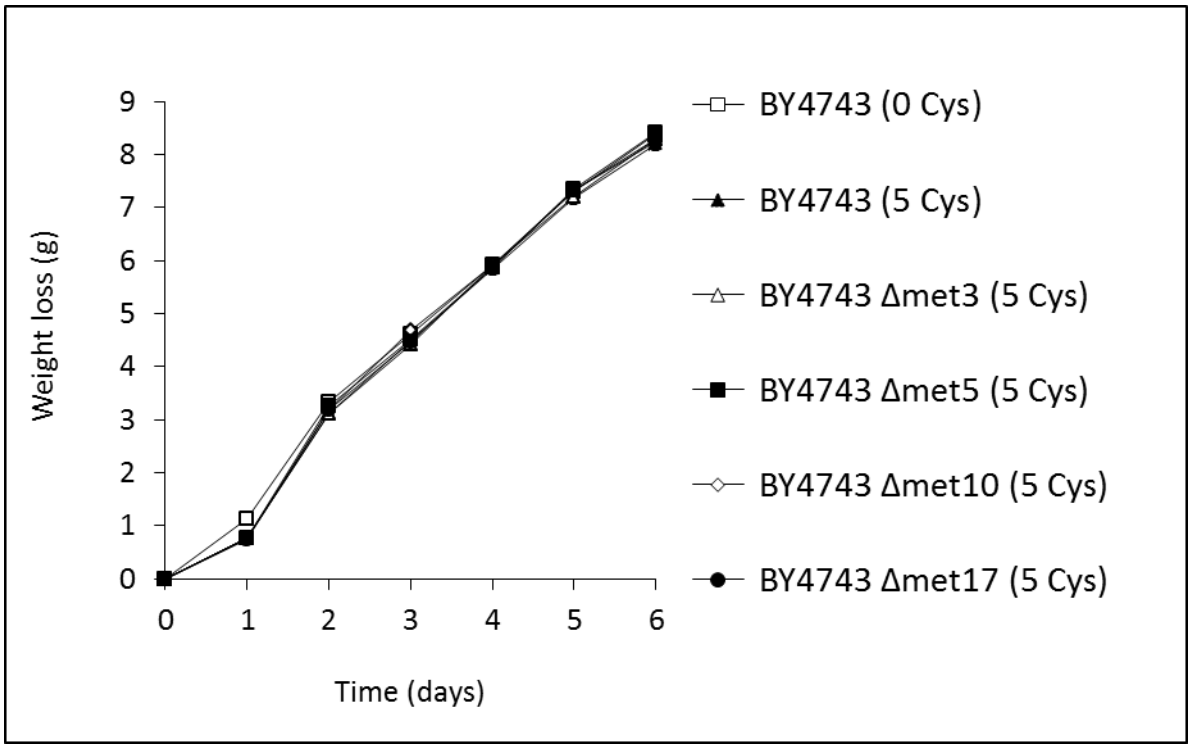


Fig. S1 Fermentation kinetics of the BY4743 and its *MET* gene deletants in 100 mL of non-sulfate CDGJM plus 0.15 mM methionine supplemented with or without 5 mM cysteine at 28 °C with shaking at 100 rpm. Data points represent mean values of triplicate fermentations ± standard error of the mean (*SEM*). NB error bars are too small to extend beyond the symbol plot.

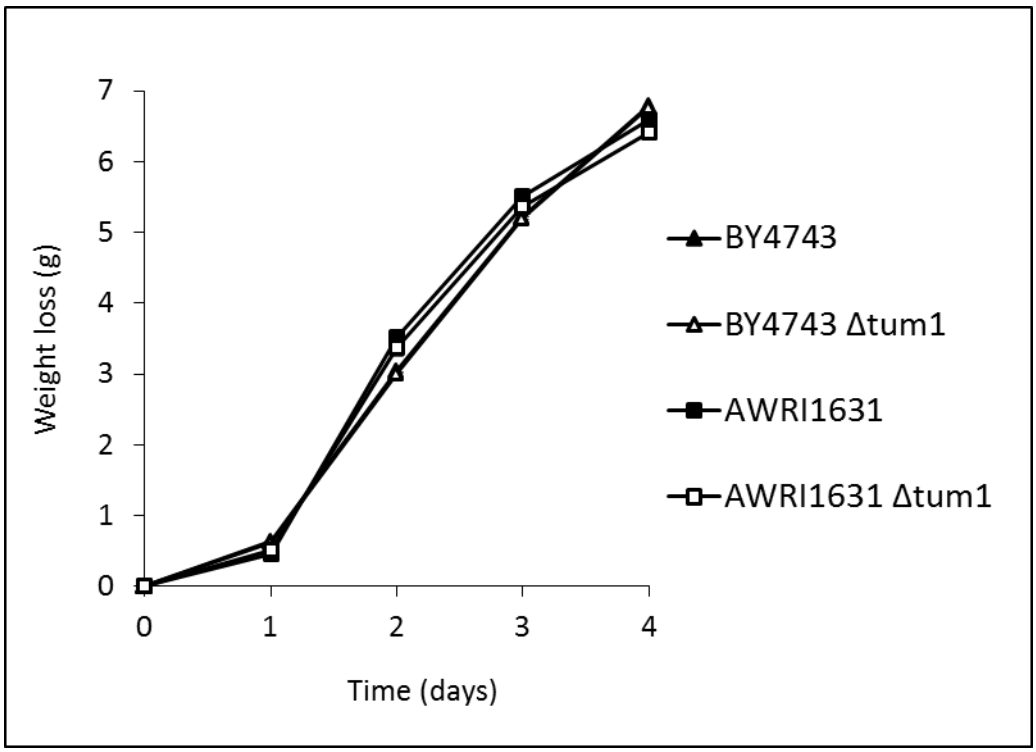


Fig. S2 Fermentation kinetics of the Δ*tum1* deletants compared to the parental strains. Fermentations were performed in 100 mL of non-sulfate CDGJM plus 5 mM cysteine and 0.15 mM methionine at 28 °C with shaking at 100 rpm. Data points represent the mean values of triplicate fermentations ± *SEM*. NB error bars are too small to extend beyond the symbol plot.

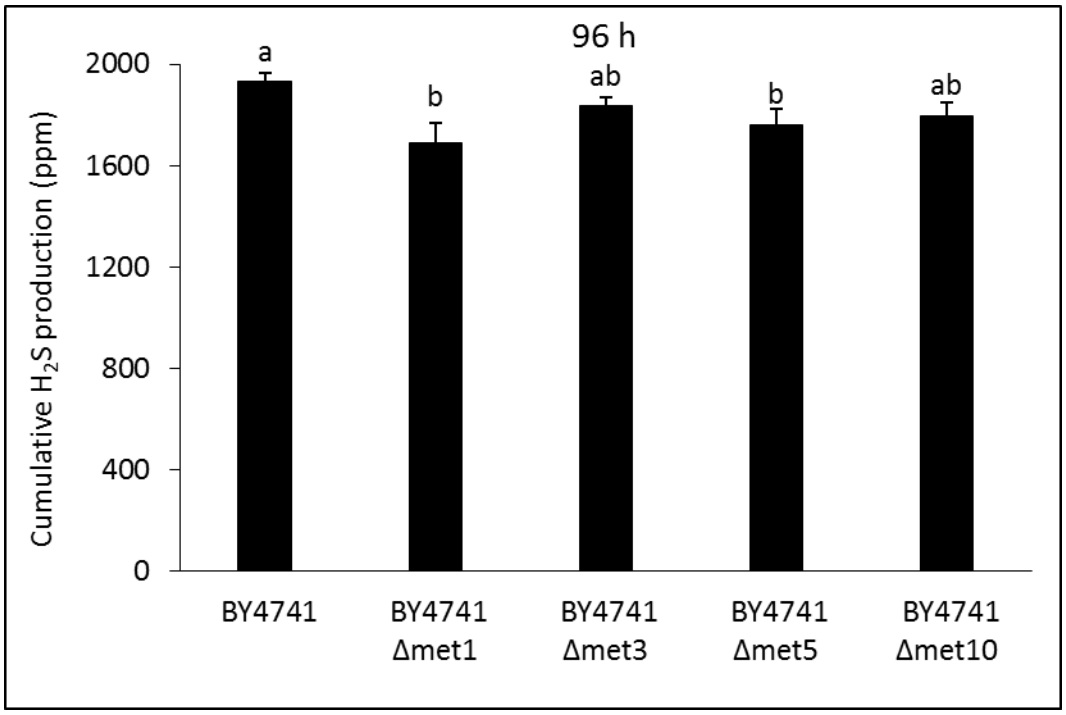


Fig. S3 Cumulative H_2S production from the individual *MET* gene deletants of BY4741 (*Δmet17*) strains**.** Fermentations were performed in 100 mL of non-sulfate CDGJM plus 5 mM cysteine and 0.15 mM methionine at 28 °C (n=3) with shaking at 100 rpm. H_2S was measured with silver nitrate (120SF: 1-1000 ppm; KITAGAWA, Japan) H_2S detector tubes, which were replaced at regular intervals. The mean H_2S released is shown and error bars show *SEM.* Samples not connected by the same letter are significantly different (ANOVA, Tukey's HSD).

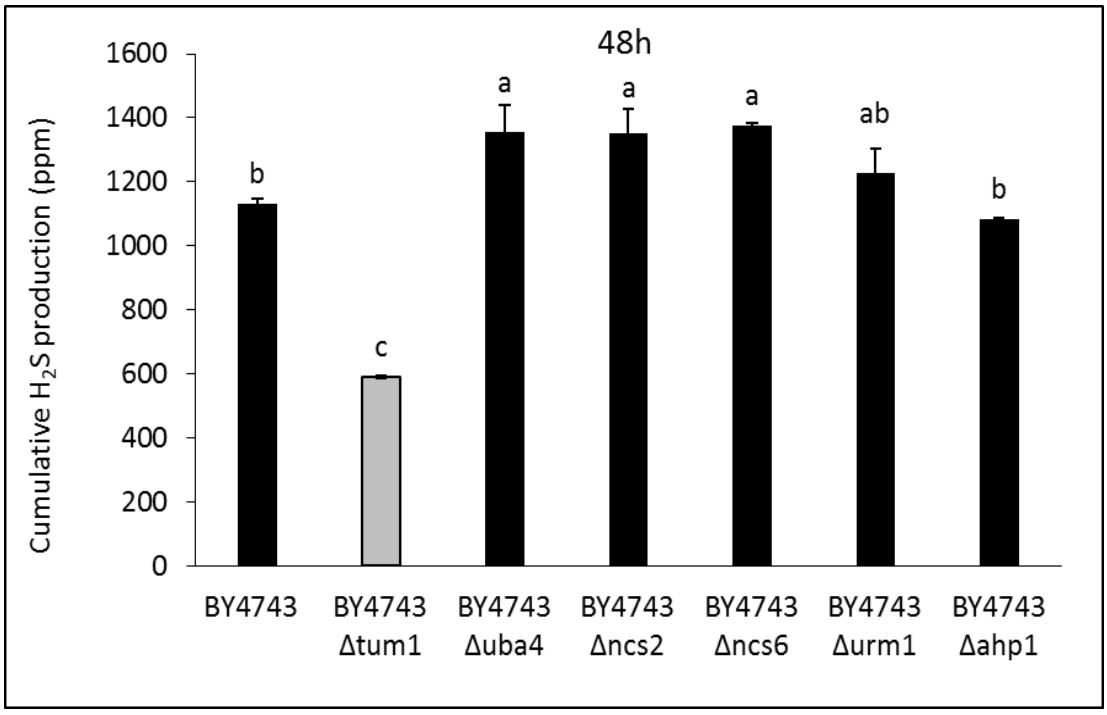


Fig. S4 Cumulative H_2S production from the tRNA thiolation deletants. Fermentations were performed in 100 mL of non-sulfate CDGJM plus 5 mM cysteine and 0.15 mM methionine at 28 °C (n=3) with shaking at 100 rpm. H_2S was measured with silver nitrate (120SF: 1-1000 ppm; KITAGAWA, Japan) H_2S detector tubes, which were replaced at regular intervals. The mean H_2S released is shown and error bars show *SEM*. Samples not connected by the same letter are significantly different (ANOVA, Tukey's HSD).

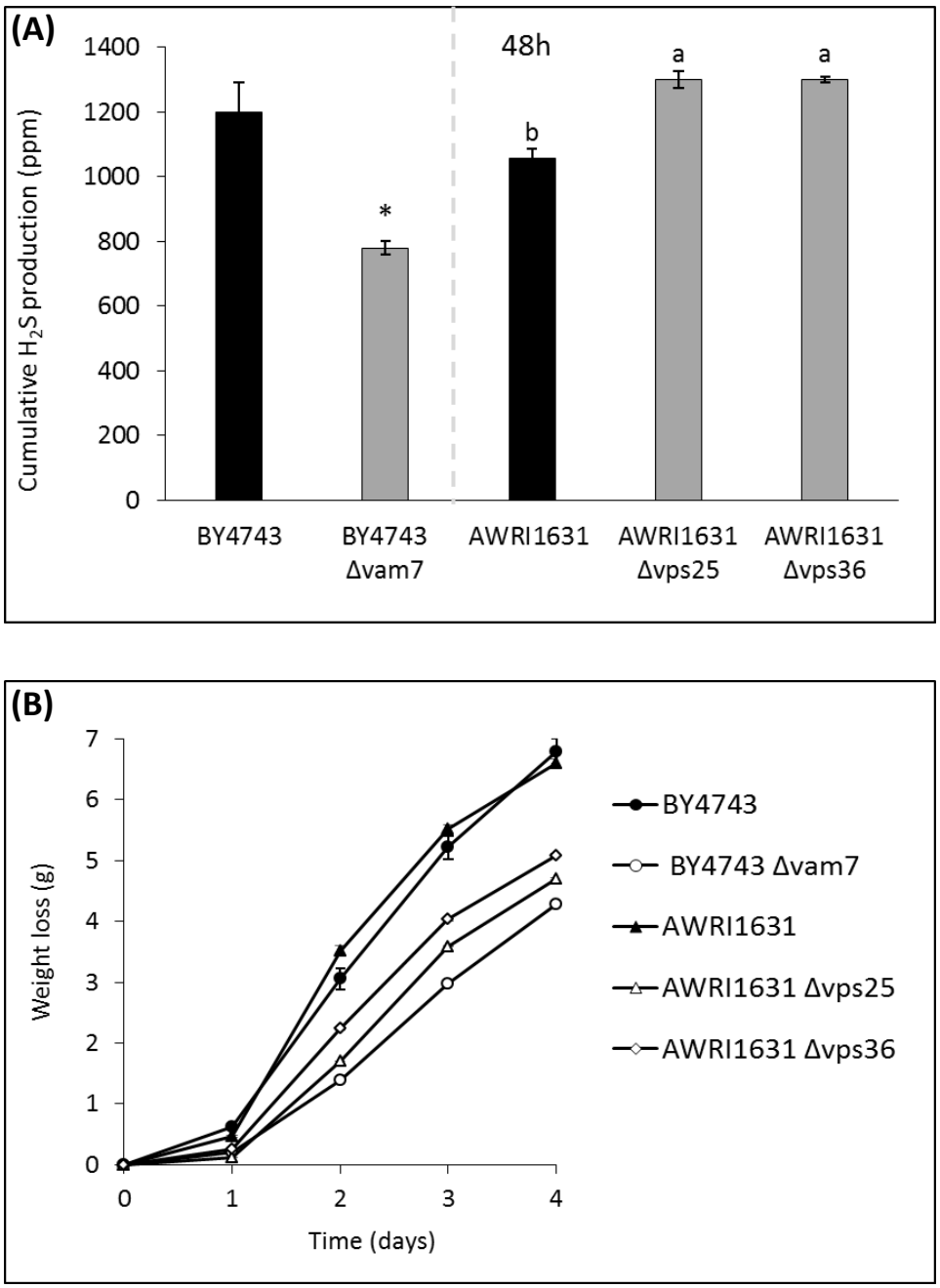


Fig. S5 (A) Cumulative H_2S production **(B)** fermentation kinetics of the vacuole related genes deletants. Fermentations were performed in 100 mL of non-sulfate CDGJM plus 5 mM cysteine and 0.15 mM methionine at 28 °C (n=3) with shaking at 100 rpm. H_2S was measured with silver nitrate (120SF: 1-1000 ppm; KITAGAWA, Japan) H_2S detector tubes, which were replaced at regular intervals. Data points represent mean values of triplicate fermentations ± standard error of the mean (*SEM*). Samples not connected by the same letter are significantly different (ANOVA, Tukey's HSD). Asterisks above bars represent significant differences compared to the wild-types ($^{*}P<0.05$, two-tailed Student's t test). NB error bars are too small to extend beyond the symbol plot.

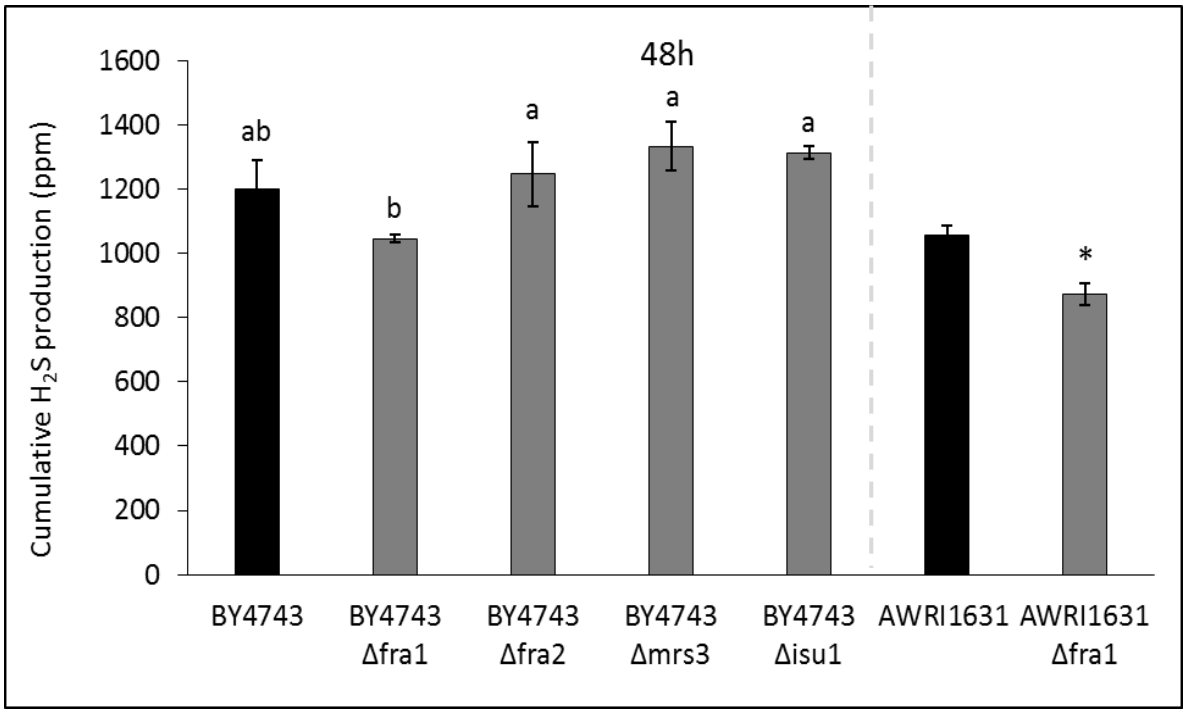


Fig. S6 Cumulative H_2S production from the iron-sulfur homeostasis related deletants. Fermentations were performed in 100 mL of non-sulfate CDGJM plus 5 mM cysteine and 0.15 mM methionine at 28 °C (n=3) with shaking at 100 rpm. H_2S was measured with silver nitrate (120SF: 1-1000 ppm; KITAGAWA, Japan) H_2S detector tubes, which were replaced at regular intervals. The mean H_2S released is shown and error bars show *SEM*. Samples not connected by the same letter are significantly different (ANOVA, Tukey's HSD). Asterisks above bars represent significant differences compared to the wild-types ($^{*}P<0.05$, two-tailed Student's t test).

Chapter 3

Development of a screening assay for detecting H_2S production from cysteine

3.1 Introduction

An early burst of hydrogen sulfide (H_2S) produced by the yeast *Saccharomyces cerevisiae* from cysteine has been proposed to be responsible for increased 3MH and 3MHA (tropical aromas) concentrations in wine (Winter *et al*. 2011). However, little is known about the mechanisms behind this. Therefore, a better understanding of the yeast genetic mechanisms involved in the formation of H_2S from cysteine could have potential industrial applications in the release of untapped thiol aromas in grape juice.

Several yeast genes affecting H_2S production from cysteine have been successfully identified through screening of a BY4742 yeast deletion library using the high-throughput methylene blue reduction method (Winter, Cordente and Curtin 2014). Based on these findings, it was decided to perform a genome-wide screen to identify other novel genes critical for this process by using different types of screening assays or different yeast deletion libraries.

Colony colour assays such as BiGGY agar (Difco) have been routinely employed to screen H_2S formation by yeast (Jiranek, Langridge and Henschke 1995; Linderholm *et al*. 2008). The development of automated plate pouring systems (e.g. Serial Filler: for high precision agar dispensing into different plates, Singer Instruments), pinning robots (e.g. ROTOR HDA: capable of pinning yeast colonies at densities of 6144, Singer Instruments) and plate readers (e.g. PhenoBooth: semi-automated for plate imaging and analysis of colony colour, Singer Instruments) have certainly made the agar-based assay an attractive and feasible option for high-throughput screening experiments (Jaeger *et al.* 2015). Alternatively, a high-throughput silver nitrate-impregnated membrane method for H_2S detection has been developed for liquid cultures (Duan *et al.* 2004).

Since the success of a screen depends on the reliability of the screening assay, in this chapter, two H_2S screening techniques: (1) bismuth-containing, grape juice-like indicator agar plates (Jiranek, Langridge and Henschke 1995) and (2) silver nitrate-impregnated membranes (Duan *et al.* 2004) were investigated with respect to their sensitivity and suitability for screening H_2S production from cysteine by yeast. The most suitable detection method identified and developed here, was employed to screen yeast deletion libraries in Chapter 4.

3.2 Materials and methods

3.2.1 Yeast strains and culture

The yeast strains used in this work are described in Table 3.1. Yeast strains were either pre-cultured overnight at 28 °C in YPD medium (10 g L^{-1} yeast extract, 20 g L^{-1} peptone and 20 g L^{-1} glucose) or starter medium (non-sulfate Chemically Defined Grape Juice Medium (CDGJM) containing 10 g L^{-1} each of glucose and fructose and 0.15 mM methionine) (Table 3.2). For solid YPD plates, 20 g L^{-1} agar (RM250; Amyl Media, Australia) was added.

Table 3.1. Yeast strains used in this study.

Strain	Genotype, phenotype and comments	Origin
BY4743	*MATa/α, his3-Δ1/his3-Δ1, leu2-Δ0/leu2-Δ0, LYS2/lys2-Δ0, met15-Δ0/MET15, ura3-Δ0/ura3-Δ0*	Euroscarf (www.euroscarf.de/)
BY4743 Δ*met17*	*met17::KanMX/ met17::KanMX*	Euroscarf
BY4741	*MATa, his3-Δ1, leu2-Δ0, met15Δ0, ura3Δ0*	Euroscarf
BY4741 Δ*met5*	*met5::KanMX*	Euroscarf
BY4741 Δ*met10*	*met10::KanMX*	Euroscarf
BY4741 Δ*met17*	*met17::KanMX*	Euroscarf
BY4741 Δ*cys4*	*cys4::KanMX*	Euroscarf
Sigma 1278b	*MATa, trp1, leu2, ura3, his3*	Prof Charles Boone, University of Toronto
Sigma 1278b Δ*met10*	*met10::KanMX*	Prof Charles Boone University of Toronto
Sigma 1278b Δ*met17*	*met17::KanMX*	Prof Charles Boone University of Toronto
AWRI1631	Wine strain; MATa haploid	Varela *et al.* (2012), AWRI
Cross Evolution®	Wine strain; Lallemand	Lab collection

Enoferm Simi White	Wine strain; Lallemand	Lab collection
Fermichamp®	Wine strain; DSM	Lab collection
FM16	Evolved derivative of L2056	McBryde *et al.* (2006), Lab collection
Lalvin L2056	Wine strain; Lallemand	Lab collection
Lalvin 71B	Wine strain; Lallemand	Lab collection
Lalvin QA23	Wine strain; Lallemand	Lab collection
Lalvin ICV GRE	Wine strain; Lallemand	Lab collection
Lalvin ICV D254	Wine strain; Lallemand	Lab collection
Maurivin AWRI796	Wine strain; AB Mauri	Lab collection
Maurivin B	Wine strain; AB Mauri	Lab collection
Oenoferm M2	Wine strain; Lallemand	Lab collection
Uvaferm 43	Wine strain; Lallemand	Lab collection
Zymaflore F10	Wine strain; Laffort	Lab collection
Zymaflore F15	Wine strain; Laffort	Lab collection
F15 (*IRC7*^{F}ox)	*ho::*P_{PGK}-*IRC7*^{F} in F15-h(α)	Roncoroni *et al*. (2011)
Schizosaccharomyces pombe		Prof Steve Oliver University of Cambridge

ox: denotes overexpression.

3.2.2 Non-sulfate CDGJM plus bismuth plates

Non-sulfate CDGJM plus bismuth agar plates (Table 3.2) were made by combining equal volumes of filter-sterilised 2x (non-sulfate CDGJM) and 2x (autoclaved, molten solidifying agents) with the addition of bismuth ammonium citrate at 5 g L^{-1} (Amresco, Astral, Australia), 0.15 mM methionine (or 0.15 mM $MgSO_4$) plus or minus 5 mM cysteine (non-sulfate CDGJM plates + Bi ± 5 mM cysteine + 0.15 mM methionine or $MgSO_4$). Excess histidine (200 mg L^{-1}), leucine (300 mg L^{-1}) and uracil (100 mg L^{-1}) were included to overcome auxotrophies (Harsch *et al*. 2010).

The following solidifying agents, in addition to bacteriological agar (20 g L^{-1}; RM250; Amyl Media), were evaluated: (1) Noble agar (20 g L^{-1}; sulfate ≤ 1%, Difco, Australia); (2) Agarose (10 g L^{-1}; BIO-41025, sulfate ≤ 0.08%, Bioline, Australia); (3) Phytagel (10 g L^{-1}; Sigma-Aldrich, Australia); (4) Gel-Gro gellan gum (20 g L^{-1}; MP Biomedical, USA); and (5) polyacrylamide gel. Polyacrylamide gel was prepared by mixing non-sulfate CDGJM with (1) 40% Acrylamide/Bis Solution at 37.5:1 (#1610149, Bio-Rad); (2) 10% Ammonium persulfate (Bio-Rad); and (3) TEMED (tetramethylethylenediamine; Bio-Rad) (Menter 2000).

Bacteriological agar (RM250; Amyl Media) was washed prior to use, by resuspending ~20 g agar in ~700 mL MQ water in a 1 L Schott bottle and allowing it to settle for ~30 min. The supernatant was discarded carefully and the washing step repeated twice.

Agarose plates with small holes were made by placing a non-skirted 96-well PCR plate (AB0600, Thermo Fisher Scientific, Australia) on top of the Nunc OmniTrays (O0764-1CS; 128 mm × 86 mm; Sigma-Aldrich, NSW, Australia) after pouring the molten agarose, which was removed when set.

The yeast precultures (5 µL) were spot-inoculated onto the plates and incubated for 96 h at 28 °C, and colony colour was examined visually against the wild-type strains. The screenings were conducted in duplicate.

Non-sulfate CDGJM plus bismuth (liquid medium; Table 3.2) was prepared by addition of 5 g L^{-1} bismuth ammonium citrate to non-sulfate CDGJM, 0.15 mM $MgSO_4$ plus or minus 5 mM cysteine. The yeast precultures (5 µL) were inoculated into 195 µL of the medium in 96-well plates (Costar 3596, Sigma-Aldrich, NSW, Australia).

Table 3.2. Composition of Chemically Defined Grape Juice Medium[a].

Component	Amount	Component	Amount
Carbon sources	**g L^{-1}**	**Nitrogen sources[b]**	**mg L^{-1}**
Glucose	100	L-alanine	100
Fructose	100	L-arginine-HCl	484
Salts	**g L^{-1}**	L-aspartic acid	50
Potassium tartrate	5	L-cysteine[c]	5
Malic Acid	3	L-glutamic acid	100
Citric Acid	0.2	L-glutamine	125
K_2HPO_4	1.14	L-glycine	5
$MgSO_4.7H_2O$[c]	1.23	L-histidine	20
$CaCl_2.2\ H_2O$	0.44	L-isoleucine	25
Trace minerals	**µg L^{-1}**	L-leucine	25
$MnCl_2.4\ H_2O$	198.2	L-lysine-HCl	6
$ZnCl_2$	135.5	L-methionine	10
$FeCl_2$	32	L-phenylalanine	40
$CuCl_2$	13.6	L-proline	300
H_3BO_3	5.7	L-serine	60
$Co(NO_3)_2.6H_2O$	29.1	L-threonine	75
$NaMoO_4.2H_2O$	24.2	L-tryptophan	10
KIO_3	10.8	L-tyrosine	10
Vitamins	**mg L^{-1}**	L-valine	30
Myo-Inositol	100	$(NH_4)_2HPO_4$	363
Pyridoxine hydrochloride	2		
Nicotinic acid	2		
Ca-panthothenate	1		
Thiamine hydrochloride	0.5		
p-amino benzoic acid	0.2		
Riboflavin	0.2		
Biotin	0.125		
Folic Acid	0.2		

[a]The composition of CDGJM medium is adapted from Henschke and Jiranek (1993).

[b]The nitrogen sources: amino acid and $(NH_4)_2HPO_4$ content was changed to reflect Marlborough Sauvignon Blanc juice (Harsch *et al*. 2010).

[c]Non-sulfate CDGJM was prepared by replacing $MgSO_4.7H_2O$ (1.23 g L^{-1}**)** with $MgCl_2.6H_2O$ (1.01 g L^{-1}**)** and without cysteine and methionine (Santiago and Gardner 2015a). A minimal concentration of 0.15 mM magnesium sulfate or 0.15 mM methionine was supplemented for yeast growth.

3.2.3 Colour analysis of yeast cultures in non-sulfate CDGJM plus bismuth

The colour of yeast cultures were assessed using the CIELAB (Commission Internationale de l´Eclairage Lab transmission values L* a* b*) method (Pérez-Magariño and González-Sanjosé 2003; Kwiatkowski *et al.* 2007). Duplicate 200 µL liquid cultures grown in a 96-well plate (Costar 3596, Sigma-Aldrich, NSW, Australia) were scanned (1 min shaking prior to measurement, 380-780 nm scan and 5 nm increments) using a Tecan Infinite M200 microplate reader. The optical density (OD) data were used to calculate L* (lightness, 0 = black), a* (positive values for red) and b* (positive values for yellow) against the un-inoculated reference.

3.2.4 Silver nitrate impregnated membranes

The silver nitrate impregnated membranes were freshly prepared prior to use, according to the protocol developed by Duan *et al*. (2004). The chromatography papers (Whatman cellulose chromatography paper 3MM Chr, 3030-221, GE Healthcare Life Sciences, Australia) were first cut 128 × 86 mm (dimension of a standard 96-well plate) and immersed in 20% silver nitrate (Chem-supply, Australia) solution. The membranes were air dried for ~2 h at room temperature in a fume hood. The dried, impregnated membranes were applied on top of either 96-well (360 µL) plates (Costar 3596, Sigma-Aldrich, Australia) or deep-well 96 (2 mL) plates (P-DW-20-C, Pacific Lab Products, Australia) containing 200 µL or 600 µL inoculated non-sulfate CDGJM, respectively. The non-sulfate CDGJM contained 0.15 mM sulfate and increasing concentrations of cysteine (0, 100, 300 and 500 mg L^{-1}). The plates were sealed with breathable sealing film (BF-400, Adelab Scientific, Australia) and incubated at 28 °C. The membranes were carefully removed after ~96 h and the colour intensity of the membrane spots (black silver sulfide) was assessed visually. The screen was conducted in duplicate.

3.3 Results and discussion

3.3.1 Non-sulfate CDGJM plus bismuth agar plates

Bismuth-containing indicator agar is a colony colour screening tool that is frequently employed to estimate the amount of H_2S produced by yeast (Jiranek, Langridge and Henschke 1995; Linderholm *et al*. 2008). Here, a modified version of grape juice bismuth indicator agar (non-sulfate CDGJM plates + Bi ± 5 mM cysteine + 0.15 mM $MgSO_4$) was evaluated with respect to its sensitivity and suitability for H_2S detection from cysteine degradation. The CDGJM was based on the recipe described by Henschke and Jiranek (1993) (Table 3.2), with $MgSO_4.7H_2O$ (1.23 g L^{-1}) being replaced by $MgCl_2.6H_2O$ (1.01 g L^{-1}) to reduce H_2S production from the sulfate assimilation pathway (SAP).

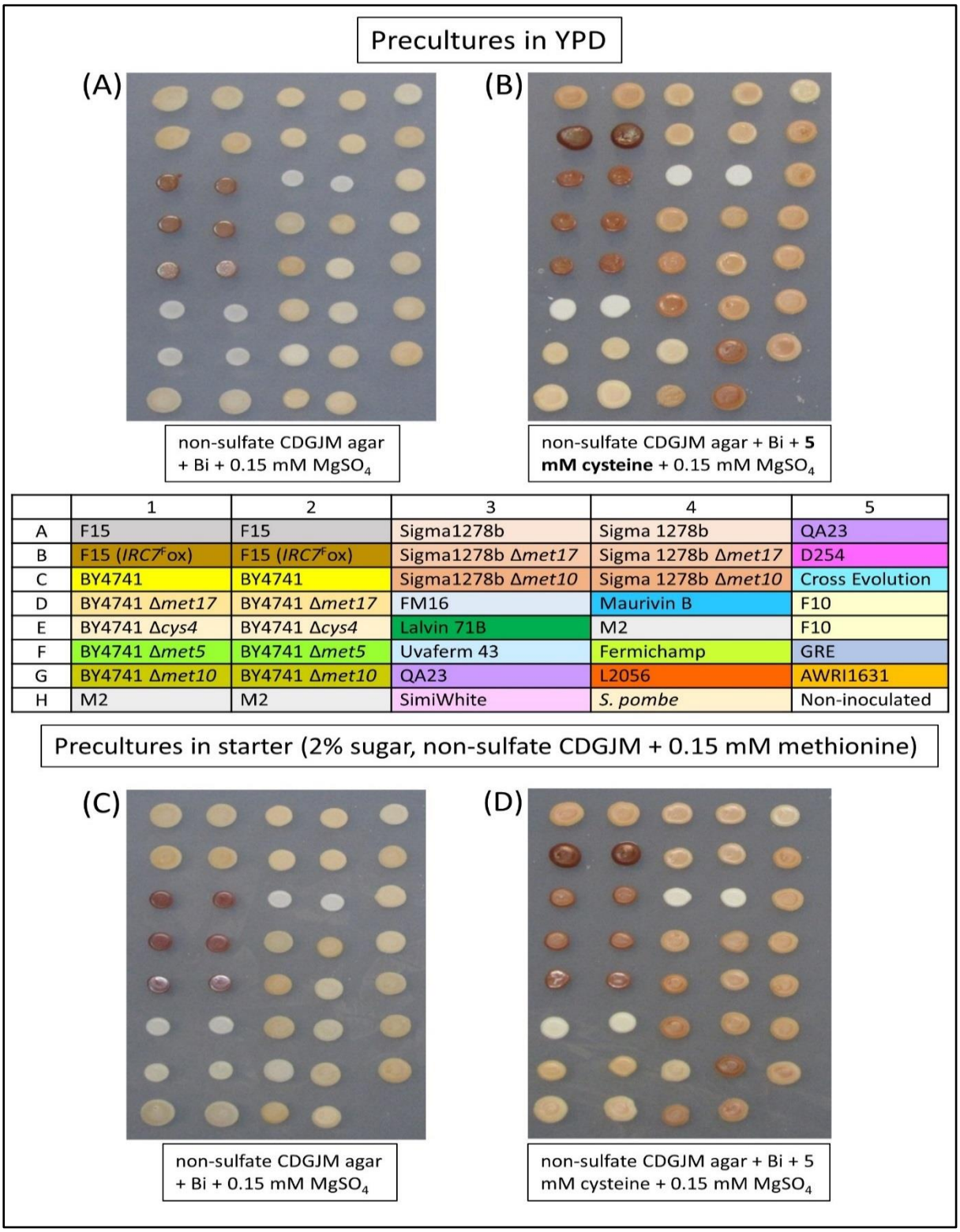
F15 (*IRC7*^{F}ox) is known to produce elevated amounts of H_2S on cysteine (Santiago and Gardner 2015b) and therefore it was used as a positive control to validate the assay for the detection of H_2S formed from cysteine degradation. F15 (*IRC7*^{F}ox) was observed to form much darker coloured colonies when 5 mM cysteine was supplemented (Fig. 3.1B), suggesting that the assay is capable of detecting H_2S generated from cysteine. In addition, the wine yeast strain AWRI1631 produced darker coloured colonies on cysteine supplemented plates, which is consistent with the H_2S production results obtained during fermentation (see Chapter 2), indicating that the assay is sensitive enough to be used for screening the AWRI1631 wine yeast deletion library (Varela *et al.* 2012).

A range of commercial wine yeast strains was included in the initial trial to investigate whether any of these strains produced distinctive or interesting colony colouration. Not surprisingly, most of strains tested were observed to produce somewhat darker coloured colonies on cysteine supplemented plates (Fig. 3.1B). In addition, Lalvin L2056 (Lallemand) had darkened more with cysteine than others (e.g. Sigma 1278b, Oenoferm M2 and Zymaflore F15), which is in agreement with the results of H_2S production by these strains during fermentation (Chapter 2). Interestingly, *Schizosaccharomyces pombe* also had darker coloured colonies on cysteine supplemented plates (Fig. 3.1B), suggesting the *S. pombe* Deletion Mutant Library (Spirek *et al.* 2010) could be an option for screening H_2S production from cysteine. This would be useful in terms of understanding the genetic basis of this pathway in two different yeasts.

It has been shown that deletion of *MET* genes does not affect H_2S from cysteine during fermentation (Chapter 2), however, the *MET* gene deletants (Δ*met5* and Δ*met10*) had lighter colouration on cysteine plates (Fig. 3.1B). Furthermore, yeast strains with a deletion of *MET17* (alias *MET15*) such as BY4741 (*MATa, his3-*Δ*1, leu2-*Δ*0, met15*Δ*0, ura3*Δ*0*) already had quite darkly coloured colonies even when cysteine was not supplemented (Fig. 3.1A). Together, these results suggest that a significant portion of the H_2S detectable by the assay conditions was from SAP.

To minimise potential interference from sulfur sources such as sulfate found in YPD, yeast strains were precultured in starter medium (2% sugar, non-sulfate CDGJM plus 0.15 mM methionine). The Δ*met17* strains grown this way, were observed to have dark colonies even in the absence of cysteine (Fig. 3.1C). Removal of all sulfate in the CDGJM by substitution of 0.15 mM $MgSO_4$ with 0.15 mM methionine had minimal effect on the colouration. BY4743 Δ*met17* still produced dark yellow colonies on non-sulfate CDGJM agar + Bi + 0.15 mM methionine plates (Fig. 3.2A).

Agar (Bacto agar) is a mixture of polysaccharides derived from red algae, which is widely used to solidify media for microbial experiments (Jaeger *et al.* 2015). Amyl Bacteriological Agar (RM 250; Amyl Media, Australia) contains up to ~1.8% of ash. Agar contains sulfate covalently bound as $O\text{-}SO_3$ (Armisen and Galatas 1987), which can be released by yeast sulfatase (Bds1p; Hall, Brachat and Dietrich 2005). This may explain why attempts to remove any potential impurities e.g. "sulfur" ash by simply washing the agar with MQ water were ineffective. Δ*met17* continued producing dark yellow colonies on the washed agar plates (Fig. 3.2A).



	1	2	3	4	5
A	F15	F15	Sigma1278b	Sigma 1278b	QA23
B	F15 (*IRC7*^{F}ox)	F15 (*IRC7*^{F}ox)	Sigma1278b Δ*met17*	Sigma 1278b Δ*met17*	D254
C	BY4741	BY4741	Sigma1278b Δ*met10*	Sigma 1278b Δ*met10*	Cross Evolution
D	BY4741 Δ*met17*	BY4741 Δ*met17*	FM16	Maurivin B	F10
E	BY4741 Δ*cys4*	BY4741 Δ*cys4*	Lalvin 71B	M2	F10
F	BY4741 Δ*met5*	BY4741 Δ*met5*	Uvaferm 43	Fermichamp	GRE
G	BY4741 Δ*met10*	BY4741 Δ*met10*	QA23	L2056	AWRI1631
H	M2	M2	SimiWhite	*S. pombe*	Non-inoculated

Figure 3.1. Colony colours of yeast strains pre-grown in YPD on **(A)** non-sulfate CDGJM agar + Bi + 0.15 mM $MgSO_4$ + no methionine, **(B)** non-sulfate CDGJM agar + Bi + 0.15 mM $MgSO_4$ + 5 mM cysteine + no methionine, and yeast strains pre-grown in starter on **(C)** non-sulfate CDGJM agar + Bi + 0.15 mM $MgSO_4$ + no methionine, **(D)** non-sulfate CDGJM agar + Bi + 0.15 mM $MgSO_4$ + 5 mM cysteine + no methionine. Images were taken after 96 h incubation at 28 °C. The known high-H_2S producer from cysteine, F15 (*IRC7*^{F}ox) was used as positive control.

3.3.2 Testing different solidifying agents

Since the sulfate content of the solidifying agents can vary considerably (~0-1.8%; Zimbro *et al.* 2009), other solidifying agents were tested for their potential to minimise H_2S production from sulfate rather than cysteine. The solidifying agents tested, in addition to Amyl bacteriological agar (RM250), were as follows: (1) Noble Agar (sulfate ≤ 1%, Difco); (2) Agarose ((BIO-4102) sulfate ≤ 0.08%, Bioline, Australia); (3) Phytagel (Sigma-Aldrich, Australia); (4) Gel-Gro gellan gum (MP Biomedical, USA); and (5) polyacrylamide gel.

Noble Agar is a washed and bleached agar which contains very low levels of impurities. It is used when clarity and purity of the agar is essential to the experiment (Zimbro *et al.* 2009). Surprisingly, despite the use of the high-purity Noble Agar, the colony colour of the *MET17* deletion strain was similarly, a dark yellow colour to that grown on Amyl bacteriological agar (Fig. 3.2A).

Agarose is a very pure agar with most of the sulfate and non-gelling portion removed through fractionation. It is mainly used for electrophoresis gels (Zimbro *et al.* 2009). As shown in Fig. 3.2A, the Δ*met17* strain formed much lighter coloured colonies on non-sulfate CDGJM agarose + Bi + 0.15 mM methionine. This result suggests that agarose could be a good alternative to agar, given the impurities (e.g. sulfate) within the bacteriological agar are primarily responsible for the dark coloured colonies produced by Δ*met17* when not supplemented with cysteine.

A major drawback to the use of agarose was the inability of the spotted yeast colonies (~2-5 μL) to hold their shape (Fig. 3.2B). Although the colony spread was somewhat reduced on a agarose plate with holes (Fig. 3.2C), it was difficult to make holes at a consistent depth and position, which was not ideal for spotting yeast cultures using a 96-channel pipette (Gilson PlateMaster P220; John Morris Scientific, Australia). Together with the higher cost of agarose, agar is still considered a better option to solidify media.

Phytagel, derived from bacterial fermentation of glucuronic acid, rhamnose and glucose, is a clear, high strength agar substitute and it is frequently used as a solidified agent for plant tissue culture (Sigma-Aldrich n.d.; Jaeger *et al.* 2015). Another agar substitute for plant tissue culture is Gel-Gro gellan gum (MP Biomedical, USA). Interestingly, dark coloured colonies were still observed for Δ*met17* on both Phytagel and Gel-Gro gellan gum plates when cysteine was not added (Fig. 3.2A), suggesting both solidified agents contained sulfur sources

that can be used by yeast to generate H_2S. Phytagel readily formed clumps at 10 g L^{-1}, even when stirred into CDGJM at ~28 °C. The amount used was higher than the producer's recommendations for plant tissue culture: ~1.5-2.5 g L^{-1}; Sigma-Aldrich).

Polyacrylamide gel electrophoresis (PAGE) is commonly used for the separation of proteins (Menter 2000). Polyacrylamide gel was evaluated as a substitute for agar without success due to the failure of the gel to polymerise even after 2 h at room temperature.

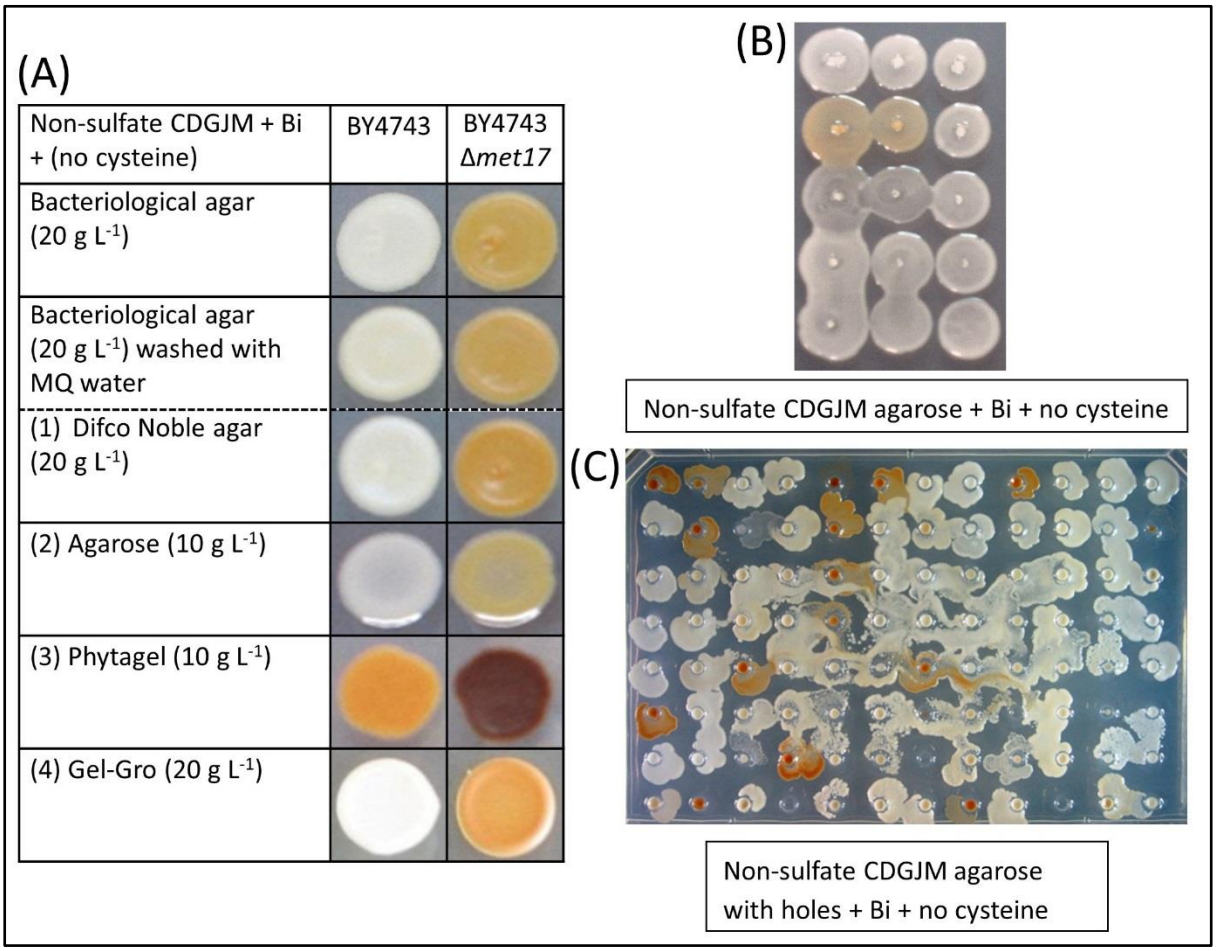


Figure 3.2. Colony colours of various yeast strains on **(A)** non-sulfate CDGJM + Bi + 0.15 mM methionine ± 5 mM cysteine containing different solidifying agents, **(B)** non-sulfate CDGJM agarose + Bi + 0.15 mM methionine and **(C)** non-sulfate CDGJM agarose with holes + Bi + 0.15 mM methionine. Images were taken after 96 h incubation at 28 °C. The known high-H_2S producer, Δ*met17*, was used as negative control as it was expected not to form dark coloured colonies.

3.3.3 Non-sulfate CDGJM plus bismuth liquid media

H_2S can be quantified spectrophotometrically in liquid media containing bismuth (Field and Oldach 1946; Dean 1966). Because the impurities contained in the solidifying agents can mask the H_2S production from cysteine, the addition of 5 g L^{-1} bismuth ammonium citrate into wells of a 96-well plate containing 200 µL CDGJM was trialled to detect H_2S formation from cysteine.

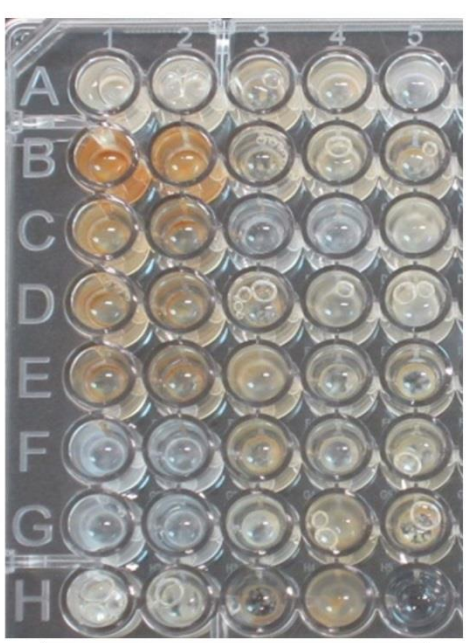
As shown in Fig. 3.3A, the colour of bismuth-containing, non-sulfate CDGJM inoculated with Δ*met17* deletants (wells: C1-E1 and C2-E2) only turned slightly yellow-brownish when cysteine was not present (0.15 mM $MgSO_4$ was added to support yeast growth), indicating that the interference caused by H_2S from other sulfur sources was considerably reduced. In addition, the colour of bismuth and cysteine-supplemented, non-sulfate CDGJM turned into a brownish colour (bismuth sulfide precipitate) in wells inoculated with the high-H_2S producer, F15 (*IRC7*^{F}ox) (wells: B1 and B2) and Δ*met17* deletants (wells: C1-E1 and B2-E2), which is consistent with previous H_2S fermentation results (Santiago and Gardner 2015b; Chapter 2).

However, no distinct colour changes were observed for the other strains tested (Fig. 3.3B), demonstrating that the assay was not sensitive enough to screen the wine yeast deletion library in AWRI 1631 for H_2S production from cysteine degradation (Varela *et al.* 2012).

Another issue with the bismuth liquid assay was quantifying the amount of H_2S being produced, as it was more difficult to visually assess the colour of vigorously fermenting cultures compared to colony colour. The CIELAB method has been used to evaluate the colour of wines (Pérez-Magariño and González-Sanjosé 2003; Kwiatkowski *et al.* 2007). Attempts were made to use CIELAB to analyse the colour intensity of the bismuth-containing ferments. Whilst the a* value (red/green) may potentially be useful, it was difficult to obtain repeatable OD values and differentiate between culture colour, ranging from white to brown (data not shown).

In previous studies, acetate buffer (Dean 1966) or citric acid buffer (Winter and Curtin 2012) were added into the H_2S detection mixes, and therefore it may be worthwhile investigating whether adding buffer would improve the assay.

non-sulfate CDGJM + Bi + 0.15 mM $MgSO_4$



non-sulfate CDGJM + Bi + 5 mM cysteine + 0.15 mM $MgSO_4$

	1	2	3	4	5
A	F15	F15	Sigma1278b	Sigma 1278b	QA23
B	F15 (*IRC7*^{F}ox)	F15 (*IRC7*^{F}ox)	Sigma1278b Δ*met17*	Sigma 1278b Δ*met17*	D254
C	BY4741	BY4741	Sigma1278b Δ*met10*	Sigma 1278b Δ*met10*	Cross Evolution
D	BY4741 Δ*met17*	BY4741 Δ*met17*	FM16	Maurivin B	F10
E	BY4741 Δ*cys4*	BY4741 Δ*cys4*	Lalvin 71B	M2	F10
F	BY4741 Δ*met5*	BY4741 Δ*met5*	Uvaferm 43	Fermichamp	GRE
G	BY4741 Δ*met10*	BY4741 Δ*met10*	QA23	L2056	AWRI1631
H	M2	M2	SimiWhite	*S. pombe*	Non-inoculated

Figure 3.3. Images of yeast strains on **(A)** non-sulfate CDGJM + Bi + 0.15 mM $MgSO_4$ + no methionine, **(B)** non-sulfate CDGJM + Bi + 0.15 mM $MgSO_4$ + 5 mM cysteine + no methionine. Images were taken after 96 h incubation at 28 °C. The known high-H_2S producer from cysteine, F15 (*IRC7*^{F}ox), was used as positive control.

3.3.4 Silver nitrate impregnated membranes

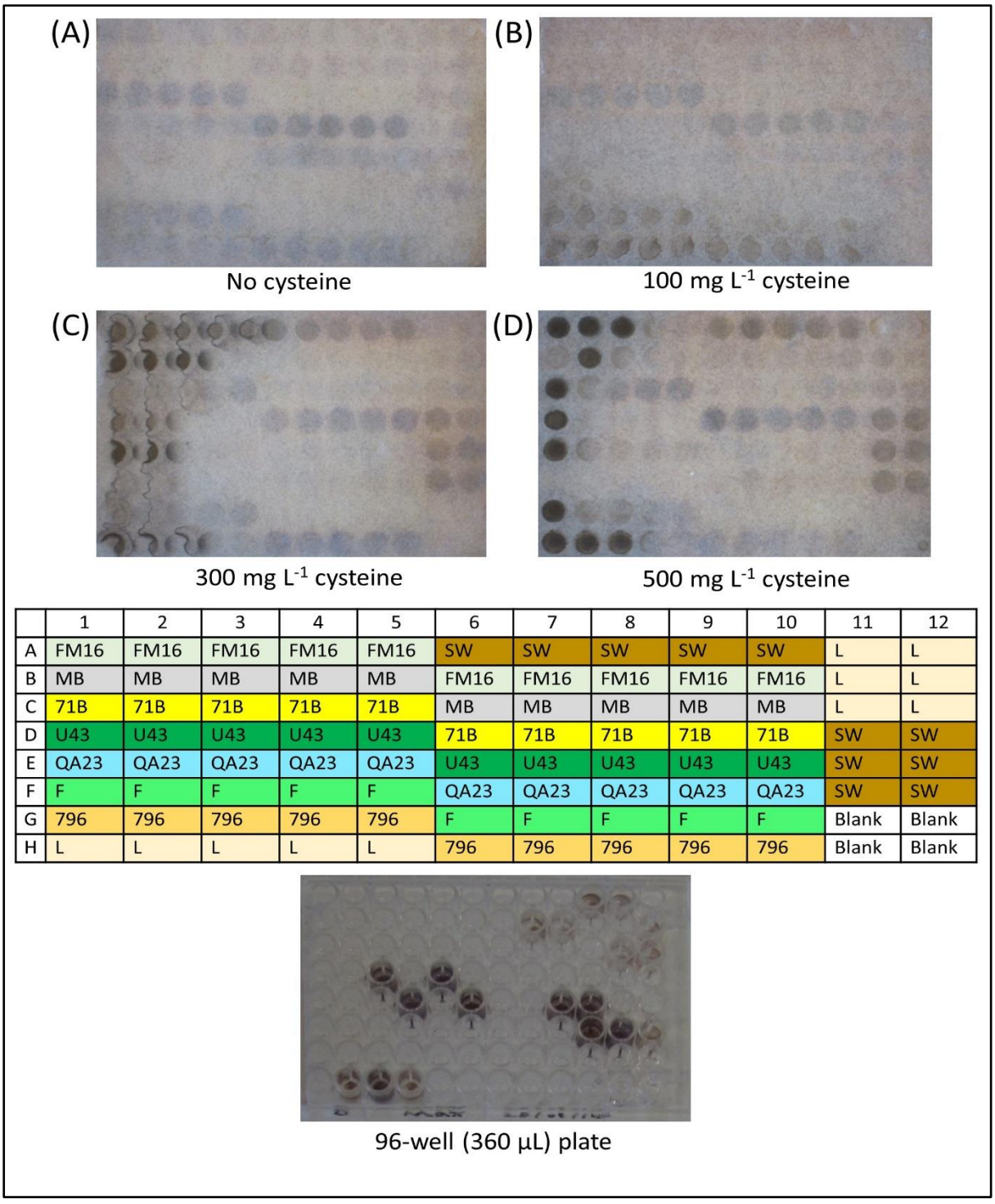
The silver nitrate impregnated membrane was used in a high through-put assay developed by Duan *et al.* (2004) for screening production of H_2S by brewing yeast. Since this method does not require a solidifying agent, it was decided to investigate whether it would be suitable for a high-throughput screen of a yeast deletion library for genes affecting H_2S formation from cysteine.

A collection of commercial yeast strains was used in the initial trial. Overall, the silver nitrate impregnated membrane made was not sensitive enough to detect H_2S produced by most of the strains with the exception of Enoferm Simi White. Dark-coloured membrane spots were consistently observed for Enoferm Simi White at both 300 and 500 mg L^{-1} cysteine (Fig. 3.4 C & D on all 11 spots: A6-A10, D11-F11 and D12-F12). Enoferm Simi White is a high H_2S producer when grown on cysteine, with H_2S levels 2-fold higher than other strains e.g. Maurivin AWRI796 (unpublished data).

In addition, while a few dark spots were observed at the left edge of the membrane (Fig. 3.4D), the dark membrane spots were not formed by the same strains at different well positions. This inconsistency may be due to more silver being impregnated at the edge of the membranes due to uneven drying.

Increasing the fermentation size from 200 µL to 600 µL, (hence more H_2S production) did not result in clearer dark spots on the membrane. Only Enoferm Simi White was again observed to produce dark membrane spots at both 300 and 500 mg L^{-1} cysteine (Fig. 3.5C & D).

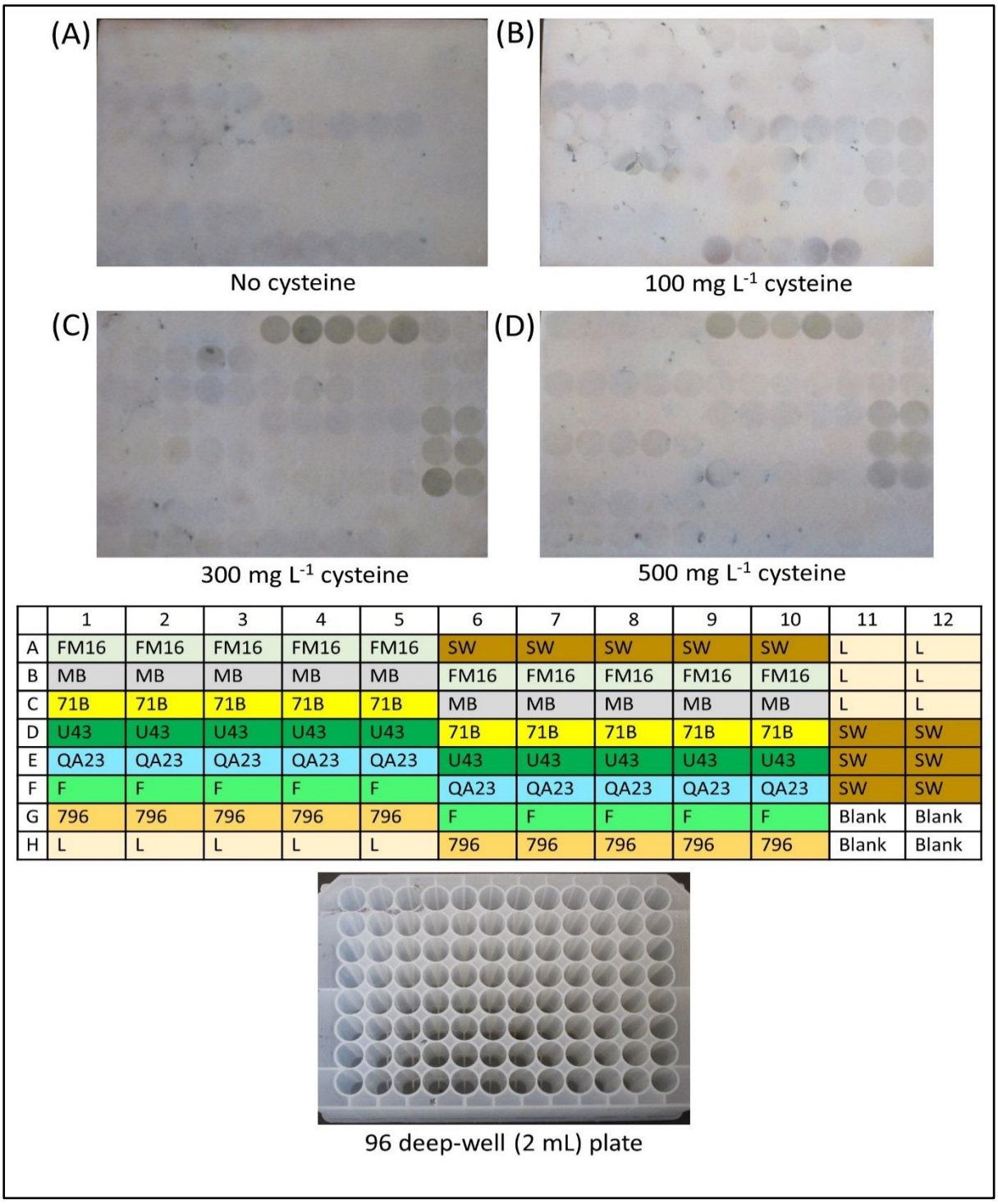
In addition to the poor sensitivity, it was difficult to remove the membrane without splashing the media within the 96-well (360 µL) plates onto the membrane (Fig. 3.4C). Some of the fermentations in the plates turned black as a result of silver sulfide dripping down from the membrane (Fig. 3.4). Natusch, Sewell and Tanner (1974), also used silver nitrate impregnated tapes, except the silver nitrate solution used was supplemented with nitric acid (HNO_3), glycerol and ethanol. Therefore, it may be worthwhile to further investigate whether addition of HNO_3, glycerol and ethanol or using different types of chromatography papers can improve the assay.



	1	2	3	4	5	6	7	8	9	10	11	12
A	FM16	FM16	FM16	FM16	FM16	SW	SW	SW	SW	SW	L	L
B	MB	MB	MB	MB	MB	FM16	FM16	FM16	FM16	FM16	L	L
C	71B	71B	71B	71B	71B	MB	MB	MB	MB	MB	L	L
D	U43	U43	U43	U43	U43	71B	71B	71B	71B	71B	SW	SW
E	QA23	QA23	QA23	QA23	QA23	U43	U43	U43	U43	U43	SW	SW
F	F	F	F	F	F	QA23	QA23	QA23	QA23	QA23	SW	SW
G	796	796	796	796	796	F	F	F	F	F	Blank	Blank
H	L	L	L	L	L	796	796	796	796	796	Blank	Blank

96-well (360 µL) plate

Figure 3.4. Images of silver nitrate impregnated membranes on top of 96-well (360 µL) plates containing 200 µL inoculated non-sulfate CDGJM, 0.15 mM sulfate and **(A)** no cysteine, **(B)** 100 mg L^{-1} cysteine, **(C)** 300 mg L^{-1} cysteine, **(D)** 500 mg L^{-1} cysteine. **Abbreviation: F** = Fermichamp, **L** = Lalvin L2056, **71B** = Lalvin 71B, **QA23** = Lalvin QA23, **796** = Maurivin AWRI796, **MB** = Maurivin B, **SW** = Enoferm Simi White, and **U43** = Uvaferm 43. Images were taken after 96 h incubation at 28 °C.



	1	2	3	4	5	6	7	8	9	10	11	12
A	FM16	FM16	FM16	FM16	FM16	SW	SW	SW	SW	SW	L	L
B	MB	MB	MB	MB	MB	FM16	FM16	FM16	FM16	FM16	L	L
C	71B	71B	71B	71B	71B	MB	MB	MB	MB	MB	L	L
D	U43	U43	U43	U43	U43	71B	71B	71B	71B	71B	SW	SW
E	QA23	QA23	QA23	QA23	QA23	U43	U43	U43	U43	U43	SW	SW
F	F	F	F	F	F	QA23	QA23	QA23	QA23	QA23	SW	SW
G	796	796	796	796	796	F	F	F	F	F	Blank	Blank
H	L	L	L	L	L	796	796	796	796	796	Blank	Blank

96 deep-well (2 mL) plate

Figure 3.5. Images of silver nitrate impregnated membranes on top of 96 deep-well (2 mL) plates containing 600 µL inoculated non-sulfate CDGJM, 0.15 mM sulfate and **(A)** no cysteine, **(B)** 100 mg L^{-1} cysteine, **(C)** 300 mg L^{-1} cysteine, **(D)** 500 mg L^{-1} cysteine. **Abbreviation: F** = Fermichamp, **L** = Lalvin L2056, **71B** = Lalvin 71B, **QA23** = Lalvin QA23, **796** = Maurivin AWRI796, **MB** = Maurivin B, **SW** = Enoferm Simi White, and **U43** = Uvaferm 43. Images were taken after 96 h incubation at 28 °C.

3.4 Conclusions

While the methylene blue reduction method has proven to be an ideal method for the high-throughput detection of H_2S during micro-scale fermentation, some concerns have been raised about the potential effects of evaporation and oxidation on the colour of methylene blue (Winter and Curtin 2012).

This chapter describes work aimed at finding whether a modified version of the bismuth-containing, grape juice-like indicator agar plate (Jiranek, Langridge and Henschke 1995) or a high-throughput screen using silver nitrate-impregnated membranes (Duan *et al.* 2004) are acceptable alternatives for screening H_2S production from cysteine by yeast.

In spite of best efforts, the silver nitrate-impregnated membrane prepared by following the protocol described in Duan *et al.* (2004) was not sensitive and reproducible enough to detect H_2S production by most of the yeast strains tested.

Although the impurities contained in Bacto agar could interfere with the interpretation of H_2S formation from cysteine (Fig. 3.2A), the bismuth-containing, grape juice-like indicator agar plate was sensitive enough to detect H_2S formed this way for both AWRI1631 and BY4741 strains (observed as darker coloured colonies; Fig. 3.1). Other advantages of this method included: (1) the spotted yeast cultures had more compact colonies on agar than on agarose (Fig. 3.2B); (2) agar is far cheaper than agarose; (3) colony colours were easier to assess visually than the colour of fermenting cultures (Fig. 3.3); (4) agar plates were simpler to prepare in large quantities than silver nitrate impregnated membranes; and (5) bismuth is relatively non-hazardous to humans or the aquatic environment compared to silver nitrate (Panyala, Peña-Méndez and Havel 2008; Rosolina, Carpenter and Xue 2016).

Whilst not perfect, the bismuth-containing, grape juice-like indicator agar plate is a viable alternative to screen for H_2S production in the AWRI1631 or BY4741 yeast deletion libraries (Chapter 4). It will be interesting to compare the results obtained using this assay to the previous study using the methylene blue reduction method (Winter, Cordente and Curtin 2014). The effects of candidate genes on H_2S production identified from the screen will be further confirmed by measuring H_2S production using H_2S detector tubes (Park 2008), in laboratory scale (100 mL) fermentations.

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Chapter 4

Yeast genes involved in regulating cysteine uptake affect production of hydrogen sulfide from cysteine during fermentation

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Principal Author

Name of Principal Author (Candidate)	Chien-Wei Huang		
Contribution to the Paper	Designed experiments, performed experiments, interpreted data, wrote the manuscript and acted as corresponding author.		
Overall percentage (%)	70		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	17/08/17

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Contribution to the Paper	Designed experiments, supervised the work, interpreted data and edited the manuscript.		
Signature		Date	17/08/17

Name of Co-Author	Bruno Fedrizzi		
Contribution to the Paper	Designed experiments, supervised the work, interpreted data and edited the manuscript.		
Signature		Date	24/08/2017

Please cut and paste additional co-author panels here as required.

Name of Co-Author	Richard C. Gardner
Contribution to the Paper	Designed experiments, supervised the work, interpreted data and edited the manuscript.
Signature	Date 23/8/17

Name of Co-Author	Vladimir Jiranek
Contribution to the Paper	Designed experiments, supervised the work, interpreted data and edited the manuscript.
Signature	Date 28.8.17

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RESEARCH ARTICLE

Yeast genes involved in regulating cysteine uptake affect production of hydrogen sulfide from cysteine during fermentation

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ABSTRACT

An early burst of hydrogen sulfide (H_2S) produced by *Saccharomyces cerevisiae* during fermentation could increase varietal thiols and therefore enhance desirable tropical aromas in varieties such as Sauvignon Blanc. Here we attempted to identify genes affecting H_2S formation from cysteine by screening yeast deletion libraries via a colony colour assay on media resembling grape juice. Both Δ*lst4* and Δ*lst7* formed lighter coloured colonies and produced significantly less H_2S than the wild type on high concentrations of cysteine, likely because they are unable to take up cysteine efficiently. We then examined the nine known cysteine permeases and found that deletion of *AGP1*, *GNP1* and *MUP1* led to reduced production of H_2S from cysteine. We further showed that deleting genes involved in the SPS-sensing pathway such as *STP1* and *DAL81* also reduced H_2S from cysteine. Together, this study indirectly confirms that Agp1p, Gnp1p and Mup1p are the major cysteine permeases and that they are regulated by the SPS-sensing and target of rapamycin pathways under the grape juice-like, cysteine-supplemented, fermentation conditions. The findings highlight that cysteine transportation could be a limiting factor for yeast to generate H_2S from cysteine, and therefore selecting wine yeasts without defects in cysteine uptake could maximise thiol production potential.

Keywords: *Saccharomyces cerevisiae*; hydrogen sulfide; varietal thiols; cysteine permease; SPS-sensing pathway; target of rapamycin (TOR) pathway

INTRODUCTION

An early burst of hydrogen sulfide (H_2S) produced by *Saccharomyces cerevisiae* during fermentation could potentially elevate the levels of varietal thiols, 3-mercapto-hexanol (3MH) and 3-mercaptohexylacetate (3MHA), and therefore enhance pleasant, tropical aromas in varieties such as Sauvignon Blanc (Schneider *et al.* 2006; Winter *et al.* 2011; Harsch *et al.* 2013; Araujo *et al.* 2016, 2017). However, the majority of H_2S liberation does

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not occur until yeast assimilable nitrogen in grape juice becomes depleted during fermentation (Jiranek, Langridge and Henschke 1995a).

Yeast could be legally induced to produce an early burst of H_2S during winemaking by supplementation with rehydration nutrients that are rich in glutathione. It was proposed that the sulfur-containing amino acid, cysteine, a constituent of glutathione (γ-L-glutamyl-L-cysteinylglycine), is degraded by yeast to generate this early H_2S production (Winter *et al.* 2011). Whilst, cysteine is naturally present in grape juice at very low concentrations (<20 mg L^{-1}) (Ugliano and Henschke 2009), commercial yeast nutrient products containing cysteine e.g. Laffort FreshArom, could be supplemented during fermentation to boost the concentration of antioxidant glutathione and therefore, preserve thiols (O'Kennedy 2013).

Yeast has been known since the 1970s to be capable of releasing H_2S from cysteine (Tokuyama *et al.* 1973). Since then, several yeast genes and mechanisms have been suggested to explain H_2S production from cysteine. Winter, Cordente and Curtin (2014) identified several vacuole-related genes, whose deletion resulted in less H_2S production from cysteine. Their findings suggest that the yeast vacuole, which is functionally similar to the mammalian lysosome, may play a crucial role in relieving cysteine toxicity by degrading cysteine to H_2S. Santiago and Gardner (2015a) demonstrated that the full-length *IRC7* gene encodes a cysteine desulfhydrase, which cleaves cysteine to generate H_2S. However, most yeast strains have a 38-bp deletion within the *IRC7* gene, which results in a truncated protein of 340 amino acids, lacking ß-lyase activity (Roncoroni *et al.* 2011). Recently, yeast *TUM1* was reported to affect H_2S from cysteine during fermentation, with yeast Tum1p thought to have enzyme activity similar to its human orthologue, sulfurtransferase, which is responsible for generating H_2S from cysteine (Huang *et al.* 2016). Despite recent progress in our understanding of yeast cysteine catabolism, much work is still required to identify other genes involved in this process and to fill knowledge gaps.

A genome-wide screen of thousands of yeast deletants from yeast single-gene deletion libraries for their H_2S production has proven to be a powerful approach to identify yeast genes responsible for H_2S formation (Linderholm *et al.* 2008; Yoshida *et al.* 2011; Winter, Cordente and Curtin 2014). To date, yeast deletion libraries have been screened using a range of H_2S screening assays including (i) BiGGY (Bismuth Glucose Glycine Yeast) agar plates (Linderholm *et al.* 2008), (ii) YPD plus lead nitrate agar plates (Yoshida *et al.* 2011) and (iii) the methylene blue reduction method (Winter, Cordente and Curtin 2014). However, studies have suggested that deletion of the genes, identified by these assays, does not necessarily have similar impacts on H_2S in a fermentation setting (Linderholm *et al.* 2008; Yoshida *et al.* 2011; Huang *et al.* 2016).

In this work, we aimed to identify other yeast genes required for H_2S production from cysteine during fermentation. Our screen differs from others, in that the AWRI1631 Wine Yeast Deletion Library (WYDL) collection (Varela *et al.* 2012) was screened using a colony colour assay that mimics a typical grape juice (Jiranek, Langridge and Henschke 1995b; Santiago and Gardner 2015a). The genes identified in this study as affecting H_2S formation from cysteine expand not only our current understanding of the cysteine transport process in yeast in a grape juice-like, cysteine-supplemented fermentation condition, but could also be valuable for the breeding of wine yeasts with potential to preserve (enhance) varietal thiols.

METHODS

Yeast strains and culture

The yeast strains used for this study are listed in Table 1. YPD media (10 g L^{-1} yeast extract, 20 g L^{-1} peptone and 20 g L^{-1} glucose) was used for standard yeast propagation at 28°C. Strains transformed with *KanMX* and *HphMX* deletion cassettes were selected on geneticin or G418 sulfate (200 mg L^{-1}; Astral, NSW, Australia) and hygromycin (300 mg L^{-1}; Astral, NSW, Australia),

Table 1. Yeast strains used in this study.

Strain	Genotype, phenotype and comments	Origin
BY4743	*MATa/α, his3-Δ1/his3-Δ1, leu2-Δ0/leu2-Δ0, LYS2/lys2-Δ0, met15-Δ0/MET15, ura3-Δ0/ura3-Δ0*	Euroscarf
BY4743 (pGP564)	BY4743 with (pGP564)	This study
BY4743 (*STP1*ox)	BY4743 with (pGP564+*YDR459C+TFB3+MFA+MRPL28+**STP1**+SPP41*)[a]	This study
BY4743 (*IRC7*ox)	BY4743 with (pGP564+*YFR056C+**IRC7**+YFR057W*)[b]	This study
BY4743 (*GAP1*ox)	BY4743 with (pGP564+*KAE+tD(GUC)K +**GAP1**+tA(AGC)K2+YKR040C+YKR041W+ UTH1+YKR043C+UIP5+YKR045C+PET10*)[c]	This study
BY4743 (*LST4*ox)	BY4743 with (pGP564+*COY1+STE3+YKL177W+**LST4** +ZRT3+YKL174C+SNU114*)[d]	This study
BY4743 (*GNP1*ox)	BY4743 with (pGP564+*tL(CAA)D +GIN4+**GNP1**+YDR509W+SMT3+YDR510CA+ACN9+ EMI1+TTR1+YDR514C*)[e]	This study
BY4743 (*MUP1*ox)	BY4743 with (pGP564+*FMP48+YGR053C+YGR054W+**MUP1**+RSC1+**LST7**+YGR058W*)[f]	This study
AWRI1631	Haploid wine strain	AWRI
AWRI1631 Δ*lst4*	*lst4::KanMX*	AWRI
AWRI1631 Δ*tum1*	*tum1::KanMX*	AWRI
AWRI1631 Δ*lst7*	*lst7::HphMX*	This study
AWRI1631 Δ*lst4*/Δ*lst7*	*lst4::KanMX; lst7::HphMX*	This study
AWRI1631 Δ*tum1*/Δ*lst4*	*tum1::KanMX; lst4::HphMX*	This study
AWRI1631 Δ*tum1*/Δ*lst7*	*tum1::KanMX; lst7::HphMX*	This study
F15 (*IRC7*^{F}ox)	*ho*::P_{PGK}-*IRC7*^{F} in F15-h(*α*)	Roncoroni *et al.* (2011)

ox denotes overexpression. Genes of interests are denoted by bold font.
Identities of plasmids from the YSC4613 Yeast Genomic Tiling Collection (Jones *et al.* 2008) are as follows: [a]YGPM-10d14; [b]YGPM-25o08; [c]YGPM-27p10; [d]YGPM-25b18; [e]YGPM-25o09; [f]YGPM-14k19.

respectively (Goldstein and McCusker 1999). Non-sulfate CDGJM medium (Huang *et al.* 2016), used in agar plates for screening H_2S production, was prepared by combining filter-sterilised 2x stock and molten 2x bacteriological agar (RM250, Amyl Media, Melbourne, Australia).

Screening of yeast deletants for effect on H_2S formation from cysteine

Cultures of the AWRI1631 WYDL collection (Varela *et al.* 2012) and BY4741 deletion collection (Euroscarf), stored at –80°C, were thawed and transferred to 96-well plates (Costar 3596, Sigma-Aldrich, NSW, Australia) containing 200 μL YPD using a 96-channel pipette (Gilson PlateMaster P220, John Morris Scientific, Australia) and incubated for 48 h at 28°C. The yeast precultures (5 μL) were then spot-inoculated using a 96-channel pipette onto Nunc OmniTrays (O0764–1CS; 128 mm × 86 mm; Sigma-Aldrich, NSW, Australia) containing non-sulfate chemically defined grape juice agar medium. The media composition contained magnesium chloride rather than magnesium sulfate (Harsch *et al.* 2010; Santiago and Gardner 2015b; Huang *et al.* 2016), 5 g L^{-1} bismuth, 0.15 mM methionine plus or minus 5 mM cysteine (non-sulfate CDGJM agar + Bi ± 5 mM Cys + 0.15 mM methionine). It should be noted that the AWRI1631 deletion library was screened on non-sulfate CDGJM agar + Bi ± 5 mM Cys + 0.15 mM magnesium sulfate + no methionine. The minimal concentration (0.15 mM) of magnesium sulfate that was initially supplemented to facilitate cell growth was later replaced with 0.15 mM methionine (non-sulfate CDGJM agar + Bi ± 5 mM Cys + 0.15 mM methionine) for the screening of BY4741 deletion library, in order to minimise H_2S production from the sulfate assimilation pathway. The screenings were conducted in duplicate. The plates were incubated at 28°C for 96 h, and colony colour was assessed visually against the wild-type strains.

Fermentations and H_2S quantification

The selected candidate genes identified from the screening were further validated in lab-scale fermentations. Yeast starter cultures were prepared by inoculating a single yeast colony into starter medium (2% sugar, non-sulfate CDGJM plus 0.15 mM methionine) for 24 h at 28°C. The starter culture was centrifuged, washed and resuspended in sterile water to inoculate 100 mL of medium (non-sulfate CDGJM ± 5 mM Cys + 0.15 mM methionine) at 2.5 × 10^6 cells L^{-1}. Elevated amounts of histidine (200 mg L^{-1}), leucine (300 mg L^{-1}) and uracil (100 mg L^{-1}) were included for auxotrophies (Harsch *et al.* 2010). Fermentations were conducted in triplicate in 250 mL flasks at 28°C with shaking at 100 rpm. Fermentation progress was monitored daily as weight loss due to CO_2 evolution (Bely, Sablayrolles and Barre 1990). Ferments were considered finished when weight loss was ≤ 0.1 g per 24 h. H_2S produced by yeast during fermentation was measured by lead acetate H_2S detector tubes (4H: 1–2000 ppm; GASTEC, Japan) that tightly fitted into the glass fermentation airlock (Park 2008).

Genetic manipulation and strain construction

PCR was performed using Velocity DNA polymerase (Bioline, Australia). Yeast deletion strains were confirmed using Kan B or Kan C primers together with gene-specific primers as reported in Table S1 (Supporting Information). Yeast transformation was conducted using the lithium acetate method (Gietz *et al.* 1992).

The deletion of *LST7* was achieved by amplifying the *HphMX* cassette in plasmid pAG32 plus ~100 bp of homologous untranslated sequence flanking *LST7* (using the primers pair Del-*lst7*-F and Del-*lst7*-R; Table S1, Supporting Information). The PCR products were used for yeast transformation with selection of transformants on YPD agar plates containing hygromycin (300 mg L^{-1}). *LST7::HphMX* deletants were confirmed by PCR using primers *Hph*-I-F and RC*lst7* (Table S1, Supporting Information). The *LST4* was deleted through a similar approach.

The overexpression of genes involved in regulating cysteine uptake was achieved by using the Yeast Genomic Tiling Collection (Jones *et al.* 2008) purchased from Open Biosystems (YSC4613, Thermo Fisher Scientific, Lafayette, CO). The leucine auxotrophic strain BY4743 was individually transformed with the plasmid pGP564 (*LEU2* selectable marker and 2-μm plasmid, as control) and plasmids containing the cloned ORFs of the candidate genes. The transformants were selected on synthetic complete leucine drop-out plates (SC-leu) (Sherman 2002). It should be noted that these overexpression strains not only overexpressed the gene of interest but also the adjacent three to four genes.

Data analysis

The mean, standard error of the mean (SEM) and *t* test (two samples assuming unequal variances) were performed using Microsoft Excel 2013 (Microsoft, Redmond, Washington, USA). Analysis of variance (ANOVA) and Tukey's honestly significant difference test were conducted using JMP software (SAS Institute, Cary, NC, USA). Statistical significance was set at the confidence level of 5%.

RESULTS

Validation of yeast deletion library genotype and screening assay for H_2S production

Several yeast genes responsible for H_2S formation from cysteine have been successfully identified from a genome-wide screen using a laboratory BY4742 yeast deletion library (Winter, Cordente and Curtin 2014). In order to identify other candidate genes affecting H_2S formation from cysteine, we conducted a genome-wide screen but using other yeast deletion libraries and an alternative screening assay.

Two yeast deletion libraries were screened in this study: (i) the AWRI1631 wine yeast deletion library (~2000 deletants) (Varela *et al.* 2012) and (ii) the laboratory BY4741 (Δ*met17*) yeast deletion library (~5000 deletants). The AWRI1631 wine yeast deletion library was selected because the deletion library was constructed using a prototrophic wine strain and the strain background is more representative of that used to conduct wine fermentations. It also has not previously been screened for H_2S production from cysteine. Deletion of *MET17* was observed to produce an additional delayed burst of H_2S from cysteine (Huang *et al.* 2016). Interestedly, this delayed burst of H_2S was not detected for the *MET5* deletant in the BY4741 (Δ*met17*) background during our initial trials (Fig. S1A, Supporting Information). Therefore, to identify other genes affecting this delayed H_2S production caused by the deletion of *MET17*, we decided also to screen H_2S production by deletants in the BY4741 (Δ*met17*) yeast deletion library.

A modified version of bismuth-containing indicator agar resembling grape juice (Jiranek, Langridge and Henschke 1995b; Santiago and Gardner 2015a) was employed in this work to

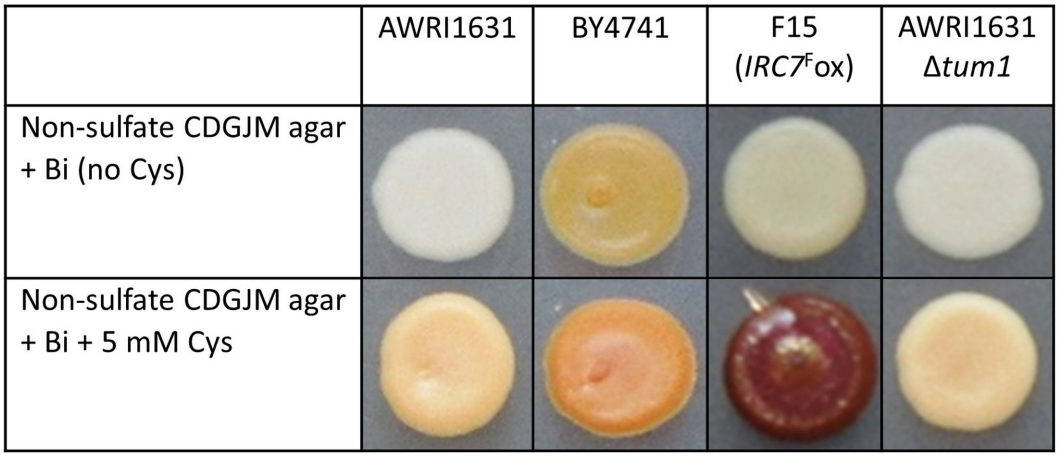

Figure 1. Colony colours of the wild-type strains on non-sulfate chemically defined grape juice agar plates containing 5 g L^{-1} bismuth, 0.15 mM methionine plus or minus 5 mM cysteine. The known high H_2S producer from cysteine, F15 (*IRC7*^{F}ox), was used as positive control. The known low H_2S producer from cysteine, AWRI1631 Δ*tum1*, was used as negative control. Images were taken after 96 h incubation at 28°C.

screen H_2S formation from cysteine. The assay is sensitive enough to detect H_2S formed this way as both AWRI1631 and BY4741 strains were observed to form darker coloured colonies when cysteine (5 mM) was added (Fig. 1). The principle of the assay is based on bismuth reacting with sulfide to form dark coloured precipitates of bismuth sulfide (Nickerson 1953). We were interested in those deletants that could still form light coloured colonies on cysteine supplemented media, as this would indicate that the genes deleted were involved in the generation of H_2S from cysteine. Since BY4741 already formed quite darkly coloured colonies on non-sulfate CDGJM agar + Bi + 5 mM Cys + 0.15 mM methionine (Fig. 1), we were primarily interested in deletants that produced light colony colours when screening the BY4741 deletion library.

Deletion of *LST4* or *LST7* resulted in lighter coloured colonies and reduced production of H_2S from cysteine

Both Δ*lst4* and Δ*lst7* deletants were identified to produce somewhat lighter coloured colonies than the wild-type strains from the screening experiment (Fig. 2A). Interestingly, the Δ*lst4* deletant was also identified as a low H_2S producer from cysteine in the previous genome-wide study using the BY4742 deletion library (Winter, Cordente and Curtin 2014). A further laboratory-scale fermentation experiment using non-sulfate CDGJM and 0.15 mM methionine plus or minus 5 mM cysteine (Huang *et al.* 2016) was thus performed to confirm the screening results.

In addition, the deletion strains in the BY4743 laboratory background have been successfully used to identify genes affecting H_2S production from cysteine during fermentation and they were also observed to ferment much better than strains in the BY4742 laboratory background (Harsch *et al.* 2010; Huang *et al.* 2016). Therefore, we decided to include deletion strains in the BY4743 background to further verify the results obtained from the screens and use them to explore other candidate genes affecting H_2S production from cysteine.

The fermentation experiment showed that H_2S was only produced by wild-type strains when cysteine was supplemented, indicating that cysteine is the most likely source of H_2S (Fig. 2B). It also confirmed that the Δ*lst4* and Δ*lst7* deletants did in fact produce significantly less H_2S on high concentrations of cysteine than the wild-type strains in different genetic backgrounds (Fig. 2B).

Although the Δ*lst4* and Δ*lst7* deletants were observed to ferment relatively slower than the wild type (Figs S2A and S2B, Supporting Information), this minor decrease in fermentation rates is unlikely to result in the observed ∼70% reduction in H_2S formation (Fig. 2B: BY4743 and AWRI1631 background). Therefore, we decided to further investigate the roles of *LST4* and *LST7* in cysteine catabolism.

Deletion of *LST4* or *LST7* further decreased H_2S in a Δ*tum1* deletant

Previous studies have suggested that multiple genes are responsible for H_2S production from cysteine (Winter, Cordente and Curtin 2014; Huang *et al.* 2016). The yeast *TUM1* gene has been recognised as one of the key genes responsible for the production of H_2S from cysteine during fermentation (Huang *et al.* 2016). We were therefore interested in the additive effect of deleting both *TUM1* and *LST4* or *LST7* on H_2S from cysteine. The amount of H_2S produced by an AWRI1631 Δ*tum1* deletant was further reduced by deleting either *LST4* or *LST7*. As shown in Fig. 3, there was no difference in H_2S production between the double deletants (Δ*lst4*/Δ*lst7*) and the single deletants of Δ*lst4* and Δ*lst7*.

Deletion of *GNP1*, *AGP1* and *MUP1* reduced H_2S production from cysteine

The yeast Lst4-Lst7 GTPase-activating protein complex is responsible for activating Gtr2p, in response to the presence of amino acids and the target of rapamycin (TOR) pathway (Péli-Gulli *et al.* 2015). This complex mediates the transport of the general amino acid permease Gap1p from the Golgi to the cell surface, with mutations in *LST4* and *LST7* leading to a decrease in Gap1p activity (Roberg *et al.* 1997). Therefore, the reduced H_2S production from cysteine observed for Δ*lst4* and Δ*lst7* is most likely as a result of reduced cysteine uptake. *GAP1* and the cysteine permease genes *AGP1*, *GNP1*, *BAP2*, *BAP3*, *TAT1*, *TAT2*, *MUP1* and *YCT1* (During-Olsen *et al.* 1999; Kosugi *et al.* 2001; Kaur and Bachhawat 2007) were chosen for further investigation. To our surprise, deletion of *GAP1* did not reduce

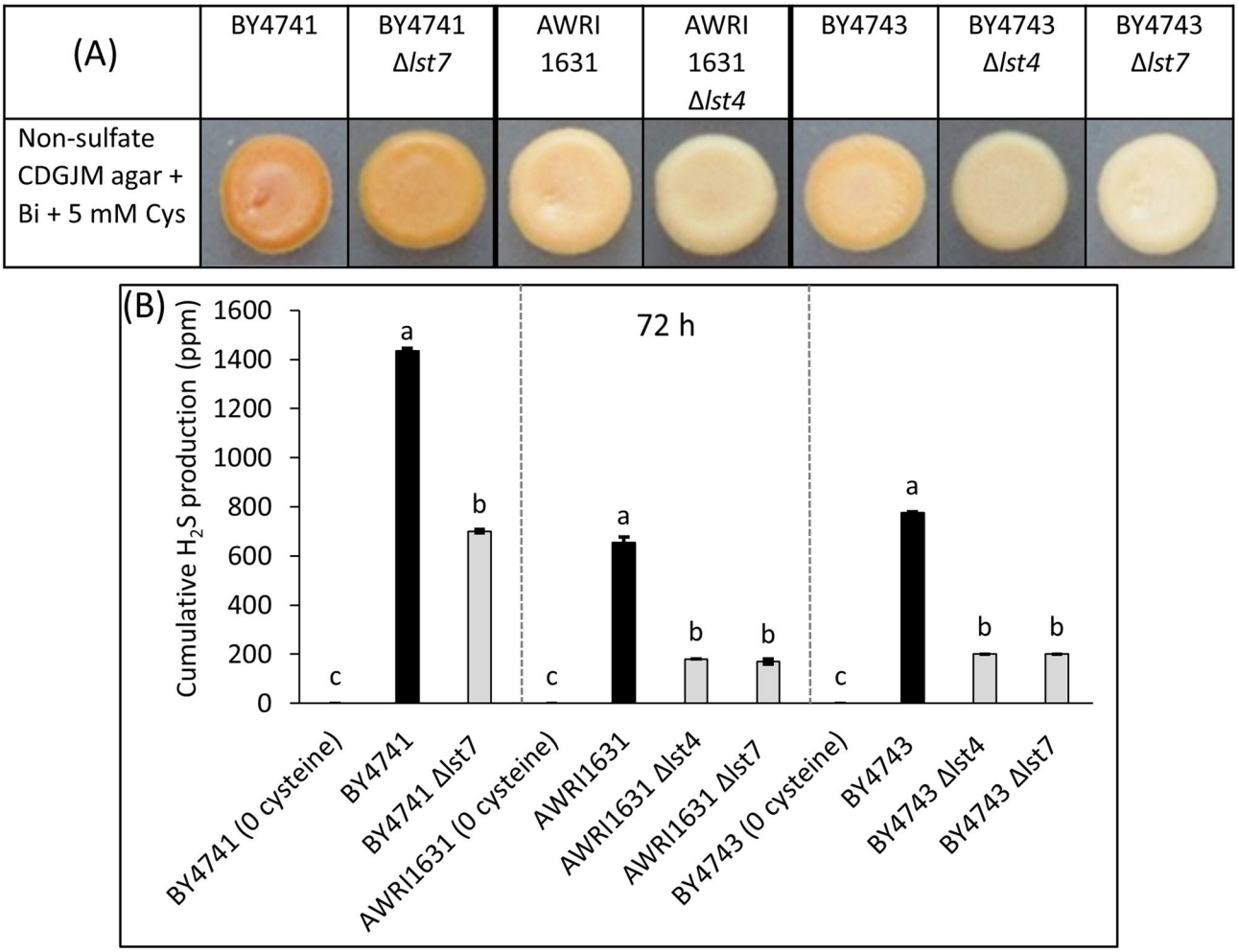

Figure 2. **(A)** Colony colours of the wild-type strains and the *LST* gene deletants on non-sulfate chemically defined grape juice agar plates containing 5 g L^{-1} bismuth, 5 mM cysteine and 0.15 mM methionine. Images were taken after 96 h incubation at 28°C. **(B)** Cumulative H_2S production of the wild-type strains and the *LST* gene deletants in different yeast genetic backgrounds (separated by dotted lines). Fermentations were performed in 100 mL of non-sulfate CDGJM and 0.15 mM methionine plus or minus 5 mM cysteine at 28°C with shaking at 100 rpm. H_2S was measured by lead acetate H_2S detector tubes (4H: 1–2000 ppm; GASTEC, Japan). Data represent mean values of triplicate fermentations ± standard error of the mean (SEM). Samples not connected by the same letter are significantly different (ANOVA, Tukey's HSD).

H_2S from cysteine but deletion of *GNP1*, *AGP1* and *MUP1* did in the laboratory strain BY4743 background (Fig. 4). A reduction in production of H_2S from cysteine was also observed for Δ*mup1* deletant in the AWRI1631 background (Fig. 4).

Deletion of *STP1* and *DAL81* involved in the SPS-sensing pathway reduced H_2S production from cysteine

The expression of yeast *GAP1* has been shown to be regulated by nitrogen (nitrogen catabolite repression) (Hofman-Bang 1999). On the other hand, *AGP1* and *GNP1* are regulated by Ssy1-Ptr3-Ssy5 (SPS) sensor (Forsberg *et al.* 2001). We were interested in knowing whether deletion of genes involved in the SPS-sensing pathway would also lead to a reduction in H_2S production from cysteine. Our results showed that deletion of *STP1* and *DAL81* did reduce H_2S from cysteine significantly (Fig. 5), and this indirectly demonstrated that the SPS-sensing pathway did play a significant role in cysteine uptake under the fermentation conditions.

Other deletants identified from the screening of AWRI1631 wine yeast deletion library

A few deletants other than Δ*lst4* and Δ*lst7* were also observed to form lighter coloured colonies than the wild-type on non-sulfate CDGJM agar + Bi ± 5 mM Cys + 0.15 mM magnesium sulfate + no methionine (Table 2); an indication that they produced less H_2S from cysteine. However, these deletants either had smaller colonies (e.g. AWRI1631 Δ*mct1*, Δ*ktr1*) or are involved in the sulfate assimilation pathway (e.g. AWRI1631 Δ*met5*, Δ*met16*; Fig. S3, Supporting Information). The smaller colony-forming deletants such as AWRI1631 Δ*mct1*, Δ*ktr1* fermented slower than the wild type (data not shown), suggesting that their inability to generate H_2S (dark colonies) from cysteine was likely due to their growth defect. Deletion of *MET* genes (e.g. *MET1*, *MET5*, *MET8* and *MET10*) has been shown to result in the formation of white colonies on BiGGY agar (Linderholm *et al.* 2008) and here, the *MET* gene deletants were also observed to form lighter coloured colonies in the presence of cysteine, although they also had lighter colouration even when cysteine was not added (Fig. S3, Supporting

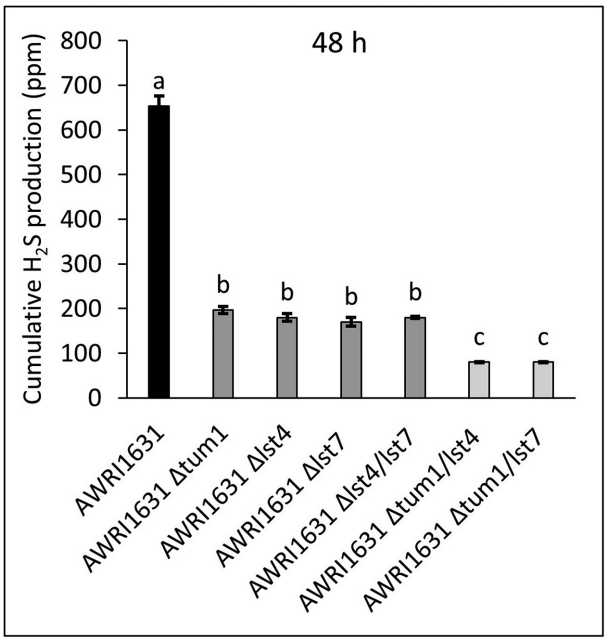

Figure 3. Cumulative H_2S production from AWRI1631 and its double *TUM1/LST* gene deletants. Fermentations were performed in 100 mL of non-sulfate CDGJM plus 5 mM cysteine and 0.15 mM methionine at 28°C with shaking at 100 rpm. H_2S was measured by lead acetate H_2S detector tubes (4H: 1–2000 ppm; GASTEC, Japan) and the mean H_2S released is shown ($n = 3$). Error bars indicate SEM. Samples not connected by the same letter are significantly different (ANOVA, Tukey's HSD).

Information). Since *MET3*, *MET5* and *MET10* do not affect H_2S from cysteine during fermentation (Huang *et al.* 2016), a significant amount of the H_2S detected by the assay conditions (0.15 mM sulfate) was likely to be from the sulfate assimilation pathway.

Some of the yeast deletants formed darker coloured colonies than the wild-type on non-sulfate CDGJM agar + Bi ± 5 mM Cys + 0.15 mM magnesium sulfate + no methionine (Table 2), indicating that they produced more H_2S from cysteine. However, most of these already formed darker coloured colonies even when cysteine was not supplemented (Fig. S3, Supporting Information) and some of the deletants (e.g. AWRI1631 Δ*hom2*, Δ*hom6*) have been identified to form darker coloured colonies on BiGGY agar (Linderholm *et al.* 2008). Therefore, their effects on colony colouration might not be cysteine specific. Interestingly, yeast deletants with smaller colonies (e.g. AWRI1631 Δ*pps1*) were observed to produce darker colonies than the wild type.

The formation of darker coloured colonies than the wild type by vacuole-related gene deletants (e.g. AWRI1631 Δ*vps4*, Δ*vps25* and Δ*vps36*) (Fig. S3, Supporting Information) is consistent with the BiGGY agar results obtained by Linderholm *et al.* (2008). However, Winter, Cordente and Curtin (2014) proposed that this elevated production of H_2S by vacuole-related gene deletants (seen as darkly coloured colonies on BiGGY agar) is not related to cysteine catabolism, and it has also been observed that deletion of *VPS25* or *VPS36* in AWRI1631 had limited effect on H_2S from cysteine during fermentation (Huang *et al.* 2016).

Deletion of *HEM25* may reduce H_2S from cysteine

The AWRI1631 Δ*hem25* deletant was observed to form lighter coloured colonies than the wild type in the initial screen (Fig. S3, Supporting Information); however, the colonies were also lighter when cysteine was not supplemented. Fermentation

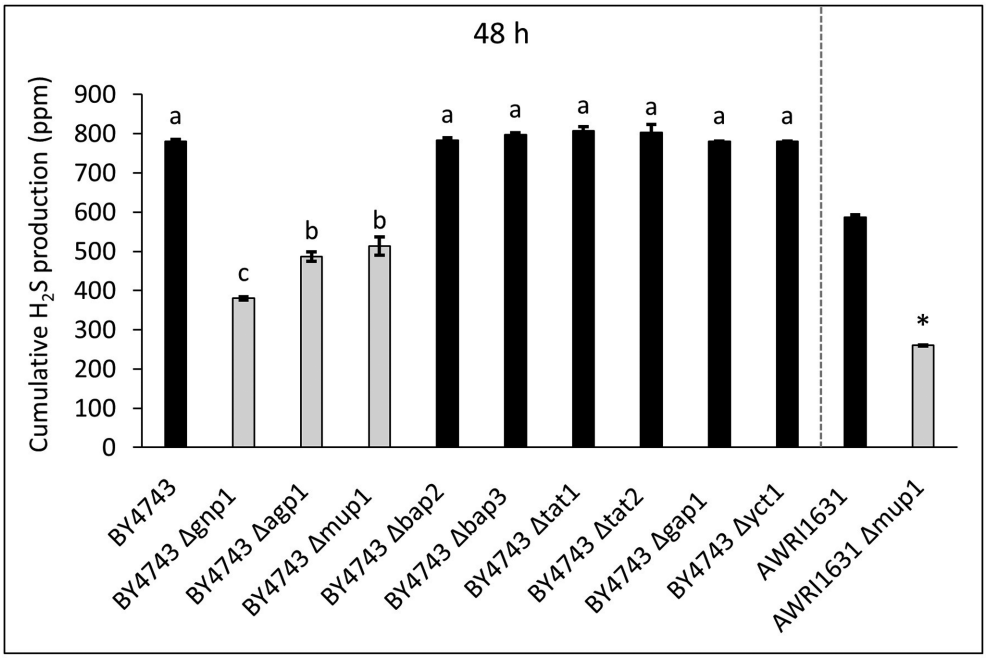

Figure 4. Cumulative H_2S production from the cysteine permease gene deletants in laboratory strain BY4743 and wine yeast strain AWRI1631 (separated by dotted lines). Fermentations were performed in 100 mL of non-sulfate CDGJM plus 5 mM cysteine and 0.15 mM methionine at 28°C with shaking at 100 rpm. H_2S was measured by lead acetate H_2S detector tubes (4H: 1–2000 ppm; GASTEC, Japan) and the mean H_2S released is shown ($n = 3$). Error bars indicate SEM. Samples not connected by the same letter are significantly different (ANOVA, Tukey's HSD). Asterisks above bars represent significant differences compared to the wild types (*$P < 0.05$, two-tailed Student's *t* test).

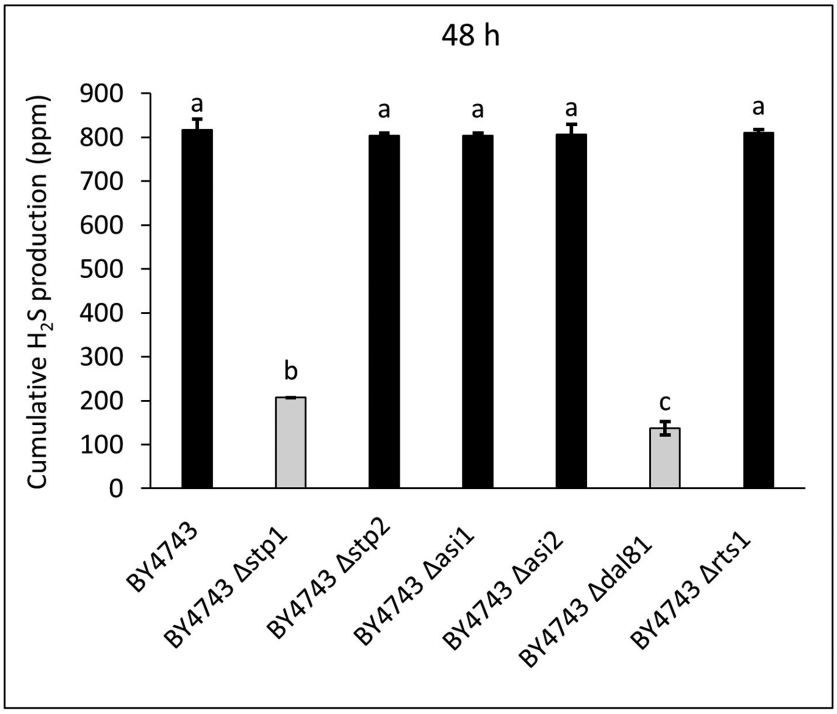

Figure 5. Cumulative H_2S production from the deletants involved in SPS-sensing pathway in laboratory strain BY4743. Fermentations were performed in 100 mL of non-sulfate CDGJM plus 5 mM cysteine and 0.15 mM methionine at 28°C with shaking at 100 rpm. H_2S was measured by lead acetate H_2S detector tubes (4H: 1–2000 ppm; GASTEC, Japan), and the mean H_2S released is shown ($n = 3$). Error bars indicate SEM. Samples not connected by the same letter are significantly different (ANOVA, Tukey's HSD).

Table 2. AWRI1631 deletants that formed lighter or darker colony colours than the wild type on non-sulfate CDGJM agar + Bi ± 5 mM cysteine + 0.15 mM $MgSO_4$ + no methionine.

AWRI1631 deletants that formed lighter coloured colonies	Functional name/group
Δ*met5*, Δ*met16*	Methionine biosynthesis
Δ*hem25*	Mitochondrial glycine transporter
Δ*mct1*	Phospholipid remodelling
Δ*lih1*	Lipase homologue
Δ*ktr1*	Kre two related
Δ*trp4*	Tryptophan biosynthesis
AWRI1631 deletants that formed darker coloured colonies	**Functional name/group**
Δ*hom2*, Δ*hom6*	Methionine biosynthesis
Δ*vps4*, Δ*vps25*, Δ*vps36*, Δ*snf8*, Δ*bro1*	Vacuolar protein sorting
Δ*ino2*, Δ*ino4*	Phospholipid synthesis
Δ*fre3*	Cellular iron ion homeostasis
Δ*npr1*	Nitrogen permease reactivator
Δ*pps1*	Protein phosphatase S phase
Δ*tom5*	Translocase of the outer mitochondrial membrane
Δ*stp22*	Sterile pseudoreversion
Δ*rrd1*	Resistant to rapamycin deletion
Δ*gdh1*	Glutamate biosynthetic process
Δ*yhl026c*, Δ*yjr120w*	Unknown

[a]Function information was obtained from *Saccharomyces* Genome Database (http://www.yeastgenome.org/).
[b]Deletants denoted by bold font indicate they were also identified to form dark coloured colonies on BiGGY agar (Linderholm *et al.* 2008).
[c]Deletants in underlined font indicate they had obvious smaller colonies (growth defects).

trials revealed that Δ*hem25* did produce less H_2S than the wild type in both BY4743 and AWRI1631 backgrounds, but deletion of *HEM25* only resulted in ~30% reduction in H_2S production in the AWRI1631 background (Fig. 6). Therefore, it is unclear whether the *HEM25* gene plays a significant role in H_2S formation from cysteine, given that the Δ*hem25* deletants were observed to ferment at a slightly slower rate relative to the wild-type strains (Fig. S4, Supporting Information). This slight growth defect was also observed by Lunetti *et al.* (2016) for BY4742 Δ*hem25* grown on YPD media.

The yeast *HEM25* gene (Heme synthesis by SLC25 family member) has recently been identified as encoding a

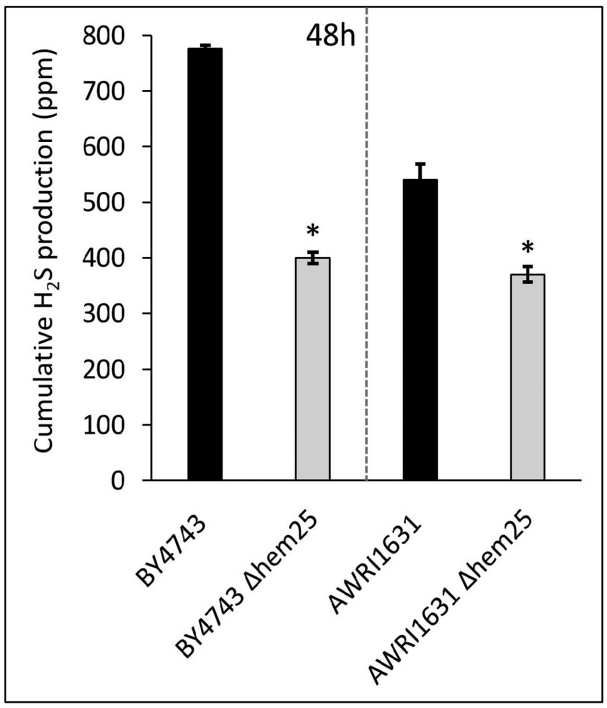

Figure 6. Cumulative H_2S production of the wild-type strains and the Δ*hem25* deletants in laboratory strain BY4743 and wine yeast strain AWRI1631 backgrounds (separated by dotted lines). Fermentations were performed in 100 mL of non-sulfate CDGJM plus 5 mM cysteine and 0.15 mM methionine at 28°C with shaking at 100 rpm. H_2S was measured by lead acetate H_2S detector tubes (4H: 1–2000 ppm; GASTEC, Japan). Data represent mean values of triplicate fermentations ± standard error of the mean (SEM). Asterisks above bars represent significant differences compared to the wild types (*$P < 0.05$, two-tailed Student's *t* test).

mitochondrial glycine transporter and is required for heme synthesis (Fernández-Murray *et al.* 2016). Given that H_2S from cysteine was affected by some of genes involved in iron–sulfur homeostasis when deleted (Winter, Cordente and Curtin 2014), makes it perhaps further investigation of the role of *HEM25* in H_2S formation from cysteine by more sensitive H_2S detection methods is worthwhile, in order to decipher the mechanism involved.

Other deletants identified from the screening of laboratory BY4741 (Δ*met17*) yeast deletion library

To identify the candidate genes affecting the delayed burst of H_2S from cysteine (Δ*met17*), the BY4741 (Δ*met17*) yeast deletion library was screened on non-sulfate CDGJM agar +Bi +0.15 mM methionine ±5 mM cysteine. However, most of the deletants that formed lighter coloured colonies (e.g. BY4741 Δ*tre1*, Δ*irc10* and *MET* genes deletants) also produced lighter coloured ones in the absence of cysteine (Table 3, Fig. S5, Supporting Information). These findings suggest that a substantial amount of the H_2S detected was from the sulfate assimilation pathway even though non-sulfate CDGJM was used. BY4741 Δ*tre1* and Δ*irc10* were observed not to affect the delayed burst of H_2S from cysteine (Fig. S1A, Supporting Information). Yeast deletants such as **Δ***mac1*, Δ*ado1* that formed smaller colonies or those noted as slower growers (e.g. Δ*coa1*, Δ*mdm32*) tended to form lighter coloured colonies. Interestingly, most of the deletants that formed lighter coloured colonies identified in this study have been observed to form lighter coloured colonies on lead nitrate agar plates (Yoshida *et al.* 2011). Collectively, the results suggested that most of the light coloured colonies identified did not specifically affect H_2S formation from cysteine.

BY4741 Δ*met5**, Δ*qdr2**, Δ*msl1** and Δ*coa1** were observed to affect the delayed burst of H_2S from cysteine (Fig. S1A, Supporting Information). However, they turned out to have a functional *MET17* gene (as identified by PCR; Fig. S1B: Δ*met5**, Supporting Information), so whilst they have decreased H_2S production, it is not related to the delayed burst of H_2S as described by Huang *et al.* (2016).

The yeast Dbf2p is a serine-threonine protein kinase that has been shown to be required for transcription, sorbic acid stress tolerance and vacuolar acidification (Liu *et al.* 1997; Makrantoni *et al.* 2007). The vacuolar acidification deletants have been

Table 3. BY4741 deletants that formed lighter or darker colony colours than the wild type on non-sulfate CDGJM agar + Bi ± 5 mM cysteine + 0.15 mM methionine.

BY4741 deletants that formed lighter coloured colonies	Functional name/group
(**Δ***cbf1*), **Δ***met1*, **Δ***met3*, **Δ***met10*, **Δ***met14*, **Δ***met16*, (**Δ***met18*), **Δ***met28*, **Δ***met5**	Methionine biosynthesis
(**Δ***ada2*)	Transcription
Δ*tre1*	Metal transporter degradation
Δ*mdm32*	Mitochondrial inner membrane protein
Δ*rsm25*	Mitochondrial ribosomal protein of the small subunit
Δ*sds3*	Component of the Rpd3L histone deacetylase complex
Δ*ldb18*	Component of the dynactin complex
Δ*paf1*	RNA polymerase II associated factor
Δ*irc10*, (**Δ***mac1*), (**Δ***ado1*), (Δ*pex4*), (Δ*ume6*), (Δ*rtg2*), (Δ*idh1*), (**Δ***fyv5*), (**Δ***fen2*), (**Δ***trk1*), **Δ***suv3*, **Δ***dfg10*, **Δ***tps2*, **Δ***srv2*, **Δ***ccs1*, **Δ***rtc6*, **Δ***qdr2**, **Δ***msl1**, **Δ***coa1**, Δ*lst7*, Δ*ssd1*	Others
BY4741 deletants that formed darker coloured colonies	**Functional name/group**
Δ*dbf2*	Ser/Thr kinase

[a]Function information was obtained from *Saccharomyces* Genome Database (http://www.yeastgenome.org/).
[b]Deletants in underlined font were also observed to form light brown colonies on YPD plus lead nitrate agar plates (Yoshida *et al.* 2011).
[c]Deletants denoted by an asterisk indicated that the *MET17* in BY4741 was not deleted within the library.
[d]Deletants denoted by bracket indicated that these were defective in growth, having smaller colonies on either 0 or 5 mM cysteine.
[e]Deletants denoted by bold font indicate they already had obvious lighter coloured colonies on plate without cysteine.

shown to produce less H_2S from cysteine (Winter, Cordente and Curtin 2014) but, interestingly, here, deletion of *DBF2* gene in the BY4741 background was observed to form darker coloured colonies when cysteine was supplemented (Table 3, Fig. S5, Supporting Information). It has been proposed that the slower fermentation rate is the major contributor to the lower levels of H_2S from cysteine produced by the vacuolar acidification deletants (Huang *et al.* 2016). The fact that these mutants form light brown colonies on YPD plus lead nitrate agar plates in the absence of cysteine (Yoshida *et al.* 2011), whilst other, vacuole-related, mutants have darker coloured colonies on bismuth-containing plates (Linderholm *et al.* 2008), suggests that the darker coloured colonies produced by Δ*dbf2* are also not directly related to cysteine. Nevertheless, the possibility that the colony colour is related to other functions of Dbf2p cannot be excluded.

DISCUSSION

In this work, we have shown that either deletion of yeast *LST4* or *LST7* reduced production of H_2S from high concentrations of cysteine (5 mM) during fermentation. This led to the identification of a new set of genes involved in regulating cysteine uptake that, in turn, impact H_2S production from cysteine during fermentation. Whilst our study is not the first to report identifying Δ*lst4* as a low H_2S producer from cysteine (Winter, Cordente and Curtin 2014), the mechanism by which *LST4* gene affects H_2S from cysteine remains to be elucidated. Yeast *LST4* and *LST7* have long been known for their important roles in the transport of the general amino acid permease Gap1p from the Golgi to the cell surface (Roberg *et al.* 1997), but it was not until recently that the Lst4-Lst7 complex was also revealed to be involved in the regulation of amino acid signalling by mediating the activity of target of rapamycin complex 1 (TORC1), a central regulator of eukaryotic cell growth (Schmelzle and Hall 2000; Péli-Gulli *et al.* 2015). Our findings suggest that for Δ*lst4* and Δ*lst7*, the reduction in H_2S derived from cysteine is most likely due to a defect in cysteine uptake/sensing, and unrelated to the degradation process, by which H_2S is formed within the yeast cell.

The deletion of *TUM1* has been observed to reduce the H_2S formed from cysteine by half during fermentation, and *TUM1* was proposed to act like a sulfurtransferase when generating H_2S from cysteine (Huang *et al.* 2016). The observation that deletion of *TUM1* further reduced the amount of H_2S produced from cysteine by the Δ*lst4* or Δ*lst7* single deletants by ∼54% (Fig. 3) is consistent with the interpretation that the effects of *LST4*/*LST7* and *TUM1* genes on H_2S formation from cysteine are caused by two different mechanisms. The observation that the Δ*lst4*/Δ*lst7* double deletant produced similar amount of H_2S as the Δ*lst4* or Δ*lst7* single deletants (Fig. 3) indicates that a disruption of either *LST4* or *LST7* genes is enough to cause a loss of Lst4–Lst7 complex function.

To date, nine cysteine permease genes *GAP1*, *AGP1*, *GNP1*, *BAP2*, *BAP3*, *TAT1*, *TAT2*, *MUP1* and *YCT1* have been identified. The effect of these genes is dependent on growth condition and yeast genotype (During-Olsen *et al.* 1999; Kosugi *et al.* 2001; Kaur and Bachhawat 2007). For example, Yct1p (yeast cysteine transporter) is the main cysteine-specific transporter at low-cysteine (<200 μM) concentrations. Its role becomes less prominent when other permeases start to take up cysteine at high-cysteine (>500 μM) concentrations (Kaur and Bachhawat 2007). In addition, the cysteine permeases Agp1p, Gnp1p and Mup1p have been reported as the major cysteine transporters in media containing ammonium and limited amino acids. Deletion of *GNP1* and *MUP1* was shown to decrease cysteine uptake by at least 40% (minimal ammonium medium + 0.3 mM L-[^{35}S] cysteine; During-Olsen *et al.* 1999) and 60% (synthetic medium + 50 μM L-[^{35}S] cysteine; Kosugi *et al.* 2001), respectively. In our study, a reduced amount of H_2S from cysteine was also observed for Δ*gnp* (∼51%), Δ*agp1* (∼38%) and Δ*mup1* (∼34%) in BY4743 but not the other cysteine permease deletants tested (Fig. 4). The results indirectly demonstrated that Agp1p, Gnp1p and Mup1p are the dominant cysteine permeases in grape juice-like, cysteine-supplemented fermentation conditions and also showed that the cysteine uptake process could be a limiting factor for yeast to generate H_2S from cysteine. Interestingly, deletion of *MUP1* led to a ∼56% reduction in H_2S from cysteine in AWRI1631, whilst in BY4743, H_2S production was only reduced by 34% (Fig. 4). It would appear that the effects of these permease genes are strain dependent, which may partly explain why different cysteine permeases have been implicated as the main cysteine permease in other studies (During-Olsen *et al.* 1999; Kosugi *et al.* 2001; Kaur and Bachhawat 2007).

The expression of yeast cysteine permease genes such as *AGP1* and *GNP1* is regulated by the plasma membrane Ssy1p-Ptr3p-Ssy5p (SPS) sensor (Forsberg *et al.* 2001). The SPS sensor is activated in response to extracellular amino acids, and induces endoproteolytic processing of the N-terminal regulatory domain of the transcription factors Stp1p and Stp2p (located in the cytoplasm). Following targeting to the nucleus, the shortened forms of Stp1p and Stp2p activate transcription of amino acid permease genes (Andréasson and Ljungdahl 2002). TORC1 is also activated by the Lst4-Lst7 complex on the vacuolar membrane (Péli-Gulli *et al.* 2015), whereby TORC1 controls the expression of the amino acid permeases through stabilisation of the transcription factor Stp1p (Shin, Kim and Huh 2009). Expression of the permease genes is further enhanced by the action of Dal81p (Boban and Ljungdahl 2007).

This study took advantage of yeast being able to generate H_2S from cysteine; the amount produced by individual yeast gene deletants related to cysteine regulation, a rough indicator of the amount of cysteine taken up. The observation that deletion of genes (in BY4743) involved in regulating amino acids uptake such as *LST4*, *LST7*, *STP1* and *DAL81* reduced H_2S formation from cysteine indirectly confirmed the findings of previous studies (Boban and Ljungdahl 2007; Shin, Kim and Huh 2009; Péli-Gulli *et al.* 2015). Figure 7 shows the model proposed regarding the regulation of cysteine uptake in *S. cerevisiae* during fermentation in a grape juice-like medium supplemented with cysteine, in which genes involved the SPS-sensing pathway and TOR pathway play central roles.

Deletion of amino acid sensor-independent genes, e.g. Δ*asi1*, has been shown to result in constitutive expression of SPS sensor-regulated genes, e.g. *AGP1* and *GNP1*, as a result of the unprocessed transcription factors Stp1p and Stp2p leaking from the cytoplasm into the nucleus and inducing the transcription of amino acid permease genes (Boban and Ljungdahl 2007). Deletion of *RTS1* was also observed to lead to constitutive amino acid signalling as the result of the increased Stp1p processing (Eckert-Boulet *et al.* 2006). Interestingly, deletion of *ASI1* or *RTS1* in BY4743 did not affect H_2S production from cysteine (Fig. 5). Results from a preliminary trial (Fig. S6, Supporting Information), whereby the genes were overexpressed using plasmids from the Genome Tiling library (Jones *et al.* 2008), suggested that overexpression of genes involved in regulating cysteine uptake may not elevate production of H_2S from cysteine. This result is not surprising, given that Kosugi *et al.* (2001) observed that overexpressing *MUP1* in a Δ*mup1* strain only restored the rate

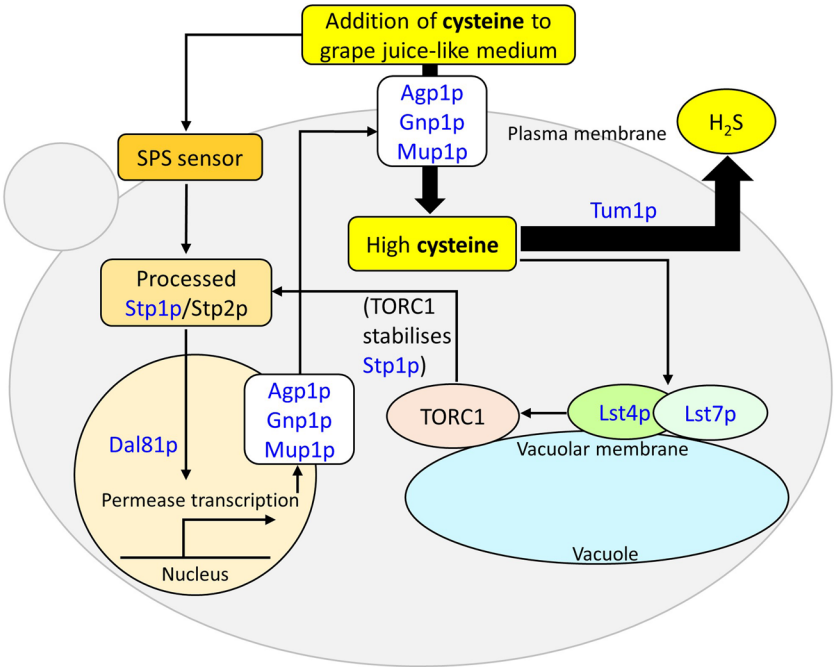

Figure 7. A model proposed for the regulation of cysteine uptake in *S. cerevisiae* under the grape juice-like, cysteine-supplemented fermentation conditions. Yeast responds to increased extracellular cysteine by activating the SPS-sensing pathway, which then leads to the transcription factors, Stp1 and Stp2 being processed and migrating to the nucleus to induce transcription of amino acid permease genes. Dal81p is required to enhance the expression of permease genes and Agp1p; Gnp1p and Mup1p are the major cysteine permeases. The TOR pathway is activated as well, with TORC1 being activated by Lst4-Lst7 complex, localised on the vacuolar membrane. TORC1 controls the expression of amino acid permeases through stabilising the transcription factors, Stp1. Disruption of proteins in the blue font resulted in reduced H_2S in this study. Whilst other permease genes may expressed, only the major cysteine permease genes identified *AGP1*, *GNP1* and *MUP1* are shown (adapted from Boban and Ljungdahl 2007; Shin, Kim and Huh 2009; Ljungdahl and Daignan-Fornier 2012; Péli-Gulli *et al.* 2015; González and Hall 2017).

of cysteine uptake to levels similar to wild type. Together, with the findings of inconsistent improvements in 3MH levels, when *MUP1* is overexpressed in yeast undergoing fermentation (Santiago 2014), we conclude that overexpression of genes, involved in regulating cysteine uptake, has limited effect on H_2S production from cysteine and on 3MH production.

Overall, most of the genes noted in this study have previously been identified in screens for genes responsible for H_2S from the sulfate assimilation pathway (Linderholm *et al.* 2008; Yoshida *et al.* 2011). This result suggested that whilst efforts to remove most of the sulfate content in the liquid medium used in the assay (non-sulfate CDGJM), a substantial amount of H_2S was still generated from the sulfate within the bacteriological agar, masking the effects of cysteine-related genes. The sulfate content in the assay could potentially be minimised by testing other solidifying agents that contain less sulfate. Alternatively, the methylene blue reduction method for H_2S detection could be an option as it does not require addition of solidified agents. However, as evaporation and oxidation may affect the colour of methylene blue, (the basis of H_2S detection), this method requires further evaluation (Winter and Curtin 2012). Of note, Δ*tum1* (a known low H_2S producer, under high cysteine conditions) only formed slightly lighter coloured colonies to the wild type on the bismuth plate assay shown in Fig. 1. Furthermore, we cannot exclude the possibility that the somewhat lighter coloured colonies produced by Δ*lst4* and Δ*lst7* deletants were due to their slightly slower growth. In fact, the identification of genes affecting H_2S formation from cysteine, other than *LST4* and *LST7*, which were identified in this study, was through a candidate gene approach and therefore, a better H_2S detection assay that accurately reflects the actual H_2S production during fermentation would be helpful.

Whilst the deletants identified as having lighter coloured colonies do not specifically affect the H_2S formed from cysteine, this study still provides interesting findings that may provide new insight into yeast sulfur metabolism. For example, BY4741 Δ*mdm32* (mitochondrial inner membrane protein), Δ*rsm25* (mitochondrial ribosomal protein of the small subunit), Δ*sds3* (component of the Rpd3L histone deacetylase complex), Δ*ldb18* (component of the dynactin complex) and Δ*paf1* (RNA polymerase II associated factor), which form lighter coloured colonies on our bismuth plate assay, exhibited an increased sulfur(1+) accumulation phenotype (Yu *et al.* 2012). The fact that these genes, having different functions can all affect the process of sulfur(1 +) accumulation and form lighter coloured colonies on bismuth plates, raises questions as to whether the colour is a result of increased sulfur(1+) accumulation. This aspect of sulfur metabolism is clearly worthy of further investigation.

In the initial screen, BY4741Δ*met5** unexpectedly produced lighter colony colours and less H_2S (Figs S1A and S5, Supporting Information). However, PCR analysis confirmed that whilst BY4741 Δ*met5** was haploid, and the *MET5* gene correctly deleted, it turned out to be a *MET17* wild type (Fig. S1B, Supporting Information). *MET17* was chosen as a selectable marker (in BY4741) because deletion of *MET17* led to excess H_2S production and resulted in dark-brown colonies on lead-containing plates (Brachmann *et al.* 1998). Interestingly, a few Δ*met17* deletants have been observed to be able to grow slowly on synthetic dextrose minimal plates but they are not considered true revertants (Brachmann *et al.* 1998). The BY4741 deletion library in our laboratory was obtained more than 10 years ago, so whilst there may be some cross-contamination, we are not sure how these *MET17* revertants may have arisen.

This work illustrated the complexity of the yeast cysteine uptake processes, with many questions still to be answered. First, it is well established that yeast synthesise glutathione (Grant and Dawes 1996), convert cysteine to methionine through the transsulfuration pathway (Hansen and Johannesen 2000) and degrade cysteine to H_2S (Tokuyama *et al.* 1973). However, high extracellular cysteine is toxic to yeast (Kumar *et al.* 2006), and therefore it is intriguing that yeast can take up excess amounts of cysteine into the cell and still maintain cysteine homeostasis. We observed that yeast is rather cysteine tolerant, being able to grow at CDGJM containing 30 mM cysteine (data not shown). Our screening results showed that none of the deletants were unable to grow as a result of the high (5 mM) concentration of cysteine. Those that did not grow already grew poorly on agar plates without cysteine.

Second, the deletants with defects in the SPS or TOR pathways tested in this study still produced limited amounts of H_2S from cysteine. Moreover, Santiago and Gardner (2015b) also showed that a yeast strain with all nine known cysteine transporters deleted could still grow on cysteine as sole nitrogen source. These findings indicate that there are more, yet unidentified, cysteine transporters or mechanisms by which yeast take up extracellular cysteine.

Another interesting question is how the Lst4-Lst7 complex is able to sense extracellular amino acids (Hatakeyama and De Virgilio 2016). The mammalian folliculin/folliculin interacting proteins (FLCN/FNIP) complex and yeast Lst7/Lst4 complex are orthologues (Péli-Gulli *et al.* 2015). The mutations in FLCN/FNIP complex have been linked to cancer (Birt-Hogg-Dubé syndrome) but the role of the FLCN/FNIP complex in cancer is not well understood currently (Nickerson *et al.* 2002; Pacitto *et al.* 2015; Péli-Gulli *et al.* 2015). The reduced production of H_2S from cysteine for Δ*lst4* and Δ*lst7* deletants, likely due to their inability to sense and transport extracellar cysteine, may provide some insight into the role of the FLCN/FNIP complex in cancer given that H_2S from cysteine has been shown to inhibit the growth of breast and prostate cancer cells (Chattopadhyay *et al.* 2012). Moreover, yeasts with heterozygous deletion of *TUM1* (the gene affects H_2S from cysteine) have also been observed to have an abnormal cell cycle phenotype, which is associated with cancer (de Clare and Oliver 2013). Further research is needed to complete the yeast cysteine uptake/sensing puzzle, and this could potentially help us understand the mechanisms behind a human cancer.

This study was initially intended to identify yeast genes that are responsible for the degradation of cysteine to H_2S. Instead, the main group of genes affecting H_2S formation from cysteine identified here are those involved in regulating cysteine uptake. Previous researches on varietal thiols have focused mainly on the transport of cysteinylated and glutathionylated thiol precursors (Cordente, Capone and Curtin 2015; Santiago and Gardner 2015b). Deletion of yeast *GAP1* (general amino acid permease gene) has been shown to reduce production of 3MH and 3MHA significantly in synthetic media, likely because it may involve in the uptake of S-3-(hexan-1-ol)-L-cysteine (Cys-3MH) (Subileau *et al.* 2008). Although it is obvious that yeast will not produce any significant amount of H_2S from cysteine, if it cannot take up the extracellular cysteine in the first place, cysteine uptake is seldom considered to have any significant impact on varietal thiol production as there are at least nine yeast cysteine transporters. But here we demonstrate that deleting genes involved in cysteine uptake reduced production of H_2S from cysteine significantly and therefore could potentially influence thiol production. To our best knowledge, this is the first time that Agp1p, Gnp1p and Mup1p (in BY4743) were indirectly shown to be the main cysteine transporters in a grape juice-like medium supplemented with high concentrations of cysteine, and that the regulation of cysteine uptake involves both the SPS-sensing and TOR pathways.

To successfully complete wine fermentation, wine yeast strains are required to respond to the rapidly changing fermentation conditions, sense and uptake the nutrients swiftly when supplemented. The polymorphisms of *AGP1* gene in wine yeasts cause differences in amino acid utilisation (Jara *et al.* 2014). In addition, polymorphisms in the promoter region of *MUP1* were observed for BY4716 and RM11 (a vineyard isolate) (Fehrmann *et al.* 2013). The new set of genes identified here would therefore be useful as markers to screen and breed yeast strains that are capable of taking up supplemented cysteine efficiently and therefore preserve (enhance) tropical fruity aromas. Most of the deletants that produced less H_2S from cysteine identified here could still ferment at similar rates as the wild type (except for Δ*lst4* and Δ*lst7*). Wort can contain excess amounts of cysteine (up to 35 mg L^{-1}) (Lawrence and Cole 1972). Therefore, these genes could be the alternative targets for reducing H_2S from cysteine, which might be desired for the brewing industry (Duan *et al.* 2004).

SUPPLEMENTARY DATA

Supplementary data are available at *FEMSYR* online.

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Conflict of interest. None declared.

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Supplementary Material

Supplementary Table S1: PCR primers and sequences

Name	**Sequence (5'–3')**	**Use**
Del-*lst4*-F	CCAGTACCATTTGCCTGTTAAAACTGT CTTGTGTGTGGCCTTGTAGAGAAGGT GAAGAGGGAGAGTTTAT**TTTAGCTTG CCTTGTCCCCGCCGGGT**	Primer pair of Del-*lst4*-R: deletion of *LST4* using the pAG32 plasmid (hygromycin B resistance).
Del-*lst4*-R	CATATATATAAAAGAAAAAAATATCGT ACACCTCTTAGGTAACTTGGAATATAT TAAACATGTAAAGAAGGAGAAAACA **TCGACACTGGATGGCGGCGTTAGTA TCG**	See above
Del-*lst7*-F	TAAAGTCCAACTAACTAGCACCTCCAT AAAATAAGTTAGCGTTTAAATGGTTGA TGAAATAGATGCATAGA**TTTAGCTTG CCTTGTCCCCGCCGGGT**	Primer pair of Del-*lst7*-R: deletion of *LST7* using the Pag32 plasmid (hygromycin B resistance).
Del-*lst7*-R	GTTCCAACAATTTTCCATGCCGTAATT TCATCGGTTATTAGGTTACCAGCATTTA TCTCAGTTTATTTGAG**TCGACACTGG ATGGCGGCGTTAGTATCG**	See above
Hph-I-F	TGCTGATCCCCATGTGTATCACTGGCA AACTG	Confirmation of deletion using the *HphMX* cassette
kanB	CTGCAGCGAGGAGCCGTAAT	Confirmation primer: Confirmation of deletion using the *KanMX* cassette
kanC	TGATTTTGATGACGAGCGTAAT	Primer pair of any Reverse Confirmation primer: Confirmation of deletion using the *KanMX* cassette
FC*bap2*	CTTCAACGGTAAATATGTCAGCAG	Primer pair of RC*bap2*: Confirmation of deletion using the *KanMX* cassette
RC*bap2*	AATATCCTTTCCATTACCCAAAGAG	See above
FC*bap3*	TTTAGTCATAATTGCCTTTTTCTGG	Primer pair of RC*bap3*: Confirmation of deletion using the *KanMX* cassette
RC*bap3*	ATCGATCATCTTGCCATGTAATAAT	See above
FC*agp1*	TGCTCCTTAGTAGTCCACAGTTCTT	Primer pair of RC*agp1*: Confirmation of deletion using the *KanMX* cassette
RC*agp1*	ATCAAACCTCTTCTGTTGCATTAAG	See above
FC*gnp1*	AAATCGGTTTTAGTGTTCGTATGTC	Primer pair of RC*gnp1*: Confirmation of deletion using the *KanMX* cassette
RC*gnp1*	TTCGATGAAAAGTGGGAAATAATAA	See above

FC*tat1*	AAACTTCACATTATCTTGACAAGGC	Primer pair of RC*tat1*: Confirmation of deletion using the *KanMX* cassette
RC*tat1*	TTTTCTTGGCACATTTACACACTTA	See above
FC*tat2*	GGTGTATCGTTAAATGGTACGTAGG	Primer pair of RC*tat2*: Confirmation of deletion using the *KanMX* cassette
RC*tat2*	TAAATTACAGTCTTGCGCCTTAAAC	See above
FC*gap1*	GTTAAATGTCAGTTTGGATGCTTTT	Primer pair of RC*gap1*: Confirmation of deletion using the *KanMX* cassette
RC*gap1*	GGGAAATCATATTGATTGATTGAAG	See above
FC*yct1*	CAATAGTGTTCGAAGTTCTTCCATT	Primer pair of RC*yct1*: Confirmation of deletion using the *KanMX* cassette
RC*yct1*	AGGCTAGAGGGGTTACTAGTCTCTG	See above
FC*mup1*	CCGTGTAGGGTTTTGTATAACAGAT	Primer pair of RC*mup1*: Confirmation of deletion using the *KanMX* cassette
RC*mup1*	AGGGTAAGGGAGCAATAAAGAACTA	See above
FC*stp1*	TGATTAAACATCAGACCATTGAGAA	Primer pair of RC*stp1*: Confirmation of deletion using the KanMX cassette
RC*stp1*	AAAAAGAAATCACAAACGTCAAAAG	See above
FC*hem25*	TACGCTGAATGGATACAAACACTAA	Primer pair of RC*hem25*: Confirmation of deletion using the KanMX cassette
RC*hem25*	CTAACAGATTTGACCTTCTCAATGG	See above
MAT	AGTCACATCAAGATCGTTTATGG	Primer pair of a and α-sextype primer to determine mating type (Huxley *et al.* 1990)
MAT-a	ACTCCACTTCAAGTAAGAGTTTG	See above (Huxley *et al.* 1990)
MAT-α	GCACGGAATATGGGACTACTTCG	See above (Huxley *et al.* 1990)
FC*met17*	CATCCTCATGAAAACTGTGTAACAT	Primer pair of RC*met17*: Confirmation of deletion using the *KanMX* cassette
RC*met17*	CCTCTTTTGTAACTTGGTCCTACAA	See above
FC*met5*	TTCATCACGTGCGTATTATCTCTTA	Primer pair of RC*met17*: Confirmation of deletion using the *KanMX* cassette
RC*met5*	TTTATTCTTCACCTCGTTTTCATTC	See above
FC*qdr2*	GGTCGTAAGACGGAGGTAATAATTT	Primer pair of RC*met17*: Confirmation of deletion

		using the *KanMX* cassette
RC*qdr2*	AAGAAACTCCAAGATCAGAAGATGA	See above

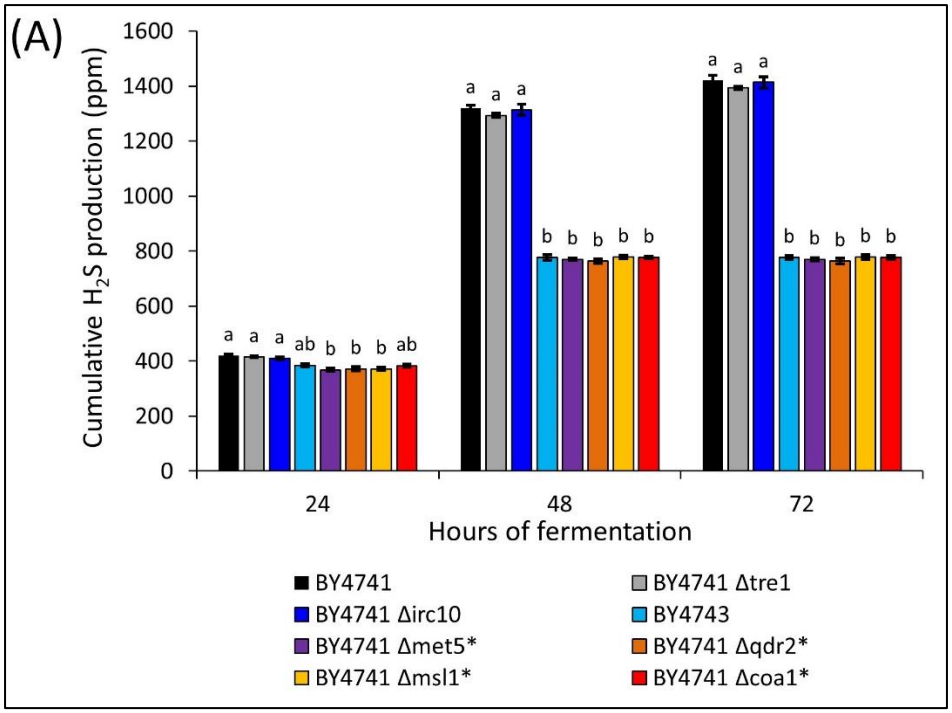
[a] PCR amplicons were confirmed with primer pairs (gene specific primer (forward) and Kan B, or gene specific primer (reverse) and KanC).

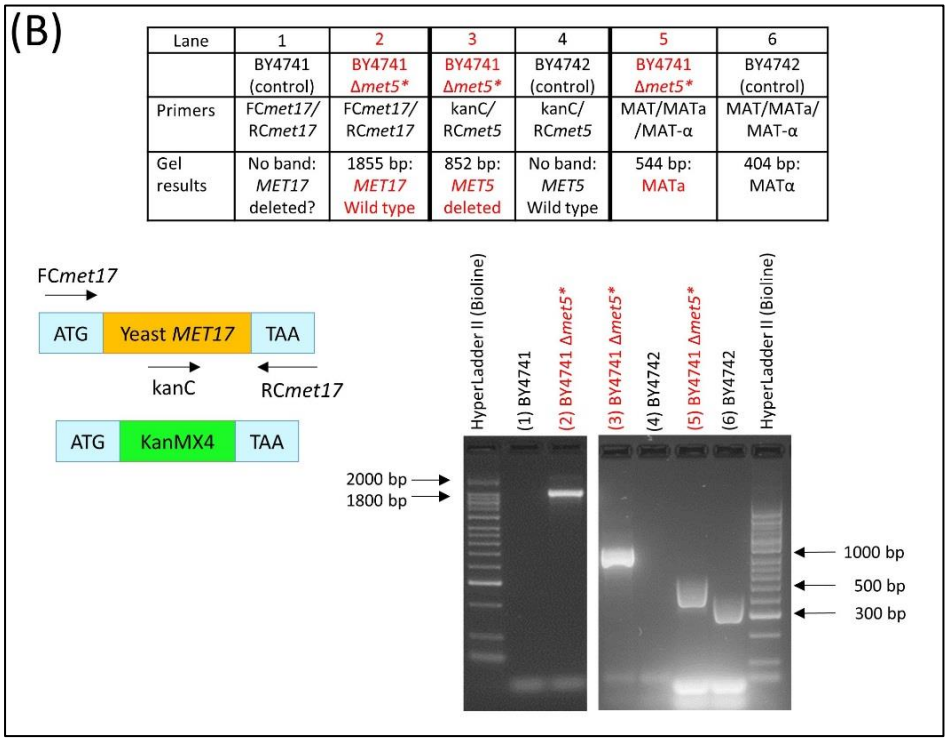
[b] KanB and KanC primers designed from Yeast Deletion Project (www-sequence.stanford.edu/group/yeast_deletion_project/deletions3.html)

[c] Bold font denotes sequence (positions 77 to 92 of *TEF1* promoter and 1245 to 1271 of terminator in plasmids pAG32 (Goldstein and McCusker 1999).

[d] *EcoRI* and *XhoI* restriction sites are underlined.

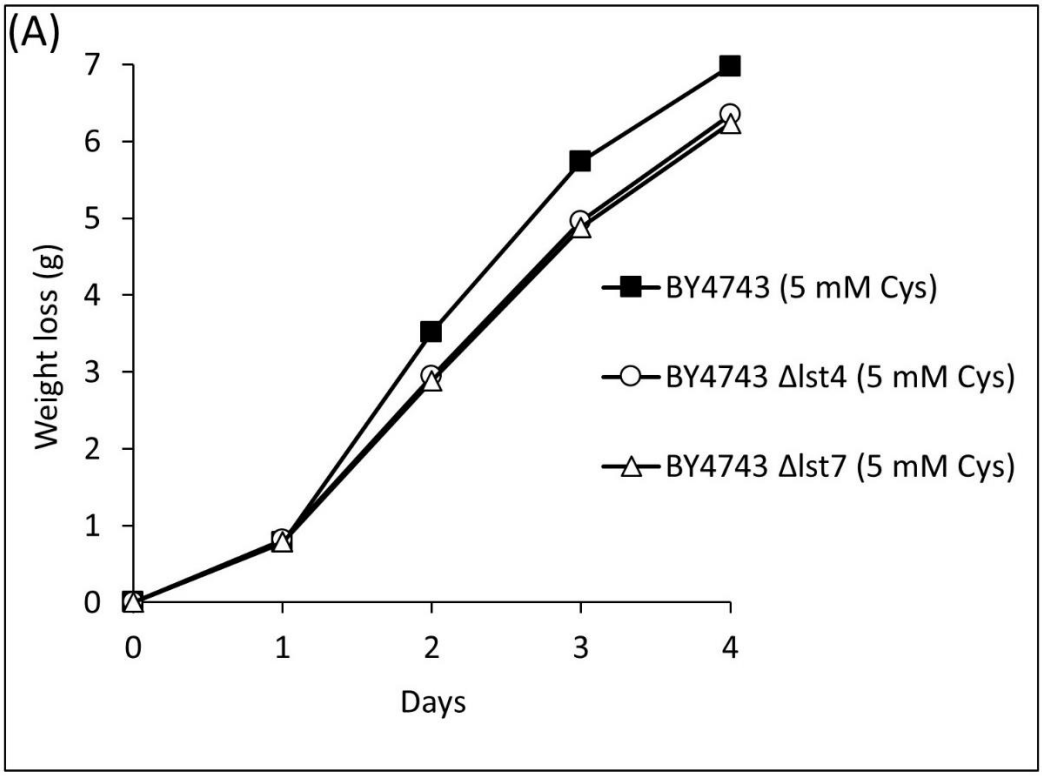
[e] MAT, MAT-a and MAT-α primers were adapted from (Huxley C, Green ED, Dunham I. Rapid assessment of *S. cerevisiae* mating type by PCR. *Trends Genet* 1990;6:236)





Lane	1	2	3	4	5	6
	BY4741 (control)	BY4741 Δ*met5**	BY4741 Δ*met5**	BY4742 (control)	BY4741 Δ*met5**	BY4742 (control)
Primers	FC*met17*/ RC*met17*	FC*met17*/ RC*met17*	kanC/ RC*met5*	kanC/ RC*met5*	MAT/MATa /MAT-α	MAT/MATa/ MAT-α
Gel results	No band: *MET17* deleted?	1855 bp: *MET17* Wild type	852 bp: *MET5* deleted	No band: *MET5* Wild type	544 bp: MATa	404 bp: MATα

Figure S1. (A) Cumulative H_2S production from the BY4741 and its candidate deletants identified from the screening. Fermentations were conducted in 100 mL of non-sulfate CDGJM and 0.15 mM methionine plus or minus 5 mM cysteine at 28 °C with shaking at 100 rpm. H_2S was measured by lead acetate H_2S detector tubes (4H: 1–2000 ppm; GASTEC, Japan) and the mean H_2S released is shown. Error bars indicate SEM. Samples not connected by the same letter are significantly different (ANOVA, Tukey's HSD). **(B)** Gel electrophoresis image analysis for *MET17*, *MET5* gene deletion and mating type in BY4741 Δ*met5** strain. The asterisk indicates that BY4741 Δ*met5** in our BY4741 deletion library is *MET17* wild type.



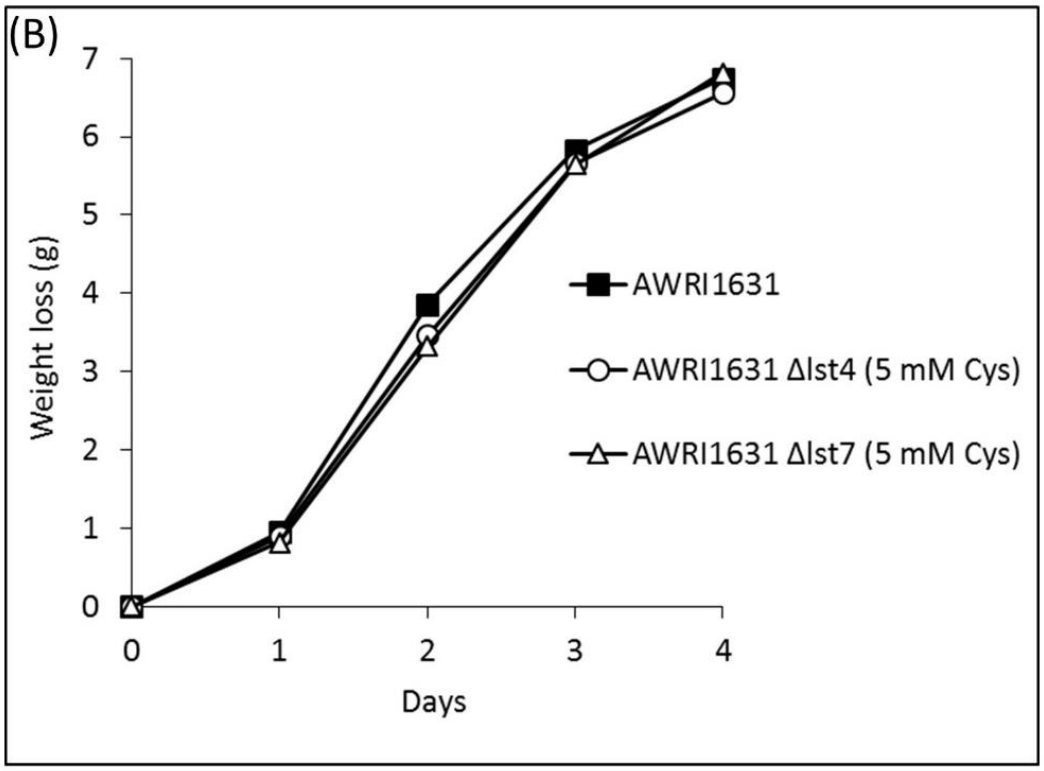


Figure S2. (A) Fermentation kinetics of the BY4743 and its *LST* gene deletants **(B)** AWRI1631 and its *LST* gene deletants in 100 mL of non-sulfate CDGJM plus 0.15 mM methionine and 5 mM cysteine at 28 °C with shaking at 100 rpm. Data points represent mean values of triplicate fermentations ± standard error of the mean (*SEM*). NB error bars are too small to extend beyond the symbol plot.

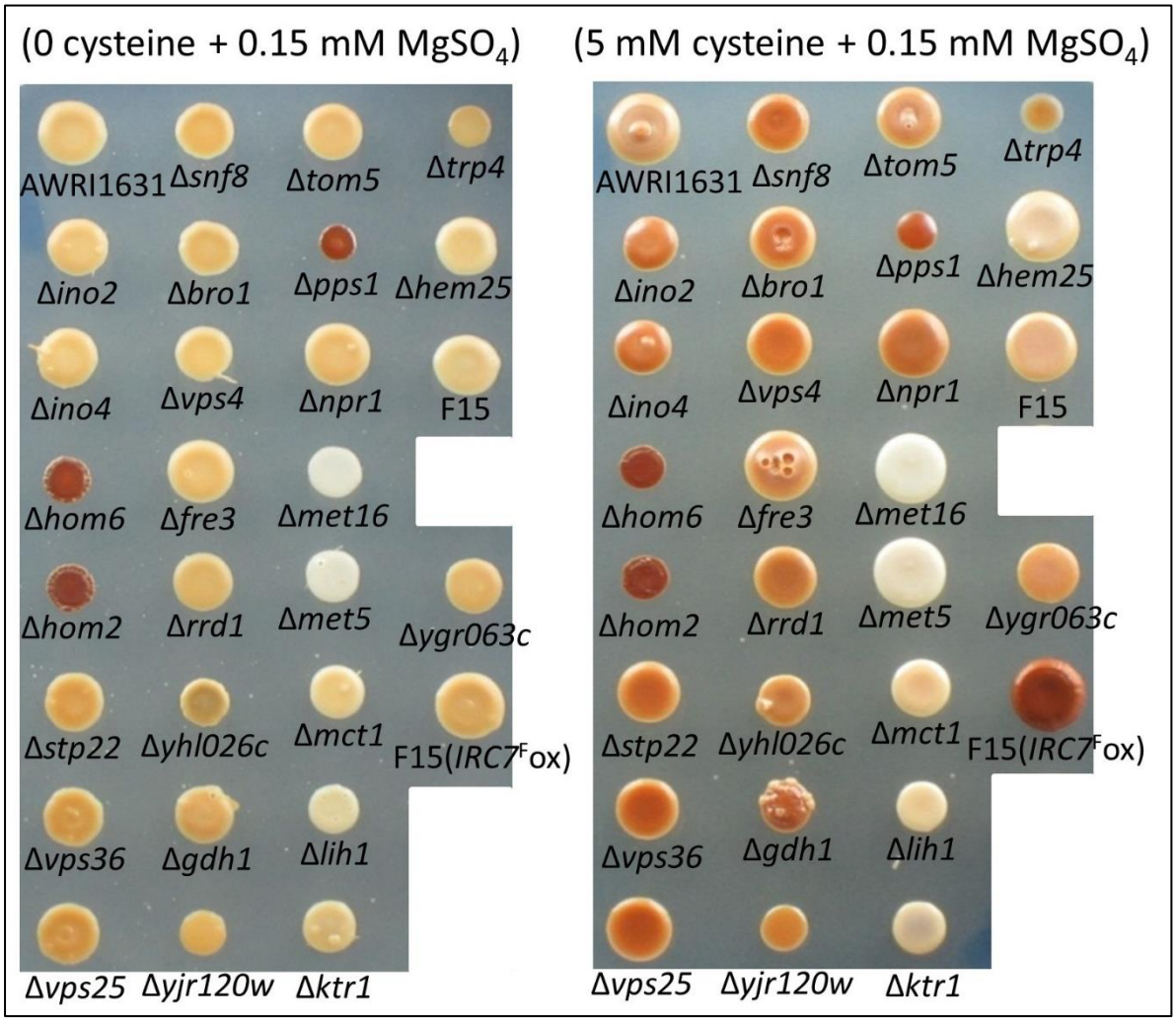


Figure S3. Colony colours of AWRI1631 deletants on non-sulfate CDGJM agar + Bi + 0.15 mM $MgSO_4$ ± 5 mM cysteine + no methionine. Images were taken after 96 hr incubation at 28 °C. The known high-H_2S producer from cysteine, F15 (*IRC7*^{F}ox) was used as positive control.

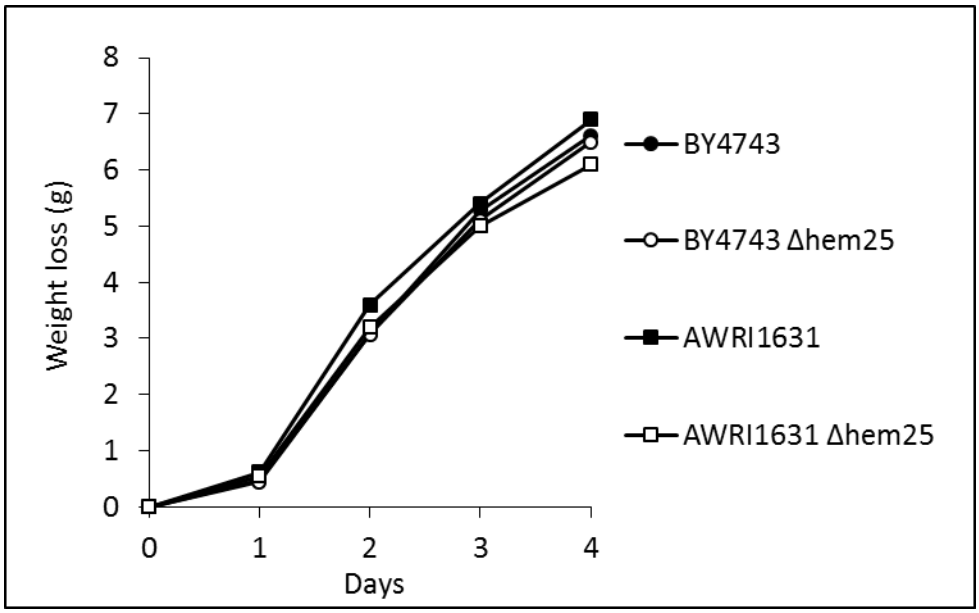


Figure S4. Fermentation kinetics of the wild types and the Δ*hem25* deletants**.** Fermentations were performed in 100 mL of non-sulfate CDGJM plus 5 mM cysteine and 0.15 mM methionine at 28 °C with shaking at 100 rpm. H_2S was measured by lead acetate H_2S detector tubes (4H: 1–2000 ppm; GASTEC, Japan) and the mean H_2S released is shown. Error bars indicate SEM. NB error bars are too small to extend beyond the symbol plot.

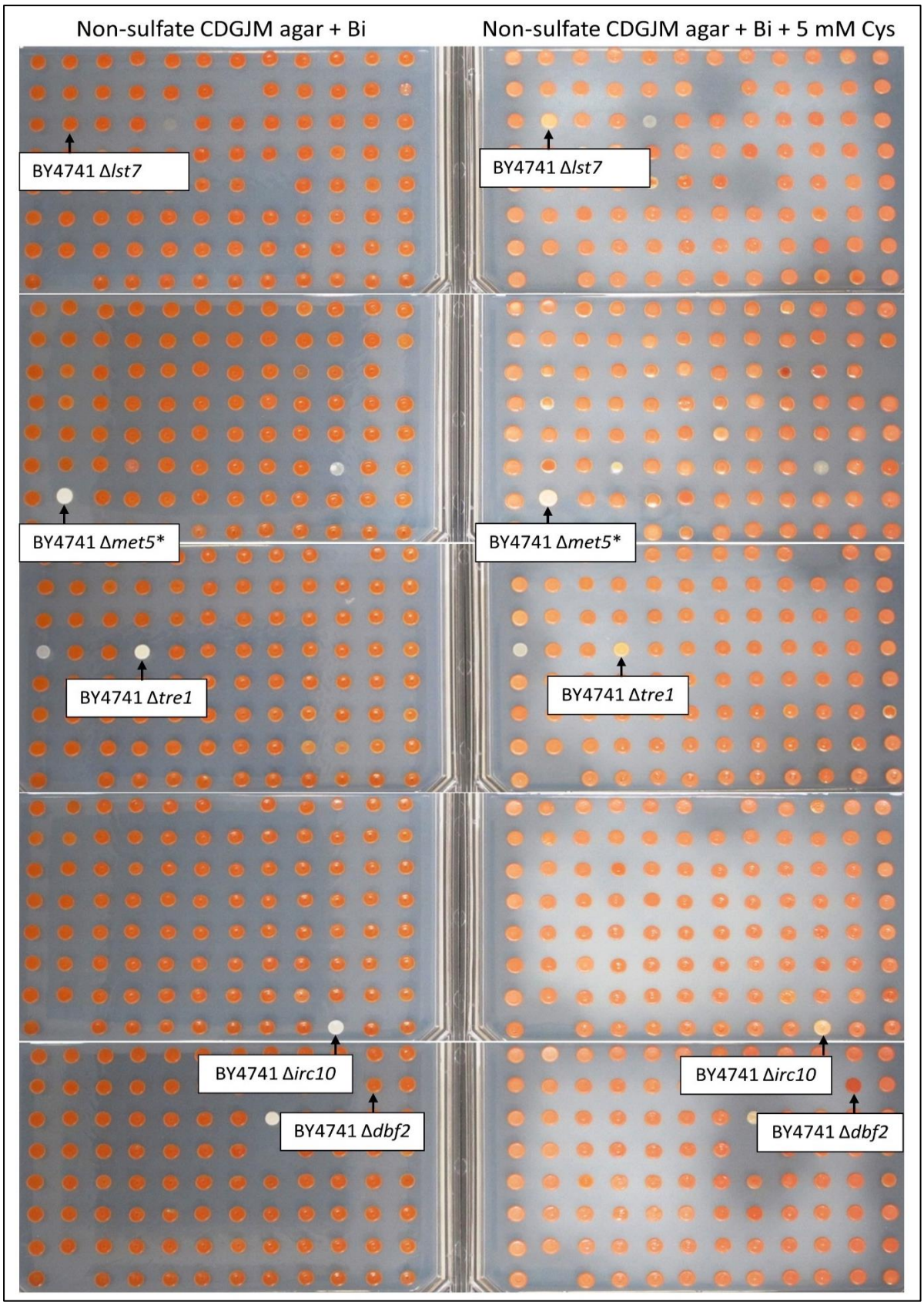


Figure S5. Colony colours of BY4741 deletants on non-sulfate CDGJM agar + Bi + 0.15 mM methionine ± 5 mM cysteine. Images were taken after 96 h incubation at 28 °C. Deletants denoted by an asterisk indicated that the *MET17* in BY4741 was not deleted within the library.

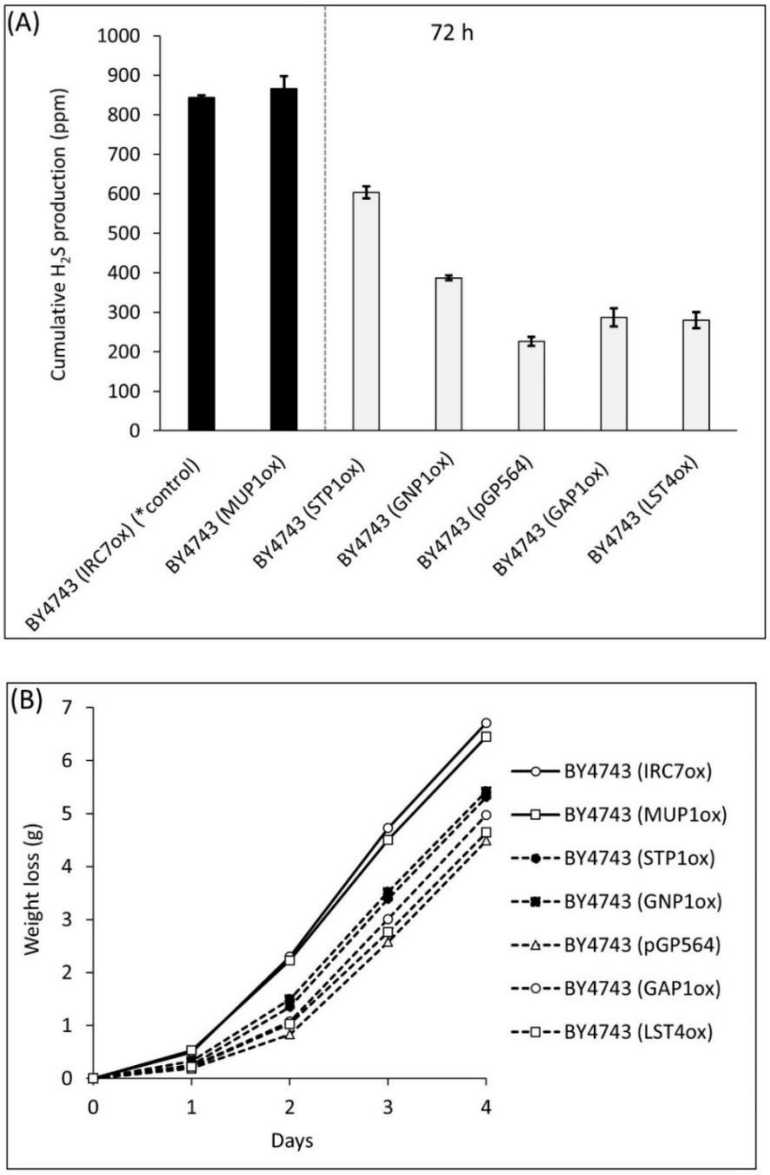


Figure S6. (A) Cumulative H_2S production (**B)** Fermentation kinetics of the BY4743 (pGP564) and the strains overexpressing genes involved in regulating cysteine uptake. Fermentations were performed in 100 mL of non-sulfate CDGJM + 5 mM cysteine + 0.15 mM methionine (with elevated amounts of 200 mg L^{-1} histidine, 100 mg L^{-1}uracil but without leucine) at 28 °C with shaking at 100 rpm. H_2S was measured by lead acetate H_2S detector tubes (4H: 1–2000 ppm; GASTEC, Japan) and the mean H_2S released is shown. Error bars indicate SEM. Most of the overexpression strains did not ferment well and because fermentation rates could affect H_2S production (Park 2008), we only compared H_2S production between strains with similar fermentation kinetics. BY4743 (*IRC7*ox) (*control) was used as control as it had similar fermentation kinetics as BY4743 (*MUP1*ox) and there was no significant differences in H_2S production between BY4743 (*IRC7*ox) and BY4743 (*MUP1*ox) ($P > 0.05$, two-tailed Student's *t* test) (The overexpressing strains that fermented relatively slower were not included in the statistical analysis, separated by dotted lines). NB error bars are too small to extend beyond the symbol plot. BY4743 (*IRC7*ox) should not increase H_2S from cysteine as its Irc7p has no *ß*-lyase activity (Santiago and Gardner 2015a). This experiment mainly served as a quick screen.

Chapter 5

H_2S, varietal thiols and polysulfides

5.1 Introduction

Varietal thiols 3-mercaptohexan-1-ol (3MH) and 3-mercaptohexyl acetate (3MHA) are the key tropical aroma compounds in many white and red wines and have very low detection thresholds of 60 ng L^{-1} (3MH, grapefruit) and 4 ng L^{-1} (3MHA, passion fruit) (Tominaga *et al.* 1998; Tominaga *et al.* 2000; Ferreira *et al.* 2002; Dubourdieu *et al.* 2006). It has been proposed that an early burst of hydrogen sulfide (H_2S) produced by the yeast *Saccharomyces cerevisiae* from cysteine during fermentation could contribute to elevated production of 3MH and 3MHA (Winter *et al*. 2011). Furthermore, adding H_2S to grape juice prior to fermentation has been shown to significantly increase concentrations of 3MH/A (Harsch *et al.* 2013). Together, these findings suggest that the tropical fruity wine styles that appeal to many consumers (Lund *et al.* 2009; Capone *et al.* 2017) could potentially be enhanced through modulating H_2S production from cysteine by yeast.

The yeast *TUM1* has been demonstrated to play a critical role in the formation of H_2S from cysteine and overexpression of *TUM1* leads to increased production of H_2S in chemically defined grape juice medium (Chapter 2). This chapter reports firstly on the evaluation of the *TUM1* overexpression strains with regards to their potential industrial application for enhancing thiol aromas in grape juice. Secondly, whether sulfane sulfurs are involved in cysteine catabolism in *Saccharomyces cerevisiae*.

Sulfane sulfurs such as cysteine persulfide (CysSSH) and polysulfides (S_x^{2-}; $x > 2$) have attracted widespread research interest because of their potential roles in signalling (Ida *et al.* 2014; Olson and Straub 2016) and the sensory quality of wine (Starkenmann *et al.* 2016). However, despite polysulfides having been detected in the fungus *Aspergillus nidulans* using the cold cyanolysis method (Wróbel *et al.* 2009), their existence in *S. cerevisiae* remains largely unexplored.

To investigate the role of polysulfides in cysteine catabolism, a fluorescent probe, SSP4 for the detection of sulfane sulfur e.g. persulfide and polysulfides (Chen *et al.* 2013) was employed to examine the presence of polysulfides in yeast undergoing fermentation in a high-cysteine, grape juice like medium. In addition, a high-performance liquid chromatography (HPLC)-based method for the detection of polysulfide was investigated (Rohwerder and Sand 2003).

5.2 Materials and methods

5.2.1 Yeast strains and culture

The yeast strains used are described in Table 5.1. Yeast strains were either grown overnight at 28 °C on YPD plates (10 g L^{-1} yeast extract, 20 g L^{-1} peptone, 20 g L^{-1} glucose and 20 g L^{-1} agar) or starter medium (non-sulfate Chemically Defined Grape Juice Medium (CDGJM) containing 10 g L^{-1} each of glucose and fructose and 0.15 mM methionine; Chapter 2).

Table 5.1. Yeast strains used in this study.

Strain	Genotype, phenotype and comments	Origin
BY4743	*MATa/α, his3-Δ1/his3-Δ1, leu2-Δ0/leu2-Δ0, LYS2/lys2-Δ0, met15-Δ0/MET15, ura3-Δ0/ura3-Δ0*	Euroscarf
Oenoferm M2 ura3Δ (pJC1)	Wine strain, MATa, ura3Δ0, ho::HphMX (pJC1)	Chapter 2
Oenoferm M2 ura3Δ (TUM1ox)	Wine strain, MATa, ura3Δ0, ho::HphMX (pJC1+TUM1)	Chapter 2
Lalvin L2056 ura3Δ (pJC1)	Wine strain, ura3Δ::KanMX (pJC1)	Chapter 2
Lalvin L2056 ura3Δ (TUM1ox)	Wine strain, ura3Δ::KanMX (pJC1+TUM1)	Chapter 2
F15 (*IRC7*^{F}ox)	*ho::*P_{PGK}-*IRC7*^{F} in F15-h(α)	Roncoroni *et al*. (2011)

ox; denotes over-expression. pJC1 plasmid gifted from Professor Alan T. Bakalinsky, Oregon State University, USA (Crous, Pretorius and Van Zyl 1995; Martin *et al.* 2003).

5.2.2 Fermentations and H_2S quantification

The yeast starter culture was centrifuged, washed and resuspended in sterile water to inoculate at 2.5×10^6 cells L^{-1} either 140 mL of non-sulfate CDGJM containing 0.04 mM (5 mg L^{-1}) or 5 mM (605.8 mg L^{-1}) cysteine, 0.15 mM methionine and 1 mg L^{-1} (*E*)-2-hexenal or Riesling juice supplemented with or without 5 mM cysteine and 1 mg L^{-1} (*E*)-2-hexenal.

The low concentration of 5 mg L^{-1} cysteine was added to reflect Marlborough Sauvignon Blanc juice (Harsch *et al*. 2010) whilst a high amount of cysteine (5 mM; 605.8 mg L^{-1}) was added to induce significant H_2S production by yeast (Chapter 2). The concentration of 1 mg L^{-1} (*E*)-2-hexenal was similar to previous studies (1.88 mg L^{-1}, Schneider *et al.* 2006; 1.5 mg L^{-1}, Harsch *et al*. 2013)

Riesling juice (~21 mg L^{-1} free SO_2), from Waite Campus, The University of Adelaide (2016 vintage), was kindly gifted by Dr Tommaso Liccioli Watson. The juice was filter-sterilised (0.2 μm; 11407-47; Sartorius, Australia). Fermentations were conducted in triplicate in 250 mL flasks with shaking at 100 rpm (28 °C). H_2S was measured by lead acetate H_2S detector tubes (4H: 1–2000 ppm; GASTEC, Japan) that tightly fitted into the glass fermentation airlock (Park 2008). Fermentation progress was monitored daily as weight loss due to CO_2 evolution (Bely, Sablayrolles and Barre 1990). Fermentations were considered finished when weight loss was ≤0.1 g per 24 h. The finished ferments were centrifuged at 6,000 x g for 10 min to separate yeast cells and solids before being stored in 120 mL sterile containers (P9246SU; Techno-Plas, Australia) at -20 °C. The finished ferments were shipped to the Wine Science Programme, University of Auckland, New Zealand for analysis of varietal thiols and reductive sulfur compounds.

5.2.3 Varietal thiol analysis

Varietal thiols 3MH and 3MHA were analysed by Dr Mandy Herbst-Johnstone (University of Auckland, New Zealand) using the ethyl propiolate (ETP) derivatisation method followed by solid phase extraction/gas chromatography-mass spectrometry (SPE/GC-MS) as described in Parish *et al.* (2017) with slight modifications to the original protocol (Herbst-Johnstone *et al.* 2013). Thiols were separated using an Agilent HP-INNOWax capillary column (60 m × 0.250 mm ID, 0.25 μm film thickness). The oven program was as follow: 150 °C; 2 min, then increased to 250 °C in 10 °C per min increments and held for 20 min. The

temperature of the interface line was set at 250 °C. The ion source, operating in electron impact mode at 70 eV, was held at 230 °C. The quadrupole temperature was set at 150 °C. Calibration curves for the varietal thiols with 10 concentration levels were obtained in triplicate by adding increasing amounts of 3MH and 3MHA to a model white wine (ultra-pure water containing 12% ethanol, 5 g L^{-1} tartaric acid, pH 3.2).

5.2.4 Reductive sulfur compounds analysis

Reductive sulfur compounds were analysed by Dr Mandy Herbst-Johnstone and Dr Matias Kinzurik (University of Auckland, New Zealand) using headspace solid phase micro-extraction coupled with gas chromatography mass spectrometry (HS-SPME/GC–MS; Nguyen, Nicolau and Kilmartin 2012; Kinzurik *et al.* 2015). It should be noted that only one sample (n = 1) of the triplicate ferments was analysed.

5.2.5 Polysulfide detection by the fluorescent probe SSP4

The fluorescent Sulfane Sulfur Probe 4 or SSP4 (3', 6'-di (O-thiosalicyl) fluorescein) was kindly supplied by Professor Ming Xian (Washington State University, USA). SSP4 reacts with sulfane sulfurs to release fluorescein (strong green fluorescence), which can be used for the highly sensitive detection and imaging of these compounds within cells.

The protocol used for detecting intracellular polysulfides was adapted from Chen *et al.* (2013) with minor modifications. Yeast cells were grown overnight as 25 mL starter cultures in 2% sugar, non-sulfate CDGJM plus 0.15 mM methionine (Day 0). The cultures (0.2 mL) were used to inoculate 100 mL non-sulfate CDGJM ± 5 mM cysteine + 0.15 mM methionine and grown for 24 h (Day 1). Cells (~300 µL) were harvested and washed once with 1x phosphate-buffered saline (PBS). The washed cells were incubated with ~50 µL of 20 µM SSP4 in dimethyl sulfoxide (DMSO) containing 500 µM cetyltrimethylammonium bromide (CTAB) for 20 min at room temperature and afterwards, washed once with 1x PBS. Fluorescence images were taken using a Nikon Eclipse 50i microscope equipped with the DS 2MBWc digital camera. Fluorescence intensity of individual 50 µL SSP4-treated yeast samples was measured using a Tecan Infinite M200 microplate reader with excitation wavelength at 482 nm and emission at 518 nm (Ida *et al.* 2014). Lime sulfur insecticide/fungicide concentrate

containing 20% calcium polysulfides (Ausgro Technologies Pty Ltd, Australia) was diluted 500-fold and used to confirm the reaction between SSP4 and polysulfides, measured as increased fluorescence attributed to fluorescein.

FACS analysis was performed with assistance from Nick van Holst (University of Adelaide). The fluorescence intensity of the SSP4 (λ_{ex} = 482 nm, λ_{em} = 518 nm) treated cells was analysed using a FACSCalibur E0250 instrument (FL1, 530/30 nm bandpass filter; BD Biosciences, Australia) and data were analysed using FCS Express (De Novo Software, USA). All measurements were performed in triplicate.

5.2.6 Polysulfide stock solution

A 0.05 M polysulfide stock solution was prepared as previously described by Ikeda *et al.* (1972). Approximately 12 g of sodium sulfide nonahydrate (Sigma Aldrich) and 1.6 g of elemental sulfur powder (Sigma Aldrich) were dissolved by stirring for ~1 h in 100 mL of oxygen-free water (Schlenk line technique). The stock solution was stored in 50 mL centrifuge tubes at ~10 °C.

5.2.7 Glutathione polysulfide stock solution

A glutathione polysulfide stock solution was prepared according to the protocol described by Rohwerder and Sand (2003) with assistance from Nina Duhamel (University of Auckland). Approximately 100 mM GSH (Sigma Aldrich) and 500 mM elemental sulfur powder (Sigma Aldrich) were dissolved in oxygen-free water (pH adjusted to 7.2 with KOH) by stirring for ~2 days under anaerobic conditions. The pH of the stock solution was reduced to 5.0 by adding HCl and stored in 50 mL centrifuge tubes at ~10 °C.

5.2.8 Analyses of the polysulfide and glutathione polysulfide stock solutions by HPLC

The polysulfide and glutathione polysulfide stock solutions were analysed by HPLC with assistance from Dr Mandy Herbst-Johnstone (University of Auckland) using an Agilent 1100 HPLC. The method was based on the protocol described by Rohwerder and Sand (2003) with modifications. Briefly, samples were filtered through 0.2 μm syringe filters and 20 μL of the filtrate was injected onto a Brownlee Aquapore RP-300 (C8) cartridge column (220 × 4.6 mm, 7 μm; Perkin Elmer, USA). Elution was performed with 30 mM phosphate buffer at pH 2.6 with a flow rate of 1 mL min^{-1} (Olejar, Fedrizzi and Kilmartin 2015). The polysulfide

fractions corresponding to the chromatographic peaks were collected manually and analysed using nuclear magnetic resonance (NMR) by Nina Duhamel (University of Auckland).

5.2.9 Data analysis

The mean, standard error of the mean (SEM) and *t* test (two samples assuming unequal variances) were performed using Microsoft Excel 2013 (Microsoft, Redmond, Washington, USA). Analysis of variance (ANOVA) and Tukey's honestly significant difference test were conducted using JMP software (SAS Institute, Cary, NC, USA). Statistical significance was set at the confidence level of 95%.

5.3 Results and discussion

5.3.1 Overexpression of *TUM1* elevated production of 3MH and 3MHA in high-cysteine, (*E*)-2-hexenal-supplemented, non-sulfate CDGJM

To determine whether overexpression of *TUM1* could elevate production of 3MH/A, fermentations were undertaken in 140 mL of non-sulfate CDGJM containing 0.04 mM (5 mg L^{-1}) or 5 mM (605.8 mg L^{-1}) cysteine, 0.15 mM methionine and 1 mg L^{-1} (*E*)-2-hexenal using either Oenoferm M2 ura3Δ (pJC1) or Oenoferm M2 ura3Δ (*TUM1*ox). Oenoferm M2 was selected because it generates large amounts of H_2S from cysteine (Chapter 2), whilst less H_2S comes from the sulfate assimilation pathway (Huang, Roncoroni and Gardner 2014).

Consistent with the work reported in Chapter 2, Oenoferm M2 ura3Δ (*TUM1*ox) produced more H_2S in high-cysteine CDGJM and no H_2S was detected when only a small amount of cysteine (5 mg L^{-1}) was present (Fig. 5.1A), indicating that the effect of *TUM1* on H_2S is cysteine-specific. Fermentation with Oenoferm M2 ura3Δ (*TUM1*ox) resulted in increased concentrations of 3MH (1115.3 vs 873.3 ng L^{-1}; Fig. 5.1B) and 3MHA (107.3 vs 27.9 ng L^{-1}; Fig. 5.1C) in high-cysteine CDGJM. These results confirm that an increase in H_2S production at the early stage of fermentation does contribute to the formation of 3MH/A (Winter *et al.* 2011; Harsch *et al.* 2013). It might also partly explain why the high-H_2S producers, the *IRC7* overexpressing strain (Roncoroni *et al.* 2011) and Δ*met17* (Harsch and Gardner 2013), were observed to increase 3MH/A concentrations in Sauvignon Blanc wine.

Whilst no H_2S could be detected (Fig. 5.1A), similar levels of 3MH were found in low-cysteine (5 mg L^{-1}) CDGJM fermented with either Oenoferm M2 ura3Δ (pJC1) or Oenoferm M2 ura3Δ (*TUM1*ox) (236.8 vs 239.1 ng L^{-1}; Fig. 5.1B), suggesting that overexpression of *TUM1* may not boost thiol aromas under the normal winemaking conditions as grape juice typically contains less than 20 mg L^{-1} of cysteine (Ugliano and Henschke 2009). Although commercial yeast nutrient products containing cysteine e.g. Laffort FreshArom, could be added during fermentation to preserve thiols (O'Kennedy 2013), excessive use of cysteine may result in the formation of other unpleasant sulfur compounds such as 2-mercapto-1-ethanol (poultry), dimethyl sulfide (cabbage) and 2-mercaptoethanol (farmyard) (Mestres, Busto and Guasch 2000; Ugliano and Henschke 2009). Therefore, further investigation into the optimal level of cysteine supplementation required to enhance fruity thiol aromas is worthwhile.

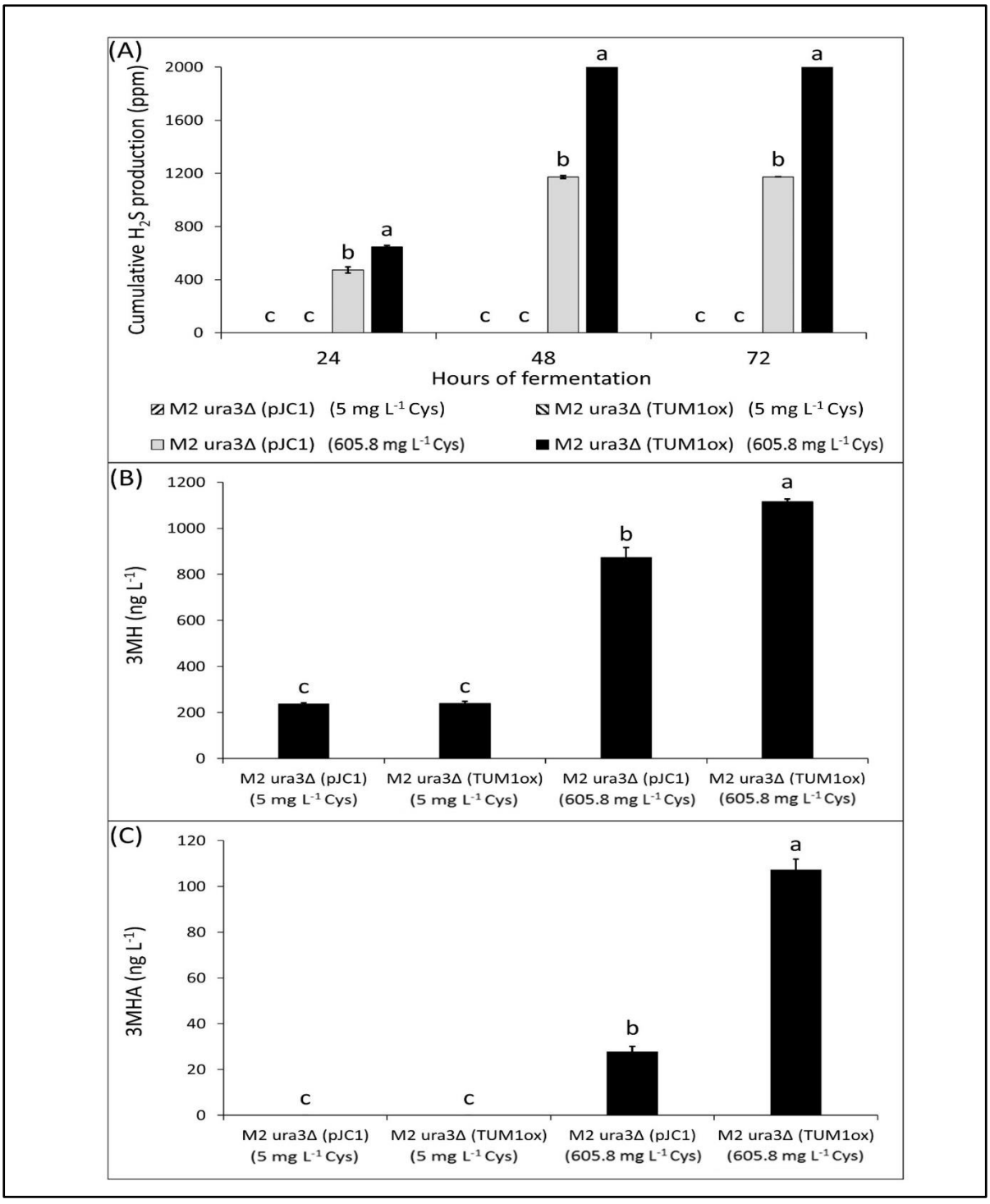


Figure 5.1. Production of **(A)** H_2S, **(B)** 3MH, **(C)** 3MHA by the wild type and *TUM1* overexpressing strain under two cysteine conditions. Triplicate fermentations were performed in 140 mL non-sulfate CDGJM plus 5 mg L^{-1} or 605.8 mg L^{-1} cysteine, 0.15 mM methionine and 1 mg L^{-1} (*E*)-2-hexenal, at 28 °C with shaking at 100 rpm. H_2S was measured by lead acetate H_2S detector tubes (4H: 1–2000 ppm; GASTEC, Japan). Error bars indicate SEM (*n* = 3). Samples not connected by the same letter are significantly different (ANOVA, Tukey's HSD).

5.3.2 Overexpression of *TUM1* may not increase 3MH/A production in Riesling juice

To investigate whether overexpression of *TUM1* can increase production of 3MH/A in grape juice, 140 mL of Riesling juice was fermented with either Lalvin L2056 ura3Δ (pJC1) or Lalvin L2056 ura3Δ (*TUM1*ox). Lalvin L2056 strain was chosen instead of Oenoferm M2 because the latter was unable to ferment the Riesling juice in the initial trials (data not shown). Oenoferm M2 is thought to be more sulfite sensitive (Roncoroni *et al.* 2013) and may not tolerate the free sulfite (~21 mg L^{-1}) in the juice.

While commercial wine strains differ in their abilities to produce 3MH/A, with most, including Lalvin L2056, capable of producing detectable amounts of 3MH/A in the Sauvignon Blanc fermentations (Swiegers *et al.* 2009). Interestingly, 3MH and 3MHA was not detectable in the Riesling juice fermentations (Table 5.2). It was suspected that some of the 3MH/A may have been lost because free SO_2 was not added to the finished ferments (Herbst-Johnstone, Nicolau and Kilmartin 2011) or the unexpected Customs delays (21 days) when sending to Auckland. Nevertheless, 3MH/A was detectable in fermentations supplemented with 5 mM cysteine. This could be due to the increased production of H_2S (Table 5.2) contributing to 3MH/A synthesis through the H_2S-C6 thiol pathway (Harsch *et al.* 2013). Alternatively, glutathione produced by yeast from cysteine could also protect thiols against oxidation (O'Kennedy 2013).

3MH/A concentrations were further increased ~2-fold upon addition of 1 mg L^{-1} (*E*)-2-hexenal to the 5 mM cysteine-supplemented Riesling juice (Table 5.2). These results demonstrated the important role of (*E*)-2-hexenal in aromatic thiol formation and supported the proposal that 3MH/A production could be enhanced by increasing concentrations of (*E*)-2-hexenal via viticulture or winemaking practices (Roland *et al.* 2010; Harsch *et al.* 2013). Normally, (*E*)-2-hexenal (ranging from a few to hundreds of micrograms per liter; Subileau *et al*. 2008) is rapidly lost during the initial stages of winemaking (Harsch *et al.* 2013), and is so not available to react with H_2S produced during fermentation. This may also partly explain why overexpression of *TUM1* did not increase 3MH/A concentrations although excess H_2S was produced during fermentation, when the juice was supplemented with 5 mM cysteine and 1 mg L^{-1} (*E*)-2-hexenal **(**Table 5.2).

Table 5.2. Effect of (*E*)-2-hexenal and cysteine supplementation on H_2S, 3MH and 3MHA production by the wild type and *TUM1* overexpressing strains in juice fermentations.

Strain and media	**H_2S (ppm)**	**3MH (ng L^{-1})**	**3MHA (ng L^{-1})**
Lalvin L2056 ura3Δ (pJC1) (Riesling juice)	1133 ± 64	nd	nd
Lalvin L2056 ura3Δ (*TUM1*ox) (Riesling juice)	1187 ± 12	nd	nd
Lalvin L2056 ura3Δ (pJC1) (Riesling juice + 1 mg L^{-1} (*E*)-2-hexenal)	1140 ± 53	nd	nd
Lalvin L2056 ura3Δ (*TUM1*ox) (Riesling juice + 1 mg L^{-1} (*E*)-2-hexenal)	1180 ± 0	nd	nd
Lalvin L2056 ura3Δ (pJC1) (Riesling juice + 5 mM cysteine)	>2000	5091 ± 138	1061 ± 31
Lalvin L2056 ura3Δ (*TUM1*ox) (Riesling juice + 5 mM cysteine)	>2000	4526 ± 73*	1056 ± 36
Lalvin L2056 ura3Δ (pJC1) (Riesling juice + 1 mg L^{-1} (*E*)-2-hexenal + 5 mM cysteine)	>2000	11308 ± 306	2422 ± 20
Lalvin L2056 ura3Δ (*TUM1*ox) (Riesling juice + 1 mg L^{-1} (*E*)-2-hexenal + 5 mM cysteine)	>2000	10759 ± 429	2318 ± 69

Fermentations were performed in 140 mL Riesling juice with and without additon of 1 mg L^{-1} (*E*)-2-hexenal and/or 5 mM cysteine. H_2S was measured by lead acetate H_2S detector tubes (4H: 1–2000 ppm; GASTEC, Japan) with maximum detection limit of 2000 ppm.
Data represent mean values of triplicate fermentations ± standard error of the mean (SEM).
***** Significant differences compared to Lalvin L2056 ura3Δ (pJC1) ($^*P < 0.05$, two-tailed Student's *t* test).
ox = overexpression. nd = not detected.

5.3.3 Overexpression of *TUM1* may increase reductive sulfur compounds in Riesling juice

Whilst H_2S generated from the sulfate assimilation pathway can lead to undesirable reductive sulfur compounds in the final wine (Kinzurik *et al.* 2016), this is not thought to be the case with H_2S production arising from rehydration nutrients (glutathione or cysteine) early in fermentation (Winter *et al.* 2011). Preliminary findings from this study suggest differently – whilst H_2S production did not differ between Lalvin L2056 ura3Δ (pJC1) and Lalvin L2056 ura3Δ (*TUM1*ox) during fermentation in Riesling juice (~1133 vs ~1187 ppm; Table 5.2), higher amounts of methanethiol, ethanethiol, carbon disulfide and methyl thioacetate were produced by the *TUM1* overexpressing strain (Table 5.3). Whilst these values are all below the aroma detection thresholds, it would be worthwhile investigating the effect of overexpression of *TUM1* upon reductive sulfur compound production in finished wine.

Table 5.3. Concentrations of reductive sulfur compounds produced by the wild type and *TUM1* overexpression strains in Riesling juice fermentations.

Reductive sulfur compound	**Threshold (μg L^{-1})**	**Odour descriptor**	**Lalvin L2056 ura3Δ (pJC1)**	**Lalvin L2056 ura3Δ (*TUM1*ox)**
Methanethiol	1.8 – 3.1[a]	rotten cabbage	0.38	0.90
Ethanethiol	1.1[a]	onion	0.04	0.09
Carbon disulfide	> 38[a]	rubber	0.03	0.05
Methyl thioacetate	50[a]	cheesy	3.89	6.36

[a] Detection threshold and descriptors were obtained from Siebert *et al.* (2010).
(*n* = 1); ox = overexpression

5.3.4 Polysulfides may be produced by *S. cerevisiae* during fermentation of high-cysteine, non-sulfate CDGJM

The fluorescent probe, SSP4 is used for the detection of sulfane sulfurs (Chen *et al.* 2013). Reaction of SSP4 with sulfane sulfurs (e.g. persulfides or polysulfides) results in increased fluorescence due to the release of fluorescein. SSP4 is specific for these compounds, and does not react with other sulfur compounds such as cysteine, glutathione and H_2S (Chen *et al.* 2013; Ida *et al.* 2014). SSP4 was tested for substrate specificity; with increased fluorescence being observed when added to a source of polysulfides – diluted lime sulfur concentrate (Fig. 5.2B).

To determine whether polysulfides are produced by *S. cerevisiae* during fermentation of high-cysteine, non-sulfate CDGJM, yeast cells were stained with SSP4 and examined under UV light using a Nikon Eclipse 50i microscope. Unexpectedly, the fluorescence from SSP4 was observed both in yeast cells from high-cysteine fermentations as well as non-supplemented ones (Fig. 5.3A). However, with a 10 msec exposure time, fluorescence was only observed in F15 (*IRC7*^{F}ox) cells from high-cysteine fermentations (Fig. 5.2A). This was consistent with the higher fluorescence intensity of SSP4-treated F15 (*IRC7*^{F}ox) cells (high-cysteine fermentations) compared to F15 (*IRC7*^{F}ox) from non-cysteine fermentations using a microplate reader (Fig. 5.2B).

However, unlike F15 (*IRC7*^{F}ox), there was no obvious difference in fluorescence intensity in BY4743 cells in the high-cysteine and non-supplemented fermentations (Fig. 5.3A and Fig. 5.3B). To investigate this further, SSP4 labelled yeast cells were analysed by flow cytometry with a FACSCalibur E0250 instrument (BD Biosciences, Australia). A 2.25-fold increase (120.55/53.51) in fluorescence was observed in SSP4 treated BY4743 cells, when cysteine was supplemented (Fig. 5.3C). This increase was less than the observed 4.99-fold increase (137.52/27.54) with F15 (*IRC7*^{F}ox) (Fig. 5.2C).

Collectively, these results suggested the possible existence of polysulfides in *S. cerevisiae* and that cysteine contributed to the formation of polysulfides. Similar observations are reported for *Aspergillus nidulans* (Wróbel *et al.* 2009). The 4.99-fold increase of fluorescence for F15 (*IRC7*^{F}ox) when cysteine was added (Fig. 5.2C) supported the proposal that Irc7^{F}p may play a crucial role in generating the sulfur donors, cysteine persulfide and glutathione polysulfides through cleavage of cystine (Santiago and Gardner 2015). The observed increase in SSP4- associated fluorescence in BY4743, which has an 38-bp deleted, non-functional ß-

lyase *IRC7* (Roncoroni *et al.* 2011), alludes to the involvement of other genes apart from the full-length *IRC7* in the formation of polysulfides. Further experiments to explore these genes e.g. *TUM1* (Chapter 2) in the formation of polysulfides would certainly be worthwhile.

The detection of SSP4-associated fluorescence in fermenting yeast cells in non-cysteine containing CDGJM, indicates that yeast either synthesize cysteine from the methionine within the medium through the transsulfuration pathway (Hansen and Johannesen 2000), or the existence of other polysulfide precursors in the non-sulfate CDGJM. The observed higher fluorescence in cells from the ferments (Day 1) compared to the starter cultures (Day 0) was also surprising (Fig. 5.2C and Fig. 5.3C). These observations suggest that polysulfides may have a role in sulfur signalling, and are induced during fermentation.

However, it should be emphasised that this is preliminary work and further studies using other detection methods are necessary to confirm these findings. This is because SSP4 not only detects polysulfides (R-S-S_n-S-R) but also persulfides (R-S-SH) (Chen *et al.* 2013), with the possibility that observed fluorescence responses are due to persulfides and not polysulfides. Comparison of multiple samples using fluorescence microscopy (with the exception of F15 (*IRC7*^{F}ox)) was made difficult with the observed rapid bleaching of the fluorescein green colour, even when mounted in glycerol or Vectashield mounting antifade medium (Vector Laboratories, USA) (data not shown). Measurement of the fluorescence intensities of the SSP4-treated yeast cultures using a microplate reader was problematic, with large variations observed in the replicates, which may be attributed to cell number variability in the SSP4-stained cells as well as pipetting error. To date, FACS analysis is the preferred method for analysis, as > 10,000 events per strain are acquired and analysed to minimise heterogeneity within the sample population. Nevertheless, better sample preparation procedures such as determining the optimal concentrations of SSP4 and CTAB, and sample temperature and processing time prior to analysis are necessary to improve the usefulness of this assay.

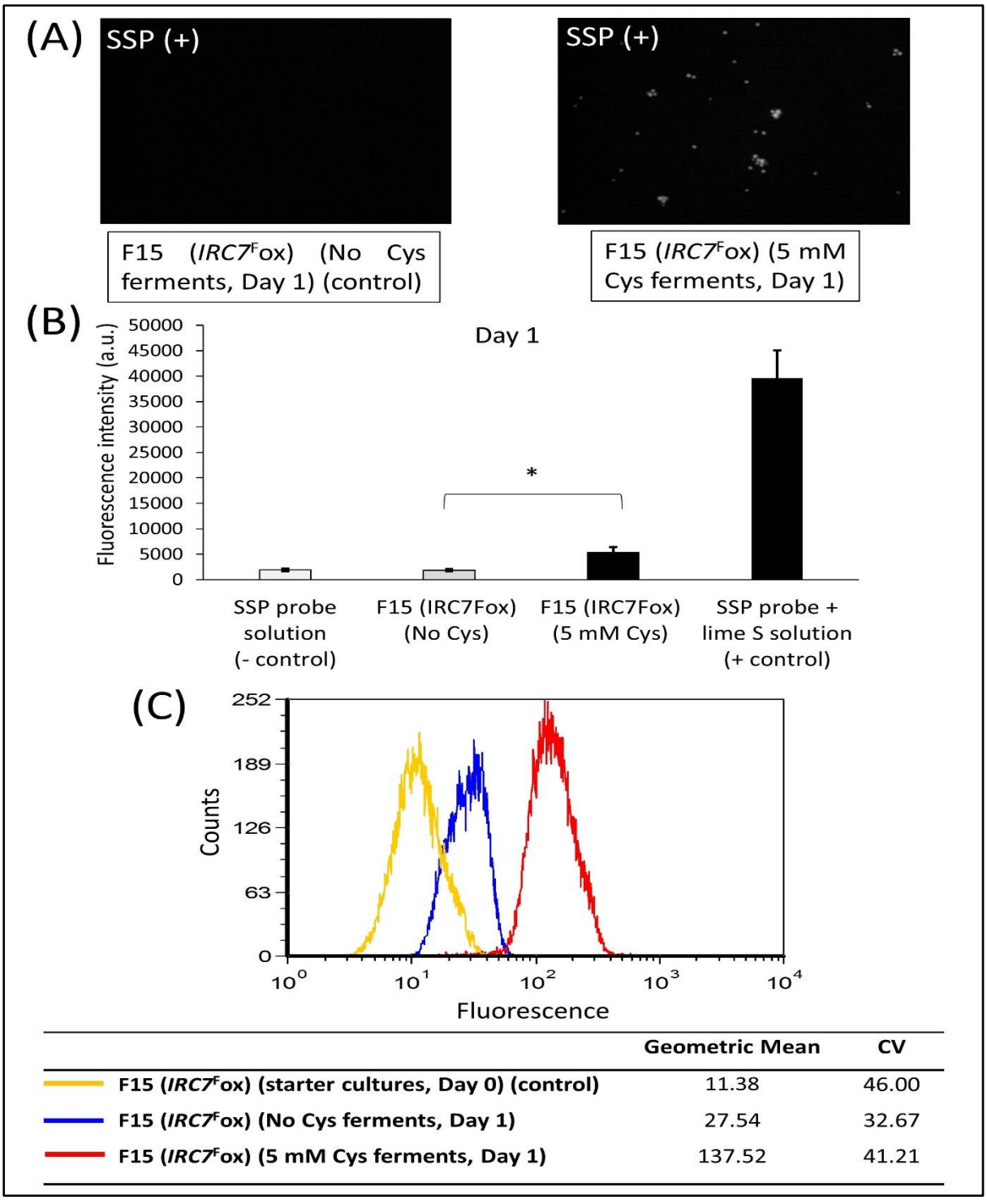


Figure 5.2. Detection of polysulfides using SSP4. **(A)** Fluorescence images of the SSP4-treated yeast cells (exposure time 10 msec). **(B)** Fluorescence intensities of the SSP4-treated samples (~50 μL) by a microplate reader (λ_{ex} = 482 nm, λ_{em} = 518 nm). Data are means ± SD (n = 3). *P < 0.05. a.u.: arbitrary unit. **(C)** Flow cytometry histogram of the SSP-treated yeast cells from non- or high-cysteine ferments. The geometric mean fluorescence is shown. CV = coefficient of variation (Standard Deviation/ mean channel number).

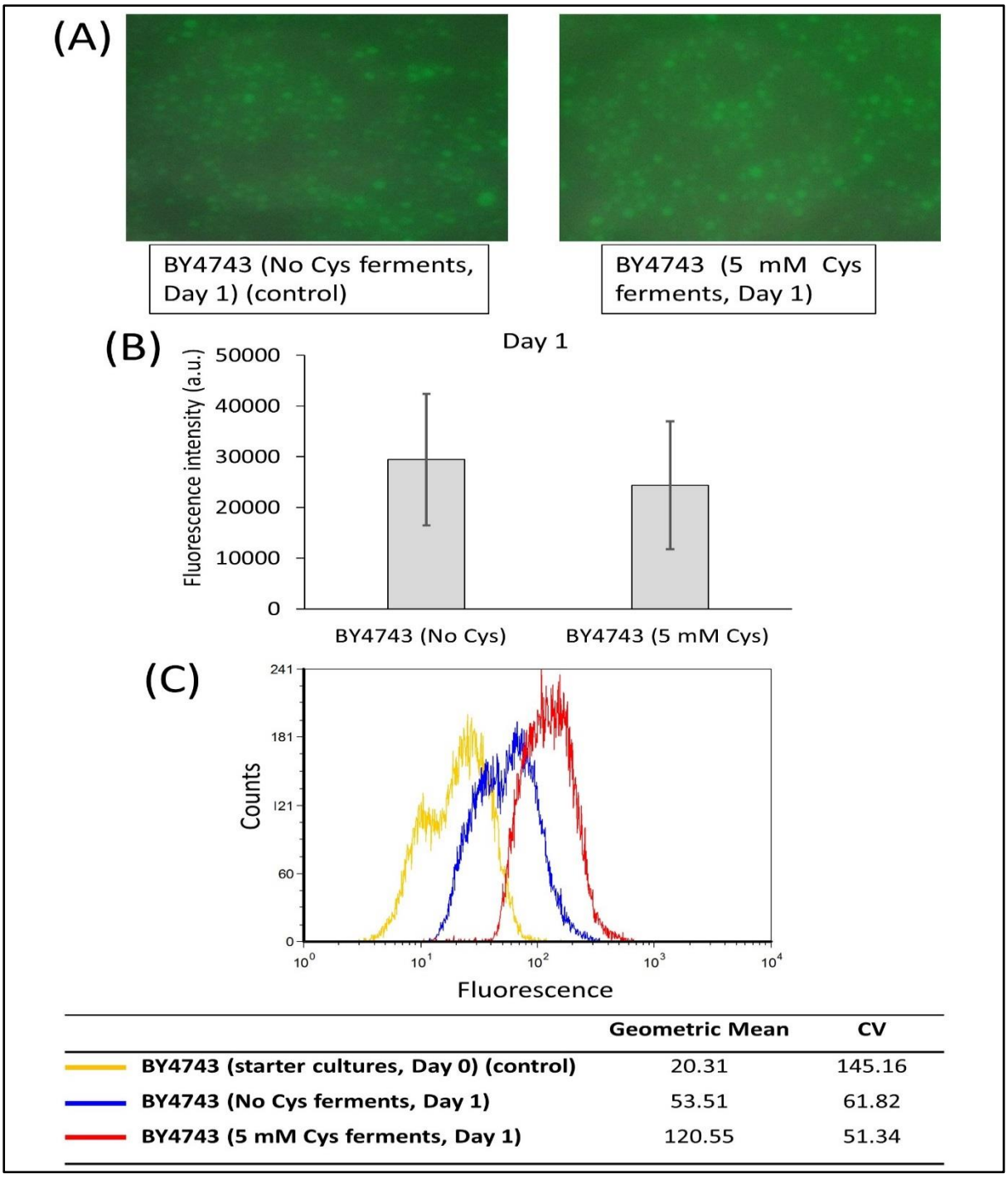


Figure 5.3. Detection of polysulfides using SSP4. **(A)** Fluorescence images of the SSP4-treated yeast cells (exposure time 10 msec). Note that the images were taken through the eyepiece of a Nikon Eclipse 50i microscope. **(B)** Fluorescence intensities of the SSP4-treated samples (~50 µL) by a Tecan Infinite M200 microplate reader (λ_{ex} = 482 nm, λ_{em} = 518 nm). Data are means ± SD (*n* = 3). a.u.: arbitrary unit. **(C)** Flow cytometry histogram of the SSP4-treated yeast cells from non- or high-cysteine fermentations. The geometric mean fluorescence is shown. CV = coefficient of variation (Standard Deviation/ mean channel number).

5.3.5 HPLC analysis of the polysulfide stock solution

Whilst SSP4 is an excellent tool for detecting the presence of polysulfides in yeast, it does not provide information about the types of polysulfide species present. An investigation was therefore conducted to test whether polysulfide species could be separated by HPLC with a C8 column (Perkin Elmer, USA) using the method described by Rohwerder and Sand (2003). The Brownlee Aquapore RP-300 C8 column (for the analysis of large biomolecules) was used because it was readily accessible.

Although a somewhat better separation was achieved through replacing the mobile phase (8% acetonitrile, 2 mM tetrabutylammonium chloride and 2 mM acetate buffer at pH 4.0) with 30 mM phosphate buffer at pH 2.6 (Fig. 5.4A and Fig. 5.4B), overall, polysulfides in both the polysulfide stock solution (Ikeda *et al.* 1972) and the glutathione polysulfide stock solution (Rohwerder and Sand 2003) could not be separated by HPLC. Further experiments using different separation columns (PLRP-S 100 Å, 8 μm, 150 × 4.6 mm; Latek Labortechnik, Germany; Rohwerder and Sand 2003) or other HPLC protocols (Kamyshny, Borkenstein and Ferdelman 2009) may be useful to resolve the different polysulfide species.

To determine what polysulfide species were present in each chromatographic peak, the fractions (chromatographic peaks): S1, S2, (Fig. 5.4A) G1, G2, G3, G4, G5 and G6 were collected (Fig. 5.4B) and analysed by NMR (Fig. 5.5). According to Nina Duhamel's analysis (University of Auckland), sample G1 is the only one with clear signals, noticeably related to glutathione. Samples G2, G3, G4 and G6 also have trace amounts of glutathione related peaks and are very similar to those in sample G1, with some small shifts in some of the peaks. In contrast, samples S1, S2 and G5 did not show any clear peaks.

The peaks in sample G1 are almost identical to those of glutathione, with the only difference being the signals of the CH_2 group adjacent to the thiol group. When glutathione is present as a free thiol, these peaks are very close and overlap on the NMR spectrum. But in G1, the signal from the two protons are very distinct, with one of the protons' giving rise to a peak about 0.36 ppm downfield from the free thiol analogue. This indicates that something had changed at the thiol position, resulting in a loss of movement at the adjacent CH_2 position. However, as no signal was observed for the thiol proton, even in the glutathione sample, further investigations using mass spectrometry could help elucidate what has happened. Nevertheless, these results indicated that species other than glutathione are present in the glutathione polysulfide stock solution (Rohwerder and Sand 2003).

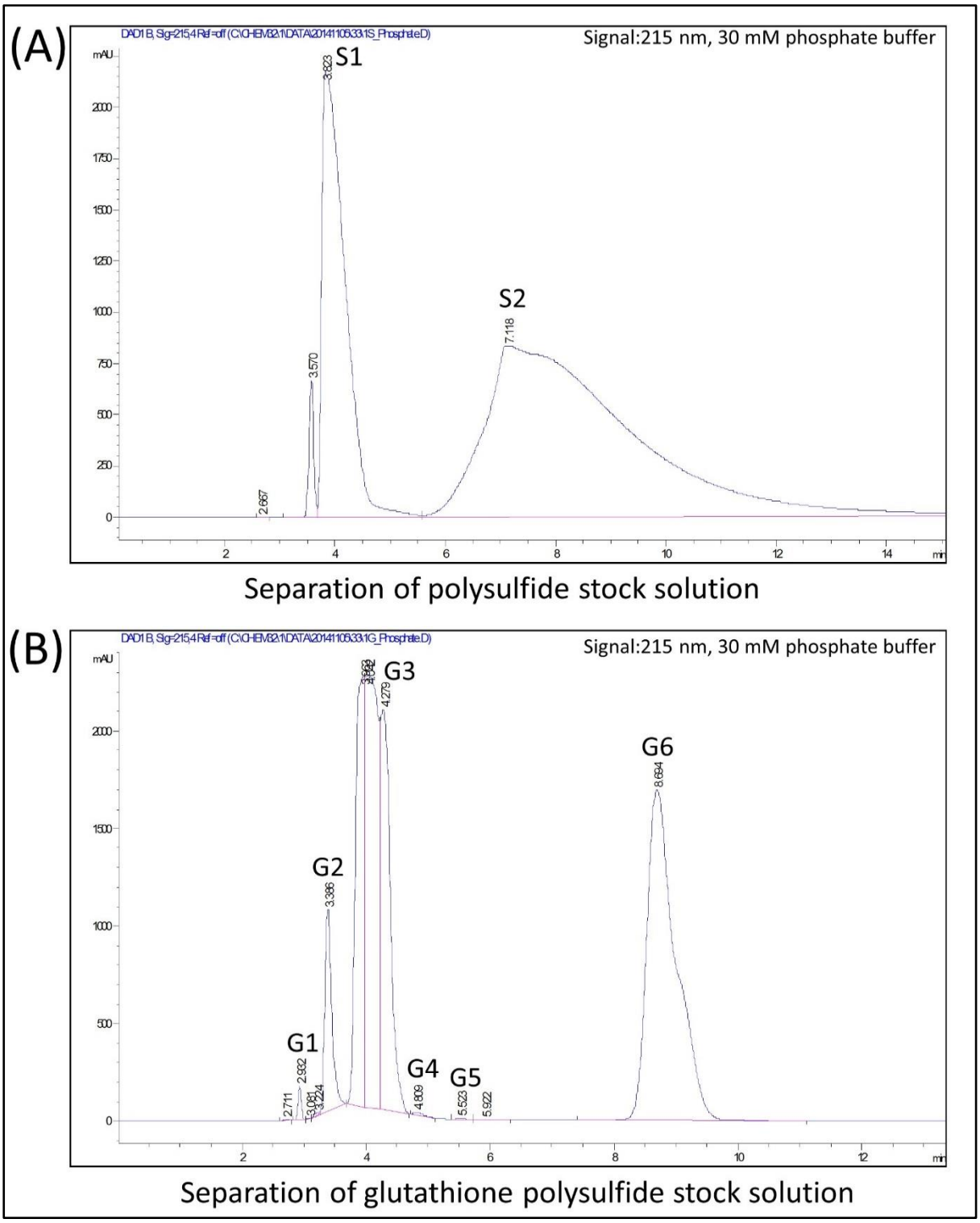


Figure 5.4. HPLC chromatogram of **(A)** polysulfide stock solution (Ikeda *et al.* 1972) and **(B)** glutathione polysulfide stock solution (Rohwerder and Sand 2003). HPLC analysis was conducted on Agilent 1100 using a Brownlee Aquapore RP-300 (C8) column.

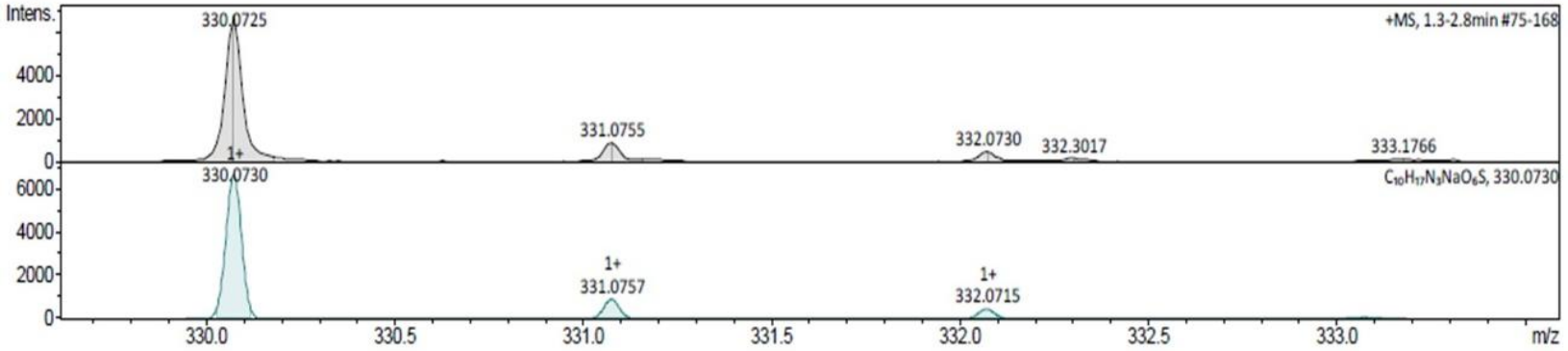
Auckland Uni Mass Spectrum SmartFormula Report

Analysis Info

Analysis Name	C:\Bruker\Data\Tony14\Tony12\14-12-10\run1\MAX16_RA3_01_1440.d	Acquisition Date	12/10/2014 11:19:11 AM
Method	may2014 - low - hplc.m	Operator	Tony
Sample Name	MAX16	Instrument / Ser#	micrOTOF-Q 228888.10191
Comment	Sample dissolved in 0.1ml H2O Sample diluted 3ul in 1ml MeOH		

Acquisition Parameter

Source Type	ESI	Ion Polarity	Positive	Set Nebulizer	0.4 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	180 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	4.0 l/min
Scan End	1000 m/z	Set Collision Cell RF	150.0 Vpp	Set Divert Valve	Waste



Meas. m/z	#	Ion Formula	m/z	err [ppm]	mSigma	# Sigma	Score	rdb	e⁻ Conf	N-Rule
330.0725	1	C9H18N2O9S	330.0728	-0.7	10.8	1	100.00	2.0	odd	ok
	1	C9H18N2O9S	330.0728	-0.7	10.8	1	100.00	2.0	odd	ok
	1	C10H17N3NaO6S	330.0730	1.5	6.2	1	100.00	3.5	even	ok
	2	C9H11N10NaOS	330.0730	1.5	9.0	2	95.49	9.0	odd	ok

Figure 5.5. Mass spectrum smart formula report of the fraction G1 (Fig. 5.4B).

5.4 Conclusions

Yeast strains capable of producing high levels of aromatic thiols are highly desirable for the wine industry (Swiegers and Pretorius 2007; Belda *et al.* 2016). Here, overexpression of yeast *TUM1* was demonstrated to elevate the production of 3MH/A in high-cysteine (605.8 mg L^{-1}), (*E*)-2-hexenal-supplemented CDGJM, likely because it produced more H_2S from cysteine. However, the positive effect of overexpressing *TUM1* on 3MH/A production was not observed in both low-cysteine (5 mg L^{-1}), (*E*)-2-hexenal-supplemented CDGJM and Riesling juice. Moreover, the preliminary results indicated that overexpression of *TUM1* resulted in elevated production of undesirable reductive sulfur compounds: methanethiol, ethanethiol, carbon disulfide and methyl thioacetate. To determine whether overexpressing *TUM1* has any practical applications, further experiments including the scale up of fermentations, use of different grape varieties (e.g. Sauvignon Blanc juice) and other genetically different *TUM1* overexpression strains (Chapter 2), as well as the addition of SO_2 to finished fermentations to prevent oxidation, would be required.

The potential role of polysulfides in sensory quality of wine has recently been recognised (Starkenmann *et al.* 2016), but whether polysulfides could be generated by *S. cerevisiae* undergoing fermentation remains unclear. To best of my knowledge, the preliminary results obtained by using SSP4 provide the first experimental evidence for the possible involvement of polysulfides in *S. cerevisiae* cysteine catabolism. Furthermore, the SSP4 results reveal the potential role of yeast $Irc7^{F}p$ in polysulfide formation. Although the attempt to separate polysulfide species using HPLC was unsuccessful, further studies using other techniques (e.g. reversed-phase ultrafast liquid chromatography coupled with quadrupole-time-of-flight mass spectrometry (UFLC-QTOF-MS); Kreitman *et al.* 2017) to distinguish the types of polysulfide species present in yeast deletants (e.g. Δ*tum1*) will certainly help to elucidate pathways leading to formation of polysulfides in *S. cerevisiae*.

In conclusion, this study shows that the desirable tropical fruity odours of 3MH/A could potentially be enhanced through modulating yeast *TUM1* gene and besides H_2S, polysulfides are also likely to be involved in the cysteine catabolism of *S. cerevisiae.* These findings not only contribute to a better understanding of *S. cerevisiae* cysteine catabolism but also could potentially improve the sensory quality of wine.

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Chapter 6

Hydrogen sulfide and its roles in *Saccharomyces cerevisiae* in a winemaking context

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Minireview

MINIREVIEW

Hydrogen sulfide and its roles in *Saccharomyces cerevisiae* in a winemaking context

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ABSTRACT

The rotten-egg odour of hydrogen sulfide (H_2S) produced by the yeast *Saccharomyces cerevisiae* has attracted considerable research interest due to its huge impact on the sensory quality of fermented foods and beverages. To date, the yeast genetic mechanisms of H_2S liberation during wine fermentation are well understood and yeast strains producing low levels of H_2S have been developed. Studies have also revealed that H_2S is not just a by-product in the biosynthesis of the sulfur-containing amino acids, but indeed a vital molecule involved in detoxification, population signalling and extending cellular life span. Moreover, polysulfides have recently emerged as key players in signalling and the sensory quality of wine because their degradation leads to the release of H_2S. This review will focus on the recent findings on the production of H_2S and polysulfides in *S. cerevisiae* and summarise their potential roles in yeast survival and winemaking. Recent advances in techniques for the detection of H_2S and polysulfides offer an exciting opportunity to uncover the novel genes and pathways involved in their formation from different sulfur sources. This knowledge will not only provide further insights into yeast sulfur metabolism, but could potentially improve the sensory quality of wine.

Keywords: *Saccharomyces cerevisiae*; hydrogen sulfide; population signalling; polysulfides

INTRODUCTION

The rotten-egg odour of hydrogen sulfide (H_2S) produced by yeast during fermentation is well known for its ability to significantly reduce the sensory quality of wine (Swiegers and Pretorius 2007; Franco-Luesma *et al.* 2016). Extensive research over the past decade has led to the identification of several yeast genes responsible for H_2S production in wine, which have now been used to breed commercial low H_2S-producing yeast strains (Cordente *et al.* 2009; Linderholm *et al.* 2010; Huang, Roncoroni and Gardner 2014; Noble, Sanchez and Blondin 2015).

Hydrogen sulfide is now recognised as not merely an intermediate of the biosynthesis of the sulfur-containing amino acids, but it has important functions in detoxification, population signalling and extending life span in yeast (Gadd and Griffiths 1977; Sohn, Murray and Kuriyama 2000; Hine *et al.* 2015). In addition, polysulfides have recently been recognised as potential mediators of signalling in mammalian cells because their degradation results in the release of H_2S (Ida *et al.* 2014; Kimura 2015; Olson and Straub 2016). The effects of polysulfides on the sensory quality of wine have also attracted a lot of attention

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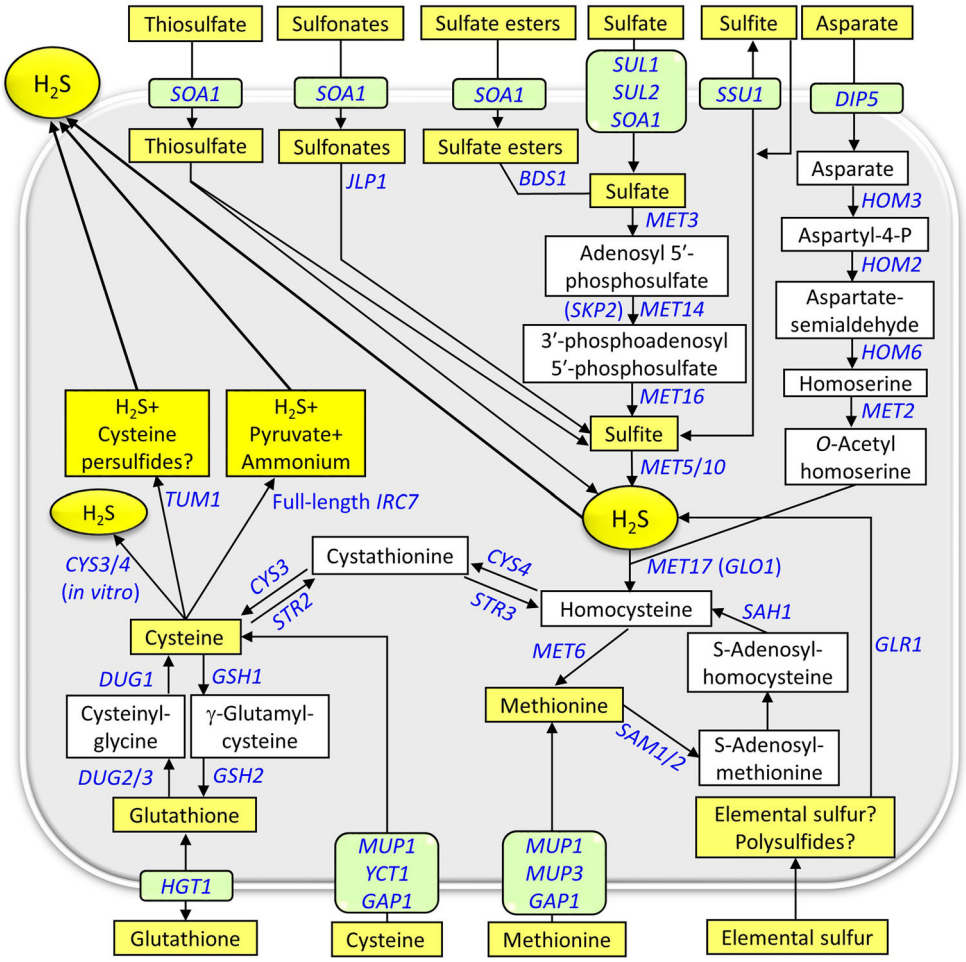

Figure 1. Sulfur metabolism in the yeast *S. cerevisiae*. Yeast can utilise a variety of sulfur sources to produce H_2S and sulfur-containing amino acids, cysteine and methionine (according to Thomas and Surdin-Kerjan 1997; Hogan, Auchtung and Hausinger 1999; Hall, Brachat and Dietrich 2005; Singh *et al.* 2009; Ugliano and Henschke 2009; Sato *et al.* 2011; Yoshida *et al.* 2011; Harsch and Gardner 2013; Hopwood, Ahmed and Aitken 2014; Santiago and Gardner 2015; Huang *et al.* 2016; Holt *et al.* 2017; Kinzurik *et al.* 2017).

lately (Kreitman *et al.* 2017) and hydropolysulfides such as H_2S_2 and H_2S_3 have been shown to contribute to the flint and mineral odour in wine (Starkenmann *et al.* 2016).

There have been numerous recent excellent reviews about the formation of H_2S and polysulfides, and their potential roles in mammals (Filipovic 2015; Kimura 2015; Olson and Straub 2016; Cuevasanta, Möller and Alvarez 2017), plants (Quirós-Sauceda *et al.* 2016) and microbes (Findlay 2016). However, there are few related reviews specifically for the model organism, *Saccharomyces cerevisiae*, which may reflect the fact that this aspect of sulfur metabolism in *S. cerevisiae* is still relatively unexplored. The goal of this review is to summarise recent work related to the production of H_2S and polysulfides in *S. cerevisiae*, to bring to the attention of researchers this emerging aspect of *S. cerevisiae* sulfur metabolism.

H_2S PRODUCTION FROM THE SAP DURING WINE FERMENTATION

The majority of H_2S produced by yeast during wine fermentation is from the sulfate assimilation pathway (SAP), where sulfate is taken up and progressively reduced to sulfide (using 2 ATPs and 4 NADPHs), the precursor of the sulfur-containing amino acids cysteine and methionine, which are required for yeast growth (Fig. 1) (Thomas and Surdin-Kerjan 1997). Grape juice usually contains plenty of sulfate (~160 to 700 mg L^{-1}) but very low concentrations of cysteine and methionine (<20 mg L^{-1}), and therefore the SAP is triggered during fermentation to support yeast growth (Rauhut 2009; Ugliano and Henschke 2009). The mechanisms by which H_2S is released from the SAP are well studied and reviewed (refer to Henschke and Jiranek 1993; Thomas and Surdin-Kerjan 1997; Swiegers and Pretorius 2007; Rauhut 2009; Ugliano and Henschke 2009).

In short, sulfur assimilation starts with extracellular sulfate entering yeast cells through the sulfate transporters, Sul1p and Sul2p (Cherest *et al.* 1997), where sulfate is first activated by the ATP sulphurylase (Met3p) to 5′-adenylylsulfate (APS), which is then phosphorylated to 3′-phospho-5′-adenylylsulfate (PAPS) by the APS kinase (Met14p). In the next step, PAPS is reduced by the PAPS reductase (Met16p) to sulfite, which is further reduced by sulfite reductase (Met5p/Met10p) to sulfide. The sulfide produced is subsequently integrated into the nitrogenous precursor, *O*-acetyl homoserine (OAH), to form homocysteine with the help of *O*-acetylhomoserine sulfhydrylase (Met17p). Finally, homocysteine is converted into cysteine, methionine and glutathione (Fig. 1) (Thomas and Surdin-Kerjan 1997). The liberation of excessive H_2S during fermentation usually occurs as a result of insufficient assimilable nitrogen (OAH) in grape juice

to combine with the sulfide generated (Jiranek, Langridge and Henschke 1995).

SULFATE TRANSPORT

Sulfate uptake is the first step of the SAP; however, the sulfate-sensing mechanisms in yeast remain poorly understood. Recent work has revealed that the yeast sulfate transporters, Sul1p and Sul2p, can also function as independent extracellular sulfate receptors (Transceptor: **trans**port and re**ceptor**) (Kankipati *et al.* 2015). The authors observed that addition of the sulfate analogue, D-glucosamine 2-sulfate, whilst not transported into the cell, triggered the activation of Sul1, Sul2-dependent PKA signalling pathway, measurable as increased trehalase activity. Furthermore, the PKA signalling pathway was activated in the two yeast mutants, $Sul1^{E427Q}$ and $Sul2^{E443Q}$, incapable of uptaking exogenous sulfate added to the medium (Kankipati *et al.* 2015).

The yeast Sul1p and Sul2p are the major sulfate transporters, but their deletion does not completely block yeast growth on high concentrations of sulfate as the sole sulfur source (~30 mM), indicating the existence of unknown sulfate transporters (Cherest *et al.* 1997; Kankipati *et al.* 2015). Recently, a third sulfate transporter, Soa1p (**s**ulf**on**ate transport; low affinity for sulfate and broad substrate specificity), was identified (Holt *et al.* 2017). The authors demonstrated that deletion of all three genes (*SUL1*, *SUL2* and *SOA1*) resulted in an inability to grow on high concentrations of sulfate as the sole sulfur source, alluding to no other sulfate transporters.

Jennings and Cui (2012) proposed that Sul2p may also act in the efflux of sulfate, based on the observed transient efflux of sulfate mediated by Sul2p in an S-starved *met3* deletant when sulfate was added. The authors suggested that this mechanism could potentially protect yeast from an excessive sulfate influx (Jennings and Cui 2012). The inability of Δ*met3*, Δ*met14* and Δ*met16* to take up sulfate gives credence to the speculation that the sulfate transporters, Sul1p and Sul2p, may form complexes with the proteins downstream of the SAP (Met3p, Met14p and Met16p) and participate in the process of activation of sulfate (Breton and Surdin-Kerjan 1977; Thomas and Surdin-Kerjan 1997). However, there is currently insufficient evidence to support this hypothesis.

SKP2, *MET2* AND *GLO1* AFFECTING H_2S PRODUCTION FROM THE SAP

SKP2 encodes an F box protein, which is predicted to be part of an SCF (Skp1p-Cullin-F-box) ubiquitin protease complex involved in biosynthesis of sulfur amino acids required for growth and H_2S and SO_2 production (Yoshida *et al.* 2011). The authors observed that the *skp2* deletant produced more H_2S and SO_2 than the wild type. As Skp2p is involved in the degradation of Met14p (APS kinase), the increase in H_2S and SO_2 production in the *skp2* deletant is likely due to a more stable Met14p, allowing extended sulfate assimilation and therefore increased H_2S and SO_2 levels.

Recently, two *SKP2* allele variants, $SKP2_{I350V}$ and $SKP2_{T357I}$, resulting in low H_2S and SO_2 production were identified by Noble, Sanchez and Blondin (2015) using a quantitative trait locus mapping strategy. It is thought that these mutational changes in Skp2p may increase its efficiency of targeting and degradation of Met14p, resulting in less sulfate through the SAP and so, reduced H_2S and SO_2.

The yeast *MET2* encodes homoserine *O*-acetyl transferase (HTA), which catalyses the conversion of homoserine to *O*-acetyl homoserine. OAH is subsequently incorporated into H_2S to yield homocysteine (Baroni *et al.* 1986) (Fig. 1). Genetic variation in *MET2* can lead to differences in H_2S production. The Δ*met2* has been shown to produce more H_2S during beer production (Hansen and Kielland-Brandt 1996). Recently, the $R_{301}G$ mutation in *MET2* was identified to be responsible for low H_2S production (Noble, Sanchez and Blondin 2015), in agreement with our findings (Huang, Roncoroni and Gardner 2014). It has been proposed that the $R_{301}G$ mutation in *MET2* may increase the activity of HTA, hence the efficiency of converting homoserine into OAH. Because of the ample supply of OAH to combine with sulfide, less sulfide will be released into wine as H_2S (Huang, Roncoroni and Gardner 2014; Noble, Sanchez and Blondin 2015).

The recently identified *SKP2* and *MET2* allele variants (Huang, Roncoroni and Gardner 2014; Noble, Sanchez and Blondin 2015), together with the *MET5* and *MET10* allele variants, identified earlier by Cordente *et al.* (2009) and Linderholm *et al.* (2010), have now been successfully employed to breed low H_2S-producing strains for winemaking.

The yeast *GLO1* encodes glyoxalase I, which is essential for the detoxification of methylglyoxal, a toxic metabolite of glycolysis (Inoue and Kimura 1996). Recently, *GLO1* has been added to the growing list of genes affecting H_2S production from the SAP. Kinzurik *et al.* (2017) showed that the *glo1* deletant grew more slowly than the wild type on sulfate as the sole sulfur source, produced more H_2S only when sulfate was added and decreased the expression level of Met17p. The authors therefore suggested that Glo1p is critical for a fully functional Met17p (*O*-acetylhomoserine sulfhydrylase). This finding is exciting in the sense that a gene not previously linked to the SAP could affect H_2S production from this pathway, illustrating the complexity of the SAP and the possibility of the existence of other unknown genes affecting the SAP (Kinzurik *et al.* 2017).

H_2S PRODUCTION FROM ELEMENTAL SULFUR

Elemental sulfur is frequently sprayed in the vineyard to fight grapevine powdery mildew, and the residual sulfur on grape has been observed to contribute to the formation of H_2S during fermentation (Thomas *et al.* 1993; Araujo *et al.* 2017). Although it has been suggested that elemental sulfur may be spontaneously converted to sulfide under the anaerobic and low pH fermentation conditions (Linderholm *et al.* 2008), Araujo *et al.* (2017) showed that no H_2S could be detected when elemental sulfur was added to grape juice model solutions (sparged with nitrogen). These findings indicate that yeast is likely to be responsible for most of the H_2S production from elemental sulfur during fermentation.

Sato *et al.* (2011) observed that a yeast with deletion of *GLR1* (glutathione reductase) produced less H_2S than wild type on sulfur (e.g. colloidal elemental sulfur and powdery elemental sulfur). Moreover, Sato *et al.* (2011) proposed that insoluble elemental sulfur is most likely converted into more soluble polysulfides first, which could then enter into yeast cells, where they are reduced by the glutathione reductase to sulfide.

H_2S PRODUCTION FROM GLUTATHIONE AND CYSTEINE

The tripeptide, glutathione (L-γ-glutamyl-L-cysteinyl-glycine), is naturally present in grape juice (~1.3 to 102 mg L^{-1}) and can also be synthesised by yeast through the SAP (Rauhut 2009). Recently, glutathione has been permitted as an additive (up to 20 mg L^{-1}) to grape juice to protect aromatic compounds from

oxidation according to the 2015 OIV resolutions (Wegmann-Herr *et al.* 2016). The addition of glutathione to grape juice has been observed to increase H_2S production (Rauhut 2009; Winter *et al.* 2011; Wegmann-Herr *et al.* 2016). The mechanism is not yet fully understood but it is generally assumed that glutathione is first hydrolysed to cysteine, which is then degraded by cysteine desulfhydrase to release H_2S under nitrogen-limited conditions (Rauhut 2009).

Adding cysteine to grape juice has long been observed to induce H_2S production (Jiranek, Langridge and Henschke 1995; Winter and Curtin 2012), and today several yeast genes affecting the formation of H_2S from cysteine have been identified. Both yeast Cys4p (cystathionine β-synthase, CBS) and Csy3p (cystathionine γ-lyase, CSE) have been reported to cleave cysteine and release H_2S *in vitro* (Singh *et al.* 2009; Hopwood, Ahmed and Aitken 2014). However, other *in vivo* studies suggested that deletion of yeast *CYS4* or *CSY3* did not reduce the production of H_2S (Linderholm *et al.* 2008; Winter, Cordente and Curtin 2014; Huang *et al.* 2016).

Santiago and Gardner (2015) demonstrated that the full-length *IRC7* gene encoding cysteine desulfhydrase was responsible for cleaving cysteine to release H_2S. However, in many yeast strains Irc7p has no functional cysteine desulfhydrase activity, as the strains carry a 38-bp deleted, non-functional version of *ß*-lyase *IRC7* (Roncoroni *et al.* 2011).

The importance of the vacuole-related genes in H_2S production from cysteine was revealed by Winter, Cordente and Curtin (2014), who showed that deletants of such genes produced less H_2S from cysteine compared to the wild type. It has been suggested that the yeast vacuole could have a vital function in the cysteine detoxification process, which involves degrading cysteine to H_2S (Winter, Cordente and Curtin 2014).

Deletion of yeast *TUM1* gene has recently been shown to reduce H_2S production from cysteine during fermentation, and it is suspected that yeast Tum1p may act like its human orthologue, sulfurtransferase, an enzyme involved in the production of H_2S from cysteine (Huang *et al.* 2016).

OTHER SULFUR SOURCES

Thiosulfate can be cleaved to sulfite and sulfide by thiosulfate reductase and utilised as a sulfur source by yeast. However, the yeast gene encoding thiosulfate reductase remains unknown (Chauncey and Westley 1983; Thomas *et al.* 1992). Funahashi *et al.* (2015) showed that yeast grew better and produced ethanol more efficiently on thiosulfate than sulfate because less energy was required for yeast to assimilate sulfite (cleaved from thiosulfate) than sulfate.

The alternative sulfur sources such as sulfonates and sulfate esters that are generally abundant in soil can also be utilised by *S. cerevisiae* (Linder 2012). Hogan, Auchtung and Hausinger (1999) demonstrated that yeast *JLP1* encodes a sulfonate dioxygenase, which is required for yeast to utilise sulfonates (e.g. isethionate and taurocholate) as a sulfur source by degrading sulfonates to sulfite. A strain with deletion of *JLP1* was shown to grow more slowly than wild type on sulfonates (Hogan, Auchtung and Hausinger 1999). The yeast *BDS1* encodes a sulfatase, which is essential for yeast to release the sulfate from the sulfate esters (e.g. sodium dodecyl sulfate), and whose deletion reduced the ability of yeast to use sulfate esters as a sulfur source (Hall, Brachat and Dietrich 2005).

There has been some doubt as to whether the alternative sulfur sources play an important part in *S. cerevisiae* sulfur utilisation as this yeast generally does not grow well on the alternative sulfur sources compared to other ascomycetes (Linder 2012). To date, only two yeast genes, *JLP1* and *BDS1*, have been associated with the utilisation of these alternative sulfur sources (Linder 2012). Recently, yeast Soa1p was found to be capable of transporting a range of sulfur compounds including sulfate, sulfite, thiosulfate, sulfonate and choline sulfate (Holt *et al.* 2017). The identification of the sulfonate and choline sulfate transporter, Soa1p, in yeast highlights that sulfonates or sulfate esters could be important sulfur sources, at least for *S. cerevisiae* living in soil. Further studies to investigate other yeast genes involved in utilisation of these sulfur sources are clearly worthwhile.

H_2S AND HEAVY METAL DETOXIFICATION

H_2S has been shown to play a critical role in detoxification of heavy metals and yeast strains that produce more H_2S were observed to be more resistant to copper (Kikuchi 1965) and methylmercury (Ono *et al.* 1991). The detoxification effect of H_2S is likely through the formation and precipitation of insoluble metal sulfides (e.g. copper sulfide) (Gadd and Griffiths 1977). Interestingly, genes (e.g. vacuole-related genes) that were recently identified to affect H_2S production from cysteine (Winter, Cordente and Curtin 2014) were similar to those previously reported to be essential for metal (e.g. cadmium, nickel) detoxification (Ruotolo, Marchini and Ottonello 2008; Arita *et al.* 2009), highlighting the central role of H_2S in detoxification.

H_2S AND POPULATION SYNCHRONY

Hydrogen sulfide's role in cell signalling was first demonstrated by Sohn, Murray and Kuriyama (2000), who established that the gas was responsible for the synchronisation of the yeast population, when grown in aerobic continuous culture. Periodic changes in H_2S production (an inhibitor of respiration) were connected to an ultradian oscillation in respiration, with H_2S being highest when respiration decreased, before declining with the onset of respiration (Sohn, Murray and Kuriyama 2000). The H_2S produced during these oscillations was shown to be generated from sulfate or sulfite by sulfite reductase through the SAP and not from cysteine or glutathione in the media (Sohn and Kuriyama 2001a). It has been suggested that the cyclic changes in H_2S production (a strong reducing agent) are a protection mechanism against oxidative stress and are triggered by the periodic depletion of glutathione and cysteine used by yeast to detoxify toxic respiration metabolites (e.g. reactive oxygen species) (Sohn and Kuriyama 2001b; Kwak *et al.* 2003). The importance of glutathione homeostasis for H_2S production and respiratory oscillation was also highlighted by Sohn *et al.* (2005), who observed that deletion of *GLR1*, encoding glutathione reductase (responsible for the reduction of oxidised glutathione), led to the yeast no longer being able to produce H_2S and undergo respiratory oscillation.

DOES H_2S GIVE *SACCHAROMYCES* A COMPETITIVE ADVANTAGE?

The ethanol and heat produced by *S. cerevisiae* during wine fermentation have been reported to give *S. cerevisiae* a significant advantage to dominate over other competing non-*Saccharomyces* species (Goddard 2008; Salvadó *et al.* 2011). Given that H_2S is also produced during fermentation and its important role in synchronising yeast populations, it has been suggested by Linderholm *et al.* (2010) that H_2S produced by *S. cerevisiae* may inhibit

respiration and oxidative metabolism of their microbial competitors, allowing *S. cerevisiae* to dominant in wine fermentation. Therefore, it seems reasonable that yeast capable of efficiently producing H_2S may confer a selective advantage under certain conditions and this may explain the difference in H_2S production across commercial and natural wine strains (Spiropoulos *et al.* 2000; Linderholm *et al.* 2008). However, whether H_2S plays a role in modifying the environment (niche construction), killing other competitors and contributing to competitive advantage of *S. cerevisiae* remains to be elucidated.

ROLE OF H_2S AND OTHER VOLATILES IN YEAST–INSECT INTERACTIONS

How yeasts survive in the wild between vintages and are transferred between the vineyard and winery is still not clearly understood. Recent studies have shown that yeast can reside in the gut of social wasps (*Polistes dominula*), alluding to the role of insects as a means of survival between vintages (Stefanini *et al.* 2016). Furthermore, the authors' ability to isolate new intraspecific (*S. cerevisiae* x *S. cerevisiae*) and interspecific (*S. cerevisiae* x *S. paradoxis*) hybrids from the wasp's gut is particularly exciting in relation to the maintenance of genetic diversity within a wild yeast population. From the many studies in yeast–insect ecology, insects are now regarded as one of the main vectors for yeast dispersal. These include bees (Goddard *et al.* 2010), *Drosophila* (Chandler, Eisen and Kopp 2012) and social wasps (Stefanini *et al.* 2012).

Another question remains as to how insects are attracted to yeast, as this relationship is clearly beneficial to both organisms: the yeast as a food source and for insects who in turn provide an efficient dispersal mechanism. Certain volatiles produced by yeast during fermentation act as attractants for insects, for example, acetate esters and acetic acid (Becher *et al.* 2012; Palanca *et al.* 2013; Buser *et al.* 2014; Günther *et al.* 2015; Dapporto *et al.* 2016). Moreover, Christiaens, Franco and Cools (2014) showed that a yeast strain with deletion of *ATF1* (encoding alcohol acetyl transferase that is responsible for acetate ester production) was less attractive to *Drosophila*, hence reducing its dispersal via this vector.

H_2S, well known for its repulsive rotten-egg smell, has also been implicated in yeast–insect interactions (Sundstrom *et al.* 2016). The preliminary results suggest that *Drosophila* fruit flies were slightly less attracted to a medium supplemented with H_2S (12.5 μM sodium sulfide); however, *Drosophila* were also seen to lay more eggs on yeast colonies supplemented with H_2S (Sundstrom *et al.* 2016). Interestingly, H_2S has been demonstrated to react with (*E*)-2-hexenal in grape juice to form the fruity varietal thiols 3-mercapto-hexanol and 3-mercaptohexylacetate (Harsch *et al.* 2013). However, only tiny amounts of thiols (<1%) are produced through this pathway as (*E*)-2-hexenal is rapidly metabolised by yeast during fermentation (Schneider *et al.* 2006; Subileau *et al.* 2008; Harsch *et al.* 2013). Nevertheless, whether H_2S exerts a repulsive or attractive effect on the insect carriers and thus affects the dispersal and survival of *S. cerevisiae* remains to be investigated.

H_2S AND YEAST LIFE SPAN

Yeast produced more H_2S on 0.5% glucose than on 2% glucose and this may explain why glucose restriction can extend yeast lifespan (Hine *et al.* 2015). Life span was also extended following supplementation with external H_2S (5 μM NaHS). The H_2S produced by yeast during glucose restriction was shown to come from cysteine or methionine and not from sulfate (SAP). This is based on the observation that *MET* gene deletants (Δ*met5*, Δ*met14* and Δ*met16*) that block sulfate assimilation (see Fig. 1 for pathway) were still capable of producing H_2S and had extended longevity on 0.5% glucose, whilst removal of cysteine or methionine from the media resulted in decreased H_2S production in a *met14* deletant.

In addition, methionine restriction has been reported to increase yeast lifespan, and interestingly, that yeast deletants Δ*met2* and Δ*met17*, known to be high H_2S producers, were observed to have a longer lifespan than wild type (Johnson and Johnson 2014; Ruckenstuhl *et al.* 2014; Hine and Mitchell 2015). Autophagy-mediated vacuolar acidification has been proposed to play a vital role in yeast longevity as deletion of genes involved in autophagy (Δ*atg5*, Δ*atg7* and Δ*atg8*) led to shortened chronological lifespan (Ruckenstuhl *et al.* 2014). Interestingly, these observations coincide with the recent findings that both Δ*met2* and Δ*met17* produce more H_2S from cysteine than wild type (Huang *et al.* 2016) and yeast vacuoles play a crucial role in formation of H_2S from cysteine (Winter, Cordente and Curtin 2014).

POLYSULFIDES IN YEAST

Polysulfides (S_x^{2-}; $x > 2$) and protein persulfides such as glutathione persulfide (GSSH) and cysteine persulfide (CysSSH) have lately emerged as potential mediators of sulfide signalling in mammalian cells (Ida *et al.* 2014; Kimura 2015; Olson and Straub 2016). To our knowledge, the formation of polysulfides in yeast remains largely uninvestigated; therefore, we have referred to studies using mammalian cells as a means to uncover any potential analogies.

Melideo, Jackson and Jorns (2014) proposed that the first step of mammalian H_2S metabolism is the conversion of H_2S (with sulfite) to thiosulfate by sulfide:quinone oxidoreductase (SQOR). In the next step, thiosulfate (with glutathione) is converted by the thiosulfate:glutathione sulfurtransferase (TST) into glutathione persulfide (GSSH), which is further converted to sulfite by sulfur dioxygenase (SDO). The sulfite produced is either oxidised to sulfate by sulfite oxidase (SO) or to thiosulfate (with H_2S) by SQOR (Fig. 2). Although mammalian TST has not been isolated, its yeast orthologue (Rdl1p) has been identified, being capable of acting like a TST and converting glutathione (with thiosulfate) to GSSH (Melideo, Jackson and Jorns 2014). Furthermore, cysteine persulfide was detected in yeast Rdl1p after its reaction with thiosulfate (Melideo, Jackson and Jorns 2014). However, whether yeast has other enzymes (e.g. SQOR, SDO) involved in the other steps of the mammalian H_2S metabolism pathway is currently unknown. Therefore, the formation of persulfides in yeast through this pathway, for example, during fermentation, remains to be explored.

Ida *et al.* (2014) showed that both mammalian H_2S-producing enzymes, CBS and CSE, are capable of converting cystine to cysteine polysulfides (e.g. CysSSSH and CysSSSCys)—precursors in the formation of glutathione polysulfides (e.g. GSSSH and GSSSG). It is proposed that the polysulfide species could be the real mediators of signalling with H_2S being released as part of persulfide degradation (Ida *et al.* 2014). Moreover, polysulfide species are much more abundant than H_2S in mammalian cells. Recently, reduced levels of protein persulfide in yeast Δ*cys4* (CBS) and Δ*cys3* (CSE) were observed using a novel persulfide detection method, suggesting that yeast *CYS4* and *CSY3* could also have roles in persulfidation (Dóka *et al.* 2016).

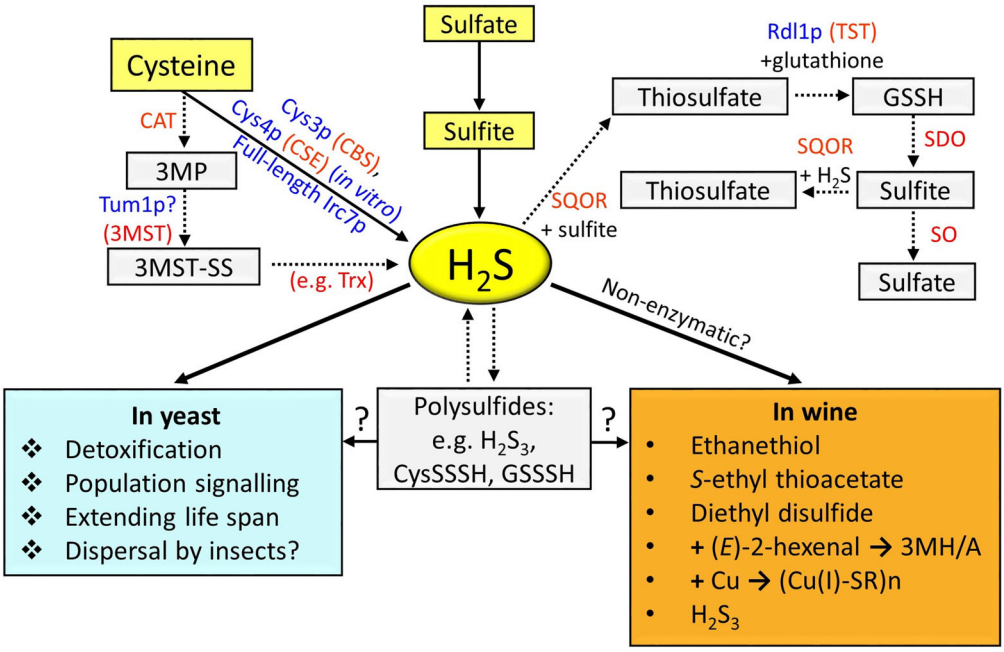

Figure 2. Potential pathways for H_2S and polysulfides production and their functions in the yeast *S. cerevisiae*. H_2S is produced from the sulfate/sulfite (SAP) and cysteine in yeast. Polysulfides are generated by CBS, CSE and 3MST in mammalian cells but currently little is known in yeast. Yeast Rdl1p can convert thiosulfate to GSSH (with glutathione). H_2S can react with Cu and (*E*)-2-hexenal to form $(Cu(I)\text{-}SR)_n$ and 3MH/A that affect the sensory quality of wine, respectively. Enzymes in *S. cerevisiae* (blue font) and in mammalian cells (red font). Dashed arrows indicate that pathways have been shown in mammalian cells but not in *S. cerevisiae*. Abbreviations: CAT: cysteine aminotransferase; 3MST: 3-mercaptopyruvate sulfurtransferase; Trx: thioredoxin; CBS: cystathionine β-synthase; CSE: cystathionine γ-lyase; SQOR: sulfide:quinone oxidoreductase; TST: thiosulfate:glutathione sulfurtransferase; SDO: sulfur dioxygenase; SO: sulfite oxidase. (according to Gadd and Griffiths 1977; Sohn Murray and Kuriyama 2000; Singh *et al.* 2009; Harsch *et al.* 2013; Hopwood, Ahmed and Aitken 2014; Ida *et al.* 2014; Melideo, Jackson and Jorns 2014; Kimura 2015; Kimura *et al.* 2015; Santiago and Gardner 2015; Hine *et al.* 2015; Huang *et al.* 2016; Kinzurik *et al.* 2016; Olson and Straub 2016; Starkenmann *et al.* 2016; Sundstrom *et al.* 2016; Kreitman *et al.* 2017).

Another mammalian H_2S-producing enzyme, 3-mercaptopyruvate sulfurtransferase (3MST), is implicated in the generation of pyruvate and protein persulfides from 3-mercaptopyruvate (3MP), which is produced by cysteine aminotransferase from cysteine. H_2S could be released from protein persulfides, once reducing systems such as glutathione or thioredoxin are available (Shibuya *et al.* 2009; Mikami *et al.* 2011). In addition, Kimura *et al.* (2015) showed that 3MST could catalyse the formation of H_2S_3 and H_2S directly from 3MP in mammalian brain cells. The human TUM1 (or 3MST) protein and yeast Tum1p are orthologues (Mathew, Schlipalius and Ebert 2011), and deletion of yeast *TUM1* has recently been observed to reduce H_2S production from cysteine during fermentation (Huang *et al.* 2016). However, whether yeast Tum1p can act like its human orthologue, 3MST, to produce H_2S and polysulfides from 3MP, is unknown. Additional indirect evidence for the potential existence of polysulfides in yeast (*S. cerevisiae*) is the ability of the yeast mitochondrial ATP-binding cassette transporter, Atm1p, to transport glutathione polysulfides (e.g. glutathione trisulfide) *in vitro* (Schaedler *et al.* 2014).

H_2S AND POLYSULFIDES RESPONSIBLE FOR 'OFF-ODOURS' IN WINE

It has long been suspected that H_2S produced by yeast during fermentation may lead to the formation of other unpleasant volatile sulfur compounds such as dimethyl tri- and tetrasulfide (reviewed by Waterhouse, Sacks and Jeffery 2016). Recently, Kinzurik *et al.* (2016) demonstrated that ^{34}S-labelled sulfate when fed to a Δ*met17* (a high H_2S producer, unable to synthesise sulfur-containing amino acids) was incorporated into ethanethiol, S-ethyl thioacetate and diethyl disulfide. This work was the first validation of H_2S (produced during fermentation) contributing to the formation of the 'cooked onion' odour of these volatile sulfur compounds. However, whether yeast enzymes are required for this process remains to be determined.

Copper fining is commonly practiced to remove H_2S responsible for the rotten-egg 'off-odour' in wine. However, growing evidence suggests that copper treatment leads to increased H_2S formation during bottle storage (Ugliano *et al.* 2011; Viviers *et al.* 2013). One of the proposed mechanisms is that H_2S reacts with copper to form copper sulfide complexes, which may then release H_2S under anaerobic storage conditions (Franco-Luesma and Ferreira 2014, 2016). The formation of (Cu(I)-SR)n complexes in wine has also been proposed (Kreitman *et al.* 2016). Furthermore, the same group showed that reaction of H_2S, copper, cysteine or glutathione led to the formation of cysteine or glutathione polysulfides in model wine, which may also contribute to H_2S production during bottle storage (Kreitman *et al.* 2017).

Hydropolysulfides such as H_2S_2 and H_2S_3 have also been reported to be associated with the flint and mineral odour in wine (Starkenmann *et al.* 2016). However, as these hydropolysulfides are unstable in wine, their effects on the sensory quality of wine are not clear.

METHODS FOR THE DETECTION OF H_2S AND POLYSULFIDES

Several detection methods for H_2S and polysulfides have been developed (see review by Takano, Shimamoto and Hanaoka

2016). Fluorescent probes for H_2S and polysulfides are particularly useful for investigating the biological functions of these highly reactive molecules because the technique is simple, non-destructive and allows real-time monitoring (imaging) of H_2S and polysulfides inside cells (Takano, Shimamoto and Hanaoka 2016). The fluorescent probes for monitoring H_2S (e.g. WSP-1; Peng *et al.* 2014) and polysulfides (e.g. SSP4; Chen *et al.* 2013) are now commercially available and have been extensively used to study the roles of H_2S and polysulfides in mammalian cells (Ida *et al.* 2014; Monti *et al.* 2016). Whilst there are no current reports on the use of these probes in yeast, preliminary data from our group suggest these probes can be used to detect polysulfides within yeast cells (unpublished results). The development of other novel and improved versions of fluorescent probes (Takano, Shimamoto and Hanaoka 2016) will allow polysulfides to be detected much earlier and more accurately, thus enabling yeast researchers to decipher the yeast genes and pathways involved in the formation of polysulfides.

Polysulfides are unstable at the low pH (Kamyshny, Borkenstein and Ferdelman 2009), and therefore it can be challenging to analyse and quantify these transient species in wine. Detection methods include sensitive but simple HPLC detection of polysulfides (Kamyshny, Borkenstein and Ferdelman 2009) to more complex ones such as reversed-phase ultrafast liquid chromatography coupled with quadrupole-time-of-flight mass spectrometry (Kreitman *et al.* 2017). Both are worthy of investigation as analytical tools for the determination of polysulfide species in wine.

CONCLUSION AND FUTURE PERSPECTIVES

Over the past years, much has been learnt about how H_2S is produced by *Saccharomyces cerevisiae* during wine fermentation but there are still aspects of H_2S in yeast waiting to be explored. For instance, low H_2S-producing yeast strains have been selected and bred for the wine industry, and whilst they have similar fermentation kinetics to the wild type (Dahabieh *et al.* 2015), it is tempting to speculate (given that H_2S may play an important role in population signalling) that the reduced ability to produce H_2S may affect the overall microbial (population) dynamics during fermentation. Further studies assessing whether H_2S provides a competitive advantage (or disadvantage) to yeast, through investigating the interactions of both low and high H_2S-producing strains with lactic acid bacteria, non-*Saccharomyces* species or *Drosophila* may be worthwhile. Other gas molecules such as nitric oxide (NO) have also been suggested to play crucial roles in signalling and various stress responses (e.g. oxidative stress) in yeast, and modulation of production of NO could improve fermentation ability of yeast (see review by Astuti, Nasuno and Takagi 2016). There is substantial evidence that NO may interact with H_2S in mammalian cells (Fago *et al.* 2012; Olson and Straub 2016). Therefore, future studies on the synergistic effect of H_2S and NO in yeast would be valuable for the fermentation industry.

Polysulfide species have recently been proposed as the central players in signalling in mammalian cells (Ida *et al.* 2014; Kimura 2015; Olson and Straub 2016) and they have been detected in the fungus *Aspergillus nidulans* (Wróbel *et al.* 2009). However, the roles of polysulfides or even their existence in *S. cerevisiae* remain largely unknown. Polysulfide production and metabolism has been suggested to be the next frontier in sulfide biology in eukaryotes (Olson and Straub 2016). Here, we propose that *S. cerevisiae* could be an excellent model organism. For such research, *S. cerevisiae* gene deletion collections and the recently developed polysulfide detection techniques will likely lead to the identification of novel genes and pathways involved in the formation of polysulfides, providing a greater understanding of the essential role of polysulfides in a wide variety of biological processes. This knowledge will not only give a more complete picture of yeast sulfur metabolism but also could potentially improve the sensory quality of wine.

Saccharomyces cerevisiae is present everywhere, being found in a wide range of habitats, including wineries, soil, oak trees, insects and human gut and not only the laboratory (Liti 2015). It has been proposed that investigation of the natural history (ecology) of *S. cerevisiae* through a population genomics approach is necessary to fully understand the *S. cerevisiae* metabolism that may have been shaped during evolution (Jouhten *et al.* 2016). A huge difference in H_2S production has been observed across yeast strains (Spiropoulos *et al.* 2000), and Olson and Straub (2016) have suggested that H_2S is likely to play a pivotal role in evolution. Future studies considering the ecological aspect of H_2S metabolism might be the key to uncovering the actual role of this exciting molecule. Finally, we would finish this review with a slightly modified version of Dobzhansky' (1973) famous quote that 'Nothing in biology (H_2S production) makes sense, except in the light of evolution.'

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Chapter 7

Conclusion and future directions

7.1 Conclusion

This project aimed to identify yeast genes and pathways responsible for the early burst of H_2S from cysteine during fermentation (Winter *et al.* 2011). Early production of H_2S – a varietal thiol precursor (Harsch *et al.* 2013), may enhance the desirable tropical aromas of wines that many consumers prefer (Lund *et al.* 2009; Capone *et al.* 2017). The major findings and contributions of this study (Chapter 2-6) are summarised below:

1) H_2S production from cysteine involves *TUM1*. Overexpression of *TUM1* elevates production during fermentation whilst its deletion reduces H_2S by half (Chapter 2).

2) Deletion of either *MET17* or *MET2* leads to an additional delayed burst of H_2S from cysteine. Triple deletants of *STR2*, *STR3* and individual *MET* genes require both *MET17* and *TUM1* to bypass the transsulfuration pathway and grow on high concentrations of cysteine as the sole sulfur source. Together, these results indicate that cysteine is not converted to sulfate or sulfite, but rather to sulfide via a novel pathway requiring the action of Tum1p. Part of the H_2S generated from cysteine is fed into the sulfate assimilation pathway to support yeast growth (Chapter 2).

3) An improved version of bismuth-containing indicator agar resembling grape juice was developed specifically to screen H_2S formation from cysteine (Chapter 3).

4) *LST4*, *LST7 AGP1*, *GNP1*, *MUP1*, *STP1* and *DAL81* regulate cysteine uptake and also affect H_2S formation from cysteine. Agp1p, Gnp1p and Mup1p are the main cysteine permeases, regulated by the SPS-sensing and rapamycin pathways, in yeast under cysteine-supplemented fermentation (Chapter 4).

5) Overexpression of *TUM1* elevates production of 3MH and 3MHA in high-cysteine, (*E*)-2-hexenal-supplemented, non-sulfate Chemically Defined Grape Juice Medium (CDGJM) (Chapter 5).

6) Polysulfides which may affect the sensory quality of wine, can be detected in fermenting yeast cells supplemented with high cysteine, using the fluorescent probe SSP4 (Chapter 5).

7) An up-to-date review is presented on the recent studies on sulfur metabolism in *S. cerevisiae*, providing suggestions for future research in this field (Chapter 6).

In conclusion, the findings of this project not only extends our current understanding of *S. cerevisiae* cysteine catabolism, but also may be applied to generate better yeast strains. The development of novel winemaking techniques to induce a transient burst of H_2S production early in fermentation may increase thiol production. Winemakers could use this strategy to fine-tune the tropical fruit character of their wines and thereby produce wines of increased quality and value. Although the contribution of volatile thiols to tropical flavour has been primarily recognised in Sauvignon Blanc, these thiols are also present in other varietals, including Chardonnay and several red wines (Ferreira *et al.* 2002; Capone *et al.* 2015, 2017). These findings therefore have the potential to increase the number of available wine styles, broadening the appeal of wines in different markets.

7.2 Future directions

To build upon this work, I recommend the following research directions to fully decipher cysteine catabolism in *S. cerevisiae* and provide high thiol-producing strains for the industry:

1) **Biochemical characterisation of yeast Tum1p**

   I have shown that yeast *TUM1* affects the production of H_2S from cysteine during fermentation (Chapter 2). It would be worthwhile further investigating whether yeast Tum1p can act like its mammalian orthologue (3-mercaptopyruvate sulfurtransferase) and generate pyruvate, protein persulfides, H_2S and polysulfides from cysteine when reducing systems such as glutathione or thioredoxin are present (Shibuya *et al.* 2009; Mathew, Schlipalius and Ebert 2011; Mikami *et al.* 2011; Kimura *et al.* 2015). Moreover, this knowledge would provide new insights into the biological role of yeast Tum1p given that H_2S and polysulfides are increasingly being recognised for their important roles in signalling and stress responses (reviewed in Chapter 6).

2) **Identification of other genes linked to H_2S formation from cysteine**

   Although no specific QTL associated with H_2S formation from cysteine was identified using a set of 96 fully sequenced M2 x F15 progeny, it would be worth attempting to use an interaction model in QTL analysis to improve the mapping (Dr Miguel Roncoroni, KU Leuven, personal communication). Alternatively, it may be useful to genetically cross a wine strain that produces low or no H_2S from cysteine with a laboratory strain with known markers (e.g. BY4742) and subsequently map the genes by bulk segregant analysis (Roncoroni *et al.* 2011). Furthermore, synthetic genetic array analysis with *TUM1* double mutants would identify other genes involved in H_2S production from cysteine (see review by Boone, Bussey and Andrews 2007).

3) **Development of an alternative assay for detecting H_2S production from cysteine**

   Whilst *LST4* and *LST7* were successfully identified as the genes affecting H_2S formation from cysteine, with the bismuth-containing, grape juice-like indicator agar plate method, an alternative H_2S detection assay that correlates well with actual H_2S production during fermentation would be useful. It would be worthwhile to test whether recently developed fluorescent probes for H_2S detection (see review by

Takano, Shimamoto and Hanaoka 2016) can be used, together with a spectrophotometer, to screen for H_2S production from cysteine during micro-scale fermentation.

4) Breeding of wine yeast strains with improved H_2S production from cysteine

Natural allelic variants in genes such as *TUM1*, *GNP1* or *MUP1* and/or novel mutants following chemical/UV mutagenesis having improved H_2S production, could provide valuable breeding material for high thiol-producing strains. These can be selected using bismuth containing plate-based screens (Chapter 3). The variants would be introduced by repeated back-crossing to commercial high thiol-producing strains (e.g. UOA Maxithiol, VL3 or X5) to produce the improved strains (Pretorius 2000).

5) Commercial-scale wine and beer fermentation trials

Further trials are required to (1) determine the optimal level of cysteine supplementation (as yeast nutrient products), (2) scale-up to winery-scale fermentations, (3) evaluate different grape varieties e.g. Sauvignon Blanc, Chardonnay and Pinot Gris and (4) evaluate the impact of early H_2S production on the overall wine aroma. The data would provide valuable information with regards to whether the findings from this project can be implemented to enhance tropical aromas under commercial winemaking conditions. In addition, brewing trials using a *TUM1* deletion strain would be worthwhile, to determine whether a similar 'industry ready' strain could be used to produce fruit-driven styles of beers through reduction in H_2S formation from cysteine (which can be up to 35 mg L^{-1} in wort; Lawrence and Cole 1972; Duan *et al.* 2004).

6) Identifying yeast genes that affect production of polysulfides

Preliminary results with the fluorescent probe SSP4 alludes to the likely existence of polysulfides in *S. cerevisiae* undergoing fermentation on high concentrations of cysteine. Polysulfides may play critical roles in yeast stress responses and sensory quality of wine (Chapter 6). The use of recently developed fluorescent polysulfide probes (Takano, Shimamoto and Hanaoka 2016) in combination with yeast deletion collections would allow the identification of genes involved in polysulfide(s) formation, which maybe applicable to industry.

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