Enhanced Wine-Making Efficiency Through Fool-Proof Malolactic Fermentation: Evolution of Superior Lactic Acid Bacteria

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Summary

Malolactic fermentation (MLF), also known as the secondary fermentation in winemaking, involves the enzymatic decarboxylation of L-malic to L-lactic acid, by lactic acid bacteria, usually *Oenococcus oeni*. This conversion improves the organoleptic properties of wine and also decreases the risk of microbial spoilage. *O. oeni* is a notoriously fastidious microbe prone to slow growth, especially in the harsh physiochemical environment of wine: high ethanol, presence of sulfur dioxide, low pH and low temperature. Each of these factors influences the growth rate and metabolism, including MLF, of this organism.

This study aimed to generate an improved strain of *O. oeni* with the ability to withstand the environmental pressures of wine, particularly high ethanol, using directed evolution (DE). Directed evolution is a non-recombinant method of generating improved strains. The process involves an organism mutating and potentially adapting to a high stress environment, in this case a high ethanol environment, over several hundred generations. This method has been used successfully to generate improved strains of other lactic acid bacteria and its efficacy as a method for the production of bacterial strains for the wine industry is detailed here.

A continuous culture of *O. oeni* was established in MRS supplemented with 20 % (v/v) apple juice medium at 30 °C and 5 % (v/v) ethanol. Over the next 290 days and approximately 260 generations the ethanol concentration in the medium was gradually increased to 15 % (v/v) ethanol. A sample of this culture was screened for malic acid consumption (MLF) compared to the original parent.

With proof of concept achieved, individual isolates from the DE culture were obtained in order to identify clones that demonstrated the ethanol tolerant phenotype to the greatest degree. An individual isolate, strain 90, was selected and its fermentation
performance was characterised under a range of different ethanol (13, 15, 17 and 19 % (v/v)) concentrations and temperatures (15, 22 and 30 °C). This strain also retained viability for 48 hours in a medium supplemented with 22 % (v/v) ethanol, a condition that lead to an almost total loss of viability in the parent strain after only one hour.

Finally the parent and two evolved strains (90 and 89) were sequenced using whole genome sequencing. 32 single nucleotide polymorphisms (SNPs) were discovered in the evolved strains compared to the parent. Twenty of these are non-synonymous mutations located in nineteen different genes; five of these are located in both strains. None of the mutations appear in known O. oeni ethanol stress response genes. GO analysis, BLAST and current literature were used to analyse these changes and propose possible reasons for the new phenotype.

This study is the first known use of DE for O. oeni strain improvement and results have confirmed DE can be successfully used as a technique for developing new strains. Furthermore these findings form the basis of exciting new studies further exploring the genetic basis for tolerance to ethanol stress.
Declaration of authorship

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

In addition, I certify that no part of this work will, in the future, be used in a submission for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree. I give consent to this copy of my thesis, when deposited in the University Library, being made available for loan and photocopying, subject to the provisions of the Copyright Act 1968.

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Alice Betteridge Date
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Onwards now to the next great adventure.
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<td>absorbance</td>
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<td>Before Christ</td>
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<td>Basic local alignment search tool</td>
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<tr>
<td>CDD</td>
<td>Conserved domain database</td>
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<td>carbon catabolite repression</td>
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<tr>
<td>CFA</td>
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<td>colony forming unit</td>
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<td>DAVID</td>
<td>Database for annotation, visualization and integrated discovery</td>
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<td>directed evolution</td>
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<td>g</td>
<td>gram</td>
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<td>gDNA</td>
<td>genomic DNA</td>
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<td>GMOs</td>
<td>Genetically modified organisms</td>
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<td>gene ontology</td>
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<td>glutamate-oxaloacetate transaminase</td>
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<td>H⁺</td>
<td>hydrogen ion</td>
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<tr>
<td>HGT</td>
<td>horizontal gene transfer</td>
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<td>heat shock protein</td>
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<td>KEGG</td>
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<td>(Kanehisa and Goto, 2000; Kanehisa et al., 2014)</td>
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<td>L</td>
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<tr>
<td>LAB</td>
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<td>L-MDH</td>
<td>L-malate dehydrogenase</td>
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<td>molar</td>
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<td>Mn²⁺</td>
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<td>ng</td>
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<td>Wine Microbiology and Microbial Biotechnology Laboratory</td>
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Chapter 1. Strategies to improve the stress tolerance of *Oenococcus oeni* to enable highly efficient malolactic fermentation

1.1 Abstract

*Oenococcus oeni* is an important micro-organism for the wine industry; the stabilisation, deacidification and organoleptic changes that it facilitates in wine through malolactic fermentation (MLF) are an integral part of the production of many wines. Wine is typically a nutritionally poor environment and has physiochemical properties that can hinder the growth of *O. oeni*: low temperature, low pH, high ethanol content and the presence of sulfur dioxide. The bacterium is notoriously fastidious in its growth and metabolic requirements, and therefore its management can add significant cost to wine production both through loss of wine quality due to spoilage, and added direct costs related to the use and maintenance of cultures. The optimisation of *O. oeni* is therefore a priority for addressing inefficiencies in processes using this organism. A range of optimisation strategies, including recombinant and non-recombinant, are reviewed with a focus on biotechnological methods of gene recombination, classical strain selection, mutagenesis, directed evolution and genome shuffling.

1.2 Introduction

Malolactic fermentation is a critical step in the production of most red, many sparkling and some styles of white wines. The efficiency of the lactic acid bacterium responsible for this fermentation, *O. oeni*, is therefore of great importance to the wine industry. This review discusses the current limitations of MLF, particularly those caused by the presence of physiochemical inhibitors of *O. oeni* in wine, specifically low temperature, high ethanol, low pH and sulfur dioxide. Strategies for manipulating
this bacterium to overcome these stresses are presented. Where wine-related information is not available the review draws on examples from other food-related lactic acid bacteria (LAB).

1.3 Lactic acid bacteria and *O. oeni*

Lactic acid bacteria are Gram positive, microaerophilic and characterised by the formation of lactic acid as a primary metabolite of sugar. The genera that have been isolated from wine are *Lactobacillus*, *Pediococcus*, *Leuconostoc* and *Oenococcus* (Dicks and Endo, 2009; Fugelsang and Edwards, 2007).

*Oenococcus* contains two species, the originally described species *O. oeni* (Dicks *et al.*, 1995) and *O. kitaharae*, which was isolated from composting distilled shochu residue (Endo and Okada, 2006). *Oenococcus* is named from the Greek *oinos*, meaning wine. *Oenococcus oeni* can be identified as Gram positive, non-motile, asporogenous, ellipsoidal to spherical in shape and usually arranged in pairs or short chains (Garvie, 1975; Lonvaud-Funel, 2000). Optimal growth occurs between 20 °C and 30 °C and at a pH between 4.8 and 5.5 (Garvie, 1975). However, even under these conditions it is a nutritionally fastidious microbe prone to slow growth and poor malic acid conversion (Kunkee, 1968; 1984; Lonvaud-Funel, 2000).

The main bacterial species found on grape skin belong to the *Lactobacillus* genus. However, the only species typically found in wine at the completion of MLF is *O. oeni*, with the population increasing throughout alcoholic fermentation and proliferating throughout MLF (Renouf *et al.*, 2008). For these reasons, as well as its acid tolerance and desirable flavour effects, *O. oeni* is the preferred species to carry out the MLF (Henick-Kling, 1993; Kunkee *et al.*, 1964; Kunkee, 1984; Lafon-Lafourcade *et al.*, 1983).
1.4 Malolactic fermentation

Malolactic fermentation is not technically a fermentation, but the enzymatic decarboxylation of the dicarboxylic L-malic acid to the monocarboxylic L-lactic acid by LAB. There are no free intermediates but the reaction requires NAD$^+$ and Mn$^{2+}$ as co-factors (Caspritz and Radler, 1983; Naouri et al., 1990; Spettoli et al., 1984). The three genes responsible for this fermentation have been isolated and sequenced. They are present in a single cluster with mleA (malolactic enzyme) and mleP (malate permease, responsible for the active transport of malate into the cell) on the same operon and mleR, the regulatory protein transcribed in the opposite direction (Figure 1.1) (Bartowsky, 2005). Optimal conditions for the enzyme activity of mleA are pH 5.0 and 37 °C, though there is a non-competitive inhibition by ethanol (Naouri et al., 1990) therefore conditions for this enzyme in wine are not optimal.

Whilst MLF increases the pH of the wine, this increase does not stimulate the growth of O. oeni (Pilone and Kunkee, 1976). Instead the increase in the intracellular pH ($pH_i$) by MLF confers an energy advantage to the cell. The resulting increase in the proton motive force (Δp) across the cell membrane combined with specific ATPases facilitates the production of ATP (Cox and Henick-Kling, 1989; 1990, 1995). Low pH inhibits sugar metabolism (Henick-Kling, 1993) and malolactic fermentation has possibly evolved as an energy-producing mechanism for LAB, particularly as this may be the only means available to the cell to generate ATP at a low pH (Bartowsky, 2005).
Figure 1.1. MLF involves the active transport of L-malic acid into the cell by malate permease (mleP; red). Decarboxylation of L-malic acid is facilitated by the malolactic enzyme (mleA) and requires NAD$^+$ and Mn$^{2+}$ as co-factors before lactate is finally transported out of the cell (green). This process is regulated by a regulatory protein MleR. The increase in the intracellular pH by MLF confers an energy advantage to the cell. The resulting increase in the proton motive force across the cell membrane combined with specific ATPases (yellow) facilitates the production of ATP. Adapted from Bartowsky (2005).
1.5 The benefits and current limitations of MLF

The removal of L-malic acid, one of the major carbon sources in wine, during MLF leads to a reduced risk of spoilage from the growth of undesirable microorganisms. In addition, the process ameliorates acidity and further contributes to highly desirable sensory outcomes, resulting in an increased complexity of wine aroma and flavour. Several compounds are formed during MLF which can lead to changes in wine aroma and flavour profiles. Most well described is the buttery flavour compound diacetyl; however, the production of esters, alcohols and other carbonyl compounds results in buttery, spicy, vanilla and smoky notes, as well as a softer, fuller mouthfeel (Malherbe et al., 2012; Sumby et al., 2010; Swiegers et al., 2005). Different strains, both in nature and available commercially, show different properties in terms of sensory compounds formed (Malherbe et al., 2012). *Oenococcus oeni* generally occurs naturally in wines and thus spontaneous MLF during or after alcoholic fermentation is common. Many wineries also inoculate with commercial starter cultures of bacteria after alcoholic fermentation is complete, in order to help ensure an efficient and timely MLF; however, even with starter cultures, the growth of LAB is often inhibited and thus MLF stalled. As explained, MLF and the growth of *O. oeni* are clearly inhibited by a number of physiochemical properties of wine. The four key wine parameters inducing stress and affecting MLF are ethanol (can exceed 16 % (v/v)), low pH (typically less than 3.5), sulfur dioxide (SO₂) (over 10 mg/L) and low temperature (can be below 12 °C) (Henick-Kling, 1993). Table 1.1 highlights the characteristics of these stressors in the wine environment that lead to inhibition of growth and MLF and can ultimately lead to cell death. In addition these stressors can affect enzyme activity of the cell leading to a loss of MLF. In combination the effect of these stressors is more severe. An almost total loss of cell viability was found after only 30 minutes of exposure to a wine-like environment, in analysing the combined effects of an acid (pH 5.5 to pH 3.5), ethanol
(0 -10 % (v/v)) and cold shock (30 °C to 14 °C) on membrane fluidity (Chu-Ky et al., 2005). For these reasons improved stress tolerance would appear to be beneficial in increasing efficiency of MLF, and in fact there is evidence that \textit{O. oeni} strains performing a faster MLF also show an increased relative expression of several stress response genes (Olguín \textit{et al.}, 2010). Similarly an increased expression of \textit{mleA} (malolactic enzyme) was found in the better performing strains. However, only the initial malolactic enzyme activity was shown to determine MLF velocity, as in the middle and at the end of MLF the slower performing strains had higher transcriptional levels of this enzyme. This finding suggests that the increase in \textit{mleA} at the onset of MLF was responsible for the increased performance. This work could be further examined by purifying and quantifying the enzyme in order to investigate a potential link between its adaptation to wine and an affinity for malic acid.
Table 1.1. Key inhibitors in wine of malolactic fermentation and their mechanisms of inhibition.

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>Comments</th>
<th>Optimal condition</th>
<th>Typical wine conditions</th>
<th>Inhibitory mechanism</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol</td>
<td>Produced during alcoholic fermentation</td>
<td>5 % is considered stimulatory to growth</td>
<td>12 – 15 % (v/v)</td>
<td>Disrupts cell membrane structure and fluidity</td>
<td>(da Silveira et al., 2002; 2003)</td>
</tr>
<tr>
<td>Low pH</td>
<td>Acidity from grape berries and winemaker intervention</td>
<td>4.8 – 5.5</td>
<td>3.0 – 4.0</td>
<td>Reduces growth and malolactic activity</td>
<td>(Henick-Kling, 1993)</td>
</tr>
<tr>
<td>Low temperature</td>
<td>Wineries often rely on ambient temperature for MLF</td>
<td>25 °C</td>
<td>12 °C – 20 °C</td>
<td>Affects growth rate and increases lag phase</td>
<td>(Henick-Kling, 1993)</td>
</tr>
<tr>
<td>Sulfur dioxide</td>
<td>Produced by yeasts and added to prevent spoilage during processing</td>
<td>0 mg/L</td>
<td>10 – 70+ mg/L</td>
<td>Reduces ATPase activity, decreases cell viability</td>
<td>(Carreté et al., 2002)</td>
</tr>
</tbody>
</table>
1.5.1 Ethanol and temperature

Ethanol tolerance is important for bacterial growth in wine (da Silveira et al., 2002). The tolerable concentration of ethanol varies depending on a number of factors including pH and temperature. Ethanol disrupts cell membrane structure and increases its fluidity, affecting membrane-associated processes such as stress-resistance and malolactic activity (da Silveira et al., 2002; 2003(Silveira et al., 2004)). Ethanol alters the physiochemical properties of the plasma membrane and affects the interactions between its proteins and phospholipids with the ratio of phospholipids to proteins within the plasma membrane decreasing in response to ethanol stress (Garbay and Lonvaud-Funel, 1996). The bacterial cell responds to ethanol shock by increasing its cyclopropane fatty acid (CFA) content, which counteracts the effect of ethanol on membrane fluidity ((Couto et al., 1996)Grandvalet et al., 2008). Tolerance to this stressor is achieved via several mechanisms. Heat shock proteins (HSP) can be important in the stress tolerance of the cell. The over-expression of three small HSPs in Lb. plantarum resulted in a clearly improved tolerance, with an increased viability in 12 % (v/v) ethanol (Fiocco et al., 2007). Interestingly, O. oeni has been reported to grow faster, and have increased metabolic activity, at 5 % (v/v) ethanol compared to 0 % (v/v) (Lonvaud-Funel, 2000); however, at concentrations above 5 % (v/v) ethanol is inhibitory.

G-Alegría and colleagues (2004) clearly showed the growth inhibitory effects of ethanol and temperature on O. oeni in wine-like conditions (18 °C and 13 % (v/v) ethanol) where the culture took an extra 96 hours to reach the same density as the control (growing at 30 °C without ethanol). Unfortunately this study did not measure L-malic consumption, and so it is not possible to determine the effects that this growth rate had on MLF. In addition, the cultures were inoculated at a density of approximately 10^8 CFU (colony forming units) /mL, whereas it is considered that MLF
is able to take place once cell densities reach $10^6$ CFU/mL (Lonvaud-Funel, 1999). It is therefore possible that, although the culture grew much slower than the control, it still may have been undergoing MLF.

The effect of ethanol concentration on *O. oeni* is inversely related to temperature. An increase in ethanol concentration decreases the optimal temperature for growth, and high temperatures decrease ethanol tolerance (Asmundson and Kelly, 1990). This is partly due to membrane rigidification of cold-shocked *O. oeni* cells, combined with the fluidising effect seen in ethanol-shocked cells. Cells undergoing the combined shock (of cold and ethanol) retain a higher viability than cells with ethanol shock alone (Chu-Ky *et al.*, 2005). Given the average ethanol concentration of wine (12 – 14 % (v/v)), the optimal temperature for the growth of *O. oeni* is between 20 °C and 30 °C. Temperatures below this increase the lag phase of *O. oeni* and decrease the growth rate. Since wines undergoing MLF are often below 20 °C, delayed and slow growth of *O. oeni* is not an unexpected problem in the wine industry. At 10 °C growth of *O. oeni* ceases almost completely (G-Alegría *et al.*, 2004).

### 1.5.2 pH

The pH of wine preferred by many winemakers is between 3.2 and 3.5 for red wine and in the order of 2.8 to 3.4 for white wines, greatly dependent upon style. Acid shock exerts a rigidification effect on the cell membrane (Chu-Ky *et al.*, 2005). *Oenococcus oeni* has a greater ability to grow at pH values below 3.4 than other wine LAB genera (Davis *et al.*, 1988). This is because *O. oeni* is able to maintain a higher pHi value at a low external pH, unlike other LAB in which intracellular systems that rely on proton motive force fail (Henick-Kling, 1993). The pH of wine can also affect the tolerance of *O. oeni* to SO$_2$ because the proportion of anti-microbial forms of SO$_2$ in solution changes with a fluctuating pH.
1.5.3 Sulfur dioxide

Sulfur dioxide can be generated by yeast or is added to wine as a preservative. At low pH values, as found in wine, the proportion of molecular sulfur dioxide; the form that diffuses freely into the cell, increases. Once inside the cell, sulfur dioxide takes the form of the bisulfite ion and can react with proteins and nucleic acids, and inhibit enzymes, leading to cell death. Sulfur dioxide also inhibits MLF by reducing ATPase activity and decreasing cell viability (Carrete et al., 2002). *Oenococcus oeni* are less susceptible to SO\textsubscript{2} lethality after being inoculated into a medium supplemented with sulfite if they are first preconditioned in a medium of pH 3.5 (wine-like) with 15 mg/L SO\textsubscript{2}, possibly because adaptation to sub-lethal levels of SO\textsubscript{2} helps the cells to maintain a constant intracellular pH (between 5.8 – 6.3) (Guzzo et al., 1998). This process could decrease the concentration of free SO\textsubscript{2} within the cell, and consequently increase cell viability (Guzzo et al., 1998). This finding implies not only a direct negative combined effect of SO\textsubscript{2} and decreased pH on cell viability, but also a possible link between resistance to these combined stressors.

1.5.4 Other stressors

A number of other stressors may also influence MLF, and recent research has investigated some of the lesser-known inhibitors of this process. The strain of yeast chosen for alcoholic fermentation can directly affect the growth of *O. oeni* and the rapidity of the resulting MLF (e.g. Patynowski et al., 2002). This effect is hypothesised to be caused by yeast-derived inhibitors of MLF. For example cryotolerant strains of yeast produce succinic acid and β-phenylethanol, which is known to inhibit growth of LAB (Caridi and Corte, 1997). Other putative yeast-derived inhibitors include fatty acids, peptides, phenolic compounds and, as discussed, SO\textsubscript{2}, which can all lead to a decrease in viability (Carrete et al., 2002; Osborne and Edwards, 2006; 2007)(Campos et al., 2009). Fatty acids have also been shown to decrease the malolactic activity of
the cell (Capucho and San Romão, 1994). Additionally, as yet unknown inhibitors are likely to be present during the wine-making process. In a study comparing the growth and malolactic activity of *O. oeni* in different wines, an unknown toxic compound was found to exist in the Chardonnay wine which decreased the viability of the bacterial cell and also seemed to directly affect the malolactic activity (Gockowiak and Henschke, 2003). For a further review of physiochemical properties affecting MLF, see Bauer and Dicks (2004).

### 1.6 Problems caused by stuck fermentation

As shown, a number of factors can inhibit MLF. Such inhibition can result in a fermentation process that is protracted, in some cases for months, or may require repeated and often costly re-inoculations. In attempting to encourage MLF the addition of SO$_2$ may be delayed thus increasing the risk of spoilage or the production of undesirable by-products (Lonvaud-Funel, 1995). In addition the wine must often be warmed, increasing the environmental impact of the winemaking process. If not borne by the producer, the costs associated with this extended and inefficient process are passed on to the consumer. Clearly, strategies are required to improve this bacterium and options for carrying this out are discussed below.
1.7 Strategies to improve *O. oeni* for better tolerance to the harsh physiochemical properties of wine

Methods for strain improvement can be divided into two main approaches, recombinant and non-recombinant, with each having its own advantages and disadvantages (Table 1.2). Recombinant techniques are highly specialised and controlled but are often focused on the addition or deletion of specific genes. This requires an intricate knowledge of gene identity as well as the understanding of functions and interactions, prior to manipulation. Non-recombinant approaches require little prior knowledge of the genetic basis of a trait, but making changes can be time consuming and often random and with pleiotropic effects. In evaluating these approaches it is important to consider uses of LAB beyond the wine industry. LAB are widely used in the production of fermented foods and constitute a majority of the volume and value of starter cultures (Hansen, 2002). The range of commercial LAB exploited for food and beverage production is diverse and makes a substantial contribution to the global economy. Globally fermented fresh dairy products represent a total economic value of USD54.2 billion annually, whilst turnover for cheese alone in 2007 was estimated at USD74.2 billion (Mills *et al.*, 2010). Hence a majority of the research on LAB is contributed from the perspective of the dairy industry. This review draws on examples from this research in addition to the work available for wine, in order to suggest possible solutions to improving the stress tolerance of *O. oeni*. 
Table 1.2. Methods of strain manipulation for use with *O. oeni* and their advantages and disadvantages.

<table>
<thead>
<tr>
<th>Method</th>
<th>Approach</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular genetics</td>
<td>Recombinant</td>
<td>Highly specific for the intended improvements</td>
<td>Requires in-depth knowledge of the relevant genetics. May need to contend with views on the use of genetically modified organisms in the food and beverage industries</td>
</tr>
<tr>
<td>Classical strain development</td>
<td>Non-recombinant</td>
<td>Strain selection based on desirable traits</td>
<td>Likely to be limited by extent of natural phenotypes</td>
</tr>
<tr>
<td>Mutagenesis</td>
<td>Non-recombinant</td>
<td>Increases genetic diversity. Potential for multiple gene changes</td>
<td>Selection can be time consuming. Requires a stringent screening method. Induces non-specific changes</td>
</tr>
<tr>
<td>Directed evolution</td>
<td>Non-recombinant</td>
<td>Potential for highly specific improvements i.e. selection and screening occur simultaneously</td>
<td>Process can be time consuming. Difficulties in defining appropriate selective conditions for desired phenotype. Possible loss of desirable attributes</td>
</tr>
<tr>
<td>Genome shuffling</td>
<td>Non-recombinant</td>
<td>Potential for multiple gene changes. The fastest non-recombinant method</td>
<td>Requires a stringent and specific screening process</td>
</tr>
</tbody>
</table>
1.7.1 Molecular genetics

Over-expression of native genes or expression of foreign genes in *O. oeni* may be achieved via introduction of plasmid DNA, as is used widely in other microbes. The first example of genetic exchange was actually demonstrated in LAB when *Pneumococcus* was transformed in 1928 (Renault, 2002). Transformation requires either the chemical formation of competent cells or the forced transfer of DNA via electroporation. Transformation is difficult in *O. oeni*, unlike other LAB. Although electroporation was used successfully to transform the plasmid pGK13 into *O. oeni* strains PSU-1, ML-34 and 19CI (Dicks, 1994), this transformation has not been confirmed in other laboratories. More recently, a new electroporation protocol using ethanol as a membrane-fluidizing agent led to the successful introduction of a foreign vector encoding a truncated form of the ClpL2 protein into *O. oeni* ATCC BAA-1163 (Assad-García et al., 2008). This work has not yet led to an increase in published accounts of molecular transformations of this bacterium, possibly due to the low copy numbers of this particular plasmid (pGID052) (Beltramo et al., 2004). Plasmid copy number is important in gene replication as an increased number increases gene dosage and therefore product yield (Spath et al., 2012). A new plasmid, pCB42, was isolated and found to be capable of replicating successfully within *O. oeni*; however, the transformation frequency was low (Eom et al., 2010). *Oenococcus oeni* contains several native plasmids (Shareck et al., 2004) which are able to replicate themselves within *O. oeni* and some of which may have higher copy numbers. Using the origin of replication from these native plasmids, modification and the inclusion of genes of interest and markers into existing plasmids may generate a plasmid more effective for over-expression.

Another method of expression of foreign genes in *O. oeni* is transduction, which is the process by which bacteriophages carry bacterial genes from one cell to
another. This is theoretically possible in *O. oeni* as bacteriophages can be one of the causes of a failed MLF (Davis et al., 1985); however, the mechanisms of infection have not yet been fully elucidated and this method needs further research before being applied to this bacterium.

The final method of genetic manipulation, conjugation, is the direct horizontal transfer of genetic material between two cells, usually on a plasmid or other mobile genetic element. Conjugative transposons are mobile genetic elements capable of independent replication and insertion of a copy within the genome. An example is the conjugative transposon Tn6098, which encodes the capacity to utilise α-galactosides in *L. lactis* isolated from plants (Machielsen et al., 2011). It was characterised and transferred into a strain of *L. lactis* derived from milk, enabling the recipient strain to grow well in soy milk (a substrate rich in α-galactosides) but retaining the flavour-forming capabilities important in dairy *L. lactis* (Machielsen et al., 2011). *Oenococcus oeni* PSU-1 contains 14 transposons (Mills et al., 2005); however, problematically the current methods of conjugation for *O. oeni* do not allow for gene replacement, as the transfer frequency is lower than the recombination frequency (Beltramo et al., 2004; Zúñiga et al., 2003).

It is difficult to apply any of these techniques to *O. oeni*, as they are most beneficial when allowing the removal or addition of genes, and therefore the individual genes associated with optimisation of stress resistance would first need to be identified. Stress resistance, the primary focus of this review, generally involves multiple genes at multiple loci. In the yeast *Saccharomyces cerevisiae*, the important oenological traits such as fermentative vigour, ethanol yield and temperature tolerance are determined by a multitude of genetic loci, which are broadly distributed throughout the genome (Giudici et al., 2005). This is likely to also be the case in *O. oeni* making targeted genetic manipulation highly complex. The main stresses affecting MLF (high
ethanol, low pH, low temperature and SO$_2$) interact at a physical level, and potentially also at a genetic level. Improving tolerance for an individual stress may thus adversely affect the organisms’ ability to survive a different stress.

Whilst the technical problems associated with molecular genetic manipulation of _O. oeni_ are numerous, another important issue relates to the purported opposition and legislative blocks to the use of genetically modified organisms (GMOs) in food and beverage production. In order to genetically modify organisms for use within the food industry, certain strictures must be applied. Selection markers need to be food grade and not based on antibiotics (Renault, 2002), and the genetic elements introduced should be derived from plasmids or genes of the same bacterial species in order to constitute a “self-cloning” system (Coffey _et al._, 1994). However, due to pressure from some consumer groups as well as government regulations, GMOs can be difficult to apply industrially, even when they are food grade (Pedersen _et al._, 2005). Alternative methods of genetic engineering, which take advantage of the diversity of existing microflora and improve strains by non-recombinant methods are therefore more practical, at the present time, for industrial applications.

### 1.7.2 Classical strain selection

The oldest and simplest method of identifying superior strains is to take advantage of natural diversity; isolate strains from nature and screen them for desired traits. There are records of fermented food/drinks dating back as far as 1500 BC (Abdelgadir _et al._, 1998) and these fermentations were typically optimised through inoculation via a small quantity of a previously performed, successful fermentation. These successive inoculations have created populations of LAB that are suited specifically to the particular fermentative environment. _O. oeni_ is a prime example of a LAB evolved to occupy a very specific ecological niche, with its relative tolerance, compared with other LAB strains, to the fluctuating environment of alcoholic
fermentation and the harsh physiochemical conditions it must survive. Intraspecific diversity amongst different strains isolated in wineries worldwide has been observed in several different phylogenetic studies (Bridier et al., 2010; de las Rivas et al., 2004; Kelly et al., 1993; Sato et al., 2001; Zavaleta et al., 1997), implying a diversity amongst the specific tolerances to different stressors facing these strains.

With the advent of the ‘omics’ era, whole genome sequencing has flourished and the amount of genomic information available has increased. Bolotin et al (2001) published the first fully sequenced LAB genome. The strain, \textit{L. lactis ssp lactis}, is now one of the most well-researched of the Gram positive bacteria, surpassed only by \textit{Bacillus subtilis} (Kok et al., 2005). At the time of this review, over 237 different LAB genomes have been fully sequenced and are available for public access. This increasing library of LAB genomes has allowed accurate representations of evolutionary pathways of the LAB, as well as comparative and functional genomics (Makarova et al., 2006). In turn, this wealth of knowledge has allowed a modern twist on this age old method of classical strain selection. Strains are sequenced and the genetic traits shown to be beneficial for specific fermentations can be identified and strains chosen for fermentation based on this (Mills et al., 2010). Bioinformatic tools for sequence analysis can identify specific sequences, such as genes encoding for enzymes required for the biosynthesis of amino acids. Strains can then be selected based on their ability to form certain amino acids, which are the precursors for desirable volatile aroma compounds (Liu et al., 2008; van Kranenburg et al., 2002). \textit{Oenococcus oeni} AWRIB429 has consistently been shown to impart more fruit-driven characters to wines, and its sequence shows novel genes, which are potential glycosidases. This finding could lead to strain selection based on specific desired flavours and aromas (Bartowsky and Borneman, 2011). In addition, specific genes may be linked to high performance, with \textit{O. oeni} that exhibited faster MLF screened in order to isolate genes that correlate with this phenomenon. Although no definitive
trends were found, there was some statistical evidence that changes in a specific portion of the genome may possibly be responsible (Bon et al., 2009). Whilst these signs are positive, it is unlikely that the technological traits sought will be found only by relying on the natural diversity in different phenotypes. Considering the difficulties still encountered with MLF, it would seem that the commonly used strains are often not optimal; however, they do provide a basis on which to apply genetic improvement programs.

1.7.3 Mutagenesis

A simple method to improve the existing genetic diversity is through the use of mutagens. Mutagenesis is relatively simple and requires no specific genomic knowledge, just an effective screening process (Parekh et al., 2000). Mutagens include a variety of chemical or physical agents and modify strains through damage to DNA. The most common methods of chemical mutagenesis involve the use of compounds such as ethyl methanesulfonate (EMS) or N-methyl-N′-nitro-N-nitrosoguanidine (NTG). Resulting mutations are caused by DNA deletions, frameshifts, base substitutions or rearrangements. The use of mutagens greatly increases the frequency of mutations and thereby diversity in a population, and is a robust and widely-used method of strain development. Mutagenesis has the potential to disrupt genes responsible for undesirable characteristics, flavour properties and stress response (Parekh et al., 2000). *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis* CNRZ 483 was subjected to mutagenesis by NTG, producing a mutant that was *ldh* (lactate dehydrogenase) deficient (Boumerdassi et al., 1997). Lactate dehydrogenase is responsible for the conversion of pyruvate into lactic acid. This deficiency causes pyruvate to be converted into substrates other than lactate, increasing the production of acetoin and diacetyl (Boumerdassi et al., 1997). Further mutagenesis by NTG produced strains deficient in α acetolactate decarboxylase, increasing the production of
α acetolactate, another important buttery aroma compound (Monnet et al., 2000). There is currently no published report of this method being applied to generate superior strains of *O. oeni*. Mutagenesis involves the random mutation of a genome; this can therefore also lead to the possible loss of desirable properties. Mutagenesis is also hampered by its need for an extensive screening of a large population after a mutagen has been applied. A more efficient way to improve strains is to combine these steps. Strains can be screened and selected simultaneously via the method of directed evolution.

### 1.7.4 Directed evolution

Directed evolution (DE) is also known as adaptive evolution, stationary phase mutation, directed mutation or stress response mutation (Rosenberg and Hastings, 2003). The best known example is the on-going long-term experimental evolution of *E. coli* for more than 55,000 generations (Kawecki et al., 2012). The process involves an organism mutating spontaneously, and potentially adapting to a high stress environment. The mutations allow the organism to prosper and proliferate under the specific stress (Foster, 1999). The process by which organisms adapt is not yet fully understood. Three possible models of adaptation are presented in the literature (Foster, 1999; Rosenberg, 2001): The first is the adaptive mutation model, in which mutations might target specific genes to relieve the stress factors. The second is the hypermutation model, in which mutation rates increase genome wide so that both adaptive and non-adaptive mutations are stimulated. The final model is the cryptic-growth model, which suggests that mutation rates do not increase at all, but that extra DNA replications simply let the normal rate of mutation acting on multiple DNA copies give the appearance of an enhanced mutation rate. Directed evolution has been used successfully to change organism function. *Lactobacillus plantarum* growth on glycerol under anaerobic conditions is too slow to be accurately measured; however,
after approximately 500 generations under continuous selection using glycerol as a limiting factor, growth rate had improved by more than an order of magnitude (Teusink et al., 2009). Another example is the replication of the natural adaptation of *L. lactis* from a plant niche to growth in milk in 1,000 generations under laboratory conditions (Bachmann et al., 2012).

Directed evolution is based upon nature’s evolutionary principle and therefore lacks many of the deleterious side effects of modern recombinant techniques (Sauer, 2001) provided the culture is adapted within its primary niche. These deleterious side effects can be summarised as secondary responses or side effects of traditional metabolic engineering, which can lead to an unusable or unstable microorganism as changes within a gene or organism may affect the viability and productivity of that organism in unforeseen ways.

Within a DE population the organism must stay viable and functional throughout the process or it will simply disappear from the population. Deleterious side effects can still arise within a DE population. In fact mutations that cause deleterious effects on fitness arise more often than advantageous mutants (Perfeito et al., 2007). Deleterious mutations accumulate over time within a population due to genome erosion, in which genes that are not necessary for a specific environment are lost. Thus a population remains viable within its specific environment, but the accumulation of deleterious mutants becomes sufficient that the strain is effectively crippled for growth in any other environment and is therefore highly specialised (Muller, 1964). This outcome is more damaging in the case of non-recombinant organisms, as without sexual reproduction they are unable to contain fewer mutations than their predecessor (Muller, 1964).

A common way of monitoring an evolving population is to monitor insertion sequences. Insertion sequences are generally small mobile genetic elements.
Monitoring of insertion elements in a batch culture of *L. lactis* with the *ldh* gene deleted, found that a transposition of the insertion sequence IS981 activated a second lactate dehydrogenase gene (*ldhB*) to restore lactic acid production under anaerobic conditions (Bongers et al., 2003). These insertion sequences and other mobile genetic elements found during sequencing demonstrate a high plasticity within the genome, which contributes to the ongoing gene decay process, allowing easier external manipulation of the genome (Kaleta et al., 2010).

The genome of *O. oeni* PSU-1 has been sequenced (Mills et al., 2005), which led to the observation of the lack of the genes *mutS* and *mutL*, which encode two key enzymes in the methylated mismatch repair (MMR) pathway (Makarova et al., 2006; Makarova and Koonin, 2007). The MMR pathway is an excision-repair system, which corrects nucleotide base pair mismatches, and the presence of *mutS* and *mutL* homologs is required for it to function (Miller, 1996; Oliver et al., 2002). The correction of mismatches by MutS and MutL decreases the spontaneous mutation rate of a species, therefore a defect in the MMR system leads to an increase in the mutation frequency (Miller, 1996). A recent study comparing spontaneous mutation rates of *O. oeni* to those of its closely related LAB species *Leuconostoc mesenteroides* and *Pediococcus pentosaceus*, which do contain the relevant encoding genes, showed a 100-fold increase in the rate of spontaneous mutations in response to rifampin and erythromycin (Marcobal et al., 2008). One possible reason for the loss of MMR was that a high mutation rate generated beneficial mutations during adaptation to a restrictive environment such as wine (Marcobal et al., 2008). During adaption an increased mutation rate leads to an increase of beneficial mutations within a population; however, once an organism has adapted to that environment, the increased mutations lead to an increased load of deleterious mutations within that population, causing a loss of fitness (Taddei et al., 1997). *Oenococcus oeni* has been shown to be a rapidly evolving organism (Yang and Woese, 1989) and this, combined with the
inhibitory properties of wine, may in fact make it a perfect candidate for this method of optimisation.

There is currently no evidence of the generation of *de novo* functions via directed evolution (Sauer, 2001); however, the functionality of a pseudogene was restored in *L. lactis* during a DE experiment (Bachmann *et al.*, 2012). Additionally Bergthorsson *et al.* (2007) do suggest a possible method for the generation of new genes. If specific genes have, prior to duplication, trace side-activity in addition to the original gene function, introduction into an ecological niche that favours that minor innovation will allow natural selection to select for that gene until amplification of the minor function occurs. This gene is then no longer constrained to maintain its original function.

### 1.7.5 Genome shuffling

A potential method to remove neutral or deleterious mutations whilst preserving useful mutations is genome shuffling. Genome shuffling allows for changes throughout an entire genome without needing genome sequence information or any knowledge of the genetic basis of desired traits (Petri and Schmidt-Dannert, 2004). It thus allows for the improvement of poorly understood and/or complex phenotypes and is a major improvement in techniques for strain improvement and metabolic engineering (Stephanopoulos, 2002). Parental strains that exhibit subtle phenotypic improvements are identified and using protoplast fusion, the genomes of the parents are shuffled. This results in new hybrid strains that exhibit the specific improvement to a greater degree (Patnaik *et al.*, 2002; Stephanopoulos, 2002; Zhang *et al.*, 2002) (Figure 1.2). Protoplasts are usually obtained via chemical treatment (polyethylene glycol (PEG)) (Cocconcelli *et al.*, 1986; Hopwood and Wright, 1978; Morelli *et al.*, 1987) but can be generated electrically (Assani *et al.*, 2005). Protoplast formation involves the removal of the cell wall, leaving a fatty membranous sac.
containing all the genetic material of the cell. These sacs are then combined or fused together allowing genetic material from multiple cells to combine. More recently, femtosecond laser and optical tweezers (Gong et al., 2008) have been applied to more efficiently pair cells. A microfluidic device has been newly patented which can be used to trap and pair fusions (Skelley et al., 2009). The strains are then allowed to naturally regenerate, with the newly recombined cell containing genetic material from more than one cell. After one round of genome shuffling, 10% of the cells are pairwise recombinants (contain genetic material from two parents), and after two rounds at least 2% of the cells have genetic information from any four parental strains (del Cardayré, 2005). Because the strains undergo a natural homologous recombination, they are not considered to be genetically modified (Zhang et al., 2002). Whole genome shuffling has been successfully applied to Lactobacillus spp.. Lactobacillus delbrueckii was successfully fused with Bacillus amyloliquefaciens in order to generate a strain that produced greater amounts of L-lactic acid from starchy wastes (John et al., 2008). Three rounds of genome shuffling in Lactobacillus produced a strain that could grow to a 70% higher density at pH 3.8 and generate 40% more lactic acid than the wild type (Patnaik et al., 2002). After a third round of genome shuffling in Lb. rhamnosus a strain was produced that could grow at pH 3.8, compared to pH 4.4 for the wild type, as well as produce up to 26% more lactic acid at this pH (Wang et al., 2007). Additionally, two rounds of genome shuffling were able to produce strains yielding up to 71.4% more lactate than the parent (Yu et al., 2008). Two rounds of genome shuffling can achieve results that previously required 20 rounds of classical strain improvement techniques (Zhang et al., 2002). That is the difference between 20 years of mutagenesis and selection on the one hand and one year of selection and genome shuffling on the other. Genome shuffling requires the screening of millions of individual cells. A stringent screening process is therefore
required making it more difficult to select strains based on performance to multiple different stresses.

Figure 1.2. Genome shuffling by recursive protoplast fusion. 1. Protoplasts are prepared, cell walls are removed in a process encouraged by PEG. The DNA is situated within a fatty membranous sac. 2. Cells are fused together. 3. Recombination of the genetic components occurs. 4. Cells regenerate their walls and are screened for the desired phenotypes. 5. The process can then be repeated. Adapted from del Cardayré (2005)
1.8 Conclusion

Malolactic fermentation remains an important step in the production of wine, and *O. oeni*, the bacterium responsible for it, is a fastidious and recalcitrant micro-organism. Methods that are currently applied to other lactic acid bacteria to optimise performance should also be applicable to this bacterium. The options vary in feasibility and specificity. Molecular methods of recombination are complex as they require an in-depth knowledge of the genes responsible for the property of interest and also an organism amenable to transformation, which *O. oeni* is currently not. Methods for transformation or gene knockouts in *O. oeni* are far from being as reliable and efficient as seen for other organisms thus this approach is difficult. Even if it were more readily achievable, the current restrictions on the use of genetically modified organisms in food and beverage production, means that genetically modified *O. oeni* are unable to be used by some industries even if they can improve the quality and efficiency of the product. Non-recombinant techniques such as strain selection and mutagenesis are possibilities for the selection of improved strains. Even though they have been useful until know they can be time-consuming and impractical due to extended screening time and the possibility that the desired strain cannot be produced this way. The technique of genome shuffling is a more recent and convenient method of genetically modifying whole genomes but requires an accurate and stringent screening process. Directed evolution is a process based on Darwin’s theory of natural selection (Darwin, 2008). This, like genome shuffling, is a genome wide method of manipulation without the necessity for gene network knowledge, whilst being a simple method of diversifying a population.

Strategies of bacterial improvement need not be used in isolation. Molecular methods, whilst not applicable industrially, are able to identify specific genes and functions, that can then be manipulated using non-recombinant methods. The
isolation of improved strains from nature coupled with mutagenesis can increase diversity. The diverse populations can then be fed into further improvement programs using genome shuffling or directed evolution which, again, coupled together are a more powerful tool of microbe manipulation than any individual strategy alone.

The primary fermentation step in wine performed predominantly by *S. cerevisiae* has been well studied and methods to improve its performance have been applied, with strains generated via mutagenesis commercially available (Schuller and Casal, 2005). Directed evolution has generated robust new strains (McBryde et al., 2006), genome shuffling combined with mutagenesis has produced an alcohol tolerant strain (Hou, 2009), and recombinant strains exist and only await consensus from the consumer before they are applied industrially (Chambers and Pretorius, 2010; Husnik et al., 2006). In stark contrast, methods of optimising *O. oeni* are more limited. The techniques analysed above could be used in combination to improve the tolerance of *O. oeni* for improved environmental tolerances, resulting in an industry ready, efficient bacterium, imperative for development of the wine industry into the future.

### 1.9 Aims and objectives of the project

In this review, the importance of MLF in winemaking and the unreliability of the bacterium responsible for it have been discussed. A range of methods for improvement of *O. oeni* have been analysed and of these, DE has been selected as the most promising method for the production of industrial strains of *O. oeni* for wine fermentation.

This PhD project will focus on DE and its efficacy as a method for the generation of strains of *O. oeni*, with a particular focus on high ethanol fermentations in order to ascertain the suitability of DE as a method for the improvement of *O. oeni*. A chosen strain will be grown in continuous culture with a stressor being applied until
an appropriate mutation(s) arises and is fixed within the population, facilitating robustness under the specific conditions.

Once an improved strain(s) has been generated, whole genome sequencing of this strain will be performed in order to analyse the genetic basis for the improved phenotype. The genome characterisation will also lead to advancements in the knowledge of the genetic basis of stress and stress resistance of *O. oeni* by determining which genes/regulons are related to survival in the presence of inhibitors in wine.
Chapter 2. Directed evolution of *Oenococcus oeni*

2.1 Introduction

*Oenococcus oeni* is an important micro-organism of the wine industry. The stabilisation, deacidification and organoleptic changes that it facilitates through MLF are an integral part of the wine-making process. This bacterium is well known to be fastidious, a fact especially relevant within an oenological context. Wine is a complex matrix, possessing physiochemical properties that inhibit the growth and malolactic activity of *O. oeni*, consequently MLF is often slow and/or fails. Common inhibitors in wine include, high ethanol content, low pH, low temperature, low nutrient availability and the presence of SO$_2$, inhibitory phenolic compounds and acetic acid (Bauer and Dicks, 2004). To alleviate these stresses a number of strain improvement methods are available that encompass both recombinant and non-recombinant protocols. To produce strains practicable for use in the wine industry it is important to focus on non-recombinant methods since recombinant methods are not favoured by some consumers. Furthermore the acquisition of stress tolerance is more than likely dependent on multiple genes thus requiring multiple manipulations at multiple loci. Directed evolution is a process based on Darwin’s theory of natural selection (Darwin, 2008), thus it is a simple genome wide method of diversifying a population therefore of the non-recombinant methods, DE is the most promising for the development of stress tolerant, industry-ready *O. oeni* strains.

Directed evolution experiments can be performed by either sequential batch or continuous culture (Sauer, 2001). The batch culture method is also known as a serial transfer where a small percentage of the population is used to inoculate fresh medium. This method is limited since only a small portion of cells, and therefore genetic material, is transferred between batches whilst the majority are removed. In addition, the multiple phases of growth mean that time is spent on lag phase limiting the number
of generations per batch. However, an advantage for some processes, including winemaking, is that a batch approach ensures that the culture is exposed to all phases of the fermentation. On the other hand, a continuous culture is grown in a chemostat and the population is maintained in a constant environment at a constant growth rate. This allows for a greater number of generations over time as the culture is continuously diluted therefore introducing more possibilities for mutations to arise. Given the likelihood of a desired strain being evolved quicker via continuous culture, this approach was selected as the preferred method for the directed evolution of *O. oeni* in this study.

The alcohol content in wines has been rising in recent decades, with mean alcohol content increasing from 12.4% to 14.4% between 1984 and 2008 in Australian red wines (Godden and Muhlack, 2010). It is speculated that factors contributing to these rising levels of alcohol include a warmer climate, vines grown with more access to nutrition and grapes picked at later stages of maturity. Malolactic fermentation in wines with a high ethanol content are known to commonly stall (Zapparoli *et al.*, 2009), consequently ethanol was selected as the focus for this DE study.

The choice of strain is a critical first step in an experiment of this kind. For instance, as described in Chapter 1, the *O. oeni* strains sequenced to date (PSU-1 and ATCC BAA-1163) lack the MMR pathway. This excision repair system requires the MutS and MutL endonucleases to correct base pair mismatches (Miller, 1996; Oliver *et al.*, 2002). The correction of mismatches by MutS and MutL decreases the spontaneous mutation rate of a species, thus a defect in the MMR system leads to an increase in the mutation frequency as well as to an increase in the horizontal gene transfer among strains (Miller, 1996). Such hypermutability could improve the diversity of the population in a DE experiment and increase the rate of evolution.
The work described in this chapter focuses on investigating the suitability of DE as a method for the generation of industrially improved bacteria, specifically *O. oeni*.

### 2.2 Materials and methods

#### 2.2.1 Bacterial strains and maintenance

The strains used in this study are listed in Table 2.1. *Oenococcus oeni* and *Lb. plantarum* strains were isolated from commercial packets, rehydrated in sterile ultrapure water (MQ) and then cultured on MRS (de Man, Rogosa and Sharpe) agar (MRSA) plates. *Lactobacillus delbrueckii* and *P. pentosaceus* were obtained from the Wine Microbiology and Microbial Biotechnology Laboratory (WMMBT) culture collection (University of Adelaide, Waite Campus, Urrbrae, SA) and were cultured on MRSA plates. An individual colony was selected and inoculated into MRS supplemented with 20% Apple Juice (MRSAJ) and incubated at 30 °C for five days (except V22 which was incubated overnight). Strains were stored at -80 °C as glycerol stocks (culture grown for five days in MRSAJ and then supplemented with glycerol; final concentration 25% (v/v)). *Escherichia coli* were cultured in lysogeny broth (LB) overnight at 37 °C.

<table>
<thead>
<tr>
<th>Name</th>
<th>Species</th>
<th>Brand name</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>SB3</td>
<td><em>O. oeni</em></td>
<td>Lactenos SB3 Instant®</td>
<td>Laffort, Woodville North, SA</td>
</tr>
<tr>
<td>VP41</td>
<td><em>O. oeni</em></td>
<td>Lalvin VP41®</td>
<td>Lallemand, Edwardstown, SA</td>
</tr>
<tr>
<td>SK3</td>
<td><em>O. oeni</em></td>
<td>BioStart® Bianco SK3®</td>
<td>Erbslöh, Altona, VIC</td>
</tr>
<tr>
<td>CH16</td>
<td><em>O. oeni</em></td>
<td>Viniflora® CH16</td>
<td>CHR Hansen, Bayswater, VIC</td>
</tr>
<tr>
<td>Oenos</td>
<td><em>O. oeni</em></td>
<td>Viniflora® Oenos</td>
<td>CHR Hansen, Bayswater, VIC</td>
</tr>
<tr>
<td>V22</td>
<td><em>Lb. plantarum</em></td>
<td>V22™ <em>Lactobacillus plantarum</em></td>
<td>Lallemand, Edwardstown, SA</td>
</tr>
<tr>
<td>LAC37Z</td>
<td><em>Lb. delbrueckii</em></td>
<td></td>
<td>WMMBT Culture collection</td>
</tr>
<tr>
<td>PED42X</td>
<td><em>P. pentosaceus</em></td>
<td></td>
<td>WMMBT Culture collection</td>
</tr>
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<td>DH5α</td>
<td><em>E. coli</em></td>
<td></td>
<td>WMMBT Culture collection</td>
</tr>
</tbody>
</table>
2.2.2 Mismatch repair identification

2.2.2.1 Buffers and solutions

*Phosphate Buffered Saline (PBS)*

In 1 L of dH₂O was dissolved 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na₂HPO₄ and 0.24 g of KH₂PO₄. pH was adjusted to 7.4 with HCl and the solution autoclaved at 121 °C for 20 minutes to sterilise.

*Tris-acetate EDTA (ethylenediaminetetraacetic acid) (TAE)*

To make one litre of 50 × stock solution of TAE 242 g of Tris Base was dissolved with 57.1 mL glacial acetic acid and 100 mL 0.5 M EDTA in one litre of MQ. For a 1× working 20 mL of solution was diluted in 980 mL water.

2.2.2.2 Isolation of genomic DNA

Genomic DNA was isolated from bacteria using the UltraClean® Microbial DNA Isolation Kit (Cat. # 12224-50, MoBio Laboratories Inc., Carlsbad, USA). A 10 mL culture of bacteria was pelleted, washed twice in PBS, resuspended in 1.8 mL of PBS and subsequently used according to the manufacturer’s instructions.

2.2.2.3 Polymerase chain reaction (PCR)

PCR amplification was performed in 12.5 µL reaction volumes typically containing 1 U/µL Velocity polymerase (Cat. # BIO-21098, Bioline, Alexandria, NSW), 5 × Hi-Fi Reaction Buffer, 200 µM dNTPs, 0.4 µM of each primer (Table 2.3), 50 ng genomic DNA. Cycling parameters are listed in Table 2.2. Cycling reactions were conducted using a BioRad S1000 Thermal Cycler (BioRad, Hercules, USA).
2.2.2.4 Gel purification

PCR products were visualised via electrophoresis on a 2% agarose gel in 1 × TAE buffer. The desired product was excised from the resulting gel and purified using the Wizard® SV Gel and PCR Clean-Up System (Cat. # A9282, Promega, Madison, USA). Purity and concentration of the resulting PCR product was determined by measuring 2 μL of sample on a Nanodrop instrument (ND-1000 spectrophotometer, NanoDrop, Thermo Scientific, Wilmington, USA). The sample was then sequenced commercially (Australian Genome Research Facility, Urrbrae, SA).

Table 2.2. PCR cycling conditions.

<table>
<thead>
<tr>
<th></th>
<th>Temperature (°C)</th>
<th>Time (seconds)</th>
<th>Repeat</th>
</tr>
</thead>
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<tr>
<td>Initial denaturation</td>
<td>98</td>
<td>120</td>
<td>1</td>
</tr>
<tr>
<td>Denaturation</td>
<td>98</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>Annealing</td>
<td>62 ± 6</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>Extension</td>
<td>72</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>Final Extension</td>
<td>72</td>
<td>240</td>
<td>1</td>
</tr>
</tbody>
</table>
Table 2.3. List of PCR primers used in this study.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence (5'-3’)</th>
<th>Comment</th>
<th>Reference</th>
</tr>
</thead>
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<tr>
<td>MutS2Fwd</td>
<td>ACMGGYCCVAAAYATGKSHGG</td>
<td>mutS degenerate primer</td>
<td>This study</td>
</tr>
<tr>
<td>MutS2Rev</td>
<td>GTDCCVCGBCCRAYYTCRTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>e.coliGSTFwd</td>
<td>CTTTGCCGTTAACCCTAAGGG</td>
<td>E. coli reference gene</td>
<td>Pfaffl, 2001</td>
</tr>
<tr>
<td>e.coliGSTRev</td>
<td>GCTGCAATGTGCTCTAACC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>e.coliYjaFwd</td>
<td>TGAAGTGTCAAGGAGACGCTG</td>
<td>E. coli reference gene</td>
<td>Clermont et al., 2000</td>
</tr>
<tr>
<td>e.coliYjaRev</td>
<td>ATGGAGAATGCGTCTCTCAAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ld1.Fwd</td>
<td>ACATGAATCGCATGATTCAAG</td>
<td>Lb. delbrueckii reference gene</td>
<td>Furet et al., 2004</td>
</tr>
<tr>
<td>Ld2.Rev</td>
<td>AACTCGGTACGCATCATTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ppe.Fwd</td>
<td>CTTTGTGGCCGGTTGGATCCT</td>
<td>P. pentosaceus reference gene</td>
<td>Cho et al., 2009</td>
</tr>
<tr>
<td>PPe.Rev</td>
<td>AAAGGCTGCAATGTAGTGTGATGCTA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ldhD Fwd</td>
<td>GCCGCAGTAAAGAACTTGATG</td>
<td>O. oeni reference gene</td>
<td>Desroche et al., 2005</td>
</tr>
<tr>
<td>ldhD Rev</td>
<td>TGCCGACAACACCAACTGTTT</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
2.2.3 L-Malic acid determination

L-Malic acid concentration was determined using a modified version of the Boehringer-Mannheim UV method (Boehringer-Mannheim, 1995). This assay is based on two reactions:

\[
\text{L-MDH} \\
1. \quad \text{L-malic} + \text{NAD}^+ \leftrightarrow \text{oxaloacetate} + \text{NADH} + \text{H}^+
\]

\[
\text{GOT} \\
2. \quad \text{oxaloacetate} + \text{L-glutamate} \leftrightarrow \text{L-aspartate} + 2\text{-oxoglutarate}
\]

L-malic acid is oxidised to oxaloacetate in the presence of L-malate dehydrogenase (L-MDH). Removal of oxaloacetate by conversion to L-aspartate occurs in a reaction catalysed by glutamate-oxaloacetate transaminase (GOT). The removal of oxaloacetate then causes the equilibrium to favour the production of oxaloacetate and NADH. The NADH formed is stoichiometric to the amount of L-malic and is measured by absorbance at 340 nm.

Each well of a 96 well micro-titre plate was filled with 150 µL of Solution 1 (0.1 M glycyglycine, 0.1 M L-glutamate (adjusted to pH 10.0 with 5 M NaOH)) and 30 µL of Solution 2 (0.0164 g/mL NAD\(^+\)) using a liquid handling robot (Corbett Robotics CAS3800). The undiluted sample (4 µL) was then pipetted into each well except for the final column which contained eight standards of L-malic acid solutions ranging from 0 to 3.5 g/L in 0.5 g/L increments. The plate was inserted into a plate reader (Infinite 200 PRO, Tecan, Männedorf, Switzerland) and 10 µL GOT solution (0.0269 U/µL), added to each well of the plate using the injectors. The plate was shaken for 3 minutes before measurement at 340 nm (A0). Finally 10 µL of the enzyme L-MDH (0.0261 U/µL) were added to each well. The plate was shaken for 25 minutes and then read again at 340 nm (A1). The change in absorbance (ΔA) was calculated (A1 – A0)
and ΔA for each of the standards and a standard curve generated for each individual plate. This equation was then applied to the ΔA for each of the samples within the plate to calculate L-malic acid concentration.

2.2.4 Directed evolution using continuous culture

A continuous culture was conducted in a Biostat A plus bioreactor (Sartorius BBI System GmbH, Goettingen, Germany) and controlled using the MFCS/DA A plus 2.1 software (Sartorius BBI System GmbH). The empty chemostat was autoclaved at 121 °C for 30 minutes and filled with 1,100 mL of MRSAJ at 5 % (v/v) ethanol and inoculated with a 10 mL culture of SB3 (2.2.1). The volume in the chemostat remained stable via a level sensor, feed/effluent pumps and control software. The feed medium was MRSAJ and the feed rate slowly decreased over the course of the experiment as the maximum specific growth rate (μ) of the population slowed. Ethanol was added separately to the culture, at a constant rate, throughout the course of the experiment. The culture was kept at 30 °C and stirred constantly at 100 rpm. The culture was fed continuously for 332 days (7959 hours) with a gradual increase in ethanol concentration to 15 % . The culture was sampled weekly and OD₆₀₀ was determined along with plating on to MRSA to determine viability. Once the culture feed had surpassed 13 % (v/v) ethanol the O. oeni population was sampled and screened for improvement in L-malic acid consumption compared to the original parent strain.

2.2.5 Screening of the evolving population

Screening was performed in quadruplicate in 200 μL of MRSAJ with 15 % (v/v) ethanol in 96 well micro-titre plates (Cat. # 3596, Corning Incorporated, New York, USA). Eight identical plates were generated from an individual source plate in order to enable sacrificial sampling over an 8 – 10 day period. This allowed a fresh plate to be analysed at each time-point for monitoring of the depletion of L-malic acid, without
influencing the fermentation performance of the small volume. Each plate was sealed with a membrane (Cat. # 100-SEAL-PLT, Excel Scientific, Victorville, USA) and incubated statically at 30 °C in an anaerobic vessel (Cat. # 260671, 260001, BD Diagnostic Systems, North Ryde, NSW). At each time point the L-malic acid concentration was determined using the enzymatic method described in 2.2.3.

2.3 Results and Discussion

2.3.1 Bacterial strain selection

All commercially available strains of *O. oeni* available in Australia at the commencement of this study were considered for inclusion (Table 2.4). A strain that appeared to be the most average, was selected based on manufacturer commentary about its tolerance to the major physiochemical stressors found in wine. As discussed in Chapter 1, inhibition of *O. oeni* by stressors is generally concomitant as is the tolerance or resistance to these stresses. Therefore selecting a strain that performs poorly to most stressors may suggest that it lacks the genes/SNPs necessary for further improvement of stress resistance, whereas a strain that is already highly stress tolerant may already be highly evolved to its specific niche and may not be able to be improved using DE. The Laffort SB3 strain of *O. oeni* was selected as the most suited for this DE experiment as it displayed the most average stress tolerance and was therefore thought to be a good candidate for DE.
Table 2.4. *Oenococcus oeni* starter cultures commercially available in Australia, including manufacturer commentary about their tolerance to the major physiochemical stressors found in wine. The strain in bold was selected as the parent for the evolution experiment. Other highlighted strains (*) were selected for screening in Section 3.2.5.

<table>
<thead>
<tr>
<th>Company</th>
<th>Strain name</th>
<th>pH (min)</th>
<th>Temperature °C (min)</th>
<th>Ethanol % (v/v) (max)</th>
<th>SO₂ mg/L (max)</th>
</tr>
</thead>
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<td>CHR Hansen</td>
<td>Viniflora® CH35</td>
<td>3.1</td>
<td>15</td>
<td>14</td>
<td>45</td>
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<td>Viniflora® CiNe</td>
<td>3.2</td>
<td>17</td>
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<td>CHR Hansen</td>
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<tr>
<td>Erbslöh</td>
<td>BioStart® Vitale SK1®</td>
<td>3.0</td>
<td>16</td>
<td>15.5</td>
<td>50</td>
</tr>
<tr>
<td>Erbslöh</td>
<td>BioStart® Bianco SK3®</td>
<td>3.0</td>
<td>13</td>
<td>13.5</td>
<td>45</td>
</tr>
<tr>
<td>Erbslöh</td>
<td>BioStart® Forte SK2®</td>
<td>3.0</td>
<td>14</td>
<td>14.5</td>
<td>45</td>
</tr>
<tr>
<td><strong>Average</strong></td>
<td></td>
<td><strong>3.2</strong></td>
<td><strong>15.5</strong></td>
<td><strong>15.1</strong></td>
<td><strong>53</strong></td>
</tr>
</tbody>
</table>
2.3.2 Confirming lack of MMR system in SB3

After selecting SB3 as the candidate for evolution, based on its oenological properties, it was important to confirm that it too lacked the MMR system. This would ensure the selected strain had an increased rate of hypermutability (Marcobal et al., 2008) and would improve the efficiency and likelihood of success in a DE experiment. Because of the role of MMR in repairing DNA damage, elimination of the MMR system in *B. subtilis* has revealed to increase stationary-phase mutagenesis indicating that the absence of MMR is also an important factor in the mutagenesis of non-growing cells. (Pedraza-Reyes and Yasbin, 2004).

A PCR approach was designed with the hypothesis that the *mutS* gene would be absent and thus unable to be amplified in *O. oeni*. The key mismatch repair gene *mutS* has been shown to be highly conserved across all prokaryotes (Jiricny, 2006). The *mutS* genes in bacteria have been well characterised and several of these from different species were selected as candidates to design degenerate primers to. These included both Gram positive and Gram negative bacteria in order to have the widest range of *mutS* gene sequences to help ensure primer specificity. The bacteria were *Pseudomonas aeruginosa* (Oliver et al., 2002), *Azotobacter vinelandii* (Le et al., 1993), *Staphylococcus aureus* (O’Neill and Chopra, 2002; Prunier and Leclercq, 2005), *B. subtilis* (Ginetti et al., 1996), *Salmonella typhimurium* (Haber and Walker, 1991; Pang et al., 1985) and *E. coli* (Li et al., 2003). A degenerate primer pair (MutS2; Table 2.3) was designed to a highly conserved region within these characterised *mutS* genes (Figure 2.1).

This primer pair was tested for specificity in *E. coli* and then used to successfully amplify *mutS* from two different lactic acid bacteria closely related to *O. oeni*; *Lb delbrueckii* and *P. pentosaceus* (Figure 2.2). These primers were subsequently tested on three different strains of *O. oeni* (VP41, SK3 and SB3) and
*mutS* was unable to be amplified suggesting it is lacking in these three strains, including SB3 (Figure 2.2). Included were also a reference gene for *O. oeni* (*ldhD*) (Figure 2.2, D) and a reference strain, *Lb. delbrueckii* (Figure 2.2, F and G). The products that were amplified by the degenerate primers in *O. oeni* were the correct size for *mutS* (Figure 2.2, A and B); however, BLAST results did not show a similarity. The possibility of designing primers based on nearby genes was considered. However whilst the MMR system is highly conserved across bacterial species, its location on the chromosome varies greatly even amongst closely related LAB, therefore genes located near the MMR locus cannot be used to help prove its existence or absence. The possibility that the designed primers do not work in *O. oeni* despite successful amplifying *mutS* in other LAB was deemed too remote to be considered further.
Figure 2.1. Nucleotide sequence alignment of mutS in P. aeruginosa, A. vinelandii, S. aureus, B. subtilis, S. typhimurium and E. coli. Location of a degenerate primer pair to amplify mutS across bacterial species is shown.
Figure 2.2. Gel image of mutS PCR amplification in O. oeni (SB3) and Lb. delbrueckii. Annealing temperatures used were: 1, 56°C; 2, 56.8°C; 3, 58.4°C; 4, 60.7 °C; 5, 63.6°C; 6, 66°C; 7, 67.3 °C and 8, 68°C. SB3 ldhD is an O. oeni reference gene. Lb. delbrueckii mutS is used as a control strain for mutS primers and Ldel as a reference. The products that were sequenced are boxed in red (A-G). D, F and G are the expected reference products. A and B are the correct size for mutS in O. oeni; however, BLAST results did not show similarity.
2.3.3 Establishing initial culture conditions

Before commencing a long-term DE experiment it is necessary to establish the optimal conditions for the production of the desired phenotype. In order to maximise the number of generations, a nutritional MRSAJ medium rather than wine was used in the chemostat. Ethanol was applied and progressively increased as a stress upon the growing culture (Section 2.2.4). If a beneficial mutation confers a 10% selective advantage it would take approximately 250 generations to become fixed in the majority of the population. For this reason experiments of this type aim to culture for at least 300 generations (Lenski, 2010). Even under highly optimal conditions, to achieve 300 generations would take at least 111 days (Table 2.5).

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>μ (h⁻¹)</th>
<th>Doubling time (hours)</th>
<th>Days to reach 300 generations</th>
</tr>
</thead>
<tbody>
<tr>
<td>21</td>
<td>0.025</td>
<td>27.84</td>
<td>348.0</td>
</tr>
<tr>
<td>25</td>
<td>0.042</td>
<td>16.46</td>
<td>205.8</td>
</tr>
<tr>
<td>30</td>
<td>0.054</td>
<td>12.77</td>
<td>159.6</td>
</tr>
<tr>
<td><strong>35</strong></td>
<td><strong>0.078</strong></td>
<td><strong>8.96</strong></td>
<td><strong>111.9</strong></td>
</tr>
<tr>
<td>40</td>
<td>Did not grow</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Temperature is well known to affect ethanol toxicity (Section 1.5.1), thus for this experiment it was important to find a suitable temperature at which to perform the experiment in order to efficiently drive the appearance of mutations. Optimal growth temperature was also sought to encourage maximum growth rate so as to keep the total experimental time to a minimum. Optimal growth of *O. oeni* has been reported to be between 20 and 30 °C (Garvie, 1967); however, in this experiment growth rate was fastest at 35 °C (Table 2.5).
No growth was observed at 30 °C once ethanol was introduced to the medium, indicated by no change in OD$_{600}$ and no viable cells observed from plating after seven days. Presumably this was because toxicity of ethanol is enhanced at 35 °C (Asmundson and Kelly, 1990), thus the working temperature was lowered to 30 °C. Initially 5 % (v/v) ethanol was added to the growing culture, which resulted in a maximum specific growth rate (µ) of 0.052 and a doubling time of 13.43 hours. Once the maximum specific growth rate was achieved ethanol was immediately increased to 6% (v/v). Considering SB3 is known to grow at 15 % (v/v) (Laffort Oenologie, 2010) this implied cells were not highly stressed at only 6 % (v/v) ethanol; however, this previously reported growth was in a low temperature wine fermentation, and as mentioned, with temperature increase ethanol toxicity becomes more severe (Chapter 1).

The culture was fed continuously for 332 days with a gradual increase in ethanol concentration to 15 %, which in turn increased the generation time to 31.8 hours, at which point the cultivation was terminated. Between 250 and 589 generations were calculated to have occurred based on the original and final generation times respectively.

The nature of a chemostat experiment involves the ability of the cell to remain viable and continue to divide under the selective pressure. It is necessary to constantly stir the population to help ensure uniformity of culture and medium and to minimise the advantage experienced by subsets of the population that may evolve mechanisms to remain within the vessel i.e. by developing an ability to attach to the surface of the vessel (Dykhuizen and Hartl, 1983). Instead it was anticipated, in this case, that selection will result in an ability to tolerate increased concentrations of ethanol. The culture was stirred continuously at 100 rpm. Since the growth of O. oeni is favoured by anaerobic conditions (Lonvaud-Funel, 1999) so it is possible that the use of inert
gas cover (e.g. N₂) could further improve the growth rate of the organism; something to be considered for future DE experiments.

2.3.4 Screening of the evolving culture

An efficient screening process was vital to confirm the presence of a superior phenotype. Three separate commercial isolates VP41, SB3 and SK3 were chosen to determine if a difference in ethanol tolerance could be established in the evolving culture. VP41 is a highly ethanol tolerant strain reportedly able to conduct MLF in wine with up to 16 % (v/v) ethanol (Lallemand, 2010). Conversely, SK3 is reported to have a lower ethanol tolerance (c.a. 13.5 % (v/v) (Erbslöh, 2010)). It was reasoned that a strain that exhibits a high tolerance to ethanol would grow better under high ethanol conditions and would also be undertaking MLF efficiently in these conditions. For this reason, a convenient assay that measured growth under different ethanol conditions was established. Initially gradient plates were considered since they have proven to be a successful and simple method of screening lactic acid bacteria (Patnaik et al., 2002). A gradient plate has a gradient of stressor from one end of the plate to the other (i.e. concentration of ethanol from low to high). In this case, uniform growth was observed in an ethanol gradient (data not shown). Presumably this occurred since during the time required to grow O. oeni (approximately seven days) the ethanol gradient across the plate had dissipated. Individual agar plates with fixed ethanol concentrations (e.g. 5 % – 15 %) were therefore trialled. Again, no significant differences were noted in the relevant ethanol tolerances of the three test strains with a majority of the population not viable at an ethanol concentration of 9 % (v/v) and above at 30 °C. This assay was not able to differentiate strain ethanol tolerance. Presumably assays such as these on solid media are not translatable to what occurs in liquid culture (such as the chemostat), where cells in a liquid culture are immersed in high ethanol whereas on a solid medium the exposure (and therefore tolerance) is
possibly different. Small scale liquid fermentations in 96 well microtitre plates were therefore investigated and the growth of the bacteria measured over time. Preliminary results showed no correlation between biomass formation and L-malic acid consumption (data not shown).

MLF occurs when the cell density reaches approximately $10^6$ cells/mL (Lonvaud-Funel, 1999) and since removal of malic acid is the most important function of *O. oeni* it was concluded that the best method of monitoring the evolving population was simply by screening L-malic acid consumption under high ethanol conditions rather than analysing cell growth.
2.3.5 An ethanol tolerant phenotype is observed

Once the culture feed had surpassed 13% (v/v) ethanol the *O. oeni* population was sampled and screened for improvement in L-malic acid consumption compared to the original parent strain. Malolactic fermentations were performed on the evolving population at 6961 hours (290 days) as described (2.2.4). The fermentation profile of the parent (SB3) and the evolved population were very similar when no ethanol was added to the growth medium (Figure 2.3). However, in the presence of ethanol (15% (v/v)) the evolved population consumed all the L-malic acid at least 69 hours sooner than the parent (89.5 hours vs 158.5 hours) showing an apparent phenotype of improved ethanol tolerance.

![Figure 2.3. L-malic acid consumption of the parent (SB3, circles) and the evolved population (squares) at 0 (closed symbols) and 15% (open symbols) (v/v) ethanol, respectively in triplicate cultures in MRSAJ at 30 °C. The evolved culture consumed available L-malic acid 69 hours prior to the parent.](image-url)
2.4 Conclusion

Directed evolution was performed on *O. oeni* SB3 and preliminary characterisation of the mixed population (grown for 290 days, reaching 13% ethanol), revealed a culture that fermented L-malic more rapidly than the parent in MRSAJ supplemented with 15% (v/v) ethanol. This is the first study targeting this *O. oeni* and ethanol tolerance and shows that DE can be successful in the generation of a population with improved MLF performance.

After generating the population that displayed superior MLF under elevated ethanol concentrations, I next sought to identify individual clones, from the evolving population, that exhibited this phenotype, to the greatest extent and to characterise them in order to understand their fermentative performances.
Chapter 3. Isolation and characterisation of an ethanol tolerant strain of *Oenococcus oeni* from a mixed DE culture

3.1 Introduction

In Chapter 2, the isolation of an evolved population from a culture of the bacterium *O. oeni* SB3 was described. This mixed culture was able to complete MLF in MRSAJ media in a shorter duration than the parent strain. This chapter will describe the isolation and characterisation of individual clones from the evolved *O. oeni* mixed population with enhanced ethanol tolerance. In an asexually reproducing micro-organism the progeny will always contain more mutations than the parent as there is no option of sexual reproduction to introduce fresh genetic material (Bachtrog and Gordo, 2004). Another method of introducing genetic diversity can be through horizontal gene transfer from other bacteria; however, in an enclosed population such as a chemostat there is not the avenue for this as there is in nature (van Reenen and Dicks, 2011). This reduction in opportunity for genetic diversity does not mean there are limited opportunities for random mutations. Whilst ethanol was chosen as the main stressor, in a chemostatic culture such as this what limits the diversification of the population is in fact its capacity to remain within the vessel and reproduce (Sauer, 2001). This could include mutations that increase vessel wall “stickiness” or the ability to grow nearer the surface and partially minimise exposure to the stress. Nonetheless it is most likely that individuals within the population arise with different mutations and thus mechanisms of surviving within the stressful environment. It is important therefore to screen a proportion of the population, in order to establish which individuals have the high-ethanol tolerant phenotype and what proportion of the population they represent and to isolate the specific individuals within the population that have this phenotype to the greatest extent. As such this chapter will describe the
isolation and characterisation of individual strains of *O. oeni* from the mixed evolved culture with enhanced ethanol tolerance.

Initially 180 isolates from the mixed population were selected and screened in micro-fermentations. From these, 27 isolates were then selected as their performance was significantly improved. These were reassessed in larger 10 mL and 50 mL fermentations. Finally four were selected based on their ability grow and conduct MLF the fastest. These four were characterised under a range of different ethanol concentrations and temperatures with one isolate, strain 90, found to be the most efficient. Strain 90 was then compared to the parent in order to determine the limits of its tolerance to ethanol.
3.2 Methods

3.2.1 Primary screening via micro-scale MLF

A sacrificial 96 well micro-titre plate experiment was designed to measure growth and L-malic acid consumption of evolved isolates in MRSAJ at 15 % (v/v) ethanol supplemented with 1 g/L L-malic acid (Figure 3.1.B). Each isolate was analysed in quadruplicate. A sample of the DE mixed culture taken at 332 days (15 % (v/v) ethanol) was plated onto MRSA plates and 180 individual colonies were selected and inoculated into individual wells of two deep-well plates each containing 1 mL of MRSAJ. Deep-well plates were sealed with a breathable membrane (Cat. # AXY BF-400, Adelab, Thebarton, SA) and incubated statically at 30 °C for three days. Strains were stored at -80 °C as glycerol stocks (final concentration of glycerol 25 % (v/v)). The remaining sample was diluted 1:5 in MRSAJ containing 15 % (v/v) ethanol and 10 different source plates were generated (Figure 3.1.A). The samples on the source plate were arranged in quadrants so that, for each quadruplicate fermentation, the influence of the position of the sample in the plate was minimised. Finally 10 μL of diluted inoculum from each source plate was transferred to a 96-well plate (each well containing 190 μL of sterile MRSAJ with 15 % (v/v) ethanol), using a liquid-handling robot (Corbett Robotics, Model # CAS3800). Six identical plates from each source plate were created and these plates were incubated statically and anaerobically, in anaerobic jars, at 30 °C. A fresh plate was then sampled at each of the six time points. The plates were sampled regularly and monitored by determining L-malic acid consumption. The concentration of L-malic acid was calculated for each time point using the enzymatic method described in Section 2.2.3.
Figure 3.1. A. Configuration of a 96 well micro-titre plate used for micro fermentation screening of individual isolates from an evolved population. Each plate contained a sample of the mixed DE culture, the parent (SB3) and 18 different isolates. B. Flow diagram representing the method for isolating 180 clones.
3.2.2 Secondary screening via small scale MLF

A subset of isolates, selected from the micro fermentation plate-based screening exercise, were evaluated in small-scale laboratory fermentations. Fermentations (10 mL and 50 mL) were performed in triplicate in MRSAJ with 15 % (v/v) ethanol at 22 °C or 13 % (v/v) ethanol at 30 °C, respectively. Strains were grown in MRSAJ for seven days to an OD$_{600}$ > 1.0 and then inoculated to fresh MRSAJ, with either 13 or 15 % (v/v) ethanol, at a dilution of 1 in 50. Samples were taken regularly and L-malic acid concentration was determined enzymatically as described in 2.2.3.

3.2.3 Characterisation of ethanol tolerance of evolved isolates

Four strains were selected from the trials described in 3.2.2, based on their increased L-malic acid consumption in two conditions. These four strains were characterised for their tolerance to ethanol at different fermentation temperatures. The parent (SB3) was used as a reference along with the mixed population from the original DE culture used for isolation of individuals and a mixed population of a natural LAB isolate from a Grenache wine MLF at 17 % (v/v) ethanol (Yalumba Wine Company, Barossa Valley – strain GJ) (Jin et al., 2013).

Strain GJ is part of a project undertaken by another PhD student monitoring lactic acid bacterial populations of uninoculated wine fermentations. Samples of fermenting Grenache were plated out and individual colonies picked and made into glycerol stocks for further analysis. For this work next 10 randomly selected glycerols were grown in 10 mL MRSAJ at 30 °C until an OD$_{600}$ > 1.0 was obtained. After strains were visualised microscopically and confirmed as O. oeni, 1 mL of each strain was mixed together to form a mixed population referred to as strain GJ. Strain GJ was stored at -80 °C as a glycerol stock (final concentration of glycerol 25 % (v/v)).
Promising single isolates from this study, mixed population GJ, parent SB3 and the DE mixed population used to obtain single isolates in this study, were grown in MRSAJ to an \( \text{OD}_{600} > 1.0 \) and inoculated in triplicate into MRSAJ at either 13, 15, 17 or 19 \% (v/v) ethanol. These fermentations were incubated at 15, 22 or 30 °C. The fermentations were sampled regularly and analysed for L-malic acid consumption (according to 2.2.3) and plated onto MRSA medium to confirm viability.

### 3.2.4 Determination of survival from exposure to high ethanol

The most promising isolate obtained from this study and the parent of the DE (SB3) were grown in MRSAJ to an \( \text{OD}_{600} > 1.0 \). An aliquot (200 µL) of each was then inoculated into 10 mL of MRSAJ with 0, 20, 21, 22, 23 or 24 \% (v/v) ethanol. These fermentations were then sampled at 1, 12, 24, 36 and 48 hours after inoculation. A dilution series of \( 10^0, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} \) and \( 10^{-5} \) was carried out and 2 µL samples were spotted onto MRSAJ agar. Plates were incubated in anaerobic jars at 30 °C for seven days prior to visualisation.

### 3.2.5 MLF performance of evolved cultures in wine

The fermentation performance of the most promising single isolate and its parent (SB3) were evaluated in a red wine fermentation to confirm that the isolates high ethanol tolerance was not restricted to MRSAJ fermentations. This strain was also compared with a number of commercially available isolates in order to evaluate its performance.

The most promising single isolate, the parent SB3 and a number of commercial isolates (Table 3.1) were grown, in MRSA, to an \( \text{OD}_{600} > 1.0 \) and inoculated into 10 mL of 0.22 µm filtered Pinot Noir wine (Tasmania, 13 \% (v/v) ethanol). In addition, the most promising single isolate and SB3 were also inoculated into the same Pinot Noir wine supplemented with ethanol to 15 \% (v/v). Samples (200 µL) were
taken regularly and analysed for L-malic acid consumption according to the methods described in 2.2.3.

Table 3.1. *Oenococcus oeni* starter cultures commercially available in Australia selected for this study, including manufacturer commentary about their tolerance to the major physiochemical stressors found in wine.

<table>
<thead>
<tr>
<th>Company</th>
<th>Name</th>
<th>pH (min)</th>
<th>Temperature °C (min)</th>
<th>Ethanol % (v/v) (max)</th>
<th>SO₂ mg/L (max)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHR Hansen</td>
<td>Viniflora® Oenos</td>
<td>3.2</td>
<td>17</td>
<td>14</td>
<td>40</td>
</tr>
<tr>
<td>CHR Hansen</td>
<td>Viniflora® CH16</td>
<td>3.4</td>
<td>17</td>
<td>16</td>
<td>40</td>
</tr>
<tr>
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<td>Lalvin VP41®</td>
<td>3.1</td>
<td>16</td>
<td>16</td>
<td>60</td>
</tr>
<tr>
<td>Erbslöh</td>
<td>BioStart® Bianco SK₃®</td>
<td>3.0</td>
<td>13</td>
<td>13.5</td>
<td>45</td>
</tr>
<tr>
<td>Lallemand</td>
<td>V22™ <em>Lb. plantarum</em></td>
<td>3.5</td>
<td>17</td>
<td>15.5</td>
<td>50</td>
</tr>
</tbody>
</table>

3.3 Results and discussion

3.3.1 Primary screening of 180 DE isolates

Directed evolution was performed with *O. oeni* SB3 and preliminary characterisation of the mixed population revealed a culture that fermented L-malic more rapidly than the parent in MRSAJ supplemented with ethanol (see Figure 2.3). DE populations are typically mixed cultures of clones, with varying phenotypes, therefore screening of clonal isolates was undertaken in order to purify and identify an individual clone of the population that displayed the highest ethanol tolerance. To this end, 180 single isolates were selected for screening. In order to facilitate the screening of this large number of isolates a high through-put method was developed and implemented. Similar studies in yeast have shown that a comparable determination can be made between the performance of a larger volume fermentation to a screen in a micro-scale (Liccioli et al., 2011). Therefore a micro-titre sacrificial sampling
experiment was designed in order to screen 180 isolates from the DE culture, in quadruplicate fermentations at six time points according to section 3.2.1. There was varied fermentation performance amongst these strains and they were chosen based on their ability to consume L-malic acid in shortest time in 15 % (v/v) ethanol MRSAJ at 30 °C (data not shown).

A drawback of such micro-scale screening can be variations amongst samples. *Oenococcus oeni* forms chains and pairs in liquid cultures so sampling of the population can be less representative than organisms that exist in a singular arrangement. When an inoculum is then divided into quadruplicate from this organism and then replicated multiple times it is possible to get some variability in total cell numbers in replicate wells due to cell aggregation which would influence the results. Additionally, in a large screen, it is difficult to inoculate all cultures at an identical cell number and therefore some isolates that grew faster initially may have a higher inoculum than others.

Twenty seven isolates of the initial 180 were selected based upon their ability to consume L-malic acid fastest. Twenty seven was decided upon as that allowed for triplicate culture samples to be analysed in a 96 well microtitre plate format.

### 3.3.2 Secondary screening

Twenty seven isolates from the mixed DE population were highlighted for screening in larger fermentations in order to isolate the most ethanol tolerant strain. The 27 isolates were cultured in 10 mL fermentations using the same conditions as the primary screening (anaerobically in 15 % (v/v) ethanol at 30 °C); however, these conditions led to immediate loss of viability of the culture. It is likely that due to the small volume of the micro-titre fermentations, combined with the breathable membrane seals, ethanol could have evaporated during the experiment, resulting in
screening actually being performed at a lower ethanol concentration. Therefore, when repeating the secondary screening of the 27 isolates in 10 mL cultures, the conditions were made less stringent by the temperature being reduced to 22 °C. In addition, the ethanol concentration of fermentations conducted at 30 °C was reduced to 13 % (v/v) and the volume was increased to 50 mL. This still resulted in highly toxic conditions with most of the selected isolates struggling to perform (Figure 3.2). In the 15 % (v/v) ethanol fermentations that were incubated at 22 °C, the majority (22) of the 27 strains were able to complete this fermentation and excitingly most had a superior performance compared with the parent (SB3).

The mixed DE strain also struggled to perform under these conditions. As this is a mixed population its performance varies amongst assays probably dependant on how ethanol tolerant the predominating members of it are in any specific inoculum. From the combined results strains 2, 76, 89 and 90 (shown in blue; Figure 3.2) were chosen as the best examples of the high-ethanol tolerant phenotype and were stringently examined under a range of different temperatures and ethanol concentrations in subsequent work.
Figure 3.2. Malic acid utilisation of 27 clonal isolates from the mixed DE population, the mixed DE population and the parent (SB3). All samples were screened in either 50 mL MRSAJ at 30 °C or 10 mL MRSAJ at 22 °C supplemented with 13 % or 15 % (v/v) ethanol, respectively. Values are the averages of three biological replicates and error bars indicate the standard deviation. Four isolates, strains 2, 76, 89 and 90 (blue) were selected for further characterisation.
3.3.3 Influence of ethanol concentration and temperature on MLF performance of four selected DE isolates

The four best performing strains, 2, 76, 89 and 90, were compared and characterised at different ethanol concentrations and temperatures according to Section 3.2.3. The ethanol concentrations tested were 13, 15, 17 and 19 % (v/v) and were examined in combination with varying temperatures; 15, 22 and 30 °C, and these fermentations were monitored for five weeks (828 hours). None of the strains grew at any of the ethanol concentrations at 30 °C (data not shown) highlighting the recalcitrant, fastidious nature of this bacterium (Chapter 1). At 22 °C, strain 90 showed a clear fermentation advantage, compared to the parent, mixed population and other single isolates, for all ethanol concentrations (Figure 3.3). The parent (SB3) was generally the poorest performing strain.

At 13 % (v/v) strain 90 completed fermentation in 65 % of the time of the next fastest strain, 89, and 15 days faster than the slowest isolate, strain 2. At 16 % (v/v) ethanol only strains 90 and GJ were able to complete fermentation in 348 and 588 hours, respectively. Strains 89 and the DE mixed population were the next fastest but stalled after consuming 75 % of the available L-malic acid. At 17 % (v/v) ethanol strain 90’s only competitor was again strain GJ, with 89 and DE consuming 1 g/L of the available L-malic acid before becoming stuck. At 19 % (v/v) strain 90 had consumed all but 15 % of the available L-malic acid before halting. This was a significant improvement on the other isolates (with the exception of GJ) which were unable to consume any L-malic acid at this ethanol concentration.
Figure 3.3. L-Malic acid consumption of four promising DE isolates strains 2 (●), 76 (■), 89 (▲) and 90 (▲) compared to the parent strain (SB3, ●), a mixed DE population sample (control, ■) and a mixed population of a wine isolated ethanol tolerant strain (GJ, ▲). Fermentations were performed in MRSAJ supplemented with ethanol and incubated at 22 °C. Values are the averages of three biological replicates and error bars indicate the standard deviation.
At a lower temperature, 15 °C, strain 90 was still able to complete MLF in the shortest duration in 13 % (v/v) ethanol, at almost 400 hours sooner than any other strain, with the next fastest strain still not finished after 828 hours (Figure 3.4). Strains 89 and GJ were the other closest to completing fermentation in the five week period. This phenomenon bears further investigation as low temperature tolerant strains would be of great value to the wine industry. The need for low temperature tolerant strains is highlighted by no strains completed fermentation under any of the other ethanol concentrations tested but did retain similar but slower fermentation profiles (data not shown).

The strain GJ included in this work was a mixed population of *O. oeni* isolated from the Yalumba winery from a 17% (v/v) Grenache fermentation. The grapes for this fermentation were from old bush Grenache vines, typically grapes from these vines are left to ripen longer in order to develop more flavoursome characteristics. As these would typically lead to higher ethanol fermentation, this may have allowed for the possibility of environmental selection, if not evolution of a LAB to niche higher ethanol fermentations. It would be interesting to compare this strain GJ to strain 90 genetically to see if they have followed similar evolutionary pathways, to adapt to high ethanol fermentations.
Figure 3.4. L-malic acid metabolism of several novel single isolates (strains 2 (●), 76 (▲), 89 (▼) and 90 (○), the parent (SB3, ■), a mixed population from the evolved culture (control, □) and a mixed population of a wine isolated ethanol tolerant strain (GJ, ■). 
Fermentations were conducted in MRSAJ (13 % (v/v) ethanol) incubated at 15 °C. Values are the averages of three biological replicates and error bars indicate the standard deviation.
3.3.3.1 Fermentation performance of strain 90

From the most promising four single isolates from the DE of parent SB3, it was clear that strain 90 was the most efficient in catabolising L-malic acid in the presence of relatively high concentrations of ethanol. The performance of strain 90 under all available conditions is presented in Figure 3.5. Interestingly the time strain 90 took to consume half the L-malic acid at 19 % (v/v) at both 15 °C and 22 °C was similar, perhaps suggesting that MLF capacity of the strain is not changed.

![Figure 3.5. Fermentation performance of strain 90 in MRSAJ supplemented with either 13 % (•), 15 % (■), 17 % (▲) or 19 % (△) (v/v) ethanol and incubated at either 15 °C (blue) or 22°C (black). Values are the averages of three biological replicates and error bars indicate the standard deviation.](image-url)
Chapter 3: Isolation and characterisation

3.3.4 Investigation of the ethanol tolerance of strain 90

The level of tolerance of strain 90 and the parent (SB3) to ethanol was investigated by analysing growth in the presence of increasing concentrations of ethanol (according to 3.2.4). An aliquot of each strain was inoculated into MRSAJ at ethanol concentrations of 0, 20, 21, 22, 23 or 24 % (v/v) ethanol. These cultures were then sampled at 1, 12, 24, 36 and 48 hours after inoculation. For both strains increasing exposure concentration and time to ethanol reduced viability, but to a much larger extent for the parent (SB3) compared to strain 90. The highest ethanol concentration (24 % (v/v) ethanol) was, as expected, most inhibitory, such that incubation times of only 1 hour resulted in no detectable CFUs from the parent culture whilst strain 90 retained some residual viability with only one or two colonies in the undiluted sample (data not shown). At 23 % (v/v) ethanol strain 90 had some growth in the $10^{-2}$ dilution after one hour but no growth at any other time points and no visible growth from the parent at any time point (data not shown). At a reduced ethanol concentration of 22 % (v/v) ethanol, strain 90 retained viability even after 48 hours, whilst SB3 had only residual viability after one hour (Figure 3.6, panel C), visually similar to strain 90 at 24 % (v/v) ethanol. At further reduced ethanol concentrations (20 % and 21 % (v/v)) similar trends were seen but with higher survival rates (Figure 3.6 – panels A and B, respectively). At 21 % (v/v) the parent (SB3) had minimal viability of the undiluted culture after 48 hours whereas strain 90 still had growth in the $10^{-2}$ dilution spot. As a control, both strains were incubated in MRSAJ without ethanol and sampled at each time-point for the duration of the experiment. No difference in viability between SB3 and strain 90 was observed at any of these time points.
Figure 3.6. The impact of exposure to ethanol, for up to 48 hours, on cell culture viability of strain 90 and SB3. Strain 90 and the parent were exposed to 20 (A) 21 (B) 22 (C) % (v/v) ethanol and sampled after 1, 12, 24, 36 and 48 hours. A dilution series of $10^0$, $10^1$, $10^2$, $10^3$, $10^4$ and $10^5$ was prepared and 2 µL samples were spotted onto MRSA. Each panel is a composite of the MRSAJ agar plates at the completion of each period of ethanol exposure as a 10-fold dilution series with the top sample an undiluted 2 µL aliquot. Only the image from one hour is shown as the control (Con).
3.3.5 MLF performance of the evolved culture in wine

MRSAJ is a highly nutritious medium especially when compared to wine. There is a possibility that as the population was evolved to a niche environment, in this case MRSAJ medium, the ability to conduct MLF in wine had been lost or reduced. To test this, the MLF performance of strain 90 was monitored in Pinot Noir wine alongside the parent (SB3) and other commercial strains as described in 3.2.4. The parent and strain 90 were inoculated into Pinot Noir wine with an ethanol concentration of 13% (v/v) as well as the same wine with ethanol supplementation to 15% (v/v). At 13% (v/v) ethanol strain 90 completed fermentation of the wine in 75% of the time required by the parent (Figure 3.7). This difference was more pronounced at 15% (v/v) ethanol with strain 90 finishing in 40% of the time of the parent (Figure 3.7). The same Pinot Noir wine was also inoculated with commercially available strains of *O. oeni*. In addition, a strain of *Lb. plantarum* V22 reported to be an ethanol tolerant strain of lactic acid bacteria (Lallemand) was included but was not as efficient at MLF as hypothesised. Strain 90 was able to consume all the L-malic acid in less than 100 hours, approximately 60% of the time of the most efficient commercially available strain tested, Oenos (Figure 3.8). Strain 90 therefore, consistently demonstrates superior performance to its parent (SB3) as well as several commercially available MLF under all conditions tested.
Figure 3.7. Utilisation of L-malic acid by strain 90 and Parent (SB3) in Pinot Noir at either 13 % or 15 % (v/v) ethanol, incubated at 22 °C. Values are the averages of three biological replicates and error bars indicate the standard deviation.

Figure 3.8. Utilisation of L-malic acid by strain 90 and LAB commercial isolates in Pinot Noir at 13 % (v/v) ethanol, incubated at 22 °C. Values are the averages of three biological replicates and error bars indicate the standard deviation.
3.4 Conclusion

One of the aims of this work was to generate an *O. oeni* strain that could be used industrially to improve MLF performance compared to other commercially available strains. In this chapter, the individual isolate, strain 90, was described. Strain 90 clearly outperformed industry leading strains in terms of tolerance to high ethanol and MLF performance. This isolate could survive in up to 23 % (v/v) ethanol and could conduct MLF in MRS AJ at 17 % (v/v) ethanol at 22 °C. This strain was also able to perform MLF faster at 15 °C an additional improvement of the strain that is desirable to industry. Strain 90 also completed MLF sooner in Pinot Noir wine at 13 and 15 % (v/v) ethanol compared to its parent. Further in 13 % (v/v) Pinot Noir was able to complete malolactic fermentation sooner than five other commercially available strains.

The next chapter describes whole genome sequencing of strain 90 and its parent SB3 in an effort to determine what genomic differences existing between the strains may be responsible for the improved MLF performance of strain 90 in high ethanol conditions.
Chapter 4. Comparative genomics of SB3 with strain 89 and strain 90

4.1 Introduction

The preceding chapters describe the generation, selection and phenotypic assessment of *O. oeni* strains with a high ethanol tolerant phenotype. This chapter describes the analysis of genomic changes within these strains and the possible mutations responsible for this phenotype. The parent (SB3) and two different isolates: strains 89 and 90 were sequenced using whole genome sequencing and the resulting genomes were aligned and analysed for changes including single nucleotide polymorphisms (SNPs), deletions, substitutions and transposons. In order to hypothesise which genetic changes were responsible for the improved phenotype these mutations were compared to previously described LAB genes and systems using NCBI, BLAST and KEGG. This approach was used as the systems and genetic interactions of other LAB strains have been more intensively studied (see Chapter 1).

Stress response in lactic acid bacteria is the responsibility of a range of genes that alter different cellular processes. The physiological response of LAB to ethanol stress has been well studied (Guzzo, 2011; Wen-Ying and Zhen-Kui, 2013). It is known that ethanol perturbs the bacterial cell membrane leading to leakage of intracellular proteins. This affects the proton gradient and then influences cellular processes dependant upon it including ATP synthesis and the transportation of amino acids (Guzzo et al., 2000). The physiological response of the cell to ethanol stress seems to consist of firstly an increase in the proportion of CFAs in the membrane by converting *cis* vaccenic acid to lactobacillic acid (Teixeira et al., 2002) to counteract the effect of ethanol on membrane fluidity (Grandvalet et al., 2008) followed by an increase in membrane protein/phospholipid ratio.
Gene expression studies have shown that a number of genes are involved in the cellular response to ethanol stress (Table 4.1). For example, Cafaro et al., (2014) reported that geranylgeranyl pyrophosphate synthase (ggpps) synthesis increased in response to increasing ethanol concentrations. This may in turn lead to an increase in carotenoid production possibly influencing cell membrane fluidity. The synthesis of genes encoding heat shock proteins (HSP) is another important response of the cell reacting to ethanol stress (Nicolaou et al., 2010). The small HSP Lo18 can bind membranes to help regulate membrane fluidity and is produced in large amounts under ethanol stress conditions (Maitre et al., 2014). Furthermore, O. oeni cells exhibiting an increased synthesis of Lo18 have shown a greater ability to survive in wine and to perform MLF (Maitre et al., 2014).

In addition to what is known about the genetic response to ethanol stress, Bon et al. (2009) used comparative genome subtractive hybridization to propose that the presence of eight stress-responsive genes (six from O. oeni) were associated with high MLF performance. It was suggested that there is a relationship between genome variation and malic acid metabolism. They also identified six different regions of plasticity, resulting from recombination or insertion/deletion events and suggested that IS30-related elements play a role in O. oeni genome plasticity with genomic enrichment of IS30 found in better performing MLF strains (El Gharniti et al., 2012). Additionally in comparing the sequence of 11 strains of O. oeni, Borneman et al. (2012) found evidence of IS element horizontal gene transfer (HGT), including IS30-mediated insertion events that appear to be transferred from Lactobacillus spp.
Literature searches lead to the generation of a list of genes that may have an effect on the ethanol tolerance of *O. oeni* (Table 4.1). In examining the basis for the enhancement of the strains generated in this study, it was hypothesised that there may be some changes in either the listed genes or their promoters, which lead to activation or increased expression. Additionally it was hypothesised that there would be some IS-mediated rearrangements of the genome.
Table 4.1. *O. oeni* genes and their predicted function that may have an effect on the high ethanol tolerance phenotype of the evolved strain.

<table>
<thead>
<tr>
<th>Locus tag</th>
<th>Gene</th>
<th>Predicted function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>OEOE_0078</td>
<td></td>
<td>Short chain dehydrogenase (3-oxo-acyl carrier protein reductase)</td>
<td>Bon <em>et al.</em>, 2009</td>
</tr>
<tr>
<td>OEOE_0086</td>
<td></td>
<td>Hypothetical protein</td>
<td>Bon <em>et al.</em>, 2009</td>
</tr>
<tr>
<td>OEOE_0184</td>
<td>hsp18</td>
<td>Heat shock protein Lo18</td>
<td>Maitre <em>et al.</em>, 2014</td>
</tr>
<tr>
<td>OEOE_0188</td>
<td>fisH</td>
<td>ATP-dependent Zn protease</td>
<td>Bourdineaud <em>et al.</em>, 2003</td>
</tr>
<tr>
<td>OEOE_0348</td>
<td></td>
<td>Cation (cadmium) transport ATPase</td>
<td>Bon <em>et al.</em>, 2009</td>
</tr>
<tr>
<td>OEOE_0353</td>
<td></td>
<td>DNA-binding ferritin-like protein (oxidative damage protectant)</td>
<td>Bon <em>et al.</em>, 2009</td>
</tr>
<tr>
<td>OEOE_0354</td>
<td></td>
<td>Copper chaperone</td>
<td>Bon <em>et al.</em>, 2009</td>
</tr>
<tr>
<td>OEOE_0355</td>
<td></td>
<td>Crp-like transcriptional regulator (Fnr-like protein)</td>
<td>Bon <em>et al.</em>, 2009</td>
</tr>
<tr>
<td>OEOE_0356</td>
<td>IS30</td>
<td>Transcriptional repressor of class III stress genes</td>
<td>Bon <em>et al.</em>, 2009</td>
</tr>
<tr>
<td>OEOE_0307</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OEOE_0513</td>
<td>ctsR</td>
<td>Transcriptional repressor of class III stress genes</td>
<td>Grandvalet <em>et al.</em>, 2005</td>
</tr>
<tr>
<td>OEOE_0514</td>
<td></td>
<td>clpA ATP-binding subunit of Clp protease and DnaK/DnaJ chaperones</td>
<td>Seydlová <em>et al.</em>, 2012</td>
</tr>
<tr>
<td>OEOE_0570</td>
<td>clpP</td>
<td>ATP-dependent Clp protease proteolytic subunit ClpP</td>
<td>Olguín <em>et al.</em>, 2010</td>
</tr>
<tr>
<td>OEOE_0572</td>
<td></td>
<td>spoVK ATP-binding subunit of Clp protease and DnaK/DnaJ chaperones</td>
<td>Seydlová <em>et al.</em>, 2012</td>
</tr>
<tr>
<td>OEOE_0640</td>
<td></td>
<td>uvrB/C ATP-binding subunit of Clp protease and DnaK/DnaJ chaperones</td>
<td>Seydlová <em>et al.</em>, 2012</td>
</tr>
<tr>
<td>OEOE_1085</td>
<td>ggpps</td>
<td>Geranylgeranyl pyrophosphate synthase</td>
<td>Cafaro <em>et al.</em>, 2014</td>
</tr>
<tr>
<td>OEOE_1176</td>
<td>cfa</td>
<td>Cyclopropane-fatty-acyl-phospholipid synthase</td>
<td>Grandvalet <em>et al.</em>, 2008</td>
</tr>
<tr>
<td>OEOE_1268</td>
<td></td>
<td>Integral membrane protein, interacts with FtsH</td>
<td>NCBI</td>
</tr>
<tr>
<td>OEOE_1309</td>
<td>dnaK</td>
<td>ATP-binding subunit of Clp protease and DnaK/DnaJ chaperones</td>
<td>Seydlová <em>et al.</em>, 2012</td>
</tr>
<tr>
<td>OEOE_1345</td>
<td>omrA</td>
<td>ABC-type multidrug transport system, ATPase and permease component</td>
<td>Bourdineaud <em>et al.</em>, 2004</td>
</tr>
<tr>
<td>OEOE_1396</td>
<td>groEL</td>
<td>Chaperonin GroEL</td>
<td>Grandvalet <em>et al.</em>, 2005; Seydlová <em>et al.</em>, 2012</td>
</tr>
<tr>
<td>OEOE_1397</td>
<td>groES</td>
<td>Co-chaperonin GroES (HSP10)</td>
<td>Grandvalet <em>et al.</em>, 2005</td>
</tr>
<tr>
<td>OEOE_1447</td>
<td>rmlB</td>
<td>dTDP-glucose 4,6-dehydratase</td>
<td>Olguín <em>et al.</em>, 2010</td>
</tr>
<tr>
<td>OEOE_1702</td>
<td>trxA</td>
<td>Thiol-disulfide isomerase and thioredoxin</td>
<td>Olguín <em>et al.</em>, 2010</td>
</tr>
<tr>
<td>OEOE_1729</td>
<td>clpX</td>
<td>ATP-dependent protease ATP-binding subunit ClpX</td>
<td>Olguín <em>et al.</em>, 2010</td>
</tr>
</tbody>
</table>
4.2 Methods

4.2.1 Genomic DNA extraction

Genomic DNA was extracted according to section 2.2.2.2 with some variations. An individual colony of the bacterium (SB3, 90 or 89) was sequentially inoculated in 10 mL, 50 mL and then 1 L of MRSAJ in order to collect enough genetic material for sequencing. The manufacturers’ method for alternate lysis of cells for reduced DNA shearing was implemented with the DNA preparations heated at 65 °C for 10 minutes and vortexed for a few seconds every two minutes.

4.2.2 Sequencing

All sequencing was performed by Dr. Mike Gardener’s group at The Flinders University of South Australia and The South Australian Museum. SB3 was sequenced using Roche 454 sequencing on 1/8th of a pico-titre plate. When the gDNA was prepared for strain 90 only Ion Torrent sequencing was available for this and at a reduced cost thus strain 89 (seemingly the second best performer from Chapter 3) was also sequenced by this method.

4.2.3 Sequence assembly and variant discovery

The genome of SB3 was assembled with the assistance of Dr. Miguel Roncoroni using MIRA 4.0 (Chevreux et al., 1999). The final assembly coverage was 32× and was contained in 52 contigs with an N50 76346 and N90 23731.

The consensus genome sequence of SB3 was finished using Contiguator 2 (Galardini et al., 2011) against the sequence of PSU-1 (Makarova et al., 2006). Shotgun reads from all strains (SB3, 89 and 90) were aligned against this consensus using bowtie2 (Langmead and Salzberg, 2012). Variant discovery was performed using SAMtools mpileup (Li et al., 2009) and variants were filtered using custom R
scripts. *SnpEff* (Cingolani *et al.*, 2012) and *SIFT* (Ng and Henikoff, 2001) were used to annotate the variants.

### 4.2.4 SNP analysis and gene ontology (GO) terms

The list of genes containing SNPs was analysed using BLAST to determine if they had similarity to genes in other organisms but with different functions. SNPs located in non-coding regions of the genome were also analysed using BLAST to ascertain if they were located in important regions in other LAB. The conserved domain database (CDD) (Marchler-Bauer *et al.*, 2013) was used to discover which protein families each of the loci belonged to and hypothesises its function based on this. KEGG pathways were utilised to study important gene interactions (Kanehisa and Goto, 2000; Kanehisa *et al.*, 2014) and lastly GO terms and gene enrichment was analysed using DAVID (Huang *et al.*, 2008, 2009).

### 4.3 Results and discussion

The genome of the parent (SB3) was assembled *de novo* from sequencing outputs that represented a high level of coverage and was contained in 52 contigs. Reads from all three strains, SB3, 89 and 90 were then assembled to this sequence. A total of 34 genomic changes (32 SNPs and 2 insertions) were discovered across the two strains when compared to the parent. The insertions were in an “A” repeat, of varying lengths across reads, in a low coverage area and are at this stage considered a false result. These insertions need to be confirmed using additional sequencing and further consideration as both are located in OEOE_1026 which is a DNA topoisomerase IV subunit B. Topoisomerase IV acts to disentangle the replicated DNA strands and is the target of two classes of antibiotic drugs: quinolones and coumarins. These antibiotics are used to treat an assortment of different diseases, by inhibiting DNA replication in the bacteria responsible. In *Lb. plantarum* isolated from various environments there was a significant difference in the protein expression of
DNA topoisomerase IV subunit B (GyrB) during lag phase which suggests a possible role in environmental adaptation (Koistinen et al., 2007).

Of the 32 identified SNPs 24 were located within the coding region of genes, with 20 resulting in non-synonymous mutations in 19 different genes (Table 4.2) and four resulting in synonymous SNPs (data not shown). Two of these SNPs, in OEOE_0544 and OEOE_1433, are also possible false positives as this is an area of some heterogeneity within the parent strain. These genes are included in the discussion below but again require further confirmation of SNPs within these genes. The eight remaining SNPs (data not shown) were located in various non-coding regions of the genome and are briefly discussed.

Generally synonymous SNPs are largely ignored in studies of this kind; however, as this is a small data set the synonymous mutations were also included. There is evidence that synonymous mutations play a role in some phenotypes, especially in disease, and frequently with an increased virility in pathogens including the LAB Staphylococcus aureus (Sauna and Kimchi-Sarfaty, 2011).
Table 4.2. Non-synonymous SNPs identified in strains 89 and 90 when compared with SB3. The locus tags and the purported gene function are included from NCBI (gene database). GO terms (Huang et al., 2008, 2009) that are enriched in the data set are included. SIFT analysis was performed to determine whether or not the amino acid changes were tolerated (score closer to 0 means it is more likely to affect protein function).

<table>
<thead>
<tr>
<th>Locus Tags</th>
<th>SNP</th>
<th>Protein Change</th>
<th>Strain</th>
<th>Putative Gene Function</th>
<th>GO Terms</th>
<th>Tolerated</th>
</tr>
</thead>
<tbody>
<tr>
<td>OEOE_0160</td>
<td>tCa/tTa</td>
<td>S51L</td>
<td>89</td>
<td>Phosphoribosylpyrophosphate synthetase</td>
<td>ATP Binding, nucleotide binding</td>
<td>N 0.00</td>
</tr>
<tr>
<td>OEOE_0183</td>
<td>Gt/Att</td>
<td>V1018I</td>
<td>90</td>
<td>Transcription-repair coupling factor</td>
<td>ATP Binding, nucleotide binding</td>
<td>Y 0.65</td>
</tr>
<tr>
<td>OEOE_0196</td>
<td>tgG/tgT</td>
<td>W74C</td>
<td>90</td>
<td>Hypothetical protein</td>
<td>ATP Binding, nucleotide binding</td>
<td>Y 0.18</td>
</tr>
<tr>
<td>OEOE_0433</td>
<td>cGg/cAg</td>
<td>P332Q</td>
<td>90</td>
<td>Translation initiation factor IF-2 (IF-2; GTPase)</td>
<td>ATP Binding, nucleotide binding</td>
<td>N/A</td>
</tr>
<tr>
<td>OEOE_0517</td>
<td>gGa/gTa</td>
<td>G60V</td>
<td>90</td>
<td>ATPase component of ABC transporter</td>
<td>ATP Binding, nucleotide binding</td>
<td>N 0.01</td>
</tr>
<tr>
<td>OEOE_0544</td>
<td>Tgc/Ggc</td>
<td>C388G</td>
<td>Both</td>
<td>YG repeat-containing glycosyl hydrolase</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>OEOE_0563</td>
<td>cAc/cGc</td>
<td>H73R</td>
<td>Both</td>
<td>Serine kinase of the HPr protein, regulates carbohydrate metabolism</td>
<td>ATP Binding, nucleotide binding</td>
<td>Y 0.06</td>
</tr>
<tr>
<td>OEOE_0665</td>
<td>gCg/gAg</td>
<td>A257E</td>
<td>89</td>
<td>F0F1 ATP synthase subunit beta</td>
<td>ATP Binding, nucleotide binding</td>
<td>N 0.00</td>
</tr>
<tr>
<td>OEOE_0738</td>
<td>gGa/gAa</td>
<td>G236E</td>
<td>89</td>
<td>ABC-type multidrug transport system, ATPase component</td>
<td>ATP Binding, nucleotide binding</td>
<td>Y 0.56</td>
</tr>
<tr>
<td>OEOE_0870</td>
<td>Gct/Act</td>
<td>A370T</td>
<td>90</td>
<td>Hypothetical protein</td>
<td>ATP Binding, nucleotide binding</td>
<td>Y 0.09</td>
</tr>
<tr>
<td>OEOE_1168</td>
<td>tAc/tCc</td>
<td>Y421S</td>
<td>Both</td>
<td>Amino acid transporter</td>
<td>ATP Binding, nucleotide binding</td>
<td>Y 0.56</td>
</tr>
<tr>
<td>OEOE_1173</td>
<td>gTa/gCa</td>
<td>V72A</td>
<td>Both</td>
<td>3-polypropenyl-4-hydroxybenzoate decarboxylase, flavoprotein (ubiX)</td>
<td>ATP Binding, nucleotide binding</td>
<td>Y 0.09</td>
</tr>
<tr>
<td>OEOE_1375</td>
<td>Gt/Ttt</td>
<td>V840F</td>
<td>89</td>
<td>DNA-directed RNA polymerase, beta subunit/140 kD subunit</td>
<td>ATP Binding, nucleotide binding</td>
<td>N 0.00</td>
</tr>
<tr>
<td>OEOE_1480</td>
<td>cGg/cTg</td>
<td>P482L</td>
<td>89</td>
<td>Transketolase</td>
<td>ATP Binding, nucleotide binding</td>
<td>N 0.00</td>
</tr>
<tr>
<td>OEOE_1581</td>
<td>tgG/tgT</td>
<td>W273C</td>
<td>90</td>
<td>Methylase of polypeptide chain release factor</td>
<td>ATP Binding, nucleotide binding</td>
<td>Y 0.13</td>
</tr>
<tr>
<td>OEOE_1629</td>
<td>atA/atG</td>
<td>I331M</td>
<td>Both</td>
<td>Polyphosphate kinase</td>
<td>ATP Binding, nucleotide binding</td>
<td>N 0.00</td>
</tr>
<tr>
<td>OEOE_1647</td>
<td>Aca/Gca</td>
<td>T381A</td>
<td>89</td>
<td>Cation transport ATPase</td>
<td>ATP Binding, nucleotide binding</td>
<td>N 0.00</td>
</tr>
</tbody>
</table>
4.3.1 Mutations in both strain 89 and 90 compared with parent (SB3)

### 4.3.1.1 OEOE_0544 YG repeat-containing glycosyl hydrolase

OEOE_0544 is a glycosyl hydrolase and is involved in two KEGG pathways; starch and sucrose metabolism (sucrose → D-fructose) and also a two-component signal transduction system. Two-component systems enable bacteria to adapt to environmental or intracellular changes (El-Sharoud, 2005). In this case a change in temperature induces a change in the bacterial cell wall and membrane composition (Figure 4.1). Additionally from KEGG pathways OEOE_0544 shows similarity with gtfB/C/D which are glucosyltranferases that catalyse the synthesis of extracellular glucan and are well studied in the oral bacterium *Streptococcus mutans* (Senadheera *et al.*, 2007). Furthermore gtfB/C/D are responsible for the adhesion of *S. mutans* to teeth (Tsumori and Kuramitsu, 1997), which is possibly translated to adhesion to bioreactor walls allowing these mutants to remain within the population additionally in *E. coli* genes associated with biofilm formation show an increased expression in response to ethanol stress (Nicolaou *et al.*, 2010).

![OEOE_0544](image)

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Figure 4.1. Two-component system (KEGG reference ooe_02020) (Kanehisa and Goto, 2000; Kanehisa *et al.*, 2014).
4.3.1.2 OEOE_0563 Serine kinase of the HPr protein, regulates carbohydrate metabolism

OEOE_0563 is the serine kinase of the HPr protein. The enzyme HPr kinase plays an important role in the regulation of carbon metabolism in Gram-positive bacteria particularly in its regulation of carbon catabolite repression (CCR). CCR is a mechanism that allows bacteria to coordinate and optimise the utilisation of abundantly available carbon sources (Poncet et al., 2004). If a mixture of carbon sources are available, bacteria have the capacity to preferentially take up and metabolise the carbohydrates that can be easily transformed. The DE experiment used MRSAJ medium, which is high in glucose and fructose, a very different environment from the wine niche the bacterium would generally be found in. The enzyme hprK may also be involved in cell adhesion (Poncet et al., 2004), another possible side effect from evolution in a bioreactor. This mutation was predicted to be tolerated thus it may not affect the function of the protein. It would be interesting to monitor expression of this gene in wine fermentations by each of the evolved strains and the parent to see if hprK is indeed involved in the evolved ethanol tolerance phenotype.

4.3.1.3 OEOE_1168 amino acid transporter

According to the CDD OEOE_1168 encodes for a transmembrane amino acid transporter protein (Marchler-Bauer et al., 2013). Ethanol toxicity acts by perturbing the cell membrane and affecting membrane processes, including amino acid transport (Chapter 1). OEOE_1168 is the only gene with two SNPs (ggA tAc / ggT tCc). The first of these is synonymous; however the second is a non-synonomous mutation predicted to affect protein function. Further investigation into the effects of this protein change on the transport of amino acids under high ethanol conditions would be beneficial. Acetate stress can be mitigated by the addition of the amino acids methionine, glycine, isoleucine and threonine and has been shown to upregulate select amino acid
transporters in *E. coli* (Nicolaou *et al.*, 2010). As generally stress response is concomitant (Chapter 1) this may also assist in ethanol stress tolerance.

### 4.3.1.4 OEOE_1173 UbiX 3-polyprenyl-4-hydroxybenzoate decarboxylase, flavoprotein

OEOE_1173 encodes for UbiX, 3-polyprenyl-4-hydroxybenzoate decarboxylase. UbiX is involved in coenzyme Q biosynthesis especially during logarithmic growth. As an aside from KEGG pathways *ggpps*, (OEOE_1085) (Table 4.1) is a part of terpenoid back bone biosynthesis that interacts with the ubiquinone and other terpenoid-quinone biosynthesis of which UbiX is a member. Even so, it does not explain the apparently enhanced ethanol tolerance or MLF capability of strains 89 and 90.

### 4.3.1.5 OEOE_1629 polyphosphate kinase (PPK)

PPK is a membrane protein that catalyses the formation of polyphosphate from ATP. KEGG pathways reveal a role in oxidative phosphorylation (Figure 4.2). Also located in the pathway for oxidative phosphorylation is OEOE_0665 (F0F1 ATP synthase subunit beta. SNP only in strain 89) (see 4.3.3.3).

PPK is also involved in bacterial RNA degradation (KEGG reference ooe03018). Beside the proteins involved specifically in the RNA degradosome, there are three associated proteins found in *O. oeni*. They are PPK, DnaK and GroEL. DnaK and GroEL are both genes found in the literature that may play a role in ethanol tolerance (Table 4.1) (Grandvalet *et al.*, 2005). Additionally both have been shown to activate under ethanol stress conditions in *B. subtilis* (Seydlová *et al.*, 2012). The disruption of *ppk* in *Lb. casei* showed a decrease in growth rate in the presence of some stressors, particularly low pH (acid stress). The study also tested the strains under ethanol stress but found no significant difference in growth rate (Alcántara *et al.*, 2010).
2013) suggesting *ppk* alone is not responsible for the high ethanol phenotype but may be an important participant.
Figure 4.2. F1F0 ATPase for the oxidative phosphorylation pathway in bacteria. (KEGG reference ooe00190; Kanehisa and Gotô, 2000; Kanehisa et al., 2014). Green labels are known gene loci from O. oeni. OEOE_1629 (2.7.4.1) is polyphosphate kinase (ppk) containing a non-synonymous mutation in both strains. OEOE_0665 (3.6.3.14) is F1F0 ATPase subunit β, a non-synonymous mutation in only strain 89.
4.3.1.6 **OEOE_0927 Acetyltransferase and OEOE_1009 GTPase**

OEOE_0927 and OEOE_1009 each contain a SNP in both strain 89 and 90. These are both synonymous SNPs and therefore do not necessarily affect protein function. OEOE_0927 is a member of the N-Acyltransferase superfamily, which includes various enzymes that characteristically catalyse the transfer of an acyl group to a substrate. In *S. cerevisiae* the gene NAT2 which encodes for a N-α-Acyltransferase was found to be amongst a list of genes essential towards resistance to high ethanol concentrations (Teixeira *et al.*, 2009). OEOE_1009 GTPase, another enzyme, binds and hydrolyses GTP, however, no clear link to ethanol tolerance is apparent.

4.3.1.7 **SNPs in non-coding regions**

Both strains contained two identical SNPs in non-coding regions. The first was located immediately downstream (13 bp) of OEOE_0493 which encodes a polysaccharide transporter (RfbX). OEOE_0494 which is located upstream of this SNP is a hypothetical protein. The second was located between OEOE_1705 and OEOE_1706 but when the region was analysed using BLAST the SNP seems to be located within OEOE_1705 which encodes methylmalonyl-CoA epimerase. According to the CDD this protein superfamily is found in a variety of structurally related metalloproteins and a group of antibiotic resistance proteins. OEOE_1706 is a hypothetical protein, predicted to be a membrane protein, as ethanol acts by perturbing the membrane and affecting membrane proteins perhaps OEOE_1706 is important to ethanol tolerance and requires further investigation.

4.3.1.8 **Summary of SNPs in both strains**

The genes that contain mutations in both sequenced strains encode for a range of proteins with different functions. OEOE_0544 and OEOE_0563 appear to possibly be related to adaption to a different environmental niche. OEOE_1629 appears
important to environmental stress response. It would be interesting to study whether the other members of the population contain this same mutation. Generating strains from SB3 with analogous mutations would be useful to confirm the importance of these SNPs.

4.3.2 Mutations only in strain 90

4.3.2.1 OEOE_0183 Transcription-repair coupling factor (Mfd)

MFD is a widely conserved bacterial protein. MFD recognises RNA polymerases stalled at a non-coding template site of DNA damage and disrupts the transcription complex (Roberts and Park, 2004). A defect shows an increased sensitivity to UV irradiation (Selby and Sancar, 1994). The gene *mfd* has been isolated and characterised and found to be involved in transcription coupled DNA repair and DNA recombination in *B. subtilis* (Ayora *et al.*, 1996). On the loci the SNP is immediately downstream of the conserved transcription repair coupling factor (TCRF) domain of the gene (Figure 4.3).

The gene overlapping OEOE_0183 is OEOE_0184 (Figure 4.3), the gene *hsp18*, that encodes a ribosome-associated heat shock protein Lo18 (Table 4.1). Lo18 is produced under ethanol stress conditions and increased synthesis has shown a greater ability, by *O. oeni*, to survive in wine and to perform MLF (Maitre *et al.*, 2014). This amino acid change is predicted to be tolerated by the protein; however, it would be beneficial to see if this SNP downstream affects the function of OEOE_0184 and subsequent production of Lo18.
Figure 4.3. Image of OEOE_0183 from ncbi (http://www.ncbi.nlm.nih.gov/gene/?term=OEOE_0183). The gene is in green, red symbolises the coding regions and the black sections are other features based on the CDD. The numbers across the top are the bp locations on the genome of *O. oeni* PSU-1. The SNP in OEOE_0183 is located in the conserved TRCF region. OEOE_0184 overlaps OEOE_0183 and is immediately upstream of this SNP.
4.3.2.2 OEOE_0433 Initiation factor 2 (infB)

The sequence for infB is highly conserved and encodes for initiation factor 2 which binds to initiator tRNA and controls the entry of tRNA into the ribosome (Shazand et al., 1990). The infB protein also interacts with GTPase (Shazand et al., 1990), including possibly OEOE_1009. Any links to ethanol tolerance and MLF are not immediately apparent.

4.3.2.3 OEOE_1581 Methylase of polypeptide chain release factor

OEOE_1581 encodes an enzyme that uses S-adenosyl methionine (SAM) as a substrate for methyl transfer. From the CDD it is a part of the protein family PrmC (Marchler-Bauer et al., 2013). PrmC activity is crucial for Pseudomonas aeruginosa to adapt to environmental stresses (Pustelny et al., 2013) and a prmC deletion mutant also showed down regulation of GroEL, which is also known as HSP10, an important stress response protein in O. oeni (Grandvalet et al., 2005). The interactions between GroEL and OEOE_1581 may therefore increase the ethanol tolerance of strain 90.

4.3.2.4 OEOE_0911 SAM-dependent methyltransferase

OEOE_0911 a synonymous SNP in a SAM-dependant methyltransferase which is a biological cofactor that has a role in the transfer of methyl groups to various biomolecules, including DNA, proteins and small-molecule secondary metabolites. An increase in CFA content is the first step in the cells response to ethanol stress (Teixeira et al., 2002) and these CFA’s are formed from unsaturated fatty acids by the transfer of a methyl group from SAM catalysed by the enzyme cfa (cyclopropane fatty acid synthetase) (Ramos et al., 1997). It would be interesting to investigate whether this SNP has an effect on the transcriptional levels of the enzyme cfa and therefore contributes to the cells response to ethanol stress.
4.3.2.5 SNPs in non-coding regions

Strain 90 contained three SNPs in non-coding regions. The sequences of these were analysed with BLAST against other LAB species; but, they did not show homology to any ORFs. The first SNP in a non-coding region is immediately downstream of OEOE_0085 (Hypothetical protein). Bon et al. (2009) found six regions of plasticity in the genome of O. oeni, thought to contribute to high performing strains. OEOE_0085 was located in region two; however, it was not considered to be a significant marker of high performance; nonetheless, the adjacent hypothetical protein, OEOE_0086 was found to be a significant marker.

The second SNP in a non-coding region was located between OEOE_t0403 and OEOE_0404. OEOE_t0403 is arginine-tRNA ligase and is involved in arginine and proline metabolism and aminoacyl-tRNA biosynthesis. OEOE_0404 encodes glyceraldehyde-3-phosphate dehydrogenase which is involved in glycolysis/glucogenesis and carbon metabolism (Figure 4.4) and the biosynthesis of amino acids (Figure 4.5).

The final SNP in a non-coding region was located between OEOE_1040 and OEOE_1041. OEOE_1040 is a hypothetical protein and OEOE_1041 is a pseudo gene. It would be interesting to apply qPCR to monitor the expression of these genes located up and down stream of the SNP to determine if expression had been affected in some way.

4.3.2.6 Summary for strain 90

Strain 90 also contains a SNP in two hypothetical proteins, OEOE_0196 and OEOE_0870 the functions of which are unknown. The mutation in OEOE_0870 is predicted to affect protein function and therefore may be important and warrants further investigation.
The non-coding SNP OEOE_0404 is involved in the biosynthesis of amino acids. Interestingly two SNP’s in genes of strain 89 also have a role in biosynthesis of amino acids, thus perhaps this pathway has a significant role in ethanol tolerance. Additionally, both strains contain the same two SNPs in the amino acid transporter OEOE_1168 implying a possible relationship with the biosynthesis and transport of amino acids and the adjustment of this in the face of ethanol stress.

Two of the genes seem to be related to SAM methylases. The possible role of this, in the formation of CFA may be why strain 90 had a better performance in high-ethanol fermentations than strain 89, which doesn’t contain these SNPs. One of these SNPs in OEOE_1581 may interact with the stress response gene groEL.

4.3.3 Mutations only in strain 89

4.3.3.1 OEOE_0160 Phosphoribosylpyrophosphate synthetase (PRPP) and OEOE_1480 Transketolase (TKtA)

Phosphoribosylpyrophosphate synthetase (PRPP) catalyses the removal of carbon atoms, from the pentose phosphate pathway, which are used for the synthesis of nucleotides, cofactors NAD+ and NADP+, and the amino acids histidine and tryptophan (Kilstrup et al., 2005) (Figure 4.5).

PRPP is a substrate in the biosynthesis of histidine and tryptophan has been characterised in the gram positive *Bacillus subtilis* (Nilsson and Hove-Jensen, 1987). Research has shown that membrane disordering resulting from ethanol exposure leads to leakage of intracellular compounds, including enzymatic co-factors (NAD / NADH) and ions essential for cell growth and fermentation (Spano and Massa, 2006). Therefore the synthesis of these compounds might contribute to the bacterium’s ethanol tolerance. In studies investigating toluene stress in the gram negative bacterium *Pseudomonas putida* it was observed that in response to toluene stress ATP synthetase was down regulated as a response to the disruption in proton motive force
caused by toluene stress. As a cellular response genes involved in NAD(P)H metabolism were then upregulated (Nicolaou et al., 2010). OEOE_0160 also interacts with OEOE_1480, a transketolase involved in the amino acid synthesis pathway.

Figure 4.4. Simplified version of the pathway for carbon metabolism (KEGG pathway ooe01200; Kanehisa and Goto, 2000; Kanehisa et al., 2014). The arrows in green are gene encoded functions found in O. oeni. Strain 90 contains a SNP in a region immediately downstream of OEOE_0404.
Figure 4.5. Simplified version of the pathway for the biosynthesis of amino acids (KEGG pathway ooe01230; Kanehisa and Goto, 2000; Kanehisa et al., 2014). The arrows in green are gene functions found in O. oeni. OEOE_1480 is responsible for the conversion of fructose-6P into erythrose 4P, as well as further intermediates into sedoheptulose-7P and then to ribose-5P. OEOE_0160 converts ribose-5P to PRPP. OEOE_0404 is responsible for the conversion of glyceraldehyde-3P for downstream reactions culminating in the formation of pyruvate. Strain 90 contains a SNP in a region immediately downstream of OEOE_0404.
4.3.3.2 OEOE_0517 ATPase component of ABC transporter

The SNP seems to be located in the conserved domain ABC_tran_2; ABC transporter. ABC transporters are transmembrane proteins that utilise ATP to carry out biological process including translocation of substances across membranes and translation of RNA and DNA repair (Nicolaou et al., 2010). ABC transporters also participate in multidrug resistance (Nicolaou et al., 2010). Perhaps this SNP improves the efficacy of the export of toxic molecules from the cell, the impact of this affect should be further investigated.

4.3.3.3 OEOE_0665 F0F1 ATP synthase subunit beta and OEOE_1647 cation transport ATPase

F0F1 ATPase active transporters are located in the cell membrane and play a role in energy conserving reactions (Figure 4.2). Protons are pumped outwards generating a proton gradient and the energy released drives further membrane bound processes (Wen-Ying and Zhen-Kui, 2013). In O. oeni, activity of H+ ATPase is induced at low pH and this activity is associated with increased acid stress resistance (Fortier et al., 2003). OEOE_1647 encodes a cation transport ATPase which are implicated in activities related to pH homeostasis (Mills et al., 2005). As ethanol seems to affect membrane associated processes and as this SNP is predicted to affect protein function it would be interesting to study this activity and see if it is reduced or increased by this mutation. It is also interesting that both these mutations target genes possibly associated with acid stress responses. The DE and screening were all performed in MRSAJ medium which has a pH of 6.2. It would be intriguing to analyse the fermentation profile of strain 89 in a low pH environment, to see if its MLF performance is better than strain 90 under acid stress.

4.3.3.4 OEOE_0738 ABC multidrug transport system
OEOE_0738 is part of a sub-family of ABC transporters that are known as ATP-dependant multidrug resistance (MDR) proteins. A protein belonging to this superfamily has been characterised in *O. oeni* (Bourdineaud *et al.*, 2004). The gene encoding this protein, *omrA* did not show increased expression at 20 % (v/v) ethanol in *O. oeni*; however, when expressed in *E. coli* *omrA* conferred significant resistance to 10 % ethanol when compared to the control (Bourdineaud *et al.*, 2004). It was postulated that MDR proteins may facilitate cell survival by maintaining membrane lipid organisation and influencing membrane fluidity (Bourdineaud *et al.*, 2004). Membrane fluidity is important in the cells response to ethanol and whilst this mutation appears to be tolerated, based on its purported function, it warrants further investigation.

### 4.3.3.5 OEOE_1375 DNA directed RNA polymerase (rpoB)

OEOE_1375 is *rpoB* that encodes the β subunit of bacterial RNA polymerase (Figure 4.6). It is the site of mutations that confer resistance to rifamycin. Marcobal *et al.* (2008) analysed SNPs in *rpoB* to determine an increasing mutation rate in MMR deficient *O. oeni* strains as communicated in Chapter 2. RNA polymerase is also a part of pyrimidine metabolism, assisting in the formation of UTP and CTP and RNA (Figure 4.7).
Figure 4.6. Bacterial RNA polymerase (KEGG reference ooe03020; Kanehisa and Goto, 2000; Kanehisa et al., 2014). OOE_1375 is \textit{rpoB} which encodes for the β subunit of RNA polymerase. The β' and β subunits contain the active centre responsible for RNA synthesis and determinants for non-sequence specific DNA
Figure 4.7. Simplified pathway for pyrimidine metabolism (KEGG reference ooe00240; Kanehisa and Goto, 2000; Kanehisa et al., 2014) The gene encoded functions in green are found in *O. oeni*. OOE_1375 assists in the formations between CTP, UTP and RNA.
4.3.3.6 OEOE_1014 hypothetical protein

OEOE_1014 is a hypothetical protein; however, CDD suggests it may be a member of the conserved DegV protein family. DegV is widely conserved within *B. subtilus*, though its function is unknown. Its structure indicates that it may play a role in fatty acid transport and metabolism (Kinch *et al.*, 2005).

4.3.3.7 Non coding SNP’s

Strain 89 contains SNPs in two non-coding regions: the first is halfway between OEOE_0463 and OEOE_0464. OEOE_0463 is a hypothetical protein and OEOE_0464 encodes phosphotransferase system, mannose/fructose-specific component IIA. OEOE_0464 is also known as ManX and KEGG pathways reveal it has a role in the phosphotransferase system (PTS) (KEGG reference ooe02060) and fructose and mannose metabolism (ooe00051). Mannose is a sugar in apple juice but it is not commonly found in wine so this could be a response, by *O. oeni* to a different nutritional environment. The second SNP, when analysed with BLAST, seems to be located in OEOE_1137 a phosphoribosylaminomimidazole carboxylase ATPase subunit, which is responsible for nucleotide metabolism, in particular purines.

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Figure 4.8. Mannose metabolism (KEGG reference ooe00051; Kanehisa and Goto, 2000; Kanehisa *et al.*, 2014). ManX (OEOE_0464) with ManY and ManZ are responsible for the conversion of mannose into mannose-6-phosphate.
4.3.3.8 Summary for strain 89

It is important to note that MLF performance of *O. oeni* strain 89 was not as efficient as that of strain 90 (Chapter 3). It is possible that some of the mutations detected have decreased its performance or that the base mutations in both strains aren’t responsible for the new phenotype and the two strains have diverged in their mechanism of response to ethanol stress with the mechanism in strain 89 not as effective as strain 90. The role of OEOE_1014, which is a hypothetical protein, would be interesting to study further. OEOE_1014 shows homology with the DegV protein family and might have an effect on fatty acid transport. Genes related to fatty acids seemed important in strain 90, and perhaps this protein, in strain 89 helps in a similar way but with a different mechanism that was not as effective.
4.3.4 Transposable element

In viewing the annotated sequences it was observed that OEOE_1495 was annotated out of order on the genomes (Figure 4.9). As PSU-1 was used as the genomic reference and because it was the first *O. oeni* genome to be sequenced, the order of the gene loci in the PSU-1 genome runs numerically. In SB3, 89 and 90 OEOE_1495 is located between OEOEt1213 and OEOEt1214 (these t loci are tRNA) (Figure 4.9). The location of OEOE_1495 on PSU-1 was confirmed using the NCBI website (http://www.ncbi.nlm.nih.gov), with OEOE_1495 located between OEOE_1494 and OEOE_1496 and not between OEOE_t1213 and OEOE_t1214 (Figure 4.10). Additionally it seems that OEOE_1495 has been putatively characterised as a transposase. Furthermore on the reference PSU-1 sequence the gap between OEOE_t1213 and OEOE_t1214 is about 90 bp whereas in SB3, 89 and 90 it is about 10 kb long with the transposase in the middle (Figure 4.9 Figure 4.10). Different elements of this putative transposon were analysed using BLAST and as well as aligning to sections of PSU-1 this genetic material showed great similarity to other *Lactobacillus* spp., including ABC transporters, 5S ribosomal RNA and transposase. Possibly this transposase has played a role in facilitating horizontal gene transfer (HGT) during the evolution of this strain (SB3) and thus the effect of OEOE_1495 on strain performance should be examined further.
Figure 4.9. The sequences of strains 89, 90 and the parent (SB3) from the genome visualisation program IGV. The top of each section is a coverage map and below are the individual aligned reads. OEOE_1495 and approximately 10kb of unannotated, genetic material is situated between OEOE_t1213 and OEOE_t1214.
4.4 Summary

The fitness advantage of specific individual mutants may only play a small role in the diversification of a DE population. Underlying genetic background variation is quickly generated which then influences the fate of any individual beneficial mutation. Therefore later beneficial mutations can depend on how well they piggyback on initial clonal variations (Lang et al., 2011). Thus mutations that may appear unhelpful may be important because they lead to the opportunity for new mutations to develop.

In theory neutral mutations should arise, within a population, by drift at a uniform rate. According to Marcobal et al. (2008) the spontaneous mutation rate of *O. oeni* to rifampin and erythromycin is $1.6 \times 10^{-6}$. In this study the mutation rate, determined according to Foster (2006) was between $1.11 \times 10^{-5}$ and $2.61 \times 10^{-5}$ depending on the growth rate. This suggests an increased mutation rate in this case and alludes to a proportion of these mutations not contributing to the high ethanol tolerant phenotype.

The adaptation of *O. oeni* to ethanol is clearly more complex than first thought. As ethanol is one of the more toxic stressors in wine a number of previous studies have focused on the response of *O. oeni* to ethanol. Additionally in the current economic and environmental climate the search for biofuels has become more important (Dunlop, 2011). The response of bacterial cells to ethanol is therefore becoming an increasingly important area of research and yet the actual genetic mechanisms are largely unknown. A number of genes have been studied and have been discovered to play a role (Table 4.1, Sumby et al., 2014); however, from this work high ethanol tolerant bacteria have been generated that are identical to the parental strain in these implicated genes. Clearly there are additional mechanisms at work. The only genes that have being reported to be involved in the ethanol stress response that interact with any in the sequenced genome were, *dnaK* and *groEL*, in the RNA degradation
pathway, which interacted with OEOE_1629, a mutation present in both strains. OEOE_1581, from strain 90, also possibly interacts with groEL.

Some hypotheses have been made but further work is needed. Five genes that should be investigated first based on the literature in this chapter are OEOE_1629 (ppK) and OEOE_1581 based on their possible interactions with known ethanol response genes dnaK and groEL. The SNP in OEOE_0911 encoding for SAM-dependant methyltransferase should also be investigated by studying cfa expression to ascertain if this SNP effects downstream fatty acid metabolism, an important step in ethanol tolerance. OEOE_1168 the amino acid transporter contains two SNPs in close proximity and deserves further investigations based on the importance of amino acids to acetate stress resistance in E. coli. Finally OEOE_0738, the ABC multidrug transport system, as part of the MDR superfamily may help enable cell survival under ethanol stress by influencing membrane fluidity.

For not only these five genes but for all mutations identified in this study it would be of interest to look at the expression of the genes using qPCR and those in regions flanking the non-coding mutations to see if expression differs in high ethanol fermentations as evidence that they may be important candidates. Another option is applying RNAseq that examines the expression of the whole genome. The change in one gene might be affecting downstream or upstream performance of other genes. As there is currently no transformation system available for O. oeni alternative methods of investigating these genes could be over-expressing or deleting these genes in Lactobacillus spp. to confirm their necessity to the high ethanol phenotype. From this data it would be interesting to use other members of the DE population and compare data from high performing strains and also those that appear to be worse in performance than the parent.
Both the synonymous and non-synonymous mutations could be analysed by checking whether protein levels are different across mutants vs the parent strains to determine if they are having an effect. Further investigation is warranted on these genes as evolution of synonymous SNPs in response to environmental pressure clearly has some relevance to this work. Additionally it would be beneficial to confirm the insertions in DNA topoisomerase using basic PCR.

In summary there were no major genomic rearrangements between the parent (SB3) and the evolved strains 89 and 90. Insertion sequence elements did not appear to play the role that was original hypothesised. Excitingly as there is a clear phenotypic difference between the strains, new avenues of response of the cell appear to be at play and must now be investigated further so as to determine their cause. Such a fuller understanding would help guide bacterial selection, cultivation and culture management techniques.
Chapter 5. Thesis summary and future directions

This thesis details the use of DE to produce a more efficient and reliable strain of *O. oeni* (strain 90) for use in industrial fermentations and it is the first such study of its kind. Ethanol was considered one of the most pertinent of the wine stressors and so was selected as a basis for adaption. With proof-of-concept achieved it would be interesting to now expand the DE studies on *O. oeni*, including further DE on strain 90 in wine using combinations of stressors and therefore a more general stress resistance might produce even more superior strains.

Strain 90 was characterised and compared to the parent (SB3) in multiple MLF’s in media under a range of temperatures and ethanol concentrations. It completed MLF at 22 °C at 13 and 15 % (v/v) ethanol and was able to consume 85 % of the available L-malic at 19 % (v/v) ethanol. The highest concentrations of ethanol in which the bacteria could remain viable was also established. With strain 90 forming CFUs at 22 % (v/v) ethanol after 48 hours unlike its parent (SB3). In a 15 % (v/v) Pinot Noir strain 90 completed MLF in 40 % of the time it took the parent, characterisation in different wine variety, alcohol and temperature is necessary. Further comparisons between strain 90 and the parent to characterise the differences in their response to ethanol would be interesting. Included would be an examination of differences in membrane fluidity under high ethanol conditions using flow cytometry (da Silveira *et al.*, 2003) and to analyse the differences in fatty acid content of the cells using gas chromatography (Teixeira *et al.*, 2002).

Lastly the genomes of the SB3, 90 and 89 were sequenced using whole genome sequencing. Some future study opportunities arising from this work have already been raised. To expand on this work it would be interesting to study a new strain from a new DE population to see its response to ethanol adaptation. Evolution can take vastly
different tracks and perhaps there are multiple genetic solutions to the same problem of ethanol toxicity. Additionally members of the population could be isolated over time based on the hypothesis that rapid genetic change as a response to adaption of the environment occurs early in the selection process (Lenski, 2010). Importantly and as previously mentioned, the transposable element OEOE_1495 needs further analysis of its role in the evolution of certain *O. oeni* to industrial MLF conditions.

Tolerance to ethanol in lactic acid bacteria, on a molecular level, is the responsibility of a number of genes including *clpX, clcP, trxA, hsp18, ftsH, omrA, groESL, dnaK, clpA, ctrS* and *rmlB*. These genes are also responsible for the response to other physiochemical stressors (Sumby *et al.*, 2014). Possibly the improvements in ethanol tolerance in strain 90 have also improved the tolerance of the strain to other stressors in wine, epitomised by strain 90 being better able than its parent to perform in a low temperature MLF at 15 °C. It would be interesting to determine if this strain performs better when faced with other stresses. Once the genes responsible for this phenotype are elucidated a clearer picture of general stress response in this bacterium will emerge.

### 5.1 Conclusion

This work demonstrated DE as an effective and novel method for the generation of new and exciting *O. oeni* strains for industrial applications. A single isolate, strain 90, was selected as a strain that showed an ethanol tolerant phenotype, being found to retain residual viability in up to 24 % (v/v) ethanol for an hour compared to 22 % (v/v) by the parent. This strain was also found to complete MLF in Pinot Noir earlier than a range of other commercially available bacteria. The whole genome sequence of this strain compared to the parent is the beginning of exciting work in studying the whole genome response of *O. oeni* to ethanol stress.
Bibliography


Bridier, J., Claissse, O., Coton, M., Coton, E., Lonvaud-Funel, A. (2010). Evidence of distinct populations and specific subpopulations within the species Oenococcus oeni. Applied and Environmental Microbiology 76, 7754-7764.


Monnet, C., Aymes, F., Corrieu, G. (2000). Diacetyl and alpha -acetolactate overproduction by Lactococcus lactis subsp. lactis biovar diacetylactis mutants that are deficient in alpha -acetolactate decarboxylase and have a low lactate dehydrogenase activity. Applied and Environmental Microbiology 66, 5518-5520.


