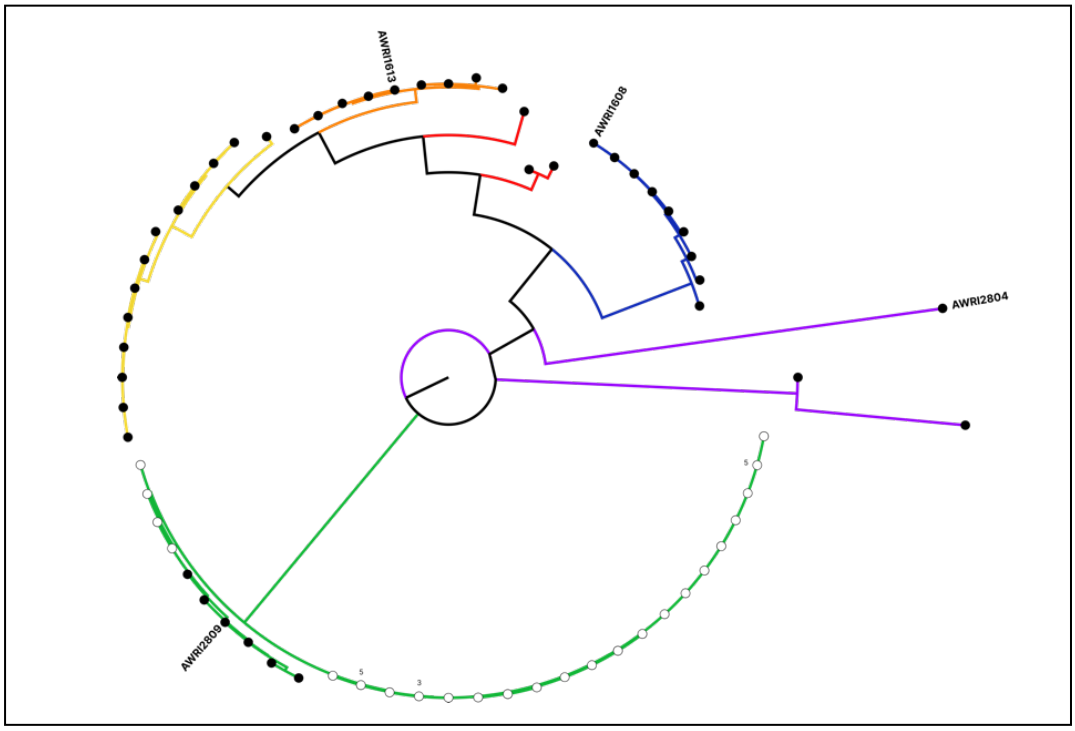
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AWRI Australian Wine Research Institute

Understanding *Brettanomyces* and its adaptation to control measures



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Table of contents

1. Abstract 4
2. Executive summary 4
3. Acknowledgements 5
4. Background 5
5. Project aims 6
6. Methods 6
7. Results and discussion 9
8. Outcome and conclusion 18
9. Recommendations 19
10. Appendix 1: Communication 21
11. Appendix 2: Intellectual property 21
12. Appendix 3: References 21
13. Appendix 4: Staff 23
14. Appendix 5: Other supporting material (including raw data, if appropriate) 23

1. Abstract

Brettanomyces bruxellensis represents a major source of microbiological spoilage in winemaking. Current control measures rely heavily on the use of SO_2 following primary fermentation; however, recent research suggests that *Brettanomyces* can evolve tolerance to this antimicrobial. This project applied a multifaceted approach to understand *Brettanomyces* spoilage. A survey of industrial isolates was conducted, which showed increasing tolerance to SO_2, while elucidating the associated genetic alterations. A framework for rapid and sensitive detection of *Brettanomyces* was established, which also predicted SO_2 tolerance levels, while additional control options, including a potential biocontrol strategy, were assessed for their efficacy in controlling tolerant *Brettanomyces* strains.

2. Executive summary

The propensity of *Brettanomyces* yeast to spoil wine remains one of the foremost microbiological problems faced by winemakers. *Brettanomyces* spoilage is currently managed through the application of a multi-faceted strategy previously developed by AWRI in collaboration with Australian winemakers. While the initiation of these efforts in the early 2000s facilitated industry-wide decreases in spoilage of finished wines, this strategy does not eliminate *Brettanomyces* yeasts from wineries and is heavily reliant upon use of the preservative SO_2 to inhibit the growth of *Brettanomyces*. To ensure that Australian winemakers can continue to manage *Brettanomyces* in a cost-effective manner, this project examined interactions between *Brettanomyces* genetics and SO_2 tolerance, and options for rapid detection and control.

More than 300 industry isolates were phenotyped, with assessments including SO_2 tolerance and the production of volatile phenols. For the volatile phenol screening, a new high-throughput method was developed that used coumaric acid consumption as a proxy for the production of ethyl phenols. A range of SO_2 tolerance levels was observed, from highly sensitive to levels that well exceed those there were considered to be the highest expected for *Brettanomyces*. Far less variation was observed in the consumption of coumaric acid, with all strains consuming amounts of coumaric acid that would be predicted to produce significant wine spoilage.

Twenty-four of the most SO_2-tolerant isolates were fully genotyped using whole-genome sequencing. All of these isolates were shown to represent members of the previously identified AWRI 1499 clade, indicating that continued adaptation of this group is occurring, as opposed to the appearance of a new genetic clade.

B. bruxellensis has been shown to have a highly variable genetic make-up. The most prevalent (and most SO_2-tolerant) genetic group that had previously been isolated from Australian wineries has a hybrid genome, which is predicted to have arisen through a 'rare-mating' event. The high level of SO_2 tolerance that was produced through this mating is predicted to have allowed for a 'selective-sweep', which allowed this new variant to largely replace other *B. bruxellensis* strains in the winemaking environment.

The discovery of contemporary strains with higher levels of SO_2 tolerance than observed previously, posed the question as to whether these new strains represented further adaptation by members of the original hybrid group, or the appearance of a completely new type of strain.

Genomic comparison of these new tolerant isolates showed that they were not a new type of *B. bruxellensis*, but represented adapted versions of the original hybrid group. More detailed analysis highlighted an amplification of a specific gene, *SSU1*, as being important for this additional adaptation to SO_2, providing a potential molecular marker for use in genetic testing for SO_2 tolerance.

While genetic transformation of *B. bruxellensis* had recently been established, which enabled the introduction of new genetic material, no methodology existed for creating specific alterations, such as gene deletions, to the existing genome. As CRISPR-based gene editing tools had been shown to provide an efficient means to perform genetic alterations in other yeast species, a CRISPR-based platform was developed for genetic engineering in *Brettanomyces*. The method that was established was shown to be able to efficiently and accurately create specific deletions in the *Brettanomyces* genome and was used to investigate genes responsible for SO_2 tolerance and the formation of volatile phenols. The production of volatile phenols represents the major detrimental impact of *Brettanomyces* on wine quality. Harnessing tools such as CRISPR-gene deletion that were developed in this project, it was possible to investigate the metabolic pathways that impacted the formation of volatile phenols by *Brettanomyces*.

Both random mutagenesis and candidate gene approaches were used to identify components of the volatile phenol pathway. While the identity of the gene that encodes the final step of the pathway (vinyl-phenol reductase) remains to be identified, it was possible to formally characterise the *Brettanomyces* gene *PAD1* as encoding the phenylacrylic acid decarboxylase enzyme that is responsible for the first step in the pathway, which converts hydroxycinnamic acids to their vinyl derivative.

Phenol-negative *Brettanomyces* strains were developed through CRISPR-based deletion of the *PAD1* gene that was identified as encoding the phenylacrylic acid decarboxylase enzyme during this project.

These phenol-negative strains were shown to be fully capable of growth in wine but did not produce detectable spoilage compounds. This key characteristic was then investigated for its potential use as a bioprotective strategy, with inoculation of the phenol-negative strain shown to inhibit both the growth and volatile phenol production of other strains of *Brettanomyces*. Phenol-negative *Brettanomyces* therefore represents an innovative means of biological control of *Brettanomyces* spoilage.

This project has developed foundational datasets on the current state-of-play in *Brettanomyces* population characteristics and genomics and combined this with a suite of potential tools to aid in addressing wine spoilage by this yeast.

3. Acknowledgements

This work was supported by Australia's grapegrowers and winemakers through their investment body Wine Australia with matching funds from the Australian Government. The AWRI is a member of the Wine Innovation Cluster in Adelaide, SA.

4. Background

Genotype analysis of >300 Australian *Brettanomyces* isolates obtained from 2000-2005 revealed that one group of strains (AWRI 1499-clade) increased in frequency of isolation from ~60% to ~95% (Curtin et al. 2007). Strains within this group were subsequently found to tolerate higher concentrations of SO_2 (Curtin et al. 2012).

When considering changing industry practices to minimise the risk of *Brettanomyces* spoilage in red wines, which rely heavily on the appropriate use of SO_2, these observations are analogous to the problem of antibiotic resistance in human pathogenic bacteria. The use of sub-lethal concentrations of SO_2, whether due to winemaker trends towards lower preservative use or increases in wine pH which result in less of the active, molecular form, provide the ideal environment for the evolution of SO_2 tolerance in this species. Indeed, previous work has shown that *Brettanomyces* is capable of significant adaptive evolution towards increased SO_2 tolerance under laboratory-based directed evolution programs (Bartel et al. 2021).

Sulfite tolerance in other fungal species, particularly *Saccharomyces cerevisiae*, has been linked to membrane protein Ssu1p that functions as a sulfite-efflux pump, reducing the intracellular concentration of sulfite (Park and Bakalinsky 2000). It is unknown whether the *B. bruxellensis SSU1* gene encoding this protein performs this function, or whether alternative mechanisms mediate sulfite tolerance in *Brettanomyces* yeasts. Knowledge of mechanisms that confer enhanced sulfite resistance in some *B. bruxellensis* strains may facilitate the design of rapid methods to test for emergence of new, potentially problematic, strains.

A weak point in many *Brettanomyces* control strategy implementations is a lack of well-validated rapid monitoring tools. 'Plating' methods, while providing the means to test for tolerance characteristics of isolates, are laborious and slow. While rapid methods are of interest, data interpretation can be difficult and information regarding specific strain backgrounds and associated tolerance characteristics is not provided.

5. Project aims

Previous research performed at the AWRI clearly demonstrated that it is possible for SO_2-resistant *Brettanomyces* strains to evolve greater levels of SO_2 tolerance (when subjected to directed evolution under laboratory conditions). This project aimed to extend investigations into SO_2 resistance through a multi-faceted approach.

Project deliverables were:

- Additional strains of *Brettanomyces* isolated, genotyped and phenotyped for sulfite tolerance, growth rate and volatile phenol production
- Investigation of improved methods for genetic engineering of *Brettanomyces* (e.g. CRISPR)
- Knowledge of the genetic basis for the production of 4-EP and 4-EG
- New molecular tests for industry to monitor and control *Brettanomyces*
- Information on the utility of non-phenol producing *Brettanomyces* strains
- Knowledge of the genetic basis for evolution of SO_2-resistant strains of *Brettanomyces*
- Additional molecular tests for industry to monitor and control *Brettanomyces*
- Practical advice and extension materials for winemakers.

6. Methods

6.1 *Brettanomyces* industry survey

6.1.1 Isolation

A 50 mL volume of sample with potential *Brettanomyces* contamination was filtered through a 0.45 µm sterile filter that was then placed onto YPD plates containing cycloheximide (100 mg/L) and chloramphenicol (50 mg/L). Plates were incubated at 28°C for up to 10 days. If colonies were visible after incubation, up to eight individual isolates per sample were streaked onto YPD agar and incubated at 28°C for five days. DNA was extracted from individual colonies (Lõoke et al. 2011) and used for ITS-RFLP identification, using the primers ITS1 and ITS4 and the restriction enzyme *Hae*III. *Brettanomyces* isolates produced two bands (375.bp and 95.bp) after digestion.

6.1.2 High-throughput sufilte tolerance screening

High-throughput SO_2 tolerance assays were performed in microplate format as described in Bartel et al. (2021).

6.1.3 Genome sequencing

Genomic DNA was extracted from liquid cultures using a QIAGEN Gentra Puregene Yeast/Bact Kit. Sequencing libraries were prepared using the Illumina Nextera library kit and barcoded and pooled in a single Miseq sequencing run using 2x300bp chemistry.

Sequencing reads were mapped to the AWRI 2804 genome (Roach and Borneman 2020), with nucleotide variant calling and phylogenetic comparison performed as detailed previously (Borneman et al. 2016).

6.1.4 Redacted due to sensitive commercial information

6.2 CRISPR engineering

6.2.1 Construction of DNA deletion cassettes

Two different types of DNA cassettes were used to transform *B. bruxellensis* and delete specific genes. Cassettes containing short flanking regions (60 bp) were obtained by amplifying the *natMX* cassette encoding clonNAT resistance from the plasmid pMA-*TDH1pr-natMX* (Varela et al. 2018) using primers containing 60 bp flanking regions corresponding to regions up- and down-stream of the specific open-reading frame (ORF) being targeted.

Cassettes containing long flanking regions (1.0 kb) were constructed in pUC19 using the method described by Gibson et al. (2009). Four PCR fragments were amplified, the first fragment contained 1.0 kb corresponding to the upstream region of the targeted gene and was obtained from *B. bruxellensis* AWRI 2804 chromosomal DNA. The second fragment contained either the *natMX* or the *kanMX* cassette, amplified from plasmids pMA-*TDH1pr-natMX* (Varela et al. 2018) and pMK-T-*TDH1pr-kanMX* (Varela et al. 2018), respectively. The third fragment contained 1.0 kb corresponding to the downstream region of the targeted ORF and was obtained from *B. bruxellensis* AWRI 2804 chromosomal DNA. The final fragment amplified plasmid pUC19. Fragments were assembled using the NEBuilder HiFi DNA assembly kit (New England Biolabs, Ipswich, MA, USA) following the manufacturer's instructions. PCR products obtained from all cassettes, with and without short and long flanking regions, were purified using the Wizard SV Gel and PCR clean-up system (Promega, Madison, WI, USA) and used for transformation as indicated below.

6.2.2 RNA guide design and formation of the ribonucleoprotein complex

RNA guides (gRNAs) were designed using the software sgRNAcas9 v2.0 (Xie et al. 2014). All RNAs were based on the genome sequence of *B. bruxellensis* AWRI 2804 (Roach and Borneman 2020). gRNAs were synthetised by Integrated DNA Technologies (Coralville, IA, USA) as CRISPR RNAs (crRNAs). crRNA is combined with the transactivating crRNA (tracrRNA) to form the gRNA. Formation of the gRNA and the ribonucleoprotein (RNP) complex was performed as described previously (Grahl et al. 2017). Briefly, 2 µL of crRNA and 2 µL of tracRNA were mixed, diluted to a final volume of 50 µL , and incubated 95°C for five minutes to form the gRNA. Alt-R S.p. Cas9 nuclease 3NLS (60 µM) (Integrated DNA Technologies, Coralville, IA, USA) was diluted to 4 µM (1.5 µL of nuclease in 21 µL final volume). A volume of 3.6 µL of cooled gRNA was then mixed with 3 µL of diluted nuclease and incubated at room temperature for five minutes to form the RNP complex. For each transformation, 6.6 µL of RNP complex were used.

6.2.3 Transformation and confirmation of positive transformants

B. bruxellensis strains were transformed by electroporation (Miklenić et al. 2015). For each transformation reaction, 6 µL of DNA cassette and 6.6 µL of RNP complex were mixed thoroughly with electrocompetent cells (50 µL) and incubated on ice for five minutes. After transformation and overnight incubation at 28°C with shaking (140 rpm), samples were spread on plates containing the appropriate antibiotic. Plates were then incubated at 28°C and transformants were visible after 5-7 days. Confirmation of positive transformants was performed by PCR with primers complementary to the DNA cassette and to the up- and down-stream sequences of the targeted ORF.

6.1 Volatile phenol formation in *Brettanomyces*

6.1.1 Creation of barcoded plasmid library in *E. coli*

Primers Bar-Ampl-F, containing *Not*I restriction site and an 18 bp random barcode and Bar-Ampl-R were used to amplify a section of the plasmid pMA, which resulted in the production of

an amplicon library containing a large number of random barcodes. The barcode-containing amplicon was then digested using *Not*I and *Pvu*I restriction enzymes and cloned into the complementary sites in the original pMA backbone. After cloning, the plasmid library was transformed into electrocompetent *E. coli*. Colonies from each plate (~18,000) were harvested in 2 mL of liquid LB medium and combined into a single mixed culture (200 mL in total). Plasmids were then extracted from the mixed culture (Maxiprep plasmid kit, QIAGEN) to form the final barcode plasmid library.

6.1.2 Random insertion mutagenesis in *B. bruxellensis*

The barcode plasmid library was digested using *Xba*I and *Spe*I restriction enzymes, to release a transformation cassette consisting of the *TDH1* promoter, *natR* selectable marker, a random 18 bp barcode, and the *TEF1* terminator, from the pMA plasmid. The insertion cassette library was then gel purified and normalised to 600 ng/µL.

B. bruxellensis strain AWRI2408 was then transformed with 1 µL (600 ng) of the purified insertion library via electroporation (Miklenić et al. 2015). Transformed cells were recovered for four hours in liquid YPD and then plated onto YPD agar plated containing CloNAT as a selection. After six days of incubation at 30°C, individual colonies were transferred into 96-well microplates containing liquid YPD+CloNAT and incubated at 30°C for six to seven days. Glycerol was then added to a final concentration of 15% and plates stored at -80°C.

6.1.3 Coumaric acid utilisation screen

Thawed glycerol stock (5 µL) was inoculated into 195 µL of YMB medium containing 5 g/L glucose in 96-well microplates. Plates were sealed (breathe-easy membrane) and incubated at 28°C for four days. Next, 5 µL of the cultures were inoculated into 195 µL YNB containing 5 g/L glucose and 100 mg/L coumaric acid. Microplates were incubated at 28°C for eight days. Following incubation, the concentration of coumaric acid remaining in the culture supernatant was estimated using absorbance (325 nm).

6.1.4 Utilisation of hydroxycinnamic acids and vinyl-phenols

PAD1 mutants were evaluated for the utilisation of hydroxycinnamic acids and vinyl-phenols in test tubes containing 2 mL of MM media supplemented with 100 mg/L of either coumaric acid, ferulic acid, 4-vinyl phenol or 4-vinyl guaiacol. After inoculation, tubes were placed in a rotating wheel and incubated at 28°C for five days. Culture supernatant was analysed to determine the concentration of hydroxycinnamic acids, vinyl- and ethyl phenols. All cultures were performed in triplicate.

6.1.5 Measuring hydroxycinnamic acids, vinyl- and ethyl phenols

Coumaric acid, ferulic acid, 4-vinyl phenol, 4-vinyl guaiacol, 4-ethyl phenol and 4-ethyl guaiacol were measured by HPLC (Agilent Technologies 1200 series) using an Alltima C18 5 µm 250 x 4.6 mm ID column. The mobile phase contained water and acetonitrile in 70:30 ratio and 0.12 g/L formic acid; flow rate was 0.8 mL/min. The column was kept at 30°C, while detection was performed by measuring absorbance at 230 nm.

6.1.6 Microbial inhibition experiments

Experiments for AWR I2804 were performed in triplicate in minimal media, synthetic wine and red wine containing 6% v/v ethanol. All media were supplemented with coumaric acid at 100 mg/L. The AWRI 2804 parental strain was inoculated at $1x10^2$ cells/mL, while AWRI 2804Δ*PAD1* was inoculated at either $1x10^4$, $1x10^5$, $1x10^6$ and $1x10^7$ cells/mL. Both strains were inoculated simultaneously and incubated at 28°C. Samples were taken regularly, plated on YPD and on YPD containing clonNAT (50 µg/L) to estimate cell concentrations for both strains, and analysed for 4-ethyl phenol production using HPLC.

Microbial inhibition experiments were also performed between AWRI 2804Δ*PAD1* and the wild type strains AWRI 1613 and AWRI 1499, and between AWRI 1613Δ*PAD1* and the wild-type strains AWRI 1613 and AWRI 1499. Experiments with AWRI 2804Δ*PAD1* were performed in red wine containing 6% v/v ethanol, whereas experiments with AWRI 1613Δ*PAD1* were performed in red wine containing 12% v/v ethanol. Red wine was supplemented with coumaric acid at 100 mg/L. Wild type strains were inoculated at $1x10^2$ cells/mL, while the mutants were inoculated at $1x10^6$ and $1x10^7$ cells/mL. Strains were inoculated simultaneously and incubated at 28°C. Cell concentrations and 4-ethyl phenol formation were determined as described above.

6.1.7 Selection and visualisation of *PAD1* mutants

Three different compounds were evaluated for their potential to aid in the selection of *PAD1* mutants. AWRI 2804 and AWRI 1613 parental strains and their corresponding *PAD1* deletants were grown in microplates containing YNB media supplemented with different concentrations of cinnamic acid (0 - 2 mM), 4-fluorocinnamic acid (0 - 2 mM) and YPD media supplemented with γ-valerolactone (0 - 3.5% w/v). Plates were incubated at 28°C for five days with cell growth determined by absorbance at 600 nm.

Two pH indicators, methyl-orange and bromophenol blue, were evaluated for their potential to help in the visualisation of *PAD1* mutants. Parental strains and mutants were inoculated individually in test tubes containing YNB media supplemented with coumaric acid (100 mg/L) and a pH indicator (0.05 mg/mL). Tubes were incubated at 28°C for five days with changes in colour evaluated spectrophotometrically.

7. Results and discussion

7.1 *Brettanomyces* industry survey

The results of previous laboratory-scale directed evolution provided direct evidence that *B. bruxellensis* has the capacity to evolve increased levels of tolerance to SO_2 (Bartel et al. 2021). However, a key question for the Australian wine industry was whether this was happening in the field.

Previous industry-based population surveys in the early 2000s had demonstrated that the AWRI1499 clade of strains, which displays the highest levels of SO_2resistance, was the most frequent *Brettanomyces* genotype isolated from Australian wineries (Curtin et al. 2012, 2007).

Since that time, Australian winemakers have become increasingly aware of the importance of SO_2 management for *Brettanomyces* spoilage control. These changing practices may have provided conditions analogous to laboratory-based selection, thereby resulting in the evolution of increased tolerance to SO_2.

Isolates of *Brettanomyces* were therefore sourced from the Australian wine industry over the period of 2017 to 2022, by microbiological plating of *Brettanomyces*-affected wines.

7.1.1 Tolerance to SO_2

The tolerance of industry isolates to SO_2 was assessed using high-throughput microplate liquid assays (Figure 1). Data were collected for 389 strains that spanned 19 years, with strains isolated prior to this project being sourced from the AWRI Wine Microorganism Culture Collection. Strains were grouped into three different time periods for comparison, 2000-2004, 2010-2014 and 2016-2019, in addition to the use of three reference isolates with known genotypes, AWRI 1499, AWRI 1613 and AWRI 2809.

Tolerance to SO_2 was assessed via a microplate growth assay that scored the ability for strains to grow in across a range of increasing SO_2 concentrations (Figure 1). The level of SO_2 tolerance observed from historical isolates did not differ between the 2000-2004 and 2010-2014 cohorts. The most tolerant isolates of these two cohorts tolerated SO_2 concentrations similar to those of AWRI 2809. Isolates obtained from the 2016-2019 period were found to have a far higher

average SO_2 tolerance and the cohort contained several strains that exhibited significantly higher SO_2 tolerance than had been observed previously (>1 mg/L mSO_2 in the YNB media used).

7.1.2 Volatile phenol production

Adaptation to specific environmental challenges can often lead to fitness trade-offs, which can influence unrelated phenotypes. While *B. bruxellensis* appears to be adapting to become more tolerant to SO_2, these strains may no longer retain the same capacity to spoil wine. High-throughput screening for the consumption of coumaric acid, the precursor to the *Brettanomyces*-derived volatile phenol spoilage compounds, was applied against the isolates that had been screened previously for tolerance to SO_2.

There was no correlation observed between SO_2 tolerance and the consumption of coumaric

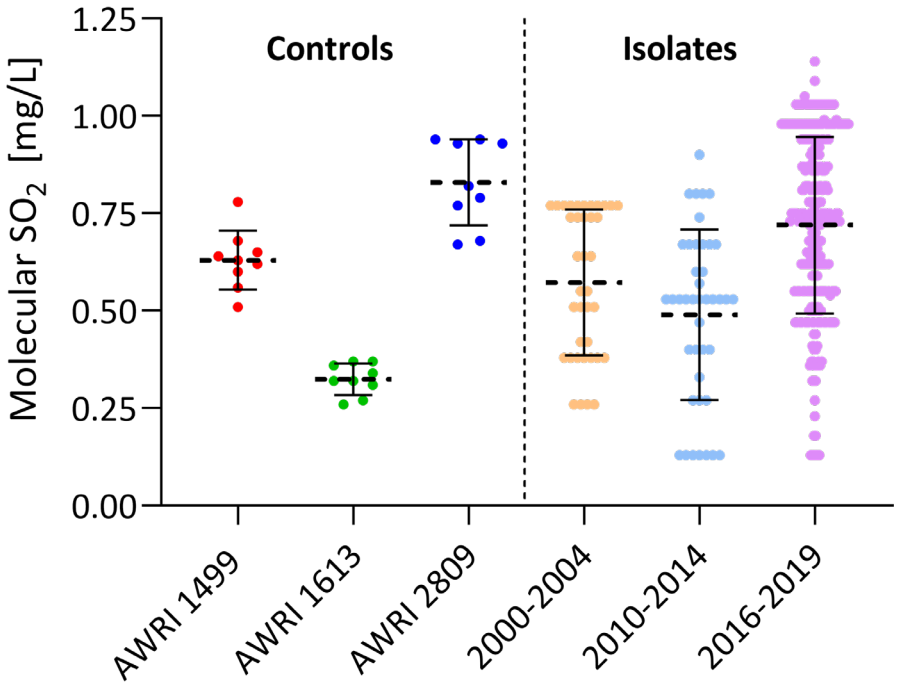


Figure 1. Sulfite tolerance of industry isolates. High-throughput SO_2 tolerance assays were performed on reference isolates (left) or industry isolates from three difference time periods (right). For reference isolates, each point represents an individual biological replicate. For the industry isolates, each point represents the results for an individual strain. Median tolerance is indicated by the dashed line with the inter-quartile range indicated by the solid lines.

acid, or trends between period of isolation and coumaric acid consumption. All strains that were tested were shown to consume coumaric acid at rates that would lead to significant wine spoilage.

The SO_2-tolerant isolates would therefore be predicted to readily spoil wine under SO_2 conditions that would inhibit spoilage by strains with lower levels of tolerance, including those that were previously assumed to represent the upper limits for *Brettanomyces*.

7.1.3 Genetics of SO_2-tolerant strains

B. bruxellensis has been shown to have a highly variable genome. Previous work in the early 2000s identified the AWRI 1499 clade as the most prevalent (and most SO_2-tolerant) genotype

that could be isolated from Australian wineries. Subsequent genome sequencing work showed that this group of *B. bruxellensis* strains possessed a triploid genome, which is predicted to have arisen through a hybridisation event between a mildly heterozygous diploid strain and a more divergent (possibly a sister species) haploid strain. Biologically, this type of hybridisation event would be considered to occur very rarely, however the high-level of SO_2 tolerance that was produced is predicted to have allowed for a 'selective-sweep' to have occurred, such that this genotype was able to largely replace other *B. bruxellensis* genotypes in the winemaking environment.

The discovery of strains with levels of SO_2 tolerance that exceeded those observed previously for members of the AWRI 1499 clade (including AWRI 2809), posed the question as to whether these new strains represented adapted members of the AWRI 1499 group, or a new genotypic group. To address this question, twenty-four of the most SO_2-tolerant isolates were chosen for whole genome sequencing. All isolates were shown to cluster into the AWRI 1499-clade,

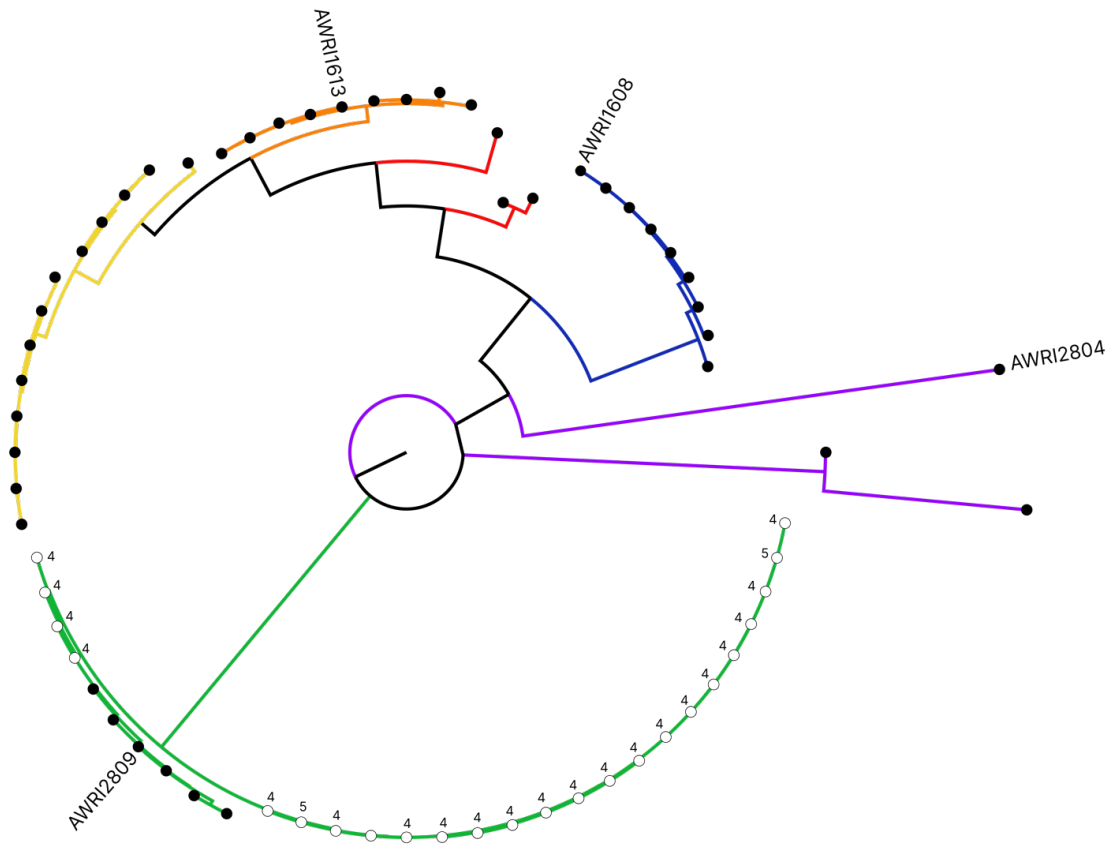


Figure 2. Phylogenetic clustering of sulfite-tolerant industry isolate genome sequences. Genomic clustering of 24 industry isolates (white nodes) on a species-tree of *B. bruxellensis*. The *B. bruxellensis* species tree can be divided into five clades, indicated by the branch colours, with the AWRI 1499 clade (known high SO_2 tolerance) shaded in green. All industry isolates except one were shown to have increased copies of *SSU1* gene (four or five copies as indicated).

irrespective of their isolation date (Figure 2).
As a triploid, AWRI 1499 has three copies of the *SSU1* gene. Analysis of *SSU1* copy-number in these isolates indicated that all but one (23/24) had undergone amplification of *SSU1*, with 91% (21/23) having four copies of SSU1 and two isolates predicted to have five.

The results of the genome sequencing were therefore consistent with further adaptation of the AWRI 1499 clade towards higher SO_2 tolerance, as opposed to a selective-sweep by a novel genotype. Furthermore, it appears that amplification of *SSU1* copy number may be a key driver in this adaptive response, as was observed in laboratory-based adaptive evolution of SO_2 tolerance in *B. bruxellensis* (Bartel et al. 2021).

7.1.4 Susceptibility to alternate control measures

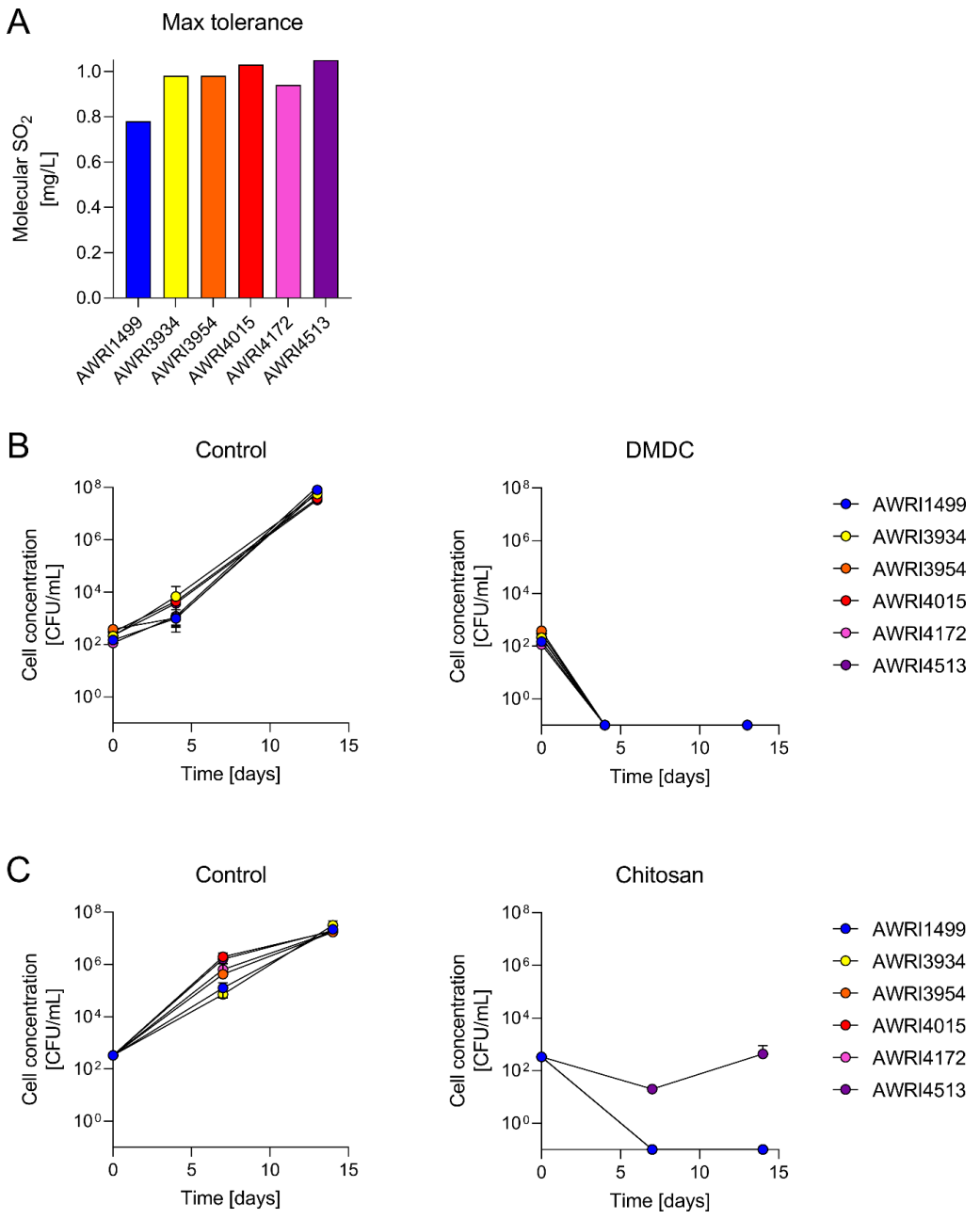


Figure 3. Susceptibility of sulfite-tolerant industry isolates to alternative control measures. (A) Maximum sulfite tolerance for AWRI 1499 and industry isolates. (B) Cell concentrations for AWRI 1499 and industry isolates in synthetic wine in response to DMDC 200 mg/L. (C) Cell concentrations for AWRI 1499 and industry isolates in CDW in response to chitosan 80 mg/L.

The results of the industry survey clearly indicate that *Brettanomyces* strains are evolving higher levels of tolerance to SO_2 in the field. To provide information on effective options for winemakers dealing with issues related to SO_2 tolerance, tolerant industry isolates were tested for susceptibility to the alternative antimicrobial agents, dimethyl dicarbonate (DMDC) and chitosan (Figure 3).

DMDC was shown to be effective against all five of the SO_2-tolerant isolates, with no viable cells observed four days after treatment of model wine containing actively growing *Brettanomyces*.

Chitosan was also shown to be highly effective against the SO_2-tolerant isolates. No viable cells were observed in model wine inoculated with four of the industry isolates (in addition to AWRI 1499) after seven days of treatment with chitosan. One strain, AWRI 4513, was shown to be resistant to killing by the addition of chitosan; however, the treatment process was able to inhibit the proliferation of this strain, keeping the level of viable cells after 14 days of incubation similar to those at the time of inoculation, while the control treatment saw extensive proliferation after inoculation.

Both DMDC and chitosan therefore remain viable alternatives for the control of the majority of SO_2-tolerant isolates of *B. bruxellensis*. The potential resistance of AWRI 4513 to both SO_2 and chitosan is concerning, especially as no broad survey into strain-specific sensitivity to chitosan has been conducted in Australia, so the prevalence of chitosan resistance is currently unknown.

7.1.5 Redacted due to sensitive commercial information

7.2 CRISPR engineering

CRISPR-based editing tools, which are expressed from plasmids, have been successfully applied for genome engineering in several non-conventional species, where they have been shown to stimulate homologous recombination via the production of double-stranded breaks (Cai et al. 2019, Shapiro et al. 2018). In addition, expression-free systems, which make use of purified CRISPR protein and guide RNA, have been used to modify the genome of several *Candida* species (Grahl et al. 2017).

7.2.1 Development of CRISPR-Cas9

As *B. bruxellensis* lacks a documented plasmid-based expression system, an expression-free CRISPR-Cas9 system was used as the basis for a genomic engineering methodology.

To begin, CRISPR was used to target two genes for deletion that display easily detected mutant phenotypes:

- *URA3*: 5-FOA resistance and uracil auxotrophy
- *ADE1*: pink colouration, adenine auxotrophy.

Targeted deletion of *URA3* and *ADE1* were both attempted using DNA cassettes with either short (60 bp) or long flanking (1 kb) regions of homology, which also contained a drug-selectable marker, such as *natMX* (nourseothricin resistance, nat^R) or *kanMX* (G418 resistance, $G418^R$).

Transformation with the appropriate short flanking DNA cassette and CRISPR-Cas9 reagents enabled the isolation of several colonies (11% for *URA3* and 10% for *ADE1*) with genome sequences consistent with a clean replacement of the ORF via homologous recombination. In addition to those colonies that had a clean replacement of the target gene, many also showed an auxotrophic phenotype due to partial deletion of the ORF, indicating that the homologous recombination machinery in *B. bruxellensis* is inefficient even when combined with the CRISPR-Cas9 system.

While very short homology (50 bp) is sufficient for homologous recombination in *S. cerevisiae* (Cai et al. 2019), very long stretches of homology (over 1 kb) are often needed in other species (Löbs et al. 2017). For *URA3*, most colonies transformed with combination of the long flanking cassette and specific CRISPR-Cas9 (10 out of 12 tested) showed a clean deletion of the gene, whereas only one colony (10 tested) showed a clean deletion of *ADE1*.

7.2.2 Targeted gene deletion of *SSU1*

As the experiments with deleting *URA3* and *ADE1* with short-flanking cassettes and CRISPR-Cas9 were effective, *SSU1*, the main gene responsible for SO_2 tolerance in yeast (Divol et al. 2012) was then targeted using this approach.

Following transformation, 30 nat^R colonies were screened by PCR for loss of *SSU1*, with 10 displaying a PCR pattern consistent with the expected gene deletion. Genome sequencing of two of these transformants showed that both contained a clean deletion of *SSU1* with no other copies of the *natMX* cassette elsewhere in the genome.

Given the expected importance of *SSU1* in tolerance of *B. bruxellensis* to SO_2, assays were performed on the two *SSU1* deletion strains to determine if an SO_2-sensitive phenotype was produced by the loss of this gene.

In media without SO_2, both the wild type and *SSU1* deletion strains showed robust growth (Figure 6A). However, the presence of SO_2 in the medium resulted in significant inhibition in the proliferation of both *SSU1* deletion strains, with 0.09 mg/L mSO_2 completely inhibiting growth (Figure 6B). This work therefore formally confirmed the role of the *SSU1* gene in providing tolerance to the inhibitory effects of SO_2 in *B. bruxellensis*.

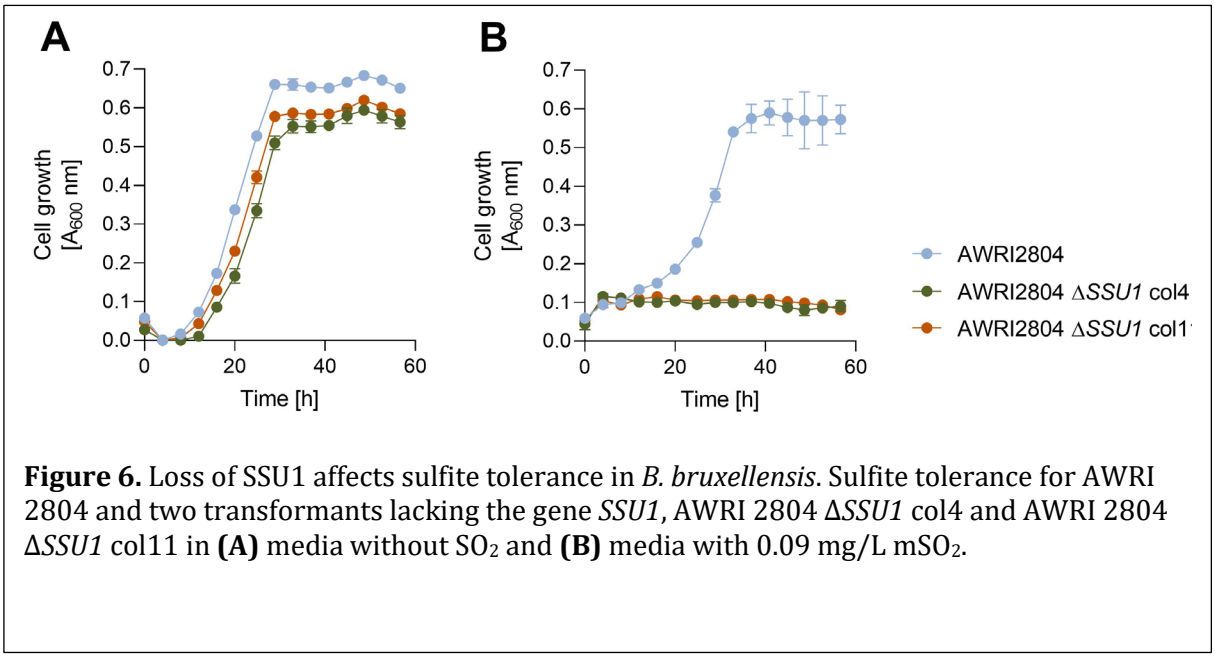


Figure 6. Loss of SSU1 affects sulfite tolerance in *B. bruxellensis*. Sulfite tolerance for AWRI 2804 and two transformants lacking the gene *SSU1*, AWRI 2804 Δ*SSU1* col4 and AWRI 2804 Δ*SSU1* col11 in **(A)** media without SO_2 and **(B)** media with 0.09 mg/L mSO_2.

7.2.3 Targeted gene deletion in a diploid background

While proof-of-concept CRISPR work was performed in a haploid strain (AWRI2804), most strains of *B. bruxellensis* exist as either diploids or triploids (Avramova et al. 2018).

To evaluate the possibility of simultaneously deleting multiple alleles in *B. bruxellensis*, targeted deletion of *URA3* was attempted in a diploid strain (AWRI 1613). Transformation of AWRI 1613 with the long-flanking *URA3* cassette and CRISPR-Cas9 delivered three nat^R colonies (96 tested), with a PCR pattern consistent with the correct integration. However, none of these nat^R colonies

also displayed uracil auxotrophy, suggesting that only one out of the two *URA3* alleles was deleted.

While it was not possible to simultaneously target both alleles in a diploid, the possibility of stepwise deletion remained as an alternative methodology. A homozygous deletion of the *URA3* locus was therefore attempted by transforming one of the heterozygous AWRI 1613 *URA3* deletion strains with a long-flanking *URA3* cassette which used *kanMX* as an alternative to the *natMX* marker used for the first transformation.

Following this process, 16/81 G418^{R}/*URA*$^{-}$ transformants were also shown to have the *kanMX* cassette correctly integrated in the remaining wild type allele of *URA3*. Genome sequencing of two potential double deletion strains confirmed a clean replacement of the remaining *URA3* gene with the *kanMX* cassette, leaving the original *URA3* deletion by the *natMX* cassette unchanged. No additional copies of either *URA3* cassette were found in the genome. Deletion of both copies of a gene within a diploid genetic background is therefore achievable via a sequential transformation strategy in *B. bruxellensis*, opening the way for genomic engineering in wine strain backgrounds.

7.3 Volatile phenol formation in *Brettanomyces*

The formation of volatile phenols is the major characteristic of wine spoilage by *B. bruxellensis*. However, the genetic pathways that underpin the formation of these compounds remain to be fully elucidated.

Two approaches were employed to identify genes in *B. bruxellensis* that contribute to the formation of volatile phenols, an insertional mutagenesis screen and a candidate gene approach.

7.3.1 Insertional mutagenesis screening

Insertional mutagenesis (IM) makes use of random integration of known sequences of DNA into the genome to induce a mutant phenotype due to the disruption of gene function by the introduced DNA.

As the majority of transformed DNA integrates randomly into the *Brettanomyces* genome (See Section 7.2), it was reasoned that transformation with an appropriately designed DNA cassette could be used to produce a library of strains with each containing a unique DNA integration event(s). This library could then be used to screen for phenotypes of interest, with the gene(s) responsible determined through mapping the insertion point of the cassette.

The *Brettanomyces*-specific marker cassette (Varela et al. 2018) was used as the starting point for the construction of the IM-cassette (Figure 7). To enable downstream use of mixed pools of transformants, an 18 bp random DNA barcode was incorporated between the resistance marker and terminator. A mixed library of IM-cassettes was ultimately produced that contained more than ~2 x 10^{6} different barcode permutations.



Figure 7. Structure of the IM-insertion cassette. The promoter of the *B. bruxellensis TDH1* gene (TDH1pr) drives expression of the nourseothricin resistance gene (natR). An 18 bp random promoter sits between natR and the transcriptional terminator of the*TEF1* gene (TEFt).

An aliquot of the mixed IM-cassette library was subsequently transformed into *B. bruxellensis* strain AWRI 2804 and ~18,000 individual transformants were isolated and arrayed in 96-well

format to form a *Brettanomyces* random deletion library (BRDL). Barcode integration was assessed in selection of 12 random strains, with individual barcodes obtained in 75% of all transformants, indicating a single integration event. Multiple cassette integrations were observed in the remaining transformants.

Once the validity of insertion within the small test set of strains was established, the strain diversity within the library was determined by performing bulk insertion mapping on the entire population. DNA was isolated from the entire library and inverse PCR was used to amplify genomic DNA that flanked the IM-cassette insertion points (while also recording the barcode sequence). Over 7,920 individual insertions points were recorded that were further categorised based upon their proximity to the coding portions of predicted genes in the AWRI 2804 genome (Table 2).

Table 2. Insertion locations in the *Brettanomyces* genome

Insertion type	**Number**
Within coding sequence	1,632
1-99 bp upstream of coding sequence	109
1-500 bp upstream of coding sequence	450

The IM library was used in a high-throughput screen to attempt to identify mutants that were no longer capable of metabolising coumaric acid as a proxy for the inability to produce volatile phenols. Each strain was screened for growth (OD_{600nm}) and coumaric acid consumption (OD_{325nm}), with four candidate strains chosen based upon >50% residual coumaric acid levels despite significant growth of the strains in the media (OD_{600nm} >0.3) (Figure 8). Volatile phenol production for these four candidate isolates was subsequently tested in shake-flask format. All four candidate strains were shown to produce significant levels of volatile phenols, indicating that these represented false positives from the screening procedure.

7.3.2 Redacted due to sensitive commercial information

7.3.3 Random mutagenesis and selection

While CRISPR-based engineering represents an established option to produce *PAD1* mutants in a variety of strain backgrounds, strains produced by this technology represent genetically modified organisms and are currently unable to be used in wine production in many parts of the world, including Australia.

However, the existence of Δ*PAD1* strains provides for a definitive positive-control to be assessed when considering potential avenues towards obtaining *Brettanomyces PAD1* (and also potentially *VPR*) mutants via classical mutagenesis and selection strategies, which produce non-GMO strains that can be used for winemaking.

Previous studies have suggested that the compounds cinnamic acid, 4-fluorocinnamic acid and γ-valerolactone interact with phenylacrylic acid decarboxylase in other yeast species and may provide a means for the positive selection of *PAD1* mutants (Bottoms et al. 2018, Mukai et al. 2010, Payne et al. 2015). To evaluate whether cinnamic acid, 4-fluorocinnamic acid and/or γ-valerolactone could be used in the selection of *PAD1* mutants, the tolerance to these compounds was determined for both the parental strains and the associated Δ*PAD1* mutants in AWRI 2804 and AWRI 1613.

In both strain backgrounds, the parental and Δ*PAD1* strains showed similar tolerance to both cinnamic acid and 4-fluorocinnamic acid. However, different profiles were obtained for γ-valerolactone, which depended on the strain background. For AWRI 2804, the Δ*PAD1* mutant

showed a higher tolerance than the parent at approximately 3% w/v, whereas for AWRI 1613, the parent showed higher tolerance than the mutant at the same concentration.

Given the lack of a distinguishing concentration for cinnamic acid and 4-fluorocinnamic acid and the conflicting results for γ-valerolactone, none of the tested compounds can be considered to be effective for the selection of *PAD1*/*VPR* mutant strains.

In addition to growth-selection strategies for mutant isolation, colourimetric strategies can also provide a means to distinguish specific mutant strains from within a non-mutant background. Accordingly, the microbial consumption of acids is often accompanied by a localised change in the pH of the media. Two pH indicators were therefore evaluated for their potential to change colour in response to the consumption of coumaric acid. Methyl-orange (pKa 3.47) and bromophenol blue (pKa 4.0) were chosen, as their pKa values were similar to the pH of the media. However, no difference in colour was found between the parental and either AWRI 2804 or AWRI 1613 Δ*PAD1* mutants for either of the pH indicators, suggesting that the pH change due to the consumption of coumaric acid is not large enough to be visually detected with these indicator compounds.

8. Outcome and conclusion

8.1 Performance against planned outputs

All project deliverables and outputs have been achieved.

8.1.1 Additional strains of *Brettanomyces* isolated, genotyped and phenotyped for sulfite tolerance, growth rate and volatile phenol production

More than 300 industry isolates were phenotyped including high-throughput assessment of SO_2 tolerance and the production of volatile phenols. For the volatile phenol screening, a new high-throughput method was developed that used coumaric acid consumption as a proxy for the production of ethyl phenols. A range of SO_2 tolerance levels was observed, from highly sensitive to levels that well exceed those there were considered to be the highest expected for *Brettanomyces*. Far less variation was observed in the consumption of coumaric acid, with all strains consuming amounts of coumaric acid that would be predicted to produce significant wine spoilage. These more tolerant strains would therefore be expected to be as capable of spoiling wine as less susceptible strains.

Twenty-four of the most SO_2-tolerant isolates were fully genotyped using whole-genome sequencing. All of these isolates were shown to represent members of the previously identified AWRI 1499 clade, indicating that continued adaptation of this group is occurring, as opposed to the appearance of a new genetic clade.

8.1.2 Investigation of improved methods for genetic engineering of *Brettanomyces* (e.g. CRISPR)

An expression-free CRISPR methodology was developed for *B. bruxellensis*. This methodology was shown to efficiently produce targeted gene deletions for a variety of genes including *URA3*, *ADE1*, *SSU1* and *PAD1*. Deletions were able to be performed both in the haploid and diploid genetic background.

8.1.3 Knowledge of the genetic basis for the production of 4-EP and 4-EG

Homology-based gene prediction was used to screen the genome sequence of strain AWRI 2804 for genes with the potential to encode phenylacrylic acid decarboxylase enzymes. CRISPR-based gene deletion was used to formally define the *Brettanomyces bruxellensis* gene *PAD1* as being required for the conversion of hydroxycinnamic acids to their vinyl derivatives.

Four genes were identified in the AWRI 2804 genome that were reported to encode enzymes with vinyl-phenol reductase activity. The function of all four of these genes was tested using CRISPR-based gene deletion. Deletion of any of the four genes did not affect the production of ethyl phenols from their vinyl-phenol derivative. The identity of the gene encoding vinyl-phenol reductase in *Brettanomyces* remains to be determined.

8.1.4 Information on the utility of non-phenol-producing *Brettanomyces* strains

CRISPR-based deletion of the gene *PAD1* was shown to produce *B. bruxellensis* strains that were unable to convert hydroxycinnamic acids into ethyl phenol spoilage compounds. These phenol-negative strains were demonstrated to inhibit the growth and ethyl phenol production of wild type *Brettanomyces* when co-inoculated at levels that would be expected when applying the *PAD1* mutant as a commercial starter strain into a background of 'wild' *Brettanomyces*.

8.1.5 Knowledge of the genetic basis for evolution of sulfite-resistant strains of *Brettanomyces*

Genomic analysis was performed for a cohort of 24 SO_2-tolerant industry isolates. All of these isolates were shown to represent members of the previously identified AWRI 1499 clade, indicating that continued adaptation of this group is occurring, as opposed to the appearance of a new genetic clade. Detailed comparison of the new isolates with the genome of the AWRI 1499 reference strain showed that all but one of the contemporary strains displayed amplification of the gene *SSU1*. Amplification of *SSU1* has also been observed in laboratory-derived SO_2-tolerant

isolates and it is likely that it is this alteration that is the main driver of the increased SO_2 tolerance of these new strains.

8.1.6 Redacted due to sensitive commercial information

8.1.7 Practical advice and extension materials for winemakers

Extension material has been refined in conjunction with the AWRI IDS team. Advice regarding the appearance of SO_2 tolerance and implications of pH on the effectiveness of SO_2 have been communicated with winemakers through AWRI workshops and AWITC presentations.

8.2 Practical implications

As flagged at the onset of this project, while the importance of sulfite in the control of *Brettanomyces* has been known to the Australian industry since the early 2000s, the trend towards higher pH wines with minimal amounts of sulfite and processing (including filtration) provides the ideal conditions for the development of sulfite tolerance. Indeed, the continued survey of isolates, combined with the high-throughput sulfite-tolerance and coumaric acid utilisation assays and genomic sequencing has confirmed that genetic adaptations are occurring in the wine industry and that these adaptations are providing increased sulfite tolerance.

By combining this knowledge with a rapid, sensitive and affordable test for *Brettanomyces*, which can also provide predictions of sulfite tolerance, this can provide winemakers with the information needed to inform risk-based winemaking practice assessment, with more conventional winemaking approaches (low(er) pH, higher SO_2, filtration, alternative control measures) warranted where sulfite-tolerant *Brettanomyces* are detected early.

The development of a phenol-negative biocontrol agent has the potential to revolutionise the management of *Brettanomyces* as this platform has the potential to provide lasting protection against spoilage, while avoiding the use of preservatives such as SO_2, DMDC and chitosan.

8.3 Benefits

Knowledge on the industry-wide occurrence of sulfite-resistant *Brettanomyces* and the genetics underpinning these changes provides a foundation for the potential refinement of best-practice *Brettanomyces* control strategies. Information on the effectiveness and mode-of-action of alternative control methods, such as chitosan and DMDC, provides winemakers with additional means to control *Brettanomyces*.

The development of a rapid, robust and economic diagnostic platform can assist winemakers to be proactive in preventing *Brettanomyces* spoilage rather than undertaking remediation efforts once spoilage has occurred. Remediation involves either blending with non-affected wine to reduce overall levels of ethyl phenols (following sterile filtration) and/or removal of spoilage compounds via reverse osmosis. While these can be effective, they generally result in significant quality reduction and economic studies (based on data from the Californian wine industry) show prevention strategies to be far more cost-effective in the long-term (Alston et al. 2021).

The establishment of a targeted gene deletion platform in *B. bruxellensis* unlocks the ability to undertake detailed investigation into the biology of this important spoilage yeast. The development of phenol-negative *Brettanomyces* is a prime example of the power of this technique; however, it also paves the way for *Brettanomyces* to be used in synthetic biology pipelines to further expand the potential uses of this species in the green biotechnology space.

9. Recommendations

The development of tolerance to SO_2, in combination with the potential existence of strains with resistance to chitosan, is a potential threat to the Australian wine industry. This situation is similar to that seen in the development of agrochemical resistance in the viticultural sector, where significant effort has been directed into industry-wide surveillance regimes. A similar framework should be established to track the development of SO_2 resistance in *B. bruxellensis*

into the future and to investigate methodologies for limiting resistance development while dealing with contamination by resistant strains. This monitoring framework should be part of a renewed extension program for *Brettanomyces* management, that highlights the combined effects of SO_2, pH, adaptation by *Brettanomyces* and developing diagnostic, prevention and remediation options.

The proof-of-concept development work using LAMPseq technology has shown that this technique can provide a field deployable methodology for the sensitive detection of *B. bruxellensis*. When combined with sequencing, it can provide genotypic information regarding likely levels of SO_2 tolerance and could potentially be multiplexed further to provide detection of a range of spoilage microorganisms in a single test.

Initial work with the phenol-negative *PAD* deletion strains of *B. bruxellensis* has the potential to provide a microbiological solution to inhibiting spoilage by wild strains. Further work should be performed to develop new strain backgrounds and to test the efficacy of strains under industry-use scenarios. The investigation of additional mutation/selection routes to obtain non-GMO variants should also be pursued to enable industrial deployment of these strains under current regulatory frameworks.

10. Appendix 1: Communication

Information was extended to industry stakeholders through roadshow presentations and workshops and presentations at the 17th and 18th Australian Wine Industry Technical Conferences in 2019 and 2022. Details of all presentations given are published in the AWRI Annual Report, accessible from: https://www.awri.com.au/about_the_awri/annual-reports/

Peer-reviewed articles published:

Bartel, C., Roach, M., Onetto, C., Curtin, C., Varela, C., Borneman, A. 2021. Adaptive evolution of sulfite tolerance in *Brettanomyces bruxellensis*. *FEMS Yeast Res.* 21(5): foab036.

Roach, M.J., Borneman, A.R. 2020. New genome assemblies reveal patterns of domestication and adaptation across *Brettanomyces* (*Dekkera*) species. *BMC Genom.* 21: 194.

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Varela, C., Bartel, C., Onetto, C., Borneman, A. 2020. Targeted gene deletion in *Brettanomyces bruxellensis* with an expression-free CRISPR-Cas9 system. *Appl. Microbiol. Biotechnol.* 104: 7105–7115.

11. Appendix 2: Intellectual property

Program IP has been communicated through presentations and publications and resides in the public domain. Copyright in any reports, posters or presentations is held by the AWRI. Copyright in publications remains with the publisher. No patentable discoveries have been identified from the work. A commercialisation evaluation of LAMPseq methodology has been conducted and this technology has been identified as a potential target for commercialisation. AWRI is exploring what business model is appropriate to present to the Management Committee for consideration.

12. Appendix 3: References

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13. Appendix 4: Staff

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14. Appendix 5: Other supporting material (including raw data, if appropriate)

Not applicable