

EXCLUSIVE

Advancing the frontline against Brett

AWRI breakthrough offers potential to transform the battle against Brett

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If there is one battle that has united the global wine industry, that battle is against 'Brett': the yeast with the potential to spoil wine, with its 'medicinal', 'Elastoplast' and 'barnyard' characters. For more than two decades, the AWRI has worked consistently to give winemakers the upper hand against Brettanomyces through a program of dedicated research, which quickly delivered a practical, application-ready control strategy. Its latest breakthrough is a world first: AWRI researchers have sequenced the genome of a Dekkera (Brettanomyces) bruxellensis strain, in fact, the most dominant strain present in Australian wineries, revealing new insight into the yeast's resistance to sulfite; examining the likelihood of a 'super' strain; and opening opportunities for winemakers to tackle spoilage more efficiently.

FRIEND OR FOE?

When N. Hjelte Claussen, of the New Carlsberg Brewery, in Denmark, first isolated a *Brettanomyces* yeast in 1904, he was not to know the enduring significance of his discovery.

Claussen was concerned with beer, primarily, and was investigating what gave British ales their unique character. His discovery led to Brett's now infamous name - *Brettanomyces*, or British sugar fungus. Claussen noted with interest that the yeast he had isolated did not 'sporulate': it did not produce or release spores. He also said it was "essential for production of English 'stock' beers", a style the Danish brewers were having trouble replicating.

Since then, Brett has been recognised as ubiquitous in the environment. It is commonly found in breweries and wineries - anywhere, in fact, that fermentation takes place. It has been found in fermented teas and detected in yoghurt drinks. Its discovery in Belgian breweries, in Lambic ales for example, led to its more recent scientific name, *Dekkera bruxellensis*, which is now used to incorporate all strains of Brett relevant to wine.

In the wine industry, it has been largely associated with red wine stored in barrels, but has also found its way into

white table wines and bottle-fermented sparkling whites. In the past, some winemakers thought that a touch of Brett could enhance sensory experience of terroir and highlight rustic, earthy characters. Today's winemakers see Brett as a persistent problem.

Studies have shown that consumers dislike the characteristic aroma and flavour produced by the yeast, which are often described as 'Elastoplast', or smelling and tasting 'medicinal'. On the palate, Brett produces a character commonly described as 'metallic' or 'excessively drying'. The verdict from consumers has been clear: its presence is unsavoury; it is not a winemaker's friend.

UNDERSTANDING THE ENEMY

The compound largely responsible for 'Bretty' characters is called 4-ethylphenol. It is now well-accepted that *Dekkera bruxellensis* is the key wine micro-organism responsible for converting phenolic acids in grape must and wine into this, and related, volatile phenols.

When levels of 4-ethylphenol and its 'sister' compounds 4-ethylguaiacol and 4-ethylcatechol reach noticeable levels, consumers say they dislike the wine. They are often unable, however, to explain why. Winemakers have been dealing

New tactics

- Sequencing the genome of the most common strain of Brett in Australian wineries is giving winemakers new 'weapons' in the fight against spoilage.
- The gene responsible for unpleasant 'Bretty' characters has been traced, offering a new target to combat the 'Bretty' taste and smell disliked by consumers.
- A key gene involved in sulfite tolerance has been found, so that variability in tolerance between different strains of Brett can be better understood.
- Clearer diagnostic tests are now more likely, identifying whether outbreaks of Brett are more or less tolerant to sulfite treatment. When available, these tests will give winemakers the upper hand in knowing what 'weaponry' to use.

with an enemy, therefore, that has been difficult to pin down and even harder to eradicate. Wine researchers throughout the world have demonstrated the scale of the problem: revealing ubiquitous, undeniable links between Brett and the spoilage of red wine.

Despite their research, the inside story of Brett has not been fully investigated. In the past, there has been little investigation into how the yeast evolved various traits to survive a harsh wine-producing environment, or why some strains of the yeast respond differently to sulfite treatments.

To address this gap in knowledge, researchers at the AWRI have embarked on a sustained program of research that now spans more than two decades. The journey of that research has seen the introduction of effective counter-measures; the dissemination of new 'intelligence' to combat Brett; and, more recently, the application of genomics technology to crack the code of the 'enemy', so that winemakers are able to target Brett more effectively.

EARLY VICTORIES

One of the key components of any successful Brett control strategy is the use of sulfite. Used in the right amounts, at the right time, sulfite can be highly effective. However, finding the right balance is not easy.

In the 1990s, winemakers were struggling to keep Brett under control. Their defence strategy was under attack; and not only by the yeast itself. There was growing concern regarding the use of sulfite in wine and its effect on some consumers, particularly in Australia's key export market, the European Union.

EU regulations put pressure on winemakers to reduce sulfite, by lowering allowable levels. At the same time, Brett continued to evolve – more isolates from Australian wineries were obtained that could tolerate higher levels of sulfite. Control strategies met resistance on all sides: resistance from the yeast, resistance from consumers and resistance through regulation.

The Australian Wine Research Institute (AWRI) entered this 'hostile environment' with its own strategy. Drawing on its world-class expertise and technology, it set to work on tracking the yeast to find out where, why and how it had the potential to create a 'Bretty' taste and smell. AWRI researchers found out, for example, that incomplete or 'stuck' fermentations can leave enough residual sugar in wine for Brett to feed on. They also found out that fewer, well-timed doses of sulfite could be used against the yeast with maximum effect in some cases, spreading the word to winemakers through their network of workshops, roadshows and other extension activities.

Within eight years, the AWRI's information campaign - combined with robust research and hard work by winemakers themselves - had won a clear victory. Levels of 4-ethylphenol dropped by a dramatic 90% in Australian Cabernet Sauvignon wines (Figure 1, see page 20). The ratio of free to total sulfite, an indicator of effective sulfite usage, had increased, while total sulfite in finished wine had not. The AWRI's Brett control strategies seemed to be working; industry was back on track.

However, the threat of another attack on Australian wines, mounted by new strains of Brett, remained a possibility.

HI-TECH WARFARE

In 2008, the Australian wine industry believed it had Brett under control. The risk of spoilage was much lower than before. Very few affected wines were reaching consumers.

Still, the AWRI remained cautious. Could Brett come back to haunt the industry? What if the dominant, sulfite-tolerant *Brettanomyces* strain in Australian wine became more tolerant to sulfite? Was this a real risk? Despite efforts to understand

the biology of Brett, these questions could not be answered. Overseas, research efforts were sporadic and focussed upon strains that were less common in Australia. There was work to be done.

At the same time, science was entering a new era of cost-effective genomics. Driven by the goal to cheaply sequence a human genome, innovation in sequencing equipment and techniques was happening at an astonishing pace. As a result, sequencing the genomes of yeast and bacteria was within the financial and technical reach of biologists, a pursuit previously confined to a few large sequencing centres.

The outcomes have already made a positive impact. Understanding of *Saccharomyces cerevisiae*, the winemaker's favourite yeast, has advanced rapidly over the past few years due to several large sequencing projects. The decision was made to bring Australian strains of *Dekkera bruxellensis* into the genomic era, in order to 'future-proof' the Australian wine industry's Brett control strategies.

Work began late in 2008. The AWRI sent DNA from Australia's most common Brett strain - AWRI 1499 - to a sequencing laboratory in the US. Technicians broke the DNA up into smaller pieces, and used a technique called 454-pyrosequencing to read each DNA fragment. The AWRI's team was among the first in the world to access leading-edge sequencing equipment: the Roche 454 GS-FLX Titanium series. This meant greater speed and efficiency, delivering significantly more data and better value for money. Timing, in particular, proved to be crucial.

Sequencing the first yeast genome in the 1990s using more traditional methods cost considerably more time and money, in comparison. The instrument used for the AWRI's sequencing of *Dekkera bruxellensis* generated the same quantity of data in



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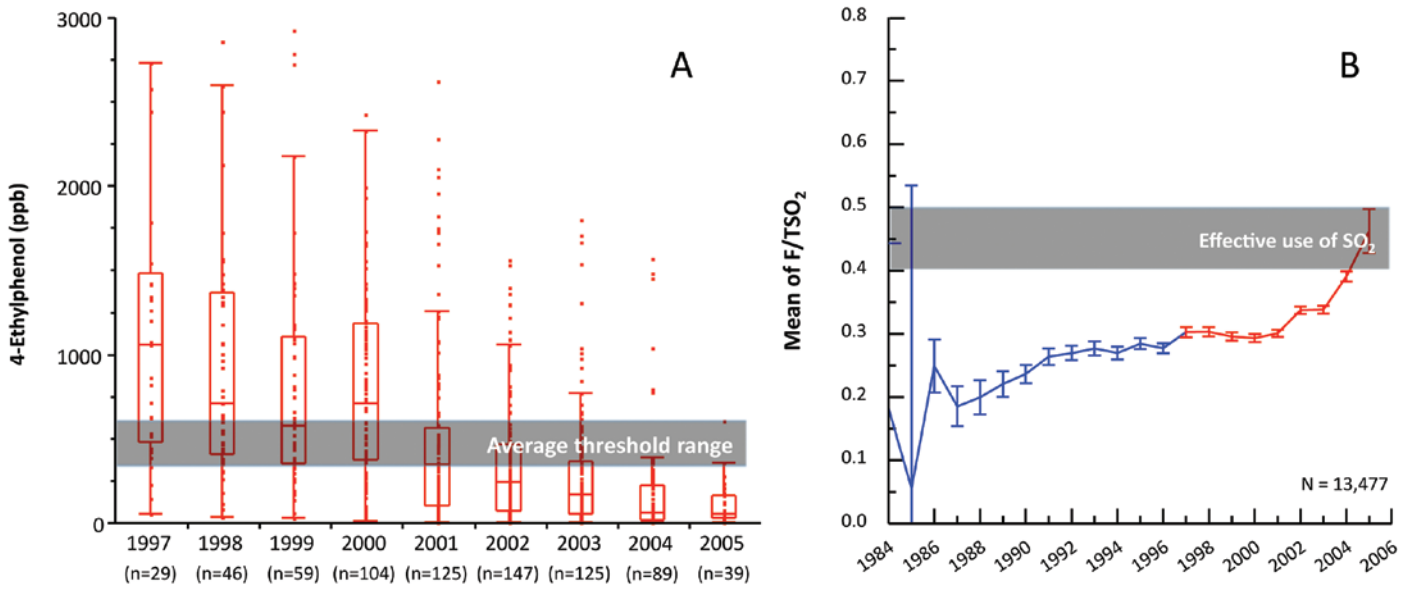


Figure 1. Incidence of 'Brett' in Australian Cabernet Sauvignon wines from five major regions (A), and ratio of free to total sulfite in Australian red wines (B). Overlapping periods in the two datasets are shown in red, highlighting that the dramatic decrease in 4-ethylphenol levels in Australian Cabernet from 2000-2005 coincided with a shift in the F/TSO₂ ratio from 0.3 to 0.45. Importantly, mean total sulfite concentrations did not increase during this period. Therefore, this shift was indicative of a widespread change in sulfite management strategies.

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Advances against Brett

- Late 1990s – AWRI researchers target Brett by launching a research and information campaign to reduce spoilage in Australian wineries. Within eight years, through the hard work of winemakers themselves, the incidence of the compound responsible for 'Bretty' characters in Australian Cabernet Sauvignon is reduced by 90%.
- 2008 – Advances in genomics make it feasible to affordably sequence the genome of wine yeast. The AWRI sets out to 'future-proof' its campaign against Brett. Sequencing of Australia's most common Brett strain - and the most resistant to sulfite – begins.
- 2009 – More than one million pieces of Brett's giant genomic jigsaw are pieced back together. The work takes many months and requires hours of painstaking work, supported by advances in bioinformatics technology.
- 2010 – In a breakthrough, three copies of many DNA regions are detected instead of one or two. The triploid structure may be important in enabling Brett to survive in wine.
- 2011 – The AWRI announces publicly that it has assembled the Brett genome; the achievement is welcomed as a world first. Researchers embark on the next stage of the project – developing targeted strategies for winemakers to eliminate Brett from their wineries.

less than a day. But, data generation was just the start of the project.

Generating masses of sequencing data was the easiest part. In the first half of 2009, the AWRI took the raw data - all 1.5 million 'sequence reads' of it - and attempted to piece the genomic jigsaw puzzle of *Dekkera bruxellensis*

back together. Upgrades were necessary to the AWRI's bioinformatics server; it needed more processing power. In August 2009, after much trial and error, 1.5 million 'reads' were assembled into some 27,000 'contigs' (contiguous DNA sequences). This meant progress, but there was still

work to do before this jigsaw puzzle would be complete.

The next stage, involving long hours over a period of almost two years, required close observation at a computer screen to decide if a stretch of DNA sequence was an 'edge piece'. If so, the question was where exactly in a pile of

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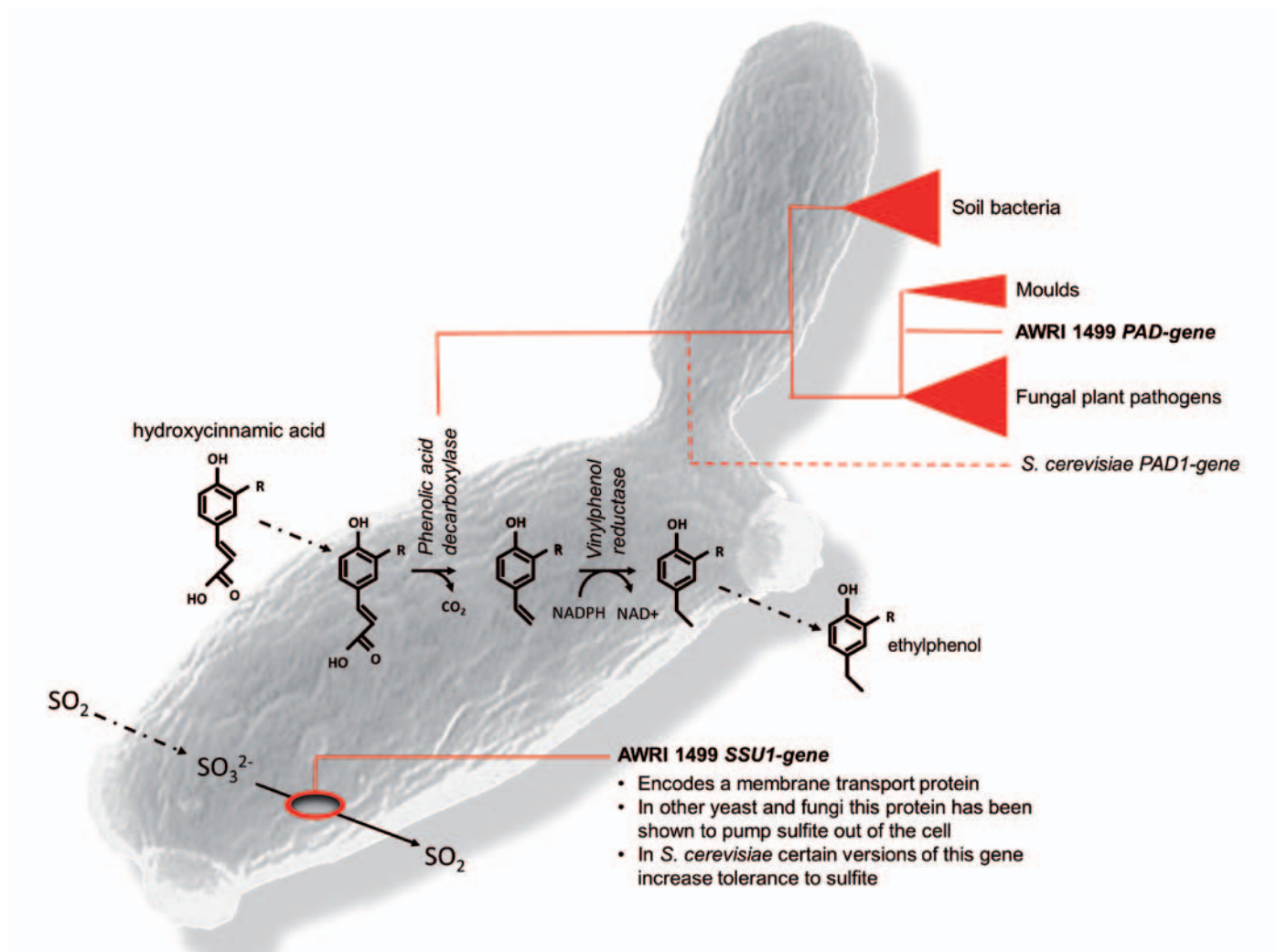


Figure 2. Gene discovery made possible by sequencing the *D. bruxellensis* genome. AWRI 1499 PAD-gene does not encode a protein with similarity to the Pad1 protein from *S. cerevisiae*, found in several other yeast and fungal species. Instead, we discovered a protein with close similarity to phenolic acid decarboxylases found in several plant pathogens and soil bacteria - this version of the protein may be more active against hydroxycinnamic acids than the *S. cerevisiae* Pad1 protein. AWRI 1499 SSU1-gene encodes a protein with similarity to sulfite pumps in several yeast and fungi. Sulfite tolerant strains of *S. cerevisiae* have higher levels of this pump present, enabling them to decrease the concentration of sulfite present inside the cell and survive in the presence of higher sulfite doses. A similar mechanism may exist in *D. bruxellensis*.

many thousands of similar-looking 'edge pieces' it belonged. When a match was found, computer software was used to 'reassemble' the two pieces. Gradually and painstakingly, the AWRI worked towards the middle of the puzzle.

Late in 2010 there was a 'lightbulb moment'. There was a reason why the puzzle was so hard to put together: there were more pieces than there should be. The genome of the *Dekkera* strain under investigation did not have two copies of each DNA region - as is the case for many genomes, including humans. There appeared to be three.

While triploid genomes do occur amongst yeast and plant species, most yeast sequencing projects have been performed using strains that have only one copy of each DNA region, to make the assembly process simpler. Without the data provided by the newly - developed Titanium series sequencing

technology, the task of assembling *Dekkera's* triploid genome may have proven impossible.

By June 2011, most of the pieces had been put back together, with 324 assembled 'contigs' in 99 'scaffolds'. The genome assembly was ready to be explored. AWRI researchers used bioinformatics tools to search the DNA sequence for genes, and compared them with databases containing all genes known to science. Drawing a blueprint for *Dekkera bruxellensis* had become a reality, with the potential to transform the way that winemakers engage with the 'enemy'.

ADVANCING THE FRONTLINE

Armed with their discoveries so far, AWRI researchers are now moving into the next stage of their *Dekkera* genomics research project.

This next phase continues to draw on the cross-disciplinary capability of the AWRI: the early victories over Brett in the 1990s were the result of extension to and engagement with winemakers in the trenches by the AWRI's Industry Development and Support Group; the sequencing project was undertaken by the Biosciences Research Team.

While key issues are still under investigation, AWRI researchers have already used this resource to make important discoveries. Significantly, one of the genes responsible for sulfite tolerance has been identified (Figure 2).

Genes involved in the manufacture of 4-ethylphenol have also been spotted - providing new targets in the battle against Brett's unsavoury characters. The genetic blueprint is also providing clues as to where the

yeast may 'hang out' in the winery. This may provide winemakers with another strategy to eliminate the enemy from their territory.

Above all, the sequencing project demonstrates the importance of combining real-world problems with world-class expertise. The focus of the AWRI's research remains the challenges faced by Australian grape and wine producers as they seek to compete globally, in a testing environment. Advancing the frontline against Brett is just one way that the AWRI is providing industry with the logistics, equipment and know-how to secure an upper hand.

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SUPPORTING PUBLICATIONS FOR FURTHER READING

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