Unravelling the capricious nature of *Oenococcus oeni*

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Exciting times lie ahead for malolactic fermentation (MLF) research and this will bring substantial benefits to winemakers. New genetic sequencing techniques are revealing the incredible diversity amongst strains of Oenococcus oeni, the bacterium primarily responsible for MLF. This new knowledge should provide extensive opportunities for Australian winemakers to choose MLF strains tailored to suit their individual conditions – optimising both fermentation efficiency and flavour release.

MLF – A KEY WINEMAKING PROCESS

MLF is critical in red wine production, where it is used to convert malic acid from grapes into lactic acid. This 'softens' the palate of a wine and decreases the likelihood of spoilage by undesirable microbes that can use malic acid as an energy source. In addition, MLF can influence wine style by changing the flavour profile; this has been one of the drivers for many winemakers adopting MLF in white wine production.

Winemakers primarily rely on the bacterium *Oenococcus oeni* to conduct MLF in the winery; one or two other species of bacteria (e.g., some strains of *Lactobacillus plantarum*) are able to perform MLF, but are not commonly used. *O. oeni* is an interesting bacterium. It is one of a few micro-organisms that can grow in the hostile environment of wine, where low pH, the presence of alcohol and a scarcity of nutrients prove far too great a challenge for most microbes. In fact, far from simply surviving in wine, *O. oeni* has only ever been isolated from winery environments, suggesting that this is its natural home.

Nonetheless, while *O. oeni* is at home in wine, it can be rather capricious; sometimes it is very slow or even fails to complete MLF. This is perhaps the biggest bugbear winemakers have with MLF; efficiency and reliability are far from guaranteed. The availability of more robust and efficient MLF strains would take some of the stress out of vintage. If such isolates could be used to shape wine style in a targeted way, so much the better.

Strains of *O. oeni* that can be accessed by winemakers vary considerably in their tolerances to stresses and, therefore, in their MLF performance. They also differ in their capacity to influence wine style. However the genetic determinants of these differences remain unknown, making it difficult to screen for new strains with properties that will benefit the winemaker. ▸

AT A GLANCE

- Malolactic fermentation is an important winemaking process for most red and some white wines, improving both sensory properties and microbial stability
- *Oenococcus oeni* is the bacterium most commonly used to conduct MLF in winemaking
- Many different strains of *O. oeni* are available, with differences in fermentation efficiency and flavour generation
- Until recently it has been difficult to conduct genetic analysis on this species; however, new techniques are making it possible to sequence its genome and start to tie winemaking-relevant properties to particular genes
- These developments are opening the door for winemakers to tailor their choice of MLF strain to specific fermentation characteristics or desired flavour outcomes
- As more *O. oeni* strains are sequenced, MLF options for winemakers will increase.

O. OENI - A DIFFICULT BACTERIUM TO STUDY

While there have been many decades of research into MLF, it has proven to be a difficult field of study for microbiologists, largely due to the intractable nature of *O. oeni*. Most microbes that are studied in the laboratory (including wine yeast) are amenable to genetic analysis. It is possible, for example, in a research setting to get yeast to exchange genes by natural means (such as mating) or through the application of genetic engineering techniques. This enables the identification and characterisation of genes that drive important processes. Knowledge of these genes then makes it easier to isolate novel non-genetically modified strains with desirable traits. However, *O. oeni* does not lend itself to traditional genetics or genetic engineering techniques.

Apart from one or two reports in the scientific literature, this species of bacterium has proven to be recalcitrant when it comes to genetic engineering techniques. Fortunately, developments over recent years in gene analysis technologies are providing a way forward. It's no longer necessary to rely on gene transfer techniques to begin to identify and characterise the genetic determinants of traits, it's now possible to go straight to the stuff that genes are made of: DNA.

Genes are essentially recipes for making proteins, which are, in turn, responsible for building, maintaining and reproducing cells. Proteins include enzymes, transporters for delivering nutrients into cells and regulators of metabolism – in fact, just about everything that a cell does is controlled and executed by proteins. In this context genes might be regarded as a cell's instructions for life. Genes are made from DNA and the full complement of DNA carrying the full set of genes of an organism is called its genome (see breakout box on facing page).

> While a considerable amount has already been learned about many aspects of *O. oeni* genetics, the sequencing completed so far has really only scratched the surface. What is required now is an intensive effort to sequence an even larger number of strains... and determine the variations in traits that are relevant to MLF performance.

Using the latest DNA sequencing technologies it is now possible to sequence the entire genome of an organism, and because bacterial genomes are relatively small, the task can be achieved in a reasonable timeframe. This has afforded scientists at AWRI and in other laboratories around the world the opportunity to sequence a number of *O. oeni* strains, and what we are learning about this bacterium is amazing.

DIVERSITY REVEALED

Genomic sequencing of bacteria in general has revealed that there is often enormous genetic diversity within a species; massively more than is found in plants and animals. This has led to the introduction of the terms 'pan genome' and 'core genome'. A core genome is the collection of genes that all members of a species have. These might be regarded as indispensible to the bacterium in question. Then there are genes that are found within a species but are dispensable; they are found only in some strains. The pan genome is the full collection of genes (dispensable and indispensable) in a species.

O. oeni has been shown to have a high level of genetic diversity (AWRI publication #1512). It has a core genome of approximately 1165 genes, but more than 2800 genes in its pan genome. Typically, for any strain of *O. oeni*, the core genome constitutes about 60-70% of its DNA, the rest carries dispensable genes, providing an enormous potential for genetic variation between strains. Presumably, while we use the word dispensable for this highly variable part of the genome, these genes will provide an advantage in some environments and not others. This might explain why, for example, some strains perform well in red wine but are less reliable in white.

How did *O. oeni* come to have this huge level of genetic diversity across strains? The answer, at least in part, appears to be due to a phenomenon that is common in the bacterial world known as horizontal gene transfer (HGT). In contrast to vertical transfer of genes, which happens during normal reproduction when parents pass on their genes to offspring, in HGT genes are transferred from one organism to another outside of a normal reproductive process, often between totally unrelated species.

In nature, it seems bacteria have been using HGT for millennia, enabling cross-species genetic engineering to improve their genetic potential. *O. oeni* has acquired genes from, for example, species of *Lactobacillus* and *Pediococcus,* both of which are found in food and beverage production. In fact, the level of transfer from *Lactobacillus* species has led to the suggestion that these bacteria provide a potential reservoir of genes for *O. oeni*.

It is humbling to recognise that biotechnologists are a few billion years behind nature in their genetic engineering skills and require sophisticated laboratory tools to achieve similar, although considerably more modest, ends. HGT can occur via any one of several mechanisms but we still don't know how *O. oeni* does it. Genomic sequencing has shown that there are clear signs of virus infection in the *O.oeni* genome. These viruses

CRACKING THE CODE: GENES AND GENOMES

Genes are recipes for making proteins. For example, your cells carry a gene/recipe for making the protein insulin, which is a hormone that regulates blood sugar level. You also have genes/recipes that instruct your cells how to make proteins that control how tall you can grow, the colour of your eyes, the general shape of your body, etc. It is these recipes of life that dictate whether you will have athletic potential or not, and, because you inherited them from your parents, you end up looking like them.

If genes are recipes, then genomes are recipe books. The human genome carries all of the recipes required for making proteins to build a human body from conception to adulthood, and repair and defend that body during its life. All of our physiology and anatomy is shaped by a collection of 20,000–25,000 genes that comprise the human genome. And, unless you have an identical twin, your recipe book differs a little from everyone else's.

The language of the genes is different to the languages we use to communicate with each other. It is based on an alphabet of only four letters (A, T, G and C) and its lexicon is limited to three-letter words, which means there are only 64 words in the genetic dictionary. However, this is more than enough to string together sets of instructions for building all of the proteins (enzymes, hormones, muscles, antibodies, cartilage, etc.) we require for life.

The 'paper' on which the words that make up the recipes of life is written is known as DNA, and when we read an entire recipe book of an organism, decoding what is recorded in its DNA, we say we are sequencing its genome. What we end up with in this process is a long sequence of millions of A, T, G, and Cs, with no spaces or obvious punctuation marks that we have to decipher. Thankfully, sophisticated computational aids can do most of this for us.

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may have brought with them genes from hosts that they had previously infected. However, it is likely that other processes are also at work and if these were better understood, it would be easier to perform genetics experiments on *O. oeni*.

SPECIFIC GENES RELEVANT TO WINEMAKING

Homing in on specific aspects of the *O. oeni* genome, considerable variation has been found in genes responsible for exopolysaccharide (EPS) production. EPSs are critical components of the shield that surrounds *O. oeni* cells, known as the cell wall, which protects against environmental assaults. EPSs, therefore, contribute to the robustness of the bacterium, which, in turn, would be expected to impact on the performance and reliability of strains in MLF. EPSs may also affect the mouthfeel of wine. EPS genes are housed in three regions of the genome and it seems that they differ between strains. The nature of the variation of these genes is consistent with reported differences in both the amount and type of EPS produced by at least three of the strains that have been sequenced. This provides some validation of genomic sequencing as a means of predicting traits in *O. oeni*.

In a nutrient deficient environment such as wine, a bacterium needs to be able to synthesise many of the molecules needed for building cellular components. Thus, the synthesis of amino acids (the building blocks of proteins) might be regarded as fundamental for survival. Interestingly, there is considerable inter-strain variation in the presence of genes for amino acid synthesis. For example, some strains lack the genes necessary for synthesis of the amino acid leucine. Others lack genes for the synthesis of threonine and/or glutamine and/or methionine. Whether these differences correlate with the ability to efficiently finish MLF has yet to be determined.

Another important observation from comparing *O. oeni* genomes concerns the genes required for sugar utilisation. It has been known for decades that different strains of *O. oeni* utilise different sugars, but the underlying reasons for this variation were not understood. From genomic sequencing we now know that strains vary in the genes that they carry for sugar uptake from the environment and for sugar metabolism. For example, only some strains have the genes necessary to utilise L-arabinose, a sugar that is found in grape juice and is not metabolised by yeast.

MORE SEQUENCING NEEDED

While a considerable amount has already been learned about many aspects of *O. oeni* genetics, the sequencing completed so far has really only scratched the surface. What is required now is an intensive effort to sequence an even larger number of strains (sequence data for about 50 *O. oeni* genomes is currently available in the public domain) and determine the variations in traits that are relevant to MLF performance. From this it will be possible to determine which genes are essential for the ability to efficiently complete MLF at low pH, in high alcohol, at low temperature and under numerous other wine-relevant conditions.

Armed with the above, it will be possible to advise on which commercially available strains of *O. oeni* are best suited to conditions that are typical of particular wine varieties, styles and regions. Large collections of *O. oeni* isolates could be screened to identify new strains with combinations of traits that are better-suited to Australian winemaking conditions than currently available commercial products. The AWRI culture collection, for example, houses approximately 900 MLF bacteria, some of which were isolated as early as the 1970s, prior to the practice of inoculating with commercially-available MLF strains. Who knows what gems we might find?

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