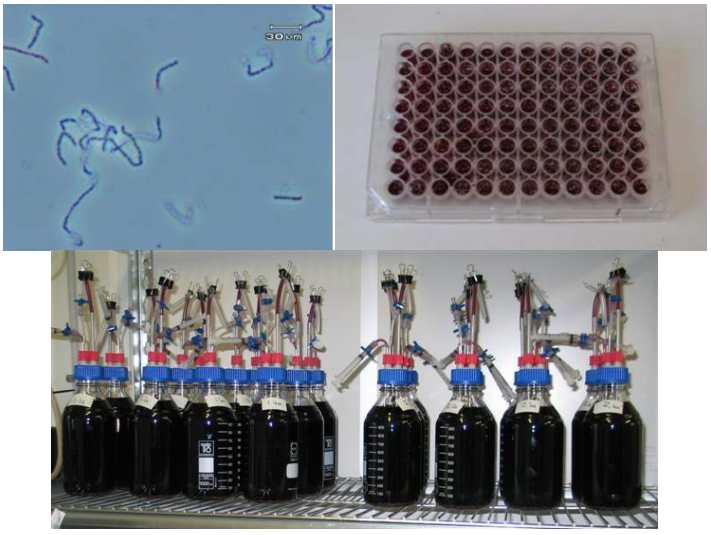
Australian Government

Australian Grape and Wine Authority

The Australian Wine Research Institute

Identifying wine-relevant genetic markers in wine bacteria to isolate robust MLF strains



FINAL REPORT to

AUSTRALIAN GRAPE AND WINE AUTHORITY

Project Number: AWR 1305

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Research Organisation:
The Australian Wine Research Institute

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1. Abstract

Over 150 Australian isolates of malolactic fermentation bacteria, collected over decades and housed in the AWRI Culture Collection (AWRI CC), were characterised by genomic (DNA) sequencing. A subgroup of these, of known provenance, was screened for tolerance to stresses encountered when grown in wine. This enabled the identification of strains with strong performance characteristics, the most promising of which will be trialled in future research at larger scale winemaking. In addition, genetic markers for stress tolerant traits have been identified. This will facilitate rapid screening of the AWRI CC to isolate candidate strains with desirable characteristics, suited to Australian winemaking conditions.

2. Executive summary

Summary of the Project's outcomes and benefits:

Currently there are no malolactic fermentation (MLF) starter culture strains that have been specifically selected for Australian winemaking conditions; development of commercially available strains is largely targeted to overseas markets. Strains of *O. oeni* isolated from Australian wineries, mostly from spontaneous MLFs, are likely to be well-suited to Australian winemaking conditions. This makes them possible candidates for development of MLF starter cultures that will be more efficient and reliable in the production of Australian wines.

This project aimed to identify Australian isolates of the MLF bacterium *O. oeni* housed in the AWRI CC that would be suited to Australian winemaking conditions. To this end, 169 wine bacteria were characterised by genomic (DNA) sequencing and a subgroup of these was screened at laboratory-scale for tolerance to stresses encountered when grown in red, white and sparkling-base wines. Thirteen genetically distinct, stress-tolerant, strains have been identified. These will be trialled in future research, including AGWA project AWR1303, for their efficacy at lab- and pilot-scale, and in wineries with a view to identifying candidates for the generation of commercially available Australian MLF starter cultures.

In addition, alignment and comparison of the genomes that were sequenced has enabled 158 strains of *O. oeni* to be compared to determine their genetic similarity. The amount of genetic variation across strains was found to be very large compared to what is found, for example, when comparing wine yeast strains. Based on the results from the sequencing work a relatedness tree was constructed, from which it is apparent that most strains cluster into three groups. One of these is a small outlying group that differs considerably from the others. There is only one previous publication that describes one strain from this group (Campbell-Sills 2015). None of the commercial strains sequenced to date are found in this group. It thus constitutes a resource with the potential to deliver novel wine-relevant traits.

Practical implications of the Project:

Novel Australian *O. oeni* isolates identified from this project as being genetically distinct from currently available commercial strains and with strong winemaking traits are potential candidates for development as starter cultures. These strains are predicted to perform well in Australian winemaking conditions and because their provenance is known, have the potential to be used to enhance regional identity of wines.

Benefits to Industry:

Improving MLF efficacy in winemaking is a significant opportunity for the Australian wine industry. Studies have shown that tolerance of commercially available wine bacteria to 'difficult' wines (e.g. low pH, high alcohol concentration, and low temperature) can vary greatly. Identification of Australian isolates of *O. oeni* strains that are efficient and reliable in Australian winemaking conditions will provide the means to develop improved MLF starter cultures for the benefit of the local industry.

In addition, there is a significant interest in Australia in developing strategies to enhance regional identity of wines. The provenance of strains trialled in this project is known. Thus winemakers from the region of origin of any strain developed as a starter culture will have the opportunity to use these "strains of provenance" and benefit from associated stylistic and marketing opportunities.

Future research:

- Strains identified from this project as strong candidates for development of Australian MLF starter cultures will be trialled at pilot and industrial scale in an ongoing AGWA-funded project (AWR1303).
- Wine regions from which the above strong candidate strains were isolated will be approached to ascertain the level of interest in development of starter cultures that can be used to enhance regional identity of wines.
- Further screening of the extensive range of Australian MLF bacterial isolates in the AWRI CC would offer great potential for identification of additional robust strains, from wine regions not captured in the current project.
- Genomic sequencing in the current project identified a group of 11 *O. oeni* strains that is genetically very different from the other clusters of strains. None of the commercially available strains sequenced to date in this (and other projects) fall into this unique group. This makes the group particularly interesting for further research and development aimed at producing novel MLF starter cultures.

3. Background

While pH, alcohol, SO_2 and temperature levels are useful guides for prediction of successful MLF, this winemaking step remains notoriously unreliable. As a result, MLF can be a major winery expense; the time and labour required for continual wine analysis and propagating and adapting bacterial cultures to initiate and complete MLF is considerable. In addition, failed and slow MLFs can lead to product downgrades. Slow MLF increases wine spoilage risks (e.g. Brett) and leads to increased costs (e.g. extended periods of heating tanks/barrel halls). Ensuring timely MLF completion is therefore paramount to reliable, cost-effective quality wine production. In addition, MLF impacts on wine style but research is required to determine how this can be reliably harnessed by winemakers to tailor their wines.

The AWRI has assisted the Australian industry in improving MLF efficiency in red wines by developing co-inoculation strategies (yeast and bacteria inoculated together) that are more efficient than standard sequential inoculations (e.g. Abrahamse 2012). However, difficulties remain for MLFs in red wine, and there is a dearth of knowledge on factors affecting MLF performance in white and sparkling base wines. Additionally, there are no MLF starter strains that have been specifically selected for Australian winemaking conditions; development of commercially available strains is largely targeted to overseas markets. In line with AGWA's strategic focus on regionality and production efficiency, there is a need for 'Australian' MLF bacteria.

Improving MLF efficacy in winemaking is a significant opportunity for the Australian wine industry. Studies have shown that tolerance of wine bacteria to 'difficult' wines (low pH, high alcohol concentration, SO_2 and low temperature) can vary greatly (Davis 1988). Consistent with this, genomic studies at the AWRI (Borneman 2010) based on sequencing the genomes of 12 *O. oeni* strains (Borneman 2012) revealed a huge level of genetic diversity across this species. Thus mapping traits to genetic loci in this bacterium will require screening of a relatively large number of strains; the link between genome and phenotype is only beginning to be unravelled (Bartowsky 2011). Once this has been achieved however, it will be possible to rapidly screen large numbers of strains for genetic markers that predict phenotypes of interest to winemakers (stress tolerance, wine sensory attributes etc).

4. Project Aims and Performance targets

The aims of the project were to:

- sequence and align the genomes of up to 200 *O. oeni* strains, to identify identical strains, remove duplicates from further evaluation and cluster according to genetic variability
- identify *O. oeni* strains that tolerate MLF-limiting wine parameters, grow rapidly and efficiently metabolise malic acid
- develop bioinformatic tools for rapid gene searching
- identify genetic markers for wine stress tolerance
- develop a comprehensive database of *O. oeni* genomes aligned with wine-relevant phenotypic

traits, that can be used to improve MLF efficiency in red, white and sparkling-base wines;
- provide the wine industry with knowledge generated from the above, particularly with regard to strategies for improving MLF outcomes
- identify potential MLF bacterial strains, suited to Australian winemaking conditions, which could be commercialised.

5. Method

The Project was carried out as outlined in the original application, with one minor variation. A variation was sought and approved (12 March 2015) to screen 30 to 36 *O. oeni* strains in only red and white wines in Year 2 wine stress tolerance screening section of the project.

- **Genome sequencing**:
  O. oeni strains characterised in this project were sourced from the AWRI Culture Collection. Genomic DNA was extracted using standard techniques optimised for *O. oeni*. Modifications included optimising bacterial lysis methodology to increase genomic DNA yield, and trialing several DNA purification kits to select the one best suited to *O. oeni* genomic DNA. Genomic DNA was sequenced through the Ramaciotti Centre (UNSW) using Illumina NextGen sequencing.

- **Genomic-bioinformatic tools**:
  Bioinformatic tools were developed enabling rapid alignment and comparison of genomic sequences from large numbers of bacteria.

  Using genomic sequence and phenotypic data, potential genetic markers for wine stress tolerance phenotypes were identified.

- **Wine stress tolerance screening**:
  Candidate bacterial strains, selected based on genome sequence analyses, were assessed for tolerance to extreme MLF-limiting wine parameters (low pH, high ethanol concentration, low temperature) using Australian red, white and sparkling base wines. Wine composition of selected wines was adjusted for pH and ethanol concentration. Bacterial strains were prepared by a two-stage cultivation procedure for direct inoculation into wines. Stress tolerance screening was conducted at lab-scale (200 μL) using the robotic liquid-handling station and a randomised bacterial strain layout in microtitre plates. MLF was monitored by determining malic acid concentration.

  Year 1: 30 bacterial strains were screened, and in conjunction with genomic sequence analyses, genetic markers were identified which were used to predict potential robust MLF strains.

  Year 2: 36 bacterial strains, chosen using prediction modelling from Year 1, were screened for wine stress tolerance in red and white wines.

- **Potential robust strains for Project AWR 1303:**
  Using data from phenotypic screens performed in years 1 and 2, potential robust *O. oeni* strains with desirable wine stress characteristics were identified and have been made available to project AWR 1303 for winemaking evaluation at lab-, pilot- and winery-scale in Australian red, white and sparkling base wines.

6. Results/Discussion

Genome sequences of wine bacteria available, aligned, compared and candidate strains selected for phenotypic screening.

1. Draft genome sequences of 169 wine bacteria were assembled, comprising 158 *O. oeni*, 8 *Lactobacillus* spp., 2 *Pediococcus* spp. and 1 *Gluconobacter oxydans*. For three bacterial strains insufficient DNA was obtained to generate a genome assembly.

2. A novel BLAST tool was developed enabling rapid alignment and comparison of genomic sequences from large numbers of bacteria. This BLAST tool was used to compare genomes of *O. oeni* strains sequenced for this project.

3. Bacterial genome sequences were analysed to identify duplicate strains. While there were very few genetically identical strains (there are almost always some nucleotide variations and variable bacteriophage integration sites separating even the closest strains), there were some very closely related strains.

   Genomes of sequenced bacteria were aligned and clustered to show genetic relatedness. A relatedness tree of these bacteria was prepared. From this, three major clusters of genetically related strains have been identified. Only two of these three clusters had been previously observed in research at the AWRI; there was one previous publication that described one strain in the third group (Campbell-Sills 2015). The newly discovered cluster is the smallest and most distant grouping of *O. oeni* strains.

   The majority (133) of the sequenced *O. oeni* strains fall into one cluster. The other two clusters are relatively small comprising 14 and 11 strains each.

4. Sixty-six candidate *O. oeni* strains from the 158 that were sequenced (30 from Year 1 and 36 from Year 2) were chosen for phenotypic screening.

Phenotypic screening of *O. oeni* strains for tolerance to wine stress (low pH, high alcohol concentration and low temperature) in red and white wines.

1. The 66 strains chosen from the 158 sequenced genomes were variously screened for MLF robustness at low pH, high alcohol, and low temperature in red and white wines.

   Thirty strains were screened in red, white and sparkling base wines in Year 1. Thirty-six strains were screened in red and white wines (Year 2).

2. A range of wine stress tolerances were observed across the 66 *O. oeni* strains screened and these differed in different wine matrices. For example, several strains that showed relatively high tolerance to low pH in white wine were less tolerant to the same stress in red wine, and strains exhibiting tolerance to alcohol in red wine did not necessarily show the same tolerance in white wine.

Identification of genetic markers for MLF stress tolerance.

1. Genomic sequence data indicated that there is enormous genetic variation across *O. oeni* strains. Tens of thousands of genetic markers, in the form of single nucleotide polymorphisms (SNPs) and nucleotide insertions and deletions (indels), were identified across the 158 strains that were sequenced.

2. While data analysis in Year 1 revealed a group of approximately 400 polymorphisms that were significantly correlated with an increase in the stress tolerance phenotype and potential genetic markers for stress tolerance were identified, by increasing the genomic sequence dataset (from 80 to 158) the analysis was further tested and refined. The polymorphisms identified were found to occur within the conserved ‘core’ genome – the set of genes shared by all the strains of *O. oeni.* From these 400, 20 highly correlated polymorphisms were identified.

3. Additional analysis was conducted to identify genetic variations occurring within the ‘pan’ genome – the full complement of genes of *O. oeni.* This analysis revealed the presence or absence of particular genes which were highly correlated with individual stress responses. Up to 82 markers per stress condition per wine type were identified in this analysis.

4. The genetic markers found within ‘core’ and ‘pan’ genomes were tested in strains that had not previously been phenotypically characterised. From this analysis two genome regions were

discovered that provide, with a high level of confidence, markers for tolerance to low pH and high alcohol concentration. No markers were found for tolerance to low temperature.

Potential robust MLF strains for further trialling.

Thirteen *O. oeni* strains that display desirable stress tolerance phenotypes and represent all clusters in the genetic relatedness tree have been identified and will be trialled in AWRI Project 3.2.4 (AWR 1303: Efficient and reliable malolactic fermentation to achieve specification wine style). This means that substantial genetic diversity will be brought into the trials to be performed in AWR 1303.

7. Outcome/Conclusion

The objectives of this project have been fulfilled. Candidate robust Australian isolates of *O. oeni* have been identified and genetic markers for traits associated with robustness in this species are now available. The methodology and technologies used in the project were appropriate to meet its Outputs.

Practical implications for the Australian grape and wine industry include:

- Australian isolates of *O. oeni* that were characterised as robust in this project have the potential to improve efficiency and reliability of MLF in Australian wine production.
- The provenance of the above robust strains is known. Thus winemakers from the region of origin of any of these strains will have the opportunity to utilise this product, if developed as a starter culture, to inoculate for MLF and benefit from associated marketing opportunities.

8. Recommendations

Future research directions for this research project:

- Strains identified from this project as strong candidates for development of Australian MLF starter cultures will be trialled at pilot and industrial scale in an ongoing AGWA-funded project (AWR1303).
- Since the provenance of the above strains is known, there is an opportunity to approach the wine regions from which they were isolated to ascertain the level of interest in development of starter cultures to enhance regional identity of wines.
- Further screening of the extensive range of Australian MLF bacterial isolates in the AWRI CC will enable identification of additional robust strains, from wine regions not captured in the current project.
- Genomic sequencing in the current project identified a group of 11 *O. oeni* strains that is genetically very different from the other clusters of strains. None of the commercially available strains sequenced to date in this (and other projects) fall into this unique group. This makes the group particularly interesting for further research and development aimed at producing novel MLF starter cultures.

9. Communication

Outcomes of this Project have been communicated via the AWRI's Annual Report, at Crush 2014 (Adelaide) and the 11th LAB symposium (31 Aug - 4 Sept 2014; The Netherlands).

The following article was published in a wine industry trade journal, to communicate about the project directly to grapegrowers and winemakers:

Borneman, A., Bartowsky, E., Costello, P., Sternes, P., Chambers, P., Herderich, M. and Johnson, D. (2015) Unravelling the capricious nature of *Oenococcus oeni*. *Wine & Viticulture Journal* 30, 34-37.

10. Intellectual Property

Knowledge has been generated in this project that may enable valuable project IP to be produced subject to further research being conducted specifically identification of genetic markers for wine stress tolerance and a group of 13 strains identified as strong candidates for development of Australian MLF starter cultures.

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