# Staying a step ahead of 'Brett'

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***Brettanomyces bruxellensis* (known in the wine industry as 'Brett') is a species of yeast that produces unpleasant medicinal and phenolic characters in wine. While there are practical steps to be taken in the winery that are currently successful at controlling 'Brett', there is a risk that a strain could emerge that is resistant to control strategies. For that reason, research is under way to understand this yeast at the genetic level and make sure winemakers can stay ahead of any 'Brett' threat.***

## A SHORT HISTORY OF 'BRETT' AND AUSTRALIAN WINE

*Brettanomyces* yeasts are found in many fermented beverages, but are particularly well-known for their role in wine, beer and cider. They have been isolated from wines made around the world, including 31 winemaking regions of Australia (AWRI publication #989). When growing in wine, these yeast produce the compounds 4-ethylphenol and 4-ethylguaiacol, which are responsible for Bandaid®, phenolic, leather, sweaty, medicinal, and barnyard aromas. Commonly known as 'Brett' character, these aromas are often combined with a metallic aftertaste. *Brettanomyces* yeasts are able to form these compounds via a metabolic pathway that is not present in other wine yeast.

Brett was a major problem for the Australian wine industry during the late 1990s and early 2000s – most red wines contained some Brett spoilage compounds, often at levels later shown to be perceived negatively by consumers (AWRI publication #1043). Extensive communication of a practical Brett control strategy (AWRI publication #756) was successful in reducing Brett effects on Australian red wines, with typical 4-ethylphenol levels in major Cabernet Sauvignon producing regions falling from approximately 1000ppb in vintage 2000 to less than 100ppb by vintage 2005. To put this in perspective, the perception threshold for 4-ethylphenol is around 300-600ppb, depending on wine style.

One key component of the Brett control strategy was more effective use of the common wine preservative sulfite. Winemakers were encouraged to add sulfite in larger quantities but less often, providing a bigger effect for the same overall amount of preservative used. The adoption of this strategy could be seen in the ratio of free to total sulfite in finished wine, which is an indicator of how sulfite has been used throughout a wine's life. Low ratios (i.e. with high amounts of bound sulfite relative to free) typically mean that sulfite has been added to the wine repeatedly in small amounts, and that microbial growth or oxidation has occurred, producing sulfite-binding compounds. During the period when the Brett control strategy was being widely communicated, the average ratio of free to total sulfite for wines analysed by the AWRI's Commercial Services group increased from approximately 0.3 to 0.45 (AWRI publication #870), indicating improved sulfite management practices and cleaner wines.

At the same time, *B. bruxellensis* strains were being collected from winemaking regions across Australia to try to get a picture of their overall diversity. The 31 strains that were eventually isolated were tested for their sulfite tolerance (AWRI publication #1447) and also analysed using a DNA fingerprinting method (AWRI publication #989) to investigate how genetically similar or different they were to each other. Interestingly, most isolates were found to belong to a sulfite-tolerant genetic group, and the relative proportion of sulfite-tolerant strains was higher in samples obtained in 2004-2005 compared with those sourced in earlier years. The concept of antibiotic resistance is well-known in the medical world – if bacteria are exposed repeatedly to a non-lethal dose of antibiotic they can evolve a survival mechanism. Could this be happening for *B. bruxellensis* in response to sulfite? How could the risk of new strains emerging and potentially leaving current control strategies ineffective be estimated? The best answer appears to lie in an examination of *B. bruxellensis* at its most basic level – a deeper understanding of its genome. ►

### AT A GLANCE

- *Brettanomyces bruxellensis* ('Brett') yeast cause wine spoilage by producing 4-ethylphenol and 4-ethylguaicol. 'Brett' spoilage was a major issue in Australian red winemaking during the late 1990s and early 2000s, and still forms the topic of around 5% of total queries to the AWRI helpdesk each year.
- 'Brett' yeast can be controlled in the winery via a combination of good sanitation, minimisation of residual sugar, effective use of sulfite and pH management. However, there is a risk that new strains could emerge that are resistant to current control strategies.
- The AWRI was first in the world to release a full genome sequence for a *Brettanomyces* yeast in 2012.
- Further studies of the 'Brett' genome have followed and revealed interesting links between yeast strain genetic composition and tolerance to the common preservative sulfite.
- AWRI researchers are working to stay ahead of 'Brett' evolution to ensure it doesn't re-emerge as a significant wine spoilage issue in Australia.

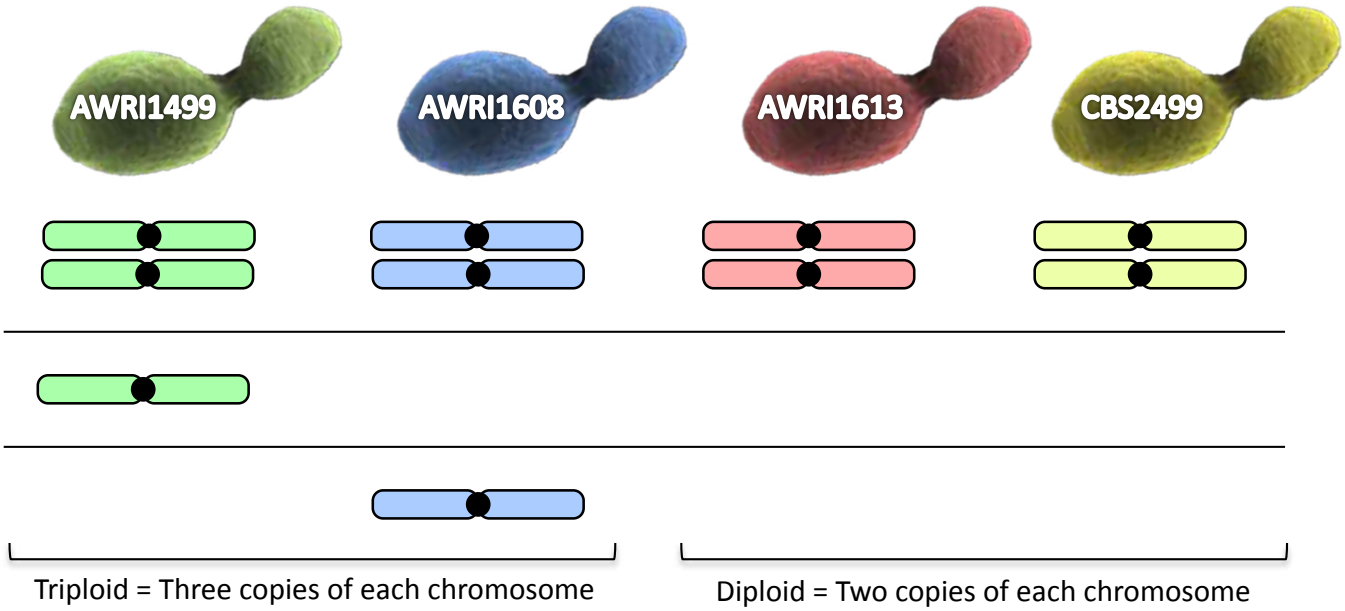


**Figure 1. Each of the sequenced *B. bruxellensis* strains contains a similar diploid genome, meaning each cell contains two copies of each chromosome. In addition, AWRI1499 and AWRI1608 both contain a third full set of chromosomes that have been inherited from more distantly related strains.**

## STRATEGIC GENOMIC SEQUENCING: DECODING THE *B. BRUXELLENSIS* GENOME

*S. cerevisiae*, the yeast used in wine, beer and bread production, amongst other industries, was one of the first organisms to have its genome fully sequenced. In the mid-2000s the same DNA sequencing technology (known as 'Sanger' sequencing) was applied to the *B. bruxellenis* genome (Woolfit *et al.* 2007). However, due to cost constraints, this study only yielded a partial genome sequence – useful for understanding where *B. bruxellensis* fits into the tree of life, but insufficient to shed light on how it had evolved. The AWRI extended this work, sequencing the DNA of an Australian *B. bruxellensis* strain (AWRI 1499) with relatively high sulfite tolerance, using a platform known as 454 pyrosequencing. The end result – a world first – was a 12.7 million base pair assembly comprising approximately 6000 genes.

What was discovered in this assembly? *B. bruxellensis* had more genes that encode membrane transport proteins and oxidation/reduction enzymes than other yeast species. These may provide an enhanced ability to take up nutrients in nutritionally barren environments, giving a greater capacity to survive for extended periods in wine. The *B. bruxellensis* genome was similar in size and gene content to that of *S. cerevisiae*. Unexpectedly, the assembly showed a triploid genome, meaning it contained three copies of its chromosomes (AWRI publication #1385), whereas most species that reproduce sexually have two sets of chromosomes (one from each parent). Furthermore, the DNA sequence of the third set of chromosomes was quite different from the other two.

To delve further into the genome composition of *B. bruxellensis*, two additional Australian strains were sequenced, representing 'intermediate' and 'sensitive' sulfite tolerance groups, while genomic data for a French wine isolate was also available for comparison (Piškur *et al.* 2012). The sulfite 'sensitive' strain (AWRI 1613) and the French wine isolate were similar in genome sequence, and both contained two rather than three copies of their chromosomes (Figure 1). They also both exhibited large regions of their genomes where both chromosomal copies had the same sequences; this usually reveals genes important for survival and reproduction. The intermediate Australian isolate (AWRI 1608) was found to be triploid, again with two sets of chromosomes that were similar to one another, and a third set that was different (Figure 1). Examination of seven genes for all four strains revealed that the divergent sequences in AWRI 1608 were not the same as those found in AWRI 1499. What does this mean? Given that AWRI 1499 and 1608 together represent approximately 92% of all isolates recovered from Australian wineries, the results imply that triploid *B. bruxellensis* strains may be 'more fit' for survival. It would also seem that the generation of these triploids happened independently; they have different evolutionary histories. Given the divergent sets of chromosomes in both strains are not the same, it is unclear whether the presence of this 'third genome' is important simply because it adds to the number of gene copies, or whether additional copies of specific genes offer particular advantage.

## *B. BRUXELLENSIS* AND SULFITE TOLERANCE

Initial work looking at functional genomics for *B. bruxellensis* has focussed on sulfite tolerance, a trait well understood for *S. cerevisiae*, both in terms of which genes are involved and what determines the relative tolerance of different strains (Park and Bakalinsky 2000; Aa *et al.* 2006; Goto-Yamamoto 1998). Central to this trait in *S. cerevisiae* is a sulfite pump encoded by the gene SSU1, which can be found across many fungal species and is present in single copy in the *B. bruxellensis* genome. If this gene is deleted from the *S. cerevisiae* genome the modified strain becomes sulfite sensitive. While there are no molecular biology tools enabling such analysis to be performed in *B. bruxellensis*, it's possible to test whether the same gene from *B. bruxellensis* (BbSSU1) complements deletion of SSU1 in *Saccharomyces*. Preliminary results showed that expression of BbSSU1 in a *S. cerevisiae* strain without the SSU1 gene brought back sulfite tolerance. Unexpectedly, the degree to which this pump is 'switched on' in *B. bruxellensis* was shown not to be different between

sulfite tolerant and sulfite sensitive strains. This was determined by studying the transcriptome (the set of all RNA molecules produced in one or a population of cells) after exposing cells to sulfite. Current work involves comparing the different sequences of BbSSU1 found in these strains, to determine whether one version of the pump confers more sulfite tolerance than another. This will provide insight into the potential for emergence of new *B. bruxellensis* strains with enhanced sulfite tolerance.

## CONCLUSION

To ensure the continued success of Brett control strategies, it is important to understand how *B. bruxellensis* has evolved to survive in wine, and how it might adapt to changing winemaking practices. Next-generation sequencing technology has been applied to decode the genomes of three Australian *B. bruxellensis* isolates, revealing that formation of triploid genomes through hybridisation may be important in determining their relative 'fitness' to survive under Australian winemaking conditions. Next-generation sequencing platforms were also used to catalogue the *B. bruxellensis* transcriptome which, combined with gene function analysis, will provide a better understanding of what makes *B. bruxellensis* tolerant to sulfite. This new knowledge will allow informed evaluation of the risk of new strains emerging that are immune to existing control strategies.

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