Brettanomyces yeasts still have potential to give wine producers a headache

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Spoilage of wine by Brettanomyces yeasts remains a challenge for winemakers. Current strategies to minimise the risk of spoilage do not eliminate Brettanomyces yeasts from wineries and are heavily reliant on the preservative sulfite to stabilise wine against Brettanomyces growth. Researchers at the AWRI have demonstrated that existing Brettanomyces wine strains have the potential to evolve greater tolerance to sulfite and have found preliminary evidence that this may be occurring in industry. These findings highlight the importance of judicious use of sulfite and the need to continue to evaluate sulfite alternatives.

BACKGROUND

Brettanomyces bruxellensis (commonly known in the wine industry as 'Brett') is a species of yeast that causes wine spoilage. The greatest impact of this yeast on wine sensory properties occurs through the production of 4-ethylphenol and 4-ethylguaiacol (Curtin *et al*. 2015). These compounds are mostly responsible for the Brett character found in wines affected by *B. bruxellensis*, often described as 'medicinal' or 'phenolic'. Wine quality scores and consumer preference ratings are lower for wines exhibiting Brett character (Lattey *et al*. 2010) and consumers dislike wines with high concentrations of 4-ethylphenol and 4-ethylguaiacol (Curtin *et al.* 2008).

The risk of Brett spoilage is currently managed through the application of a multi-faceted strategy developed by the AWRI in collaboration with Australian winemakers, which facilitated industry-wide decreases in the levels of Brett spoilage compounds in finished wines (Curtin *et al.* 2008). This strategy does not, however, eliminate Brettanomyces yeasts from wineries, and is heavily reliant on the use of preservative sulfite (at an appropriate pH) to stabilise wine against Brettanomyces growth. Previous work has shown that the majority of Brett strains isolated in Australia belong to a relatively sulfite-tolerant genetic group, and that the overall proportion of sulfite-tolerant strains was higher in samples obtained in 2004-2005 when compared with those sourced in earlier years (Curtin *et al.* 2012).

To ensure Australian winemakers' continued ability to minimise the risk of Brett spoilage, it is important to evaluate and understand the potential for Brett strains to evolve increased tolerance to sulfite. Much like the evolution of antibiotic resistance in bacteria, where repeated exposure to a non-lethal dose of antibiotic can trigger the evolution of increased tolerance for that antibiotic, Brett strains may be able to evolve survival mechanisms to thrive under increasing concentrations of sulfite.

EVOLUTION OF SULFITE RESISTANCE IN LABORATORY EXPERIMENTS

Long-term repeat batch culture, or 'adaptive evolution', experiments are an effective way to study the ability of microorganisms to evolve tolerance through adapting to challenging environments. In this technique, a microbial culture is repeatedly grown over a long period of time under ever-increasing concentrations of the inhibitor under investigation, ultimately generating a microbial population that can tolerate a greater concentration of the inhibiting compound. This closely mimics the way microbes grow in nature (or in wineries). During adaptive evolution experiments, microbes can sometimes grow better by adapting to the experimental conditions (nutrient source, pH, temperature, etc) and not necessarily to the inhibiting substance. To account for this, parallel experiments with control populations evolving without the inhibitor are usually also conducted for comparison purposes.

To use adaptive evolution to study sulfite tolerance in Brettanomyces, batch cultures were established for three different Brett strains: AWRI1613, AWRI1499 and AWRI2804. Increasingly

AT A GLANCE

- *Brettanomyces bruxellensis* spoils wine by producing 4-ethylphenol and 4-ethylguiacol, which are responsible for 'Band-Aid', 'phenolic', 'leather', 'sweaty', 'medicinal' and 'barnyard' aromas, often combined with a 'metallic' aftertaste.
- Although a combination of strategies is used to limit the growth of Brettanomyces yeasts in wineries, the effective use of sulfite is the primary control measure used to avoid wine spoilage by these yeasts.
- Laboratory experiments have revealed that common Australian winery strains of B. *bruxellensis* have the potential to evolve greater tolerance to sulfite. Strikingly, comparing new B. *bruxellensis* industry isolates to strains isolated over the past two decades suggests that selection for sulfite tolerance may already be occurring.
- AWRI researchers are seeking samples of finished but unfiltered red wine from around Australia to gain a broader picture of sulfite resistance in current populations of Brettanomyces.

higher concentrations of sulfite were introduced to the cultures over a period of almost 12 months. At the end of this long adaptive period, all of the *B. bruxellensis* experimental populations displayed increased tolerance to sulfite, although both the speed of adaptation and the ultimate level of tolerance was highly dependent on the starting strain (Figure 1). The highest concentrations of the molecular form of sulfite, sulfur dioxide (SO_2), at which evolved populations were successfully sub-cultured were: 0.71mg/L for AWRI1613, 1.00mg/L for AWRI1499, and 0.27mg/L for AWRI2804. As a comparison, a wine with 16mg/L of free SO_2 at pH3.5 and 14%v/v ethanol would have 0.58mg/L of molecular SO_2.

For each strain, individual colonies from each of the evolving populations (A, B and C) and from control populations (not exposed to sulfite, labelled D, E and F in Figure 2, see page 38) isolated after 50 and 100 generations were individually assessed for sulfite tolerance. For AWRI1613 at 100 generations, the three evolving populations showed a remarkably increased sulfite tolerance compared with the control population (Figure 2, top). For AWRI1499 (the most sulfite-tolerant parental strain) at 100 generations, all three populations exposed to sulfite stress (A, B and C) included individual isolates with higher sulfite tolerance. However, only in AWRI1499 population C were sulfite-tolerant individuals the dominant type of strain. As indicated by the broad distribution of individual sulfite tolerances, both populations A and B appeared to retain individuals that were not more tolerant to sulfite than the founding strain AWRI1499 and may therefore represent 'cheater' strains that have simply adapted to the general media conditions to increase their overall growth rate (Figure 2, bottom).

Adaptive evolution experiments with AWRI2804 (a non-wine, sulfite-sensitive strain) displayed modest increases in sulfite tolerance in only one of the populations (data not shown), suggesting that AWRI2804 does not have the requisite genetic adaptability required to improve sulfite tolerance under the conditions tested. Given the varied behaviour of the individual isolates and the differential evolution of this trait observed across strains, it is likely that the initial genetic make-up of the population influences the evolutionary extent of sulfite tolerance in *B. bruxellensis*. The focus of the AWRI's research on Brett is now shifting towards identifying and characterising

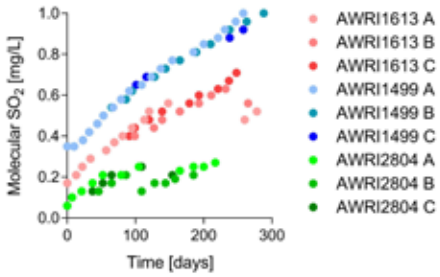

Figure 1. Laboratory adaptive evolution experiments. Replicate populations (A, B and C) for three Brettanomyces strains (AWRI1613, AWRI1499 and AWRI2804) were subjected to increasing concentrations of sulfite over time. The dots on the graph show the maximum SO_2 concentration at which viable cells could be cultured for each population at a particular time (i.e., the maximum concentration of SO_2 each population could tolerate at that time).

the mechanisms by which *B. bruxellensis* gains tolerance to sulfite to understand the basis of these changes.

SULFITE TOLERANCE OF NEW INDUSTRY ISOLATES

The results of the laboratory-scale directed evolution experiments provide the first direct evidence that *B. bruxellensis* strains have the capacity to adapt to the use of sulfite and increase their level of sulfite tolerance. However, the key question for the Australian wine community is whether this could happen in the field. Previous industry-based population surveys in the early 2000s had already shown that the AWRI1499-like strains, which display the highest levels of sulfite resistance, were the most frequent Brettanomyces genotype isolated from Australian wineries (Curtin *et al*. 2008, Curtin *et al*. 2012). Since then, Australian winemakers have become increasingly aware of the importance of sulfite management for controlling the risk of Brettanomyces spoilage. Their changing practices could potentially be providing selective conditions similar to those imposed in the laboratory, thereby causing evolution of sulfite tolerance.

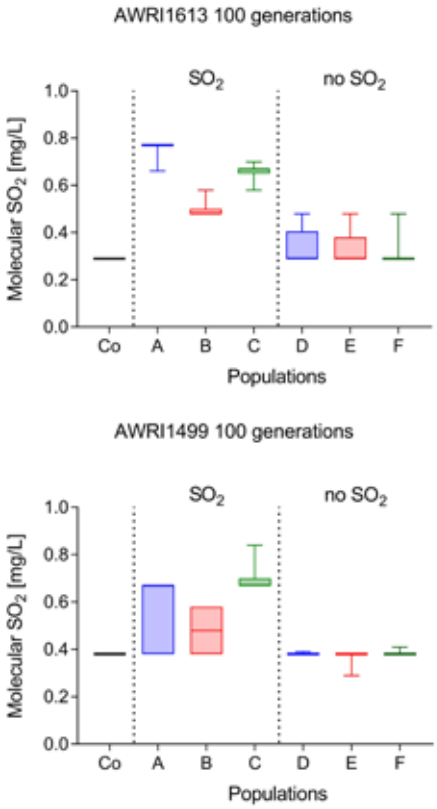

Figure 2. Sulfite tolerance for individual isolates obtained at 100 generations during adaptive evolution experiments for AWRI1613 (top) and AWRI1499 (bottom). Populations exposed to increasing concentrations of sulfite (A, B and C), control populations without sulfite (D, E and F) and control original strain (Co).

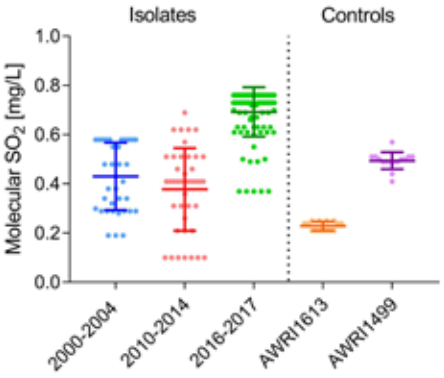

Figure 3. Sulfite tolerance for industry isolates obtained during the years 2000-2004, 2010-2014, and 2016-2017. AWRI1613 and AWRI1499 are included as controls.

Historical industry isolates were therefore sourced from the AWRI wine microorganism culture collection (including those isolated during the original Curtin 2008 *et al*. study), in addition to current industry isolates sourced from a commercial partner in 2016-2017. All of these strains were subjected to sulfite tolerance assays to determine if levels of sulfite resistance had changed over time (Figure 3).

Isolates from 2000-2004 displayed levels of sulfite tolerance that ranged between those observed for AWRI1499 and AWRI1613 and broadly represented the range of tolerances observed in the original study (Curtin *et al*. 2012a). Isolates from 2010-2014 did not show a significant difference in their median sulfite resistance, although there were a small number of isolates that displayed higher levels of sulfite resistance than those seen in the 2000-2004 cohort. Interestingly, the 2016-2017 isolates, sourced from 16 different wines, displayed greater tolerance to sulfite, growing at concentrations significantly higher than those observed from the two previous cohorts. It should be noted that the 2016-2017 isolates were sourced from only two wineries (although one is a multi-site producer). As such, they represent a small part of the overall industry. However, this preliminary finding suggests that strains with significantly higher levels of sulfite resistance are currently present in the field. Further sampling is required to determine the extent of resistance development and to determine if this is a wide-reaching problem requiring significant attention by the Australian wine industry. The AWRI is therefore seeking samples of finished wine (prior to any filtration, with or without suspected Brettanomyces spoilage) from around Australia in order to gain a broader understanding of current levels of sulfite resistance. Any assistance would be greatly appreciated, and winery anonymity will be maintained at all times for those that can provide samples.

CONCLUSION

Adaptive evolution experiments conclusively demonstrated that Brettanomyces strains have the capacity to evolve greater tolerance to sulfite, though the extent to which this is possible depends on the genetic make-up of individual strains. A small number of new Brettanomyces industry isolates (from 2016-2017) are, on average, significantly more sulfite tolerant than isolates obtained during earlier studies (2000-2014). Although these results might not reflect a wide-reaching resistance problem, further sampling is required to determine if additional wineries are experiencing Brett populations with increased sulfite tolerance. In any case, this work highlights the need to evaluate other agents that may hinder Brettanomyces growth by themselves or in conjunction with sulfite.

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