Use of directed evolution to generate multiple-stress tolerant *Oenococcus oeni* for enhanced malolactic fermentation

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Abbreviations

∆p	proton motive force
ABC	ATP binding cassette
ACE	Australian Centre for Ecogenomics
ADI	arginine deiminase pathway
AF	alcoholic fermentation
AGRF	Australian Genome Research Facility
AJ	apple juice
AP	antagonistic pleiotropy
ATP	adenosine-5'-triphosphate
BLAST	Basic Local Alignment Search Tool
C8	octanoic acid
C10	decanoic acid
C12	dodecanoic acid
CDGJM	Chemically Defined Grape Juice Medium
CFA	cyclopropane fatty acid
CFU	colony forming unit
DAVID	Database for Annotation, Visualization and Integrated Discovery
DE	directed evolution
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
GMOs	genetically modified organisms
GOT	glutamate-oxaloacetate transaminase
HGT	horizontal gene transfer
INDELs	small insertions and deletions
IS	insertion sequence
KEGG	Kyoto Encyclopaedia of Genes and Genomes
LAB	lactic acid bacteria
L-LDH	L-lactate dehydrogenase
L-MDH	L-malate dehydrogenase
MA	mutation accumulation
MCFA	medium chain fatty acids

MDR	multidrug resistance
ME	malic enzyme
MLE	malolactic enzyme
MLF	malolactic fermentation
MMR	methylated mismatch repair
MRS	De Man, Rogosa and Sharpe
MRSAJ	MRS supplemented with 20% (v/v) apple juice
N/A	not applicable
NAD^+	nicotinamide adenine dinucleotide, oxidised
$NADP^+$	nicotinamide adenine dinucleotide phosphate, oxidised
NADPH	nicotinamide adenine dinucleotide phosphate, reduced
NCBI	National Center for Biotechnology Information
OD	optical density
ORF	open reading frame
PCR	polymerase chain reaction
PPP	pentose phosphate pathway
PSI	Position-specific Iterated
RFCDGJM	Red Fermented Chemically Defined Grape Juice Medium
RNA	ribonucleic acid
SAM	S-adenosyl methionine
SIFT	Sorting Intolerant from Tolerant
SNP	Single Nucleotide Polymorphism
SS	Sanger sequencing
TA	titratable acid
TAE	tris-acetate EDTA
TDP	thymidine-5'-diphosphate
tRNA	transfer RNA
UDP	uridine-5'-diphosphate
UniProt	universal protein resource
WGS	whole genome sequencing
WIC	Wine Innovation Cluster

Abstract

This study aimed to optimise *Oenococcus oeni* for more efficient malolactic fermentation in wine with multiple stressors. First, a previously evolved ethanol tolerant strain, A90, was characterised for resistance to combined pH and ethanol stress in both MRSAJ and Red Fermented Chemically Defined Grape Juice Medium (RFCDGJM). A90 showed a similar viability in RFCDGJM compared to its parent, SB3, indicating the need for further improvement. With the success of the previous proof-of-concept directed evolution (DE) in *O. oeni*, a new DE was carried out to determine 1) if DE can be applied to further improve A90 in a wine-like environment using combinations of stressors to generate more superior strains with better general stress resistance; 2) how much further can A90 be developed, and how stable the new phenotype would be; 3) possible new patterns of stress response through study of the genetic basis for the superior phenotype.

A continuous culture of A90 was established in a bioreactor and grown in a wine-like environment for approximately 350 generations with increasing ethanol and sulfur dioxide (SO_2), and decreasing pH over time. Samples of the population in the bioreactor were collected at three significant times during the DE to screen for improved isolates based on L-malic acid consumption and growth. Three strains, namely 1-161, 2-49 and 3-83, outperformed from a total of 378 isolates.

With a view to applying these strains to the industry, in-depth physiological characterisations were undertaken. Aspects examined included tolerance to various oenologically related stressors such as ethanol, pH, SO_2 and medium chain fatty acids, as well as phenotype stability and fermentation ability under more realistic winemaking conditions, i.e. un-filtered wine and winery scale fermentation. Overall, 2-49 and 3-83 constantly displayed better growth and

malolactic activity than the parent strain A90 in either lab-scale or winery-scale trials.

Finally, whole genome sequencing of strains SB3, A90, 2-49 and 3-83 and genetic characterisation were utilised to investigate changes during DE in *O. oeni*. A total of 19 single nucleotide polymorphisms (SNPs) were found in 2-49 and 3-83 strains compared to A90. The SNPs identified may affect cell envelope and fatty acids biosynthesis, DNA translation and homeostasis of internal pH, leading to the improved performance of DE strains. Sequences were also compared to the available sequence for commercial strain VP41. Several mutations were identified in stress response genes, indicating VP41 and SB3-related strains might have different responses to stressors. SNPs in the predicted *mleA* promoter sequence may suggest a new mechanism of MLF activation. Additionally, Nucleotide BLAST was used to analyse the presence of genes with oenological traits in SB3-related strains. Genes associated with the release of desirable aromas were found, whilst genes involved in the formation of biogenic amines were absent.

This study expands the knowledge regarding optimisation of *O. oeni*, and may be helpful for the further improvement of food-related microbes with enhanced performance.

Chapter 1 Literature review[a] and thesis structure

1.1 Introduction

Successful malolactic fermentation (MLF) conducted by lactic acid bacteria (LAB) is beneficial to the sensory quality of wines. However, LAB are notoriously fastidious microbes prone to slow growth due to the numerous inhibitors in wine, leading to poor fermentation performance. The fermentation process is usually inhibited by several physicochemical properties of the wine, especially high ethanol content, low pH and temperature, and the presence of SO_2 (Henick-Kling 1993). Failure of MLF can also result from low nutrient availability and the presence of phenols and acetic acid (Bauer and Dicks 2004).

A problematic MLF may require repeated re-inoculation with LAB starter cultures. Consequently, the duration of fermentation can be extended, which adds to the cost of production. Further risks include the increasing likelihood of bacterial spoilage and producing undesirable sensory compounds (Bartowsky 2005). Thus, the selection of more efficient LAB strains for optimised MLF is of great importance to the wine industry.

The criteria for the selection of LAB strains with improved MLF performance are strict (Volschenk et al. 2006; Krieger-Weber 2009). Specifically, these criteria include tolerance to low pH, high ethanol content, low temperature, the presence of SO_2, compatibility with yeasts as well as production of aroma compounds that are attributed to a pleasant wine aroma profile (Henick-Kling 1993; Marcobal et al. 2004; Volschenk et al. 2006). Several mechanisms are involved in conferring the ability of LAB to survive and function in wine. A better understanding of stress response of LAB can aid the development of superior new strains.

[a]This literature review was written during the first 6 months of PhD candidature (March 2014), as part of the Core Component Structured Program (CCSP) and thereby only contains references published to that point.

Considerable research has been carried out on the selection of improved LAB strains (Wang et al. 2007; John et al. 2008; Yu et al. 2008; Guzzon et al. 2009; Ruiz et al. 2010). This review will also discuss current methods utilised to optimise LAB, which include acclimation, classical strain selection, molecular methods and directed evolution.

1.2 Malolactic fermentation and lactic acid bacteria

MLF is a secondary fermentation conducted by LAB in wine. It can occur due to the action of indigenous LAB during or after alcoholic fermentation (AF). However, many winemakers choose to inoculate with commercial LAB after AF (Lonvaud-Funel 2001; du Toit et al. 2011; Ruiz et al. 2012). LAB are currently defined as a group of bacteria that are gram positive, catalase negative, anaerobic, and utilise sugars as their main carbon and energy source and generate lactic acid during sugar metabolism (Muñoz et al. 2011). LAB are either 'cocci' (spherical-shaped bacteria) or 'bacilli' (rod-shaped bacteria) based on their cell morphology.

Generally, the intra cellular organisation of LAB species is similar, however, their characteristics vary due to different physiological features (Ribéreau-Gayon et al. 2006a). LAB can be classified based on morphological, genetic and biochemical aspects (Ribéreau-Gayon et al. 2006a; Hornsey 2007). Four genera have been identified as the dominant bacteria involved in the winemaking process: *Lactobacillus*, *Leuconostoc*, *Oenococcus* and *Pediococcus* (Dicks et al. 1995; Dicks and Endo 2009). Of all the species of LAB that have been isolated and characterised, only a few are capable of surviving in the complex and harsh environment of grape must and wine (Table 1.1).

Table 1.1 The main wine-related lactic acid bacteria (adapted from Dicks and Endo 2009; Pozo-Bayón et al. 2009)

Genus	Species
Pediococcus	*P. damnosus, P. inopinatus, P. parvulus, P. pentosaccus*
Leuconostoc	*Leuc. mesenteroides*
Oenococcus	*O. oeni*
Lactobacillus	*Lb. brevis, Lb. boballius, Lb. buchneri, Lb. casei, Lb. collinoides, Lb. fermentum, Lb. fructivorans, Lb. hilgardii, Lb. kunkeei, Lb. lindneri, Lb. mali, Lb. nagelii, Lb. paracasei, Lb. paraplantarum, Lb. plantarum, Lb. uvarum, Lb. vini*

The main bacterial strains that are used to conduct MLF in wineries belong to the species of *O. oeni* (López et al. 2007; Ruiz et al. 2008), because they have the ability to adapt to the wine environment, while producing satisfactory sensory results (Lafon-Lafourcade et al. 1983; Kunkee 1984; Henick-Kling 1993). *Lactobacillus* spp. can be found during MLF whilst *Pediococcus* spp. are more likely to occur in wines after MLF when pH is higher (Costello et al. 1983; du Toit et al. 2011; Lafon-Lafourcade et al. 1983). *Lactobacillus* is the most common genera found on the grape skin from several Australian vineyards (Bae et al. 2006). Recent studies also reported that strains of *Lb. plantarum* can proliferate at low pH (G-Alegría et al. 2004), and therefore have the potential to conduct a successful MLF. Additionally, genes encoding for enzymes involved in aroma modification were found in *Lb. plantarum* strains isolated by Lerm et al. (2011), which imply that *Lb. plantarum* is also able to produce desirable organoleptic profiles in wine.

During MLF, L-malic acid is decarboxylated to L-lactic acid and CO_2 catalysed by the malolactic enzyme (MLE). MLE also requires NAD^+ as a co-enzyme and Mn^{2+} as a catalyst (Caspritz andRadler 1983; Spettoli et al. 1984). MLF confers an energetic advantage to LAB, which can stimulate their growth

(Cox and Henick-Kling 1995; Poolman et al. 1991; Olsen et al. 1991). During MLF, the intracellular pH of cells increases, which leads to a greater proton motive force (Δp) across the cell membrane (Cox and Henick-Kling 1995; Salema et al. 1996). Increased Δp can then combine with membrane ATPase to generate ATP (Henick-Kling 1986; Olsen et al. 1991).

While L-malic acid is typically converted to L-lactic acid directly, it can also be metabolised indirectly (Fig. 1.1). Whilst malolactic enzyme (MLE) has commonly been found in LAB, the pathway utilising the malic enzyme (ME) is somewhat less reported (Denayrolles et al. 1994; Landete et al. 2013; London et al. 1971). *Lactobacillus casei* is the only LAB described to decarboxylate L-malic acid using both MLE and ME pathways (Landete et al. 2010). Landete et al. (2013) determined that the ME pathway supported *Lb. casei* growth whereas the MLE pathway was only related to rapid degradation of L-malic acid.

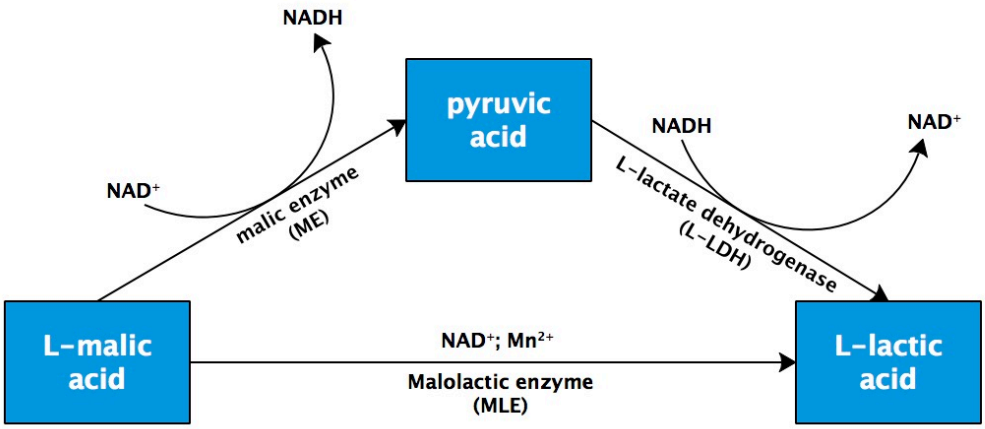

Fig. 1.1. Two MLF pathways for the conversion of L-malic acid to L-lactic acid in wine-related LAB. (adapted from Ribéreau-Gayon et al. 2006b; Hornsey 2007).

The results of MLF can have a positive influence on the chemical, microbiological and sensory properties of wine. After the conversion of L-malic acid to L-lactic acid, the pH is increased by 0.1–0.2 units and titratable acidity (TA)

is decreased. The wine can have a much softer mouthfeel due to the reduction in TA and the production of lactic acid. Additionally other compounds formed during MLF, such as higher alcohols, volatile fatty acids, volatile phenols, glycosides and esters, can enrich wine body and contribute to a more desirable aroma (Swiegers et al. 2005; Sumby et al. 2010). Wines that have undergone MLF are considered to be more round and full in mouthfeel (Pozo-Bayón et al. 2005). Henschke et al. (2002) concluded that MLF could lead to buttery, nutty, vanilla, fruity, vegetative, toasty and wet leather featured aromas, while Henick-Kling (1993) demonstrated that MLF strengthened the fruity and buttery smell but reduced the vegetative and grassy aromas. Complete consumption of L-malic acid also reduces the availability of a carbon source for other bacteria, thus enhancing the microbial stability of the wine (Bauer and Dicks 2004; Swiegers et al. 2005). In addition, it has been demonstrated that MLF can lead to a decrease in wine colour (Costello et al. 2012; Martínez-Pinilla et al. 2012; Aleixandre-Tudó et al. 2013; Burns and Osborne 2013), probably due to the decreased content of stable anthocyanin-derived pigments (Burns and Osborne 2013). Metabolism of acetaldehyde by *O. oeni* can reduce the formation of these pigments since acetaldehyde provides an ethyl bridge to link anthocyanins to tannins or other anthocyanins (Cheynier et al. 2006). These studies suggested that winemakers can take advantage of MLF by inoculating LAB that are able to produce pleasant aromas whilst do not metabolise acetaldehyde to minimise colour loss during MLF.

1.3 Factors inhibitory to LAB growth

Even though MLF increases complexity of wine aroma and flavour, it is often challenging for LAB to go through MLF completely. Wine is a complex environment with numerous compounds such as organic acids, phenols, SO_2 and

yeast products, which can have a negative impact on the growth of LAB and consequently MLF performance (Table 1.2).

Table 1.2 Wine-related LAB growth inhibitors and their inhibitory mechanisms

Inhibitors	Sources of the inhibitors	Inhibitory mechanisms	Reference
Ethanol	Produced by yeast during AF	Damage to cell membrane, leakage of intracellular materials	Da Silveira et al. 2002, 2003
SO_2	Produced by yeast or added by winemakers	Affect growth by reacting with co-factors like NAD^+; reduce ATPase production	Bauer and Dicks 2004; Romano and Suzzi 1993; Carreté et al. 2002
Temperature	Low fermentation temperature is preferred by winemakers	Affect growth rate and lag phase	Henick-Kling 1993
pH	Grape berries	Damage to cell membrane; affect ATP production by inhibiting sugar and malic acid metabolisms	Cotter and Hill 2003; Davis et al. 1986; Rosi and Canuti 2003
Phenolic compounds	Grape berries or added by winemakers	Damage to cell membrane	Campos et al. 2009
Yeast	Indigenous yeast from grape berries or commercial yeast inoculated by winemakers	Limited nutrients left in wine after AF; inhibitory products produced by yeast during AF	Fleet 2003; Osborne and Edwards 2007
Medium chain fatty acids	Produced by yeast during AF	Decrease in the intracellular pH; increase membrane permeability and decrease ATPase activity	Capucho and San Romão 1994; Carreté et al. 2002
Bacteriocins	Other bacteria	Damage to cell membrane	Sahl 1991 Knoll et al. 2008

1.3.1 Ethanol

Ethanol is the main product formed during AF from grape sugars and is generally considered to be the principal stressor on the growth of LAB and thus influences MLF rate. Henick-Kling (1993) determined that growth of *O. oeni* is inhibited by ethanol shock when the alcoholic concentration is above 14% (v/v). Nowadays MLF is becoming more challenging in high alcohol wines resulting from greater ripening of grapes, i.e. due to climate change (De Orduna 2010). Thus, ethanol tolerance is an essential feature for the activity of LAB in wine.

Ethanol interacts with the cell membrane at the lipid water interface by weakening the hydrophobic barrier to the permeability of polar molecules, and consequently perturbs membrane structure and function (Rigomier et al. 1980; Barry and Gawrisch 1994; Da Silveira et al. 2002; Weber and de Bont 1996). Da Silveira et al. (2002; 2003) showed that ethanol can affect the stress-resistance

and malolactic activity of *O. oeni* by increasing membrane fluidity and destroying cell membrane structure.

The viability of LAB in ethanol concentrations is strain dependent (Britz and Tracey 1990). Generally, *Lb.* spp. are more tolerant to higher ethanol concentrations than *O. oeni* and *Pediococcus* spp. (Davis et al. 1988).

1.3.2 Sulfur dioxide

Due to its chemical properties, SO_2 is used to control the growth of undesirable microorganisms and prevent oxidation at grape crushing and later stages of winemaking (Fleet and Heard 1993). In wine, SO_2 binds with carbonyl compounds (acetaldehyde, α-ketoglutaric acid and pyruvic acid) and others to generate the bound form (Rankine & Pocock 1969, Azevedo et al. 2007) whilst the rest, known as the free forms, including molecular SO_2, bisulfite (HSO_2^-) and sulfite (SO_3^{2-}) (Fugelsang and Edwards 2007). In addition to deliberate addition by winemakers, SO_2 can also be produced during AF by yeast (Dott et al. 1976; Wells and Osborne 2011).

At lower pH values, sulfite increasingly takes the form of molecular SO_2, the only form able to diffuse into the cell and therefore the most inhibitory form. After crossing the cell membrane by diffusion, molecular SO_2 is converted into bisulfite, which may react with compounds in the cell, thus inhibiting bacterial growth (Bauer and Dicks 2004). SO_2 can also affect the production of energy in cells and in turn LAB growth by reacting with cofactors like NAD^+ and FAD (Romano and Suzzi 1993) as well as imposing a stress on ATPase activity (Carreté et al. 2002).

The inhibitory effect of bound SO_2 is generally considered to be much weaker than molecular SO_2. However, recently several researchers have argued

that the inhibition of the bound form may be more significant than previously believed (Larson et al. 2003; Osborne and Edwards, 2006; Wells and Osborne 2011). Some forms of bound SO_2 can be degraded by bacteria, and consequently, the free SO_2 released can have an antimicrobial impact (Osborne et al. 2006; Jackowetz and de Orduña 2012). The inhibition by different forms of bound SO_2 has also been studied. Acetaldehyde-bound SO_2 was reported to be the main bound SO_2 inhibitor by Hood (1983) and Osborne et al. (2006) whilst Larsen et al. (2003) reported that inhibition was more significant when SO_2 was bound to compounds other than acetaldehyde. Wells and Osborne (2011) also reported that *Lb. hilgardii* was more sensitive to acetaldehyde-bound SO_2 while *O. oeni* was more sensitive to pyruvic acid-bound SO_2. The impact of different forms of bound SO_2 on bacterial growth has not been fully studied, and requires further investigation.

The ability to tolerate SO_2 differs among LAB species. *O. oeni* is reported less tolerant to total (free and bound) SO_2 concentrations than *Lactobacillus* spp. and *Pediococcus* spp. (Davis et al. 1988).

1.3.3 Temperature

Temperature has a great impact on the growth of LAB (Britz and Tracey 1990), mainly influencing growth rate, length of lag phase and maximum bacterial population (Henick-Kling 1993; Bauer and Dicks 2004). The optimal temperature for LAB growth and MLF in wine is 20˚C (Ribéreau-Gayon et al. 2006a), with temperatures below 16˚C being inhibitory to the growth of *O. oeni* and also leading to decreased cellular activity (Henick-Kling 1993; Ribéreau-Gayon et al. 2006a). Interestingly, Chu-Ky et al. (2005) found that although cold shocks at 8 and 14˚C strongly rigidified the plasma membrane, cell viability was unaffected.

1.3.4 pH

The pH of wine plays an essential role in LAB growth and the success of MLF, with the optimum pH for *O. oeni* being 4.5–5.5 (Salou et al. 1991). Clearly, the pH of wine is much lower than this, usually between 2.8 and 3.8, depending on wine style. *O. oeni* was found to have better growth or viability at low pH compared to other wine-related LAB species since *O. oeni* can maintain a higher intracellular pH level at low extracellular pH than other species (Davis et al. 1988; Henick-Kling 1993). Viability of LAB can be reduced at lower pH (Rosi and Canuti 2003). pH values ranging from 3–4 did not influence the survival of *O. oeni* regardless of rigidifying the cell membrane (Chu-Ky 2005) whereas at pH values of less than 3.0, cell growth is difficult in wines (Lonvaud-Funel 1995).

For gram-positive bacteria, low external pH can cause a reduction in intracellular pH, leading to damage of the cell membrane and reduction of ATP production (Cotter and Hill 2003). Furthermore, at low pH, sugar metabolism by *O. oeni* is inhibited, therefore, the energy obtained in this manner is decreased resulting in growth inhibition (Davis et al. 1986; Henick-Kling 1988). Additionally, acidity inhibits MLE activity resulting in extended MLF (Henick-Kling 1993; Rosi et al. 2003).

1.3.5 Medium chain fatty acids

Medium chain fatty acids (MCFA) such as decanoic acid (C10) and dodecanoid acid (C12) produced by yeast during AF are also inhibitory to LAB (Capucho and San Romão 1994) on both growth and MLF activity. The concentration of C10 in wines often ranges between 0.6 and 14 mg/L depending on must composition, yeast strain and physiochemical conditions during AF

(Lafon-Lafourcade et al. 1984). Minimum inhibition of MCFA to LAB is highly dependent on the type of MCFA present, the medium and LAB strains (Edwards and Beelman 1987; Capucho and San Romão 1994; Lonvaud-Funel et al. 1988).

The undissociated molecule is the most toxic form since this form is soluble in cell membrane and can cross the membrane by passive diffusion (Eliaz et al. 1976; Warth 1988) This can lead to the membrane acidification and increased membrane permeability, which consequently would result in the loss of intracellular components that are beneficial for growth or fermentation. The uptake of MCFA leads to the accumulation of ions inside the cells, therefore, the intracellular pH is decreased, which eventually could lead to a decrease in MLE activity (Capucho and San Romão 1994). Apart from these inhibitory mechanisms, Carreté et al. (2002) reported that ATPase activity of *O. oeni* was also inhibited by the presence of MCFA, which caused loss of cell viability.

1.3.6 Phenolic compounds

Phenolic compounds are naturally present in grape berries and wines, some of which such as gallic acid and free anthocyanins activate bacterial growth (Vivas et al. 1997) whilst others can have a negative effect on LAB growth. For example, hydroxycinnamic acids can be inhibitory at high concentrations (Reguant et al. 2000) and ferulic acid appeared to be more inhibitory than ρ-coumaric acid when controlling the growth of *Lb. plantarum* (García-Ruiz et al. 2008). Vanillic acid has a slight effect on inhibiting *O. oeni* growth while protocatechuic acid showed no effect (Vivas et al. 1997). Apart from the inhibitory effect on growth, phenolic acids can also delay the metabolism of glucose and citric acid by *O. oeni* and *Lb. hilgardii* (Campos et al. 2009). García-Ruiz et al. (2011) proposed that a

phenolic extract might be used as an alternative to SO_2 for controlling growth of LAB in wines.

Phenolic acids primarily act at the membrane level by passive diffusion (Kouassi and Shelef, 1998; Wen et al. 2003) and then alter membrane structure and acidify the cell cytoplasm. Cell membrane damage caused by phenolic acids results in ion leakage as well as high proton influx in *O. oeni* and *Lb. hilgardii* leading to decreased viability (Campos et al. 2009). However, as the latter study was conducted in a laboratory medium, which was rich in nutrients and had a higher pH (4.5) than typical wines, more investigation are needed in wine or wine-like media since low pH can facilitate passive diffusion of phenolic acids (Campos et al. 2009).

1.3.7 Other factors

Yeast-bacteria interaction are an additional factor that can affect LAB growth, the basis of which involves competition for nutrients and the production of inhibitory metabolites by yeast (Fleet 2003). For example, the depletion of amino acids by yeast can delay the onset of *O. oeni* growth (Nygarrd and Prahl 1996). Yeast products that inactivate bacterial growth include ethanol (Capucho and San Romão 1994), medium chain fatty acids (Edwards and Beelman 1987; Edwards et al. 1990), some peptides (Osborne and Edwards 2007; Nehme et al. 2008) and proteins (Comitini et al. 2005; Mendoza et al. 2010).

The requirement for particular nutritional compounds can also impose a stress on bacterial growth since LAB are fastidious (Fugelsang and Edwards 2007; Terrade and Mira de Orduña 2009). Ten key compounds including carbon, nitrogen and phosphate sources, Mn^{2+} and vitamins are essential nutrients for

both *O. oeni* and *Lb.* spp., but different in specific requirements (Terrade and Mira de Orduña 2009). *O. oeni* requires L-glycine, L-tyrosine and L-tryptophan while *Lb. buchneri* and *Lb. hilgardii* do not. The presence of riboflavin is a necessity for *Lb. hilgardii* and *Lb. buchneri* (Terrade and Mira de Orduña 2009).

Bacteriocins (Díez et al. 2012; Radler 1990), pesticides (Cabras et al. 1994; Vidal et al. 2001) and copper ions (Vidal et al. 2001) have also been reported to inhibit the growth of LAB. Nisin (one of the bacteriocins) can interact with the bacterial cytoplasmic membrane and form transient pores, which allows efflux of ions and loss of molecules, leading to cell death (Sahl 1991). The presence of bacteriocin encoding genes in the genome of *Lb. plantarum* isolated from South African red wines has been reported (Knoll et al. 2008). Putative genes encoding bacteriocins were also identified in *O. oeni* strains, raising the possibility of the existence of some new bacteriocins (Knoll et al. 2008). The basis of inhibition of pesticides on wine-related LAB has not been fully investigated, but Johnston et al. (2003) showed that single and combined biocides treated *Staphylococcus aureus* can lead to membrane damage.

1.3.8 Multiple stressors

Multiple stressors would result in a more severe inhibitory effect on bacterial growth compared to individual stressors. For example, temperature can impact the growth of LAB in conjunction with ethanol. The optimal growth temperature is lower at higher ethanol concentrations and vice versa (Asmundson and Kelly 1990; Henick-Kling 1993; Bauer and Dicks 2004). When *O. oeni* and *Lb. plantarum* were grown at 18°C and 13% ethanol (v/v), growth was slower than the control group of bacteria incubated without ethanol at 30˚C (G-Alegría et al. 2004). When decanoic acid and low pH or ethanol were both present, ATPase activity was reduced,

leading to loss of viability in *O. oeni* (Carreté et al. 2002). Additionally, the effect of SO_2 is greater when in combination with lower pH (Kunkee 1967). Different forms of sulfites are at equilibrium in wine, and a decrease in pH favours molecular SO_2 (Carr et al. 1976), the key inhibitory form.

1.4 Methods to optimise lactic acid bacteria for enhanced performance in wine

As previously described, several factors can inhibit the growth and viability of LAB, therefore impeding the completion of MLF. This can incur great cost for winemakers, with the need for re-inoculation, the increased risk of spoilage by other microbes, and quality loss through production of undesirable by-products. Thus, many winemakers tend to inoculate with commercial strains to carry out MLF since they are thought to be more reliable than indigenous strains. Even so, MLF is not always successful, thus it is important to enhance the robustness of wine-related LAB to ensure a more reliable and efficient MLF. Optimised performance of LAB can be achieved by either pre-adaptation to sub-optimal conditions or by strain improvement, which may be via recombinant or non-recombinant methods.

1.4.1 Acclimation to sub-optimal ethanol or pH

Recent studies report that pre-adaptation in media containing ethanol ranging from 4%–10% (v/v) or pH between 3.5 and 4.6 for one to two hours enhances ethanol tolerance of *O. oeni* and *Lb. plantarum* during subsequent MLF (Bravo-Ferrada et al. 2014; Cecconi et al. 2009; Chu-Ky et al. 2005). As previously discussed, the membrane damage from ethanol leads to loss of intracellular compounds that are essential for fermentation activity and growth (Da Silveira et al.

2002). Thus maintaining the integrity of the membrane is crucial for a successful MLF. Acclimation of *Lb. plantarum* strains with 6% and 10% (v/v) ethanol lead to less cell damage compared to non-acclimated cells, which in turn lead to better growth and MLF performance than the non-acclimated cells in a wine-like medium (Bravo-Ferrada et al. 2014). Additionally, Cecconi et al. (2009) showed that 20 proteins were differentially produced in *O. oeni* cells acclimated with 10% (v/v) ethanol compared to non-acclimated cells. Most of the proteins were identified as stress proteins and proteins involved in sugar and amino acid metabolism (Cecconi et al. 2009), thereby hinting at target.

Even though acclimation can improve resistance of LAB to wine related stressors, this method may not be suitable for winery-scale fermentations. This is because pre-adaptation of adequate cell numbers to commence an industrial scale MLF requires both technical expertise and considerable amount of a food-grade acclimation medium prior to each inoculation. Instead pre-acclimated strains supplied as freeze-dried preparations are available commercially.

1.4.2 Recombinant methods

Recombinant approaches involve specific modification of the genome of an organism by deletion or modification of native genetic elements or their replacement with sequences from other sources. This method requires solid knowledge of the genetic basis of a phenotype. Transformation, transduction and conjugation are three common recombinant approaches for strain improvement.

Transformation involves the uptake of dissociative DNA fragments directly from the surrounding environment using calcium chloride or electroporation to increase the efficiency of DNA uptake. Transformation is widely reported in

Lactobacillus spp. (Chassy et al. 1987; Aukrust et al. 1992, 1995; Fiocco et al. 2007; Spath et al. 2012), but is problematic in *O. oeni*. There are only limited reports of successful transformation in *O. oeni* (Dicks 1994; Assad-García et al. 2008), however neither was reported to be used successfully on a regular basis and nor had good transformation efficiency. Clearly, developing a new method to improve DNA uptake in *O. oeni* is a necessity. Recently, several new plasmids have been found (Beltramo et al. 2004a; Eom et al. 2010; Favier et al. 2012), which might be used as cloning vectors for potential genetic transfer into *O. oeni*.

Transduction refers to a process whereby DNA is transferred from one bacterium to another via a virus. Bacteriophages can code for genes such as toxins to increase fitness of the host (reviewed by Wagner and Kaldor 2002); however, *O. oeni* invaded by bacteriophages resulted in failure of MLF (Davis et al. 1985). It might be more suitable to apply a different phage to *O. oeni*, but currently there are no reports of using such a phage on *O. oeni*.

Conjugation refers to the transfer of mobile genetic fragments that are capable of independent replication between two cells. For example, a conjugation transposon, Tn*6098* has been transferred into a milk related isolate, *Lactococcus lactis* (Machielsen et al. 2011). The transposon encoded proteins that allow metabolism of α-galactosides in *L. lactis* that was originally isolated from plants, thereby the manipulated strains can grow well in soy milk whilst also allowing the maintaining of the desired flavour. However, this method is considered unsuitable with *O. oeni*, as gene replacement can fail due to the less transfer frequency compared to recombination frequency (Zúñiga et al. 2003; Beltramo et al. 2004a).

1.4.3 Non-recombinant methods

Compared to recombinant methods, non-recombinant methods require little knowledge of the genetic basis of traits, but stringent screening methods for selection of improved strains are a necessity (Parekh et al. 2000). Four commonly used non-recombinant approaches are discussed below.

Classical strain selection

This is one of the oldest methods of selecting superior strains and is often used to obtain improved strains for sale to the wine industry (Guzzon et al. 2009). The basis of this method is to isolate fitter strains that arise through natural variations in a desired character. The best example is the recovery of *O. oeni* in wine where survival is dependent on traits that help resist physicochemical stresses. Therefore, much of the work in selecting indigenous LAB from wines has focused on *O. oeni* (Solieri et al. 2010; Guzzon et al. 2009; Capozzi et al. 2010; Ruiz et al. 2010; Bordas et al. 2013).

Genomes of a number of LAB strains have been sequenced (Mills et al. 2005; Bartowsky and Borneman 2011; Borneman et al. 2012), which increases the accuracy of the classical strain selection process. After sequencing, strains can be selected according to genetic traits (Bolotin et al. 2001; Makarova et al. 2006; Mills et al. 2010). For example, *O. oeni* AWRIB429 (also known as VP41) was selected for generation of more fruity characters and the new genes were associated with potential glycosidases (Bartowsky and Borneman 2011).

Although this method is straightforward and achievable, it is time consuming and requires large amounts of laboratory work to characterise the isolates. Additionally, as classical strain selection largely relies on natural diversity

in different phenotypes, it would be difficult to select a perfect strain with desired traits using this method. Otherwise, such a strain would have already been isolated.

Mutagenesis

Mutagenesis is a process that changes the genetic information of an organism. It may occur naturally, or as a result of exposure to a mutagen. A number of chemical compounds (eg. nitrous acid, hydroxylamine, N-methyl-N'-nitro-N-nitrosoguanidine (NTG) and ethyl methanesulfonate (EMS)) and physical agents (eg. X-rays, γ-rays and ultraviolet rays) can be used as mutagens. Mutagenesis can increase the genetic diversity in a population by introducing DNA deletions, frame shifts or substitutions, thus strains with desired features can be isolated through appropriate screening methods. An example includes the mutagenesis of *L. lactis* subsp. *lactis biovar diacetylactis* where in 483 mutants generated with the assistance of NTGs that were lactate dehydrogenase deficient (Boumerdassi et al. 1997). However, although the frequency of mutation is high, mutations occur randomly, and could not necessarily yield the phenotype of interest.

Genome shuffling

The desired phenotypes are obtained through several rounds of protoplast fusion or recursive fusion, which allows recombination between genomes (Stephanopoulos 2002; Petri and Schmidt-Dannert 2004; Zhang et al. 2002). Protoplast fusion can be encouraged by chemical (polyethylene glycol) or electrical treatment (Cocconcelli et al. 1986; Morelli et al. 1987; Assani et al. 2005). Genome shuffling has been applied by Patnaik et al. (2002) and Wang et al. (2007)

to improve the acid tolerance of *Lb.* spp.. In addition, Yu et al. (2008) successfully used genome shuffling to enhance L-lactic acid productivity by improving glucose tolerance of *Lb. rhamnosus*. Currently, there are no publications reporting the use of genome of shuffling with *O. oeni*, but this method is theoretically applicable with this bacterium.

Directed evolution

Directed Evolution (DE), which is also known as Adaptive Evolution (AE), involves cultivating microbes in the presence of increasing amounts of inhibitory compound(s) over a period of time, during which stable phenotypes accumulate, making it possible for the selection of improved strains (Bennett and Hughes 2009; Dragosits and Mattanovich 2013; Rosenberg and Hastings 2003). DE is a common method in microbiology studies, and the most typical organisms associated with DE are *Escherichia coli* and *Saccharomyces cerevisiae* (Paquin and Adams 1983; Bennett et al. 1990; Sleight and Lenski 2007; Sleight et al. 2008; Wisselink et al. 2009). Three features of microorganisms, namely simple nutrient requirement, easy cultivation in the laboratory, as well as fast growth make it convenient and advantageous to use DE (Dragosits and Mattanovich 2013). Time span is an important factor during DE. Generally, the time of DE ranges from several weeks to many months as each evolution typically requires 100–200 generations (Ibarra et al. 2002; Hua et al. 2007; Barrick et al. 2009; Charusanti et al. 2010; Conrad et al. 2010).

DE has been used by Teusink et al. (2009) and Bachmann et al. (2012) to improve the growth of *Lb. plantarum* on a poor carbon source and *L. lactis* isolated from mung-bean sprouts in milk, respectively. Directed evolution has also been applied to improve acid tolerance of *Lb. casei* (Zhang et al. 2012). When exposed

to acid stress, the evolved strains had higher intracellular pH, NH_4^+ concentrations and lower inner membrane permeability compared to the parent (Zhang et al. 2012), suggesting these mechanisms are important for acid tolerance (O'Sullivan and Condon 1997; Siegumfeldt et al. 2000). These three experiments were conducted using a sequential transfer approach. By contrast, a continuous culture was used with *O. oeni* to improve ethanol tolerance (Betteridge et al. 2013) since *O. oeni* has relatively slow growth. The commercial strain SB3 was grown in MRS supplemented with 20% apple juice (MRSAJ) and increasing ethanol for approximately 290 generations. The resulting mixed population completed MLF in MRSAJ supplemented with 15% (v/v) ethanol in 50% of the time it took the parent (Betteridge et al. 2013). However, MRSAJ is rich in nutrients and is a different niche from wine. Whether the ethanol tolerant strains possess better multiple-stressor tolerance in typical red wines is unknown, and needs to be further characterised.

Spontaneous genetic mutations such as single nucleotide polymorphisms (SNPs), small insertions and deletions (INDELS) and large deletions and insertions that result in the genomic changes, e.g. increased or decreased gene dosage, altered or diminished gene function, and finally modified the phenotypes (Conrad et al. 2011; Dhar et al. 2011; Adamo et al. 2012; Dragosits et al. 2013).

Candidate gene sequencing has been used to identify the nature of the mutations arising during DE (Lapierre et al. 2002; de Visser et al. 2004). Insertion sequence (IS) mediated mutations, which can affect transcription levels of individual genes, were reported in two DE experiments with LAB (de Visser et al. 2004; Bongers et al. 2003). This method relies on prior knowledge to select an appropriate target gene. Additionally, there might be undetected mutations in other

loci responsible for improved fitness of the evolved microbe, which cannot be identified using this method. In order to fully define potential beneficial mutations, whole genome sequencing and genome comparison are widely used in DE studies (Bachmann et al. 2012; Araya et al. 2010; Barrick et al.2009; Lee and Palsson 2010; Minty et al. 2011). Table 1.3 summarises beneficial genetic mutations in recent DE studies with LAB.

Table 1.3 Directed Evolution studies with lactic acid bacteria and beneficial genetic mutations in the evolved strains

Species	Selective pressure/condition	Beneficial genetic mutations	Reference
L. lactis	Growth/starvation/shaking/non-shaking	IS*981*-mediated mutations (INDELS)	de Visser et al. 2004
L. lactis	Grew in milk	Point mutations in loci involved in nitrogen metabolism for growth in milk; point mutation in *mutL*	Bachmann et al. 2012
L. lactis	Propagated in water-in-oil emulsion	ND	Bachmann et al. 2013
L. lactis	*ldh*-deficient cells select for fast growth	Insertion of IS*981*-like element upstream of *ldhB*. This mutation activated *ldhB* in the evolved strain	Bongers et al. 2003
L. lactis	Grew in chemically defined medium with lactose	SNP in promoter region of the *cel* cluster. This SNP activated transcription of the cryptic *cel* cluster	Solopova et al. 2012
L. lactis	Grew on plates of Fast Slow differential agar medium with hydrogen peroxide	ND	Rochat et al. 2005; 2012
L. lactis	Grew in LM17 broth at 34 ˚C	Mutations in *llmg_1816*. *llmg_1816* encodes a membrane-bound stress signalling protein of the GdpP family, and displays cyclic dimeric AMP-specific phosphodiesterase activity	Smith et al. 2012
Lb. plantarum	Grew in murine intestine	Mutations in genes encoding cell envelope associated functions and energy metabolism	Van Bokhorst-van de Veen et al. 2013
Lb. plantarum	Grew in chemically defined medium with glycerol	ND	Teusink et al. 2009
Lb. casei	Grew in MRS with increasingly low pH	ND	Zhang et al. 2012
Lb. delbrueckii	Grew in Milk	IS*L4* and IS*L5* inserted upstream of the *lac* operon	Lapierre et al. 2002

ND: not determined

1.5 Response of lactic acid bacteria to wine-related stressors

In order to overcome the inhibitory stressors in wine (Section 1.3), LAB display numerous responses. Generally, three mechanisms are involved for LAB to survive, proliferate and function: (1) the proton motive force produced during

MLF (Section 1.2); (2) the synthesis of stress proteins; and (3) the activation of membrane-bound ATPases (Drici-Cachon et al. 1996; Salema et al. 1996). With advances in technology, many tools, like metabolomics, genomics, proteomics and transcriptomics, are available to study the adaptive responses of LAB to oenologically related stressors. A number of genes involved in the adaptation and stress response of wine-related LAB have been characterised (Table 1.4)

Table1.4 Genes involved in the stress response in wine-related lactic acid bacteria

Genes	Gene description	Stressors	References
Malate/citrate metabolism			
mleA	Malolactic enzyme[a]	pH	Beltramo et al. 2006
citE	Citrate lyase beta subunit[a]	pH	Olguín et al. 2009
maeP	Putative citrate transporter[a]	Ethanol	Olguín et al. 2009
Amino acid metabolism			
arcA	Arginine deiminase[a]	pH	Bartowsky 2005
arcB	Ornithine carbamoyltransferase[a]	pH	Bartowsky 2005
arcC	Carbamate knase[a]	pH	Bartowsky 2005
arcD	Arginine-ornithine antiport protein[a]	pH	Bartowsky 2005
arcR	Putative regulatory protein[b]	Heat and osmotic	Bourdineaud 2006
Cytoplasmic and membrane			
cfa	Cyclopropane-fatty-acyl-phospholipid synthase[a]	Ethanol and pH	Grandvalet et al. 2008
ggpps	Geranylgeranyl pyrophosphate synthase[b]	Ethanol	Cafaro et al. 2014
rmlB	DtdP-glucose-4,6-dehydratase[b]	Ethanol	Da Silveira et al. 2004
Heat shock proteins			
Hsp18	Heat shock protein Lo18[a]	Heat, ethanol and pH	Guzzo et al. 2000; Desroche et al. 2005; Beltramo et al. 2006; Maitre et al. 2014
groES	10 kDa chaperonin[a]	Heat and ethanol	Desroche et al. 2005; Grandvalet et al. 2005
grpE	Protein GrpE[a]	Heat, ethanol and pH	Desroche et al. 2005; Grandvalet et al. 2005
clpL	ATP-dependent Clp protease ATP-binding subunit[a]	Heat, ethanol and pH	Beltramo et al. 2004b; Desroche et al. 2005; Grandvalet et al. 2005; Beltramo et al. 2006
clpX	ATP-dependent Clp protease ATP-binding subunit ClpX[a]	Heat	Guzzo et al. 2000; Desroche et al. 2005
ctsR	Transcriptional regulator CtsR[a]	Heat, ethanol and pH	Desroche et al. 2005; Grandvalet et al. 2005
Redox protein			
trxA	Thioredoxin[a]	Heat, pH and oxidative stressor (H_2O_2)	Jobin et al. 1999 Guzzo et al. 2000; Beltramo et al. 2006
ATP binding cassette protein			
clpP	ATP-dependent Clp protease proteolytic subunit[a]	Heat and ethanol	Beltramo et al. 2004b; Desroche et al. 2005
omrA	Multidrug resistance ABC transporter, ATP binding and permease protein[a]	Heat, osmotic, and toxic salt (sodium laurate)	Bourdineaud et al. 2004
horA	Multidrug transporter HorA[a]	Heat, osmotic, and toxic salt (sodium laurate)	Bourdineaud et al. 2004
ftsH	ATP-dependent zinc metalloprotease FtsH[a]	Heat and osmotic	Bourdineaud et al. 2003
Plasma membrane H^+-ATPase activity			
atpB	ATP synthase subunit beta[a]	pH	Beltramo et al. 2006

[a] Gene descriptions were taken from the UniProt database (http://www.uniprot.org).
[b] Gene descriptions were taken from corresponding literature.

Arginine is a major amino acid in wine, which many LAB have the ability to metabolise through the arginine deiminase pathway (ADI). This catabolism is

important for LAB, as ATP formed during ADI allows bacteria to survive longer in harsh environments (Stuart et al. 1999). The ADI pathway has been shown to enhance pH resistance of oral bacteria, such as *Streptococcus rattus* and *S. sanguis* (Curran et al. 1995). NH_3 produced during ADI at low pH can bind with H^+ to form NH_4^+, leading to an increase in intracellular pH to protect against acid inhibition (Marquis et al. 1993; O'Sullivan and Condon 1997; Siegumfeldt et al. 2000). In another study conducted by Bourdineaud (2006), the expression of *arcR*, which encodes a regulatory protein, increased after heat and osmotic shocks in *O. oeni*. Even though the ADI pathway helps LAB to adapt to harsh environments, it can also lead to the production of undesirable ethyl carbamate (Ough et al. 1988) and putrescine (Guerrini et al.2002) that can have adverse health effects: ethyl carbamate is a carcinogen while putrescine can be toxic for sensitive consumers.

Heat shock proteins are produced by LAB to protect them against harsh environments. The *hsp18* gene encoding a small heat shock protein Lo18 has been well studied (Jobin et al. 1997; Delmas et al. 2001) and found to play an important role in maintaining membrane stabilisation, and is involved in both the early and later stages of the ethanol stress response (Maitre et al. 2014).

The caseinolytic protein (Clp) ATPases contains two classes: the first class also known as HSP1000 includes ClpL and ClpA, has two ATP nucleotide-binding domains whilst proteins (ClpX) in the second class only contain one. ClpL or ClpX, acts as ATPase regulatory component (Gottesman et al. 1990, 1993) of ClpP and ClpATPases. ClpP, an ATP-dependent proteolytic component (Woo et al. 1989), when associated with ClpATPases, can degrade abnormal proteins, which is one of the responses of bacteria to stress (Maurizi et al. 1990). *Oenococcus oeni* has Clp protein family homologous to those in other bacteria, and might have similar

functions relating to stress adaptation. In agreement with this, mRNA levels of *clpL* increased at low pH (Beltramo et al. 2004b); however, the mechanism regarding regulation of the *clpL* gene needs further investigation.

Three other ATP-binding cassette (ABC) proteins, OmrA, HorA and FtsH, are also involved in stress tolerance and response. Several multiple drug resistance (MDR) transporters belonging to the ABC protein family have been identified in LAB, which includes HorA in *Lb. brevis* and OmrA in *O. oeni* (Bolhuis et al. 1997; Sakamoto et al. 2001; Bourdineaud et al. 2004). Changes in *omrA* gene expression are not significant in response to ethanol, sulfite or ethidium bromide whereas the expression of *omrA* was reported to increase when *O. oeni* was submitted to high temperature or osmotic shock (Bourdineaud et al. 2004). Another ABC protein identified in *O. oeni*, the FtsH protein, is also associated with stress adaptation with a higher expression being observed in *O. oeni* under heat and high osmotic conditions (Bourdineaud et al. 2003). FtsH is involved in degradation of short-lived proteins or proteins that are misassembled (reviewed by Ito and Akiyama 2005) as well as sporulation and cell division (Deuerling et al. 1995; Wehrl et al. 2000)

Proton extrusion by ATPases play an important role in acid tolerance (Tourdot-Maréchal et al. 1999). The membrane-bound H^+-ATPases pump protons out of the cell, thus maintaining pH homeostasis. The activity of H^+-ATPase is induced in *O. oeni* when pH is low. A recent study showed that expression of *atpB* (encoding the β-subunit of the F_1F_0 ATPase) increased three-fold after acidic adaptation (Beltramo et al. 2006). Unfortunately, as H^+-ATPases are also required during MLF to pump hydrogen ions out of the cell, the mechanism of the ATPases response at low pH is still not clear.

Apart from the above responses, maintaining membrane integrity is also important during adaptation to stressors. One strategy that bacteria have developed is to adjust membrane fluidity via modification of membrane fatty acid composition (Maitre et al. 2014). High plasma membrane fluidity is suggested to be related to ethanol tolerance in bacteria (Couto et al. 1996). The importance of the *O. oeni* plasma membrane regarding response to ethanol stress has been studied by Teixeira et al. (2002), with results indicating that the accumulation of lactobacillic acid (C19 cyclo) can maintain appropriate membrane fluidity, thereby providing protection against ethanol toxicity. Moreover, cell viability of *O. oeni* under ethanol stress is related to the expression of genes, like *ggpps* that encoding the geranylgeranyl pyrophosphate synthase and *cfa* (Grandvalet et al. 2008; Sico et al. 2009; Cafaro et al. 2014).

Such discoveries could assist *O. oeni* selection by a sequencing-based method to check if the selected strains have SNPs in these stress response genes (Table 1.4) compared to the parent.

1.6 Project summary and thesis structure

In this review, the benefit and limitations of LAB to conduct MLF have been discussed. To overcome the multiple inhibitors and eliminate problematic MLF, more robust LAB for efficient MLF are needed. They can be optimised through various methods. As *O. oeni* plays a central role in winemaking, this project was focused on enhancing the performance of *O. oeni*. As described in the review, for *O. oeni*, DE is likely to be the most practical approach for strain optimisation since *O. oeni* is a rapidly evolving organism (Yang and Woese 1989). Additionally, no prior knowledge of the genetic basis for the improvement is needed and the resulting strains are non-GMO.

Two strains with high ethanol tolerance in MRSAJ have already been generated via DE (Betteridge et al. 2013). This project aims to further characterise these strains for multi-stress resistance as well as broaden the resistance of these strains to multiple stressors present in typical red wines, namely the combination of low pH, high ethanol concentrations, the presence of phenolic compounds and SO_2. In addition, this project also aims to study the genetic basis for differences between the improved strains and the parent.

In order to accomplish the project aims, the following objectives were pursued and are described in detail in the following chapters of this thesis;

1. Characterise two ethanol-resistant *O. oeni* strains in MRSAJ and a wine-like medium with combined ethanol and pH stressors (Chapter 2)
2. Develop Directed Evolution methodology for generating multi-stress resistant *O. oeni* (Chapter 3).
3. Characterise evolved strains for stress tolerance, phenotypic stability and MLF in the presence of yeast (Chapter 4).
4. Study the genetic features of the evolved *O. oeni* and determine the changes between the genomes of the evolved strains and the parent strain to investigate the genetic basis for their different phenotypes (Chapter 5).

Chapter 2 Characterisation of ethanol tolerant *Oenococcus oeni* strains in MRSAJ and synthetic wine media with multiple stressors

2.1 Introduction

A number of LAB, such as *Lactobacillus spp*., *Pediococcus spp*. and *Oenococcus oeni* have been isolated and identified as the dominant bacteria involved in malolactic fermentation during winemaking (Dicks et al. 1995; Dicks and Endo 2009; Franquès et al. 2017). *Oenococcus oeni* is able to adapt to the harsh and complex environment of grape must and wine (López et al. 2007; Ruiz et al. 2008) while producing pleasant aroma and mouthfeel (Lafon-Lafourcade et al. 1983; Kunkee 1984; Henick-Kling 1993). For these reasons, strains of *O. oeni* are the main bacteria used to carry out inoculated MLF in the wine industry.

Even though strains belonging to *O. oeni* are widely used in the wine industry, their MLF performance can sometimes be poor. Several physiochemical properties of wine such as high ethanol concentration, low pH, phenolic compounds, proteins, peptides, etc. (Davis et al. 1985; Rosi and Canuti 2003; Campos et al. 2009; Rizk et al. 2016) can have a negative effect on bacterial growth, and as a consequence, influence the progress of MLF (Arena and Manca de Nadra 2005). Additionally, *O. oeni* have fastidious nutritional requirements and relatively slow growth rates.

Ethanol is generally considered to be the principal inhibitor of LAB growth. *Oenococcus oeni* growth is inhibited when ethanol concentration is above 14% (v/v) (Henick-Kling 1993). Ethanol negatively impacts the physicochemical state

and biological function of cell membranes (Weber and de Bont 1996) by weakening the hydrophobic barrier at the lipid water interface (Rigomier et al. 1980; Barry and Gawrisch 1994; Da Silveira et al. 2002). Membrane fluidity is also altered via changes in fatty acid composition in response to high ethanol (Couto et al 1996; Da Silveira et al. 2003; Da Silveira and Abee 2009).

Low pH can also have an inhibitory effect on cell viability. *Oenococcus oeni* is best suited to growth at pH values between 4.8 and 5.5 (Henick-Kling 1993) whilst wine pH is usually between 2.8 and 3.8. Growth of *O. oeni* was found to be reduced at pH 3.2 compared to that at pH 3.5 and 3.7 (Davis et al. 1986). ATPase activity has been linked to the mechanism of pH resistance in *O. oeni*. Mutant strains that had a low ATPase activity showed a reduced viability at pH 5.3 and failed to grow at pH 3.2 (Tourdot-Maréchal et al. 1999).

Wine is a complex matrix that contains multiple stressors. Growth and malolactic activity of *O. oeni* is greatly inhibited by a combination of stressors such as those encountered in wine (Britz and Tracey 1990; Chu-Ky et al. 2005), potentially leading to unsuccessful MLF. Failure of MLF can cause a series of problems, including extension of fermentation, the need for re-inoculation, and an increased risk of microbial spoilage. The extra workload required for slow or stuck MLF and increased spoilage risk will impose financial burdens on the winemaker. In order to reduce the possibility of unsuccessful and inefficient MLF, more robust strains that have better tolerance to wine related stressors are needed. For these reasons, numerous researchers have attempted to improve the phenotypes of LAB with different strain improvement methods (reviewed by Betteridge et al. 2015).

Betteridge (2015) isolated *O. oeni* strains with high tolerance to ethanol using DE. A continuous culture of a commercially available strain, SB3, was established in MRSAJ at 30 ˚C with the ethanol concentration in the medium increasing steadily over 290 days from 5% (v/v) to 15% (v/v). Individual isolates were obtained from the DE culture and screened for improved ethanol tolerance in MRSAJ medium. In this way, two improved *O. oeni* strains; A89 and A90 were identified.

Ethanol tolerance assays were performed on strains A89 and A90, revealing that they can tolerate up to 20% (v/v) ethanol in MRSAJ (Betteridge 2015). However, pH tolerance and combined pH and ethanol tolerance of these strains was not evaluated. In this study, strains A89 and A90 were grown in MRSAJ and Red Fermented Chemically Defined Grape Juice Medium (RFCDGJM) with ethanol at different pH levels.

2.2 Materials and methods

2.2.1 Strains

Three strains of *O. oeni* (SB3, A89 and A90) were used in this study. SB3, a commercial strain (Laffort Oenologie), is reported to have average ethanol tolerance (up to 15% (v/v), datasheet from Laffort Australia). Strains A89 and A90 are DE strains from SB3 with improved ethanol tolerance and more efficient MLF in MRSAJ (Betteridge 2015).

2.2.2 Media

Strains were cultured from MRSAJ glycerol stocks kept at -80 ˚C. Tolerance assays were carried out in modified MRSAJ (Amyl Media) and a synthetic wine medium; RFCDGJM made from Chemically Defined Grape Juice Media (McBryde

et al. 2006) supplemented with 5% (v/v) grape tannin extract (Tarac Technologies, GSKINEX) and fermented with *Saccharomyces cerevisae* until dry (total sugar < 2.0 g/L).

MRSAJ was supplemented with 5g/L D/L-malic acid. MRSAJ and RFCDGJM were then modified by adding analytical grade ethanol. The pH of the medium was adjusted with 10M NaOH or 37% (v/v) HCl. Final ethanol concentration and pH of the medium was determined by a Wine ME/DMA 4500M alcolyser (Anton Paar) and a CyberScan 1100 pH meter (Eutech). Modified MRSAJ medium and RFCDGJM were sterilised (0.22 µm) before use. Table 2.1 summarises the conditions and the media used in this study.

Table 2.1. Media used in stress tolerance assays

Media	pH range or fixed value	Ethanol range or fixed value (% (v/v))	Malic acid (g/L)
MRSAJ-1	2.8–3.6 (0.2 increments)	0	5
MRSAJ-2	2.8–3.6 (0.2 increments)	12	5
MRSAJ-3	3.5	12–18 (2% (v/v) increments)	5
RFCDGJM-1	2.8–3.6 (0.2 increments)	12	3.5
RFCDGJM-2	3.5	12–18 (2% (v/v) increments)	3.5

2.2.3 Growth conditions

Pre-cultures from glycerol stocks were incubated at 30 ˚C for 4 days in 8 mL of MRSAJ medium. The liquid cultures were then diluted with fresh MRSAJ to an optical density 600 nm (OD_{600}) of 1.0 (Helios Cuvette spectrophotometer, Thermo Scientific) before 500 µL was added to 8 mL fresh MRSAJ. After a 24-hour incubation at 30 ˚C, cells were in the middle of exponential phase (OD_{600} =

0.8–1.0) and harvested. The cultures were then re-diluted with MRSAJ to adjust their OD_{600} to 0.5 before being inoculated into experimental media (250 µL into 8 mL) as described in Table 2.1. All cultures were incubated at 22 ˚C for 10 days. Each culture was prepared in triplicate.

2.2.4 Cell enumeration

After inoculating *O. oeni* into experimental media, samples were taken at time points between 0 and 240 hours. A ten-fold dilution series of each sample (10^{-1}–10^{-5}) was made and 5 µL of culture spotted onto the surface of MRSAJ agar plates, which were then incubated at 30 ˚C with 20% (v/v) CO_2 for 7 days. Colony forming units (CFUs) were counted.

2.2.5 Data analysis

Data was first processed with Microsoft Excel 2011. Graphing, two way-ANOVA and Fisher's LSD tests were conducted with GraphPad Prism 7 to test the impact of time and strain on bacterial viability under each condition. The confidence interval applied in all statistical analysis was 95%.

2.3 Results

2.3.1 pH stress tolerance in MRSAJ

All *O. oeni* strains maintained high viability at all tested pH levels with viable cell numbers remaining over 10^6 CFU/mL (Fig. 2.1). Viable cell numbers of all strains were typically greater at pH 3.2 and above; however, reduced immediately after inoculation at pH 3.0 and lower (Fig. 2.1 C–E). At pH 3.2, cell numbers remained constant at approximately 4×10^7 CFU/mL for each strain (Fig. 2.1C). At pH 3.4 and 3.6, bacterial growth was improved, with cell numbers increasing

gradually up to 10^9 CFU/mL (Fig. 2.1D and E). Typically both strains A90 and A89 had significantly better growth than their parent SB3 in MRSAJ between pH 2.8 and pH3.4 ($p<0.05$).

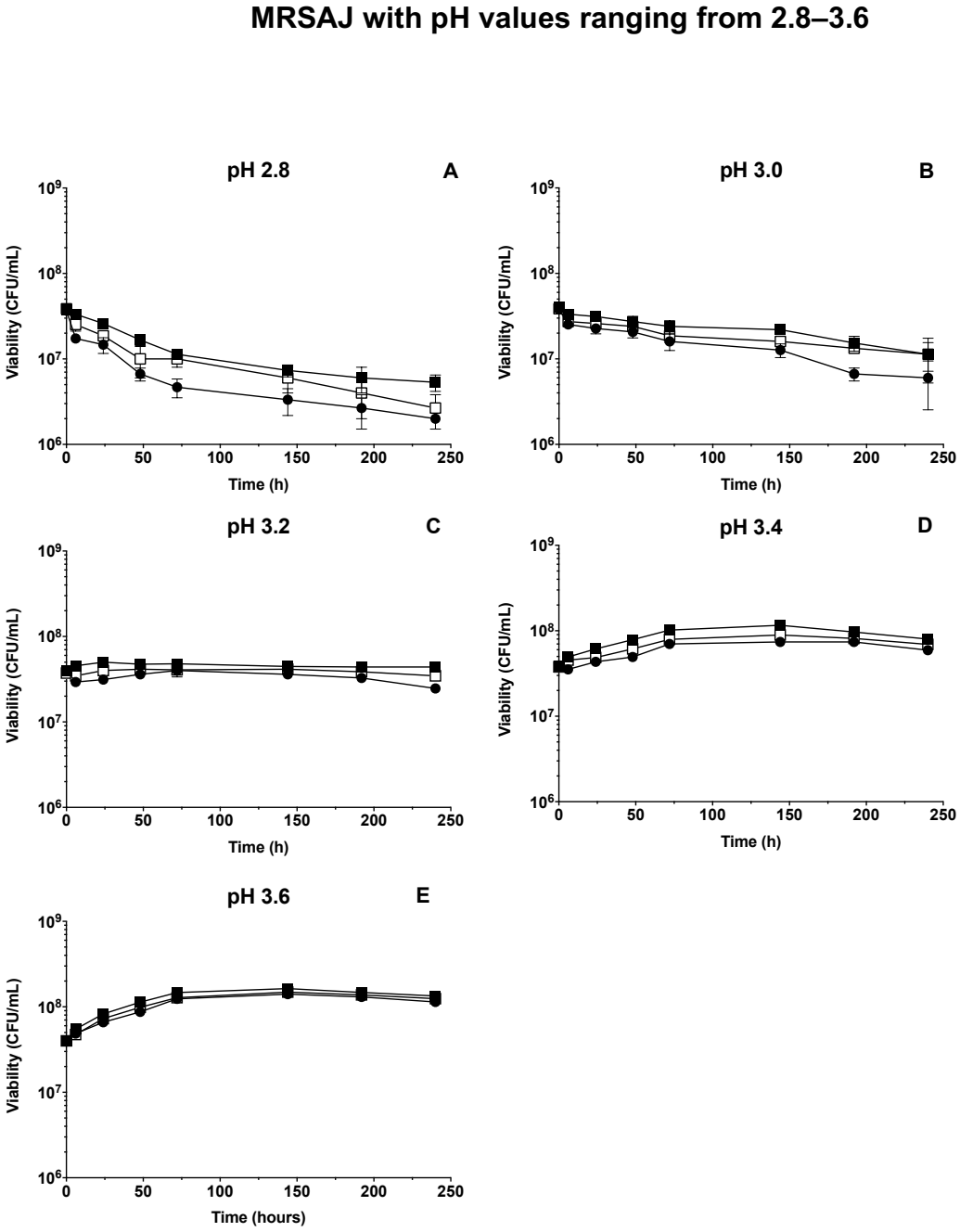

Fig. 2.1. Viability of SB3 (●), A90 (■) and A89 (□) in MRSAJ. Values are the averages of three biological replicates and error bars indicate the standard deviation.

2.3.2 Multi-stress tolerance in MRSAJ

Whilst no increase in viable cell numbers was observed, in MRSAJ with a combination of ethanol and pH stressors (Fig. 2.2 and Fig. 2.3), there were differences between strains.

In the experiment with fixed ethanol concentration (12% (v/v)), viable cell numbers of three tested strains were maintained at ~10^7 CFU/mL at pH 3.6 but decreased at lower pH levels with cell numbers declining by 10^3-fold at pH 2.8 by Day 10 (Fig. 2.2). Overall, viability of strain A90 was significantly greater than strain SB3 under all conditions tested ($p < 0.05$).

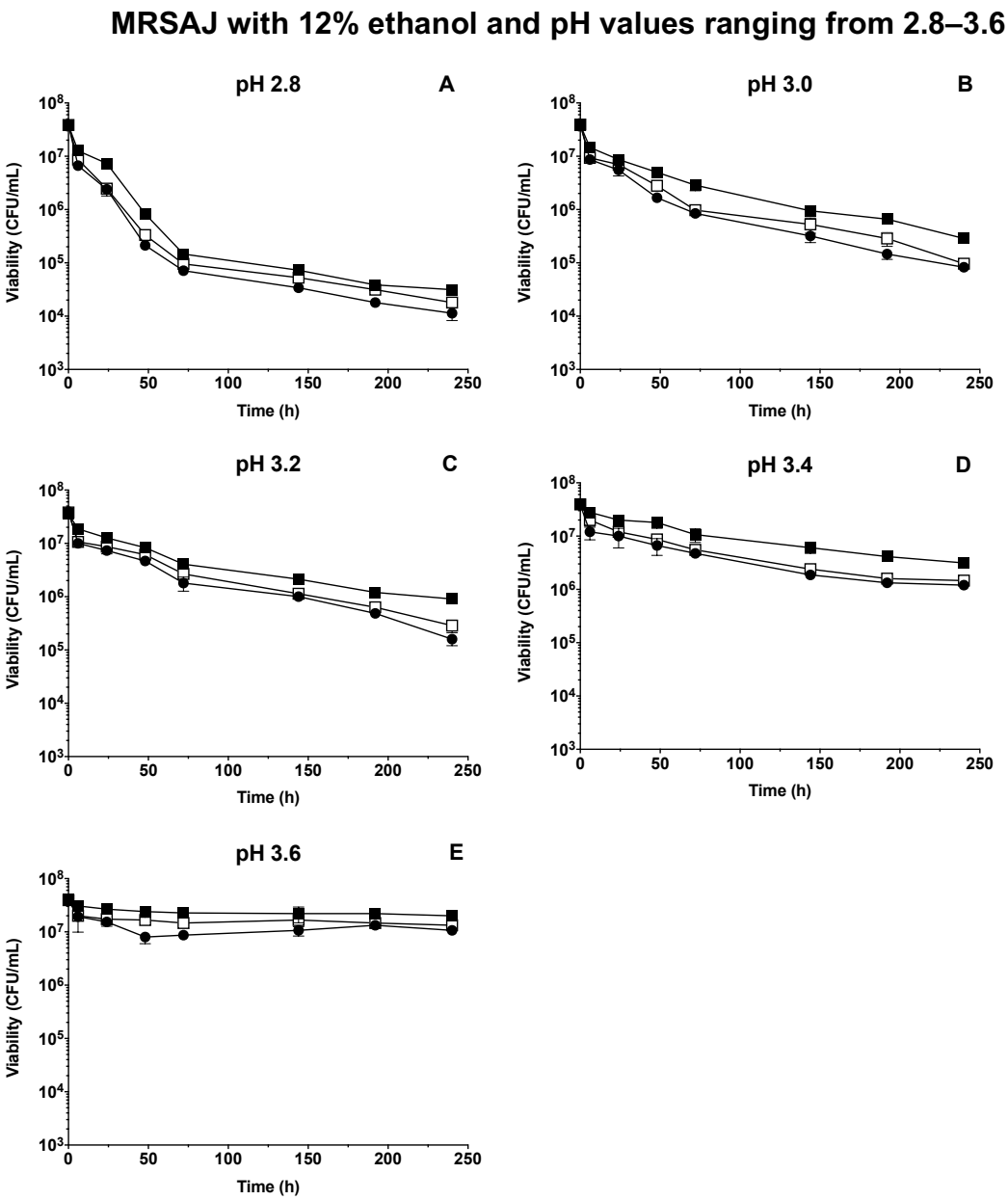

Fig. 2.2. Viability of SB3 (●), A90 (■) and A89 (□) in MRSAJ with 12% (v/v) ethanol and pH ranging from 2.8–3.6. Values are the averages of three biological replicates and error bars indicate the standard deviation.

When pH was fixed at 3.5, viability decreased as ethanol concentration increased (Fig. 2.3). In MRSAJ containing 12% (v/v) ethanol, viable cell numbers of A90 remained over 10^7 CFU/mL throughout the experiment while those of strains SB3 and A89 decreased to less than 10^7 CFU/mL (Fig. 2.3A). By contrast, when

ethanol increased to 18% (v/v), a 10^3–10^4-fold decrease was observed by Day 10 (Fig. 2.3D). An intermediate decrease in viability was seen at 14% and 16% (v/v) ethanol (Fig. 2.3B and C). Strain A90 again performed better than its parent SB3 in medium with 12% ($p = 0.0001$) and 14% (v/v) ($p = 0.0032$) ethanol.

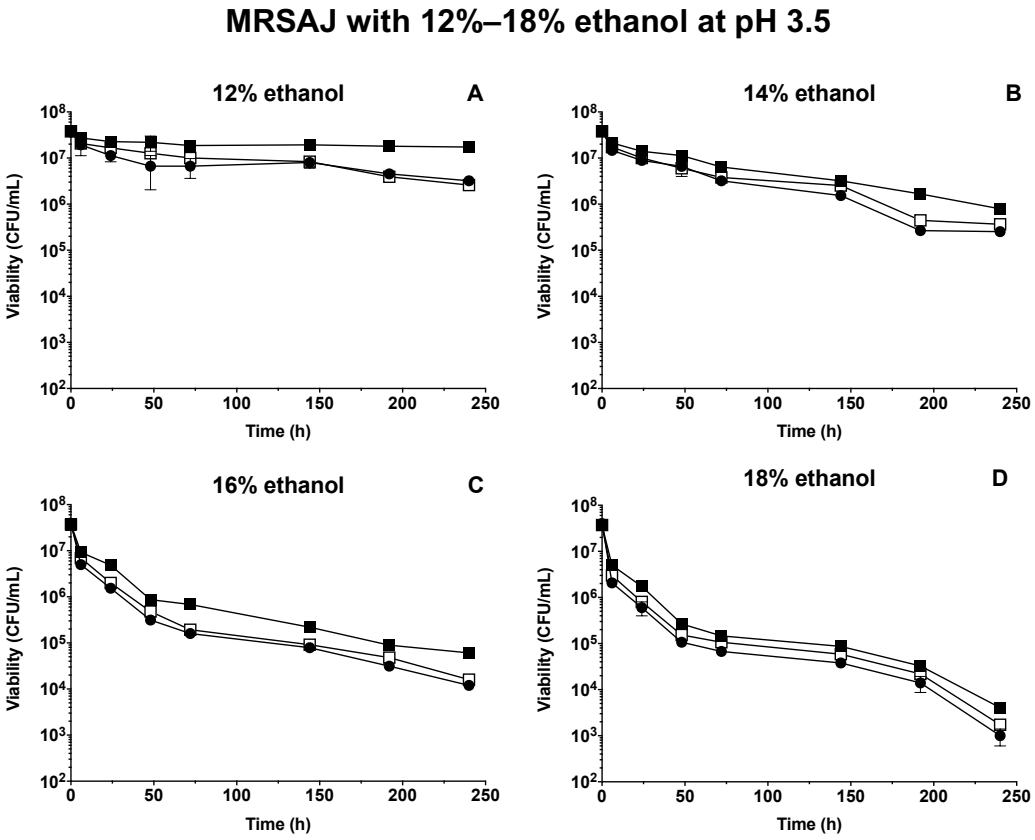

Fig. 2.3. Viability of SB3 (●), A90 (■) and A89 (□) in MRSAJ with 12–18% (v/v) ethanol at pH 3.5. Values are the averages of three biological replicates and error bars indicate the standard deviation.

2.3.3 Multi-stress tolerance in RFCDGJM

In order to evaluate the performance of the strains under conditions more similar to an oenological environment, similar trials were conducted in RFCDGJM, which is more comparable to wine and was fermented by yeast.

With the presence of 12% (v/v) ethanol, pH below 3.2 had an inhibitory impact on *O. oeni* growth and the drop of viability was the fastest and furthest at the lowest pH value. Viable cell numbers reduced by 10 and 100 folds by the last sampling point at pH 3.0 and 2.8 respectively. By contrast, an increased viable population was observed at pH 3.2 and above (Fig. 2.4). Overall, growth or viability of three strains were similar to each other under all tested conditions ($p > 0.05$).

RFCDGJM with 12% ethanol and pH values ranging from 2.8–3.6

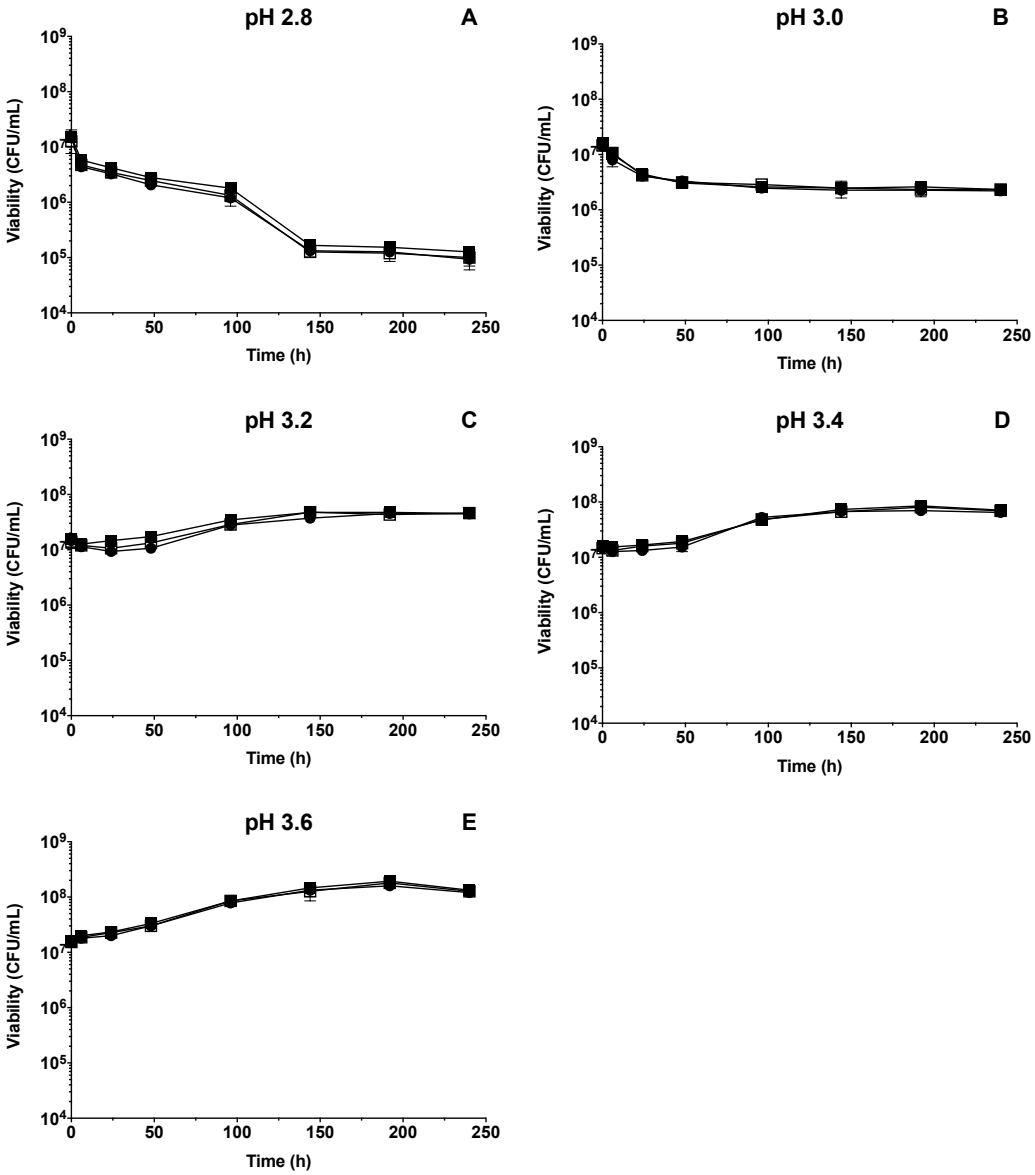

Fig. 2.4. Viability of SB3 (●), A90 (■) and A89 (□) in RFCDGJM with ethanol fixed at 12% (v/v). Values are the averages of three biological replicates and error bars indicate the standard deviation.

All strains survived in RFCDGJM with up to 18% (v/v) ethanol at pH 3.5, but bacterial viability was the greatest in RFCDGJM with the lowest ethanol concentration (Fig. 2.5). Viable cell numbers of three strains increased to ~10^8 CFU/mL at 12% and 14% (v/v) ethanol whereas decreased to ~10^7 and ~10^5 CFU/mL at 16% and 18% (v/v) ethanol (Fig. 2.5). Under all RFCDGJM tested conditions, the strains performed similarly ($p > 0.05$).

RFCDGJM with 12%–18%ethanol at pH 3.5

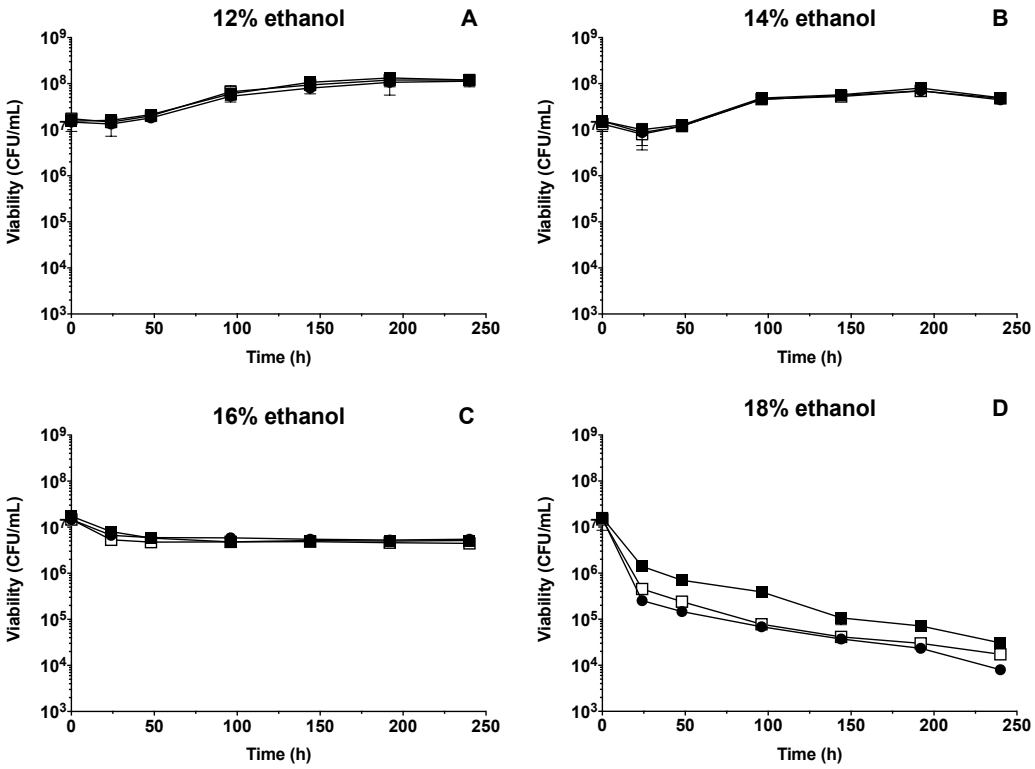

Fig. 2.5. Viability of SB3 (●), A90 (■) and A89 (□) in RFCDGJM ethanol ranging from 12–18% (v/v) at a fixed pH of 3.5. Values are the averages of three biological replicates and error bars indicate the standard deviation.

2.4 Discussion

Whilst it has been previously reported that *O. oeni* are able to grow and survive under certain suboptimal conditions (Britz and Tracey 1990; G-Alegría et al. 2004; Capozzi et al. 2010; and this study). Growth and malolactic activity of *O.*

oeni can be negatively impacted when cells experience stressful conditions, leading to unsuccessful MLF. Two optimised *O. oeni* strains with improved ethanol tolerance in MRSAJ compared to the commercial parent strain, were generated via DE (Betteridge 2015). These strains can survive in MRSAJ with up to 20% (v/v) ethanol (Betteridge 2015). In this study here, tolerance assays were carried out with these improved ethanol tolerant strains to investigate their tolerance to low pH and combinations of ethanol and pH in MRSAJ and a synthetic, wine-like medium, RFCDGJM.

The tolerance assays showed that directed evolution not only improved ethanol tolerance of strain A89 and A90 (Betteridge 2015), but also enhanced their pH, and combined pH and ethanol tolerance in MRSAJ. Strain A90 showed significantly better viability than SB3 in MRSAJ under most tested conditions, indicating tolerance to a broad range of stress. Previous studies have also reported that *S. cerevisae* generated by DE have cross-protective properties (Çakar et al. 2009; Almario et al. 2013).

All strains were culturable in MRSAJ media at pH 3.2 and above with no ethanol. This was in agreement with what has been reported by others (Britz and Tracey 1990; G-Alegría et al. 2004). However, these previous studies did not test *O. oeni* growth at pH values less than 3.2. Short-term acid shock at pH 3.0 has been reported not to affect *O. oeni* viability but increased membrane rigidity (Chu-Ky et al. 2005). Three *O. oeni* strains in this study possessed a good resistance to low pH in MRSAJ since they survived in pH values down to 2.8 for 10 days. The combination of low pH and ethanol had a synergistic inhibitory effect on cell numbers. In this study, viability of strain A90 dropped to 65% of the initial inoculation rate within 6 h in MRSAJ containing 14% ethanol at pH 3.5. Whereas

under the same levels of pH and ethanol, viability of strain *O. oeni* ATCC BAA-1163 was decreased more quickly by 45% in just 5 minutes with total cell death after 30 minutes (Chu-Ky et al. 2005). Different media in this study could lead to the difference in the results. Nonetheless, strain A90 was likely to be more stress tolerant than strain ATCC BAA-1163.

Bacterial growth was different in RFCDGJM, when compared to in MRSAJ with the same stressors. Cell numbers of all strains declined to different extents in MRSAJ with combined ethanol and pH. However, viability of the strains was only reduced in RFCDGJM under the more severe conditions (eg. ethanol 12% (v/v), pH 2.8–3.0; ethanol 16–18%, pH 3.5). Under other conditions, all strains increased in viable cell numbers, implying that RFCDGJM has a more complex composition both in formulation of the base medium and having been fermented by yeast. Some nutrients such as amino acid and Mn^{2+} in RFCDGJM may stimulate bacterial growth (Terrade and Orduña 2009). Overall, growth in RFCDGJM was similar across the three strains, which suggested that previously optimised strains might not have a significant advantage of consuming malic acid in wines.

2.5 Conclusion

This study further characterised previously improved *O. oeni* strains for low pH tolerance and combined ethanol/pH tolerance of in MRSAJ and RFCDGJM. Strain A89 and A90 typically performed better than SB3 in MRSAJ, but similarly to SB3 in RFCDGJM. Whilst the composition of RFCDGJM is more comparable to wine than MRSAJ, the results suggest that strains A89 and A90 are unlikely to have satisfactorily improved multi-stressor tolerance in wine/synthetic wine media. These results indicate that there is still scope to further improve *O. oeni* strains to tolerate multiple stresses typical of winemaking. Nevertheless, strain A90 grew

significantly better than strain A89 in MRSAJ conditions, and was therefore chosen as a parent strain to be further optimised to allow it to possess desirable MLF performance under multiple stressors in oenological conditions. The next chapter describes the application of directed evolution to A90, isolation and characterisation of single clones from the evolving cultures.

Chapter 3 Directed evolution of *Oenococcus oeni* strains for more efficient malolactic fermentation in a multi-stressor wine environment

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Principal Author

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Contribution to the Paper	Performed the experiment, analysed data and wrote the manuscript		
Overall percentage (%)	70%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	06/09/2017

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Abstract

High concentrations of ethanol, low pH, the presence of sulfur dioxide and some polyphenols have been reported to inhibit *Oenococcus oeni* growth, thereby negatively affecting malolactic fermentation (MLF) of wine. In order to generate superior *O. oeni* strains that can conduct more efficient MLF, despite these multiple stressors, a continuous culture approach was designed to directly evolve an existing ethanol tolerant *O. oeni* strain, A90. The strain was grown for ~350 generations in a red wine-like environment with increasing levels of stressors. Three strains were selected in screening experiments based on their completion of fermentation in a synthetic wine medium with 15.1% (v/v) ethanol, 25 mg/L SO_2 at pH 3.3 within 160 h, while the parent strain fermented no more than two thirds of L-malic acid in this medium. These superior strains also fermented faster and/or had a larger population in four different wines. A reduced or equivalent amount of the undesirable volatile, acetic acid, was produced by the optimised strains compared to a commercial strain in Mouvedre and Merlot wines. The above findings demonstrate the feasibility of using directed evolution as a tool to generate more efficient MLF starters tailored for wines with multiple stressors.

Key words: directed evolution; *Oenococcus oeni*; multi-stressor; malolactic fermentation; wine

1. Introduction

Malolactic fermentation (MLF), which involves the conversion of L-malic acid to L-lactic acid by lactic acid bacteria (LAB), is required in most red wines and some white wines (Davis et al. 1985). This process not only deacidifies the wine, to produce a softer mouthfeel, but also increases microbial stability of the wine by removing biologically active organic acids. The LAB strains that typically conduct MLF due to their better tolerance of wine-related stressors belong to the species *Oenococcus oeni* (Wibowo et al. 1985).

While MLF is desirable because of the benefits it confers to wine, it is often difficult for bacteria to complete due to poor growth or malolactic activity in wine. High ethanol, low pH, SO_2, medium chain fatty acids and some phenolic compounds are common inhibitors to bacterial growth. For example, Capucho and San Romão (1994) reported that an ethanol content between 8 and 12% (v/v) strongly inhibited *O. oeni* cell growth. Even though 8–12% (v/v) ethanol had a negative effect on bacterial growth, it had little effect on malolactic activity. When ethanol concentration was increased to 18% (v/v) and more, the malolactic activity was also eliminated (Capucho and San Romão 1994). Ethanol-stressed cells of *O. oeni* exhibit increased membrane fluidity and a reduced production of ATP (Da Silveira et al. 2003; Da Silveira and Abee 2009). The presence of SO_2, medium chain fatty acids and low pH also impairs the ATPase activity of *O. oeni*, resulting in a reduced population and prolonged fermentation (Tourdot-Maréchal et al. 1999; Carreté et al. 2002). Medium chain fatty acids can be produced by yeast during alcoholic fermentation. SO_2 is often added during winemaking to avoid oxidation and restrict the growth of unwanted microorganisms (Ough and Crowell 1987; Takahashi et al. 2014). Phenolic compounds have a dual effect on bacterial

growth. Some hydroxybenzoic acids and their derivatives, phenolic aldehydes, flavonoids and tannins inhibit *O. oeni* growth and viability (Figueiredo et al. 2008; Campos et al. 2009; García-Ruiz et al. 2011), while gallic acid and free anthocyanins activate cell growth (Vivas et al. 1997). The growth of *O. oeni* can also be restricted by nutrient limitation, with several amino acids such as cysteine being essential (Saguir and Manca de Nadra 2002).

The combination of stressors, such as found in the complex matrix that is wine, can produce an even more severe inhibitory effect on *O. oeni* growth compared to a single stressor (reviewed by Betteridge et al. 2015). For example, a combination of pH at 3.2 and 26 mg/L of SO_2 is more inhibitory of MLF compared to the individual stressors of either pH or SO_2 (Nielsen et al. 1996). Exposure to 10% (v/v) ethanol at pH 3.5 for 30 minutes leads to near total cell death whereas 75% and 70% cell viability were retained upon exposure to pH at 3.0 or 10% (v/v) ethanol, respectively, for the same time (Chu-Ky et al. 2005).

Given the multi-stressor environment of wine, and hence potential delays in completion of wine processing and stabilisation, more robust LAB strains with improved tolerance under such conditions are needed to improve the efficiency of MLF. Torriani et al. (2010) summarised the criteria for selection of improved LAB, which included that more efficient LAB starters must not only grow and ferment desirably in an environment with ethanol content (over 14% (v/v)), low pH (under 3.0), high SO_2, but also produce pleasant aromas to enhance the sensory profile of the wine.

Two main approaches can be used for strain improvement: recombinant and non-recombinant methods (Betteridge et al. 2015). Recombinant methods require a good knowledge of the genetic basis of the attribute in question, while

non-recombinant methods do not necessarily require such knowledge (Hindré et al. 2012; Betteridge et al. 2015). A few transformation experiments have been conducted successfully, however, they either could not be repeated by other laboratories, or may have low transformation efficiency for *O. oeni* (Dicks 1994; Assad-García et al. 2008). Recently, successful transformation experiments with *O. oeni* were reported, however, transformation efficiencies were not provided (Darsonval et al. 2016a; b). Also, even though recombinant methods are precise, some consumers may reject food or wine produced by genetically modified microorganisms (Pretorius and Bauer 2002).

Non-recombinant methods, including mutagenesis, classical strain selection and directed evolution (DE), require no specific genetic knowledge, but need effective screening processes to select the best candidate(s). UV mutagenesis has been successfully applied to *O. oeni*, and a strain with greater malic acid utilisation and enhanced softness index as well as reduced wine decolouration was selected from treated isolates (Li et al. 2015). Even though mutagenesis is easy to perform, disadvantages relate to the fact that the random mutations might not generate the desired phenotype or produce undesired ones. Classic strain selection involves the isolation and screening of LAB from environments such as wineries. Many strains that show promise for carrying out MLF have been selected using this method (Capozzi et al. 2010; Berbegal et al. 2016; Iorizzo et al. 2016). However, strains with excellent combinations of technological traits (e.g. high performance in MLF, desired flavour attributes) are unlikely to be identified only by relying on natural diversity, otherwise such strains might have already been found (Betteridge et al. 2015).

Directed evolution (DE) can be straightforward to establish, however, it can be time consuming. Optimal strains are generated as the population struggles to adapt to the stressors applied in the DE system. Two approaches of DE, a sequential batch system or continuous culture, can be performed with LAB based on the time frame of DE and the generation time of the target bacteria. An acid-resistant mutant of *Lactobacillus casei* was obtained by sequentially transferring a culture into MRS with decreasing pH levels over 70 days (Zhang et al. 2012). A sequential batch system has also been successfully applied to *Lb. plantarum* and *Lactococcus lactis* (Teusink et al. 2009; Bachmann et al. 2012). In terms of generating more robust *O. oeni* for ethanol resistance, DE has been successfully performed on a commercially available strain LACTOENOS SB3 (henceforth SB3) in De Man, Rogosa and Sharpe medium (MRS) (de Man et al. 1960) supplemented with 20% (v/v) of apple juice (MRSAJ) using a continuous culture system (Betteridge et al. 2017). In that work, the evolved strain A90 had a higher ethanol tolerance (at 22% (v/v)) than the parent SB3 in MRSAJ.

While the previous proof-of-concept study on optimising *O. oeni* by DE focused on ethanol tolerance in MRSAJ, some of the factors described above, such as low pH and the presence of SO_2 in wine, can also be inhibitory to bacterial growth and MLF, and should be factored into strain optimisation. DE offers significant potential to derive novel *O. oeni* strains suited to the multiple stressors present during winemaking. Since *O. oeni* with good ethanol resistance can be generated by DE, it was hypothesised that deliberate application of multiple selective pressures through long-term laboratory evolution could be used to produce *O. oeni* strains more adapted to the multi-stress winemaking environment to yield strains more reliable for completing MLF.

2. Materials and methods

2.1 Bacterial cultures

SB3 is a commercial *O. oeni* strain from Laffort Oenologie. *O. oeni* A90 (A90) was generated from SB3 via DE and has improved ethanol tolerance in MRSAJ (Betteridge et al. 2017). A90 was used as the parental strain for DE performed in this study.

2.2 Media and wine

MRS (AM 103, Amyl Media, Australia) supplemented with 20% (v/v) apple juice was used to culture bacteria. The medium was sterilised at 121˚C, 0.1 MPa for 20 minutes before use. A synthetic wine medium was prepared from a chemically defined grape juice medium (McBryde et al. 2006) supplemented with 5% (v/v) grape tannin extract (GSKINEX, Tarac Technologies, Australia) and fermented by *Saccharomyces cerevisiae* yeast to produce Red Fermented Chemically Defined Grape Juice Medium (RFCDGJM). This RFCDGJM was then supplemented with analytical reagent grade ethanol and L-malic acid and used as the DE feed and in the first and second round screening experiments (Table 1).

A Shiraz wine made in 2015 at the University of Adelaide's Waite Campus and in which MLF became stuck at 0.8 g/L malic acid was considered suitable as a medium for strain improvement. This wine was supplemented with L-malic acid and ethanol for DE feed and the third screening experiment (Table 1). Wines used to further characterise the best DE isolates were Mouvedre from McLaren Vale, as well as Shiraz and Merlot from the Waite Campus, Urrbrae, and Pinot Noir from the Adelaide Hills, SA, from the 2016 vintage. All wines, except the Pinot Noir, had not undergone MLF. An extra 2.70 g/L of L-malic acid was added to the Pinot Noir

to test the performance of evolved *O. oeni* strains under nutrient deficient conditions (Table 1). RFCDGJM and all wines were filter sterilised (0.22 µm) before use.

Table 1. The key compositional features of RFCDGJM and wines used in this study

Main parameters	RFCDGJM	2015 Shiraz	2016 Mouvedre	2016 Shiraz	2016 Merlot	2016 Pinot Noir
Total SO_2 (mg/L)	0	34	3	0	1	10
Glucose (g/L)	2.41	0.06	1.07	0.55	1.00	0.98
Fructose (g/L)	0.36	0.52	1.17	2.11	1.72	1.87
Citric acid (g/L)	3.19	0.69	0	0	0	0
Tartaric acid (g/L)	2.08	2.07	2.07	1.84	1.95	0.88
Succinic acid (g/L)	0.88	8.6	4.02	4.38	2.49	1.66
L-Malic acid (g/L)	1.50 – 3.22	1.90 – 2.93	2.47	2.33	2.41	2.70
Ethanol (% (v/v))	10.9 – 16	13.7 – 20	13.9	16.7	15.6	14.2
pH	3.30 - 3.50	3.30 - 3.50	3.37	3.51	3.55	3.51

2.3 Preparation and inoculation of *O. oeni* strains

All the strains were cultured in MRSAJ before inoculation into the experimental medium. Except for the micro-scale screening experiments, for which a simplified inoculation regime was used (see below), growth of bacterial starter cultures was monitored at 600 nm (OD_{600}) using a Helios Cuvette spectrophotometer (Thermo Scientific). Once OD_{600} reached 1.5, the liquid cultures were diluted with MRSAJ to OD 0.7 or 1.2, depending on the purpose of the fermentation experiments. A final volume of 2% (v/v) of starter culture was then inoculated into each experimental medium.

2.4 Bacterial enumeration

Viable cell numbers were determined by serially diluting the sample with ultra-pure deionised water following by spot-plating 5 µL droplets on MRSAJ agar plates. These were incubated at 30 ˚C with 20% (v/v) CO_2 for 7 days before the colonies were counted.

2.5 Chemical analyses

The concentration of ethanol was determined by an Alcolyser Wine ME/DMA 4500M (Anton Paar, Australia). L-malic acid was measured using an enzymatic test kit (4A165, Vintessential laboratories, Australia) with modifications so that a Tecan spectrophotometer (Infinite 200 PRO, Tecan, Männedorf, Switzerland) could be used to read absorbance. Specifically, each well of a 96 well micro-titre plate was dosed with 70 µL buffer, 14 µL nicotinamide adenine dinucleotide, 70 µL distilled water, 0.7 µL glutamate oxaloacetate transaminase and 5 µL sample or one of the standards (ranging from 0–3.0 g/L). The plate was incubated at 22 ˚C for 3 minutes and the first absorbance was read at 340 nm; 7 µL of the 1:10 diluted L-malate dehydrogenase was added and mixed into each well; the plate was incubated at 22 ˚C for 15 minutes before the second absorbance was measured at 340 nm. L-malic acid in each sample was calculated from standard curves prepared with known concentrations. Fructose and glucose were quantified enzymatically using commercially available kits (K-FRUGL, Megazyme, Ireland) following methods modified by Walker et al. (2014). Citric, tartaric, lactic and acetic acids were determined by HPLC (Agilent 1100, USA). An Aminex HPX-87H column (Bio-Rad, USA) was used under the following conditions: column temperature, 60 ˚C; mobile phase, 2.5 mM H_2SO_4; flow rate, 0.5 mL/min, and 20 µL volume injection. The organic acids were measured by a DAD-1C

detector at 210 nm. Concentrations of the organic acids were calculated from calibration curves using Agilent ChemStation (Agilent, USA). The concentration of free and total SO_2 was measured by the aspiration method (Rankine and Pocock 1970).

2.6 Directed evolution of *O. oeni* for optimised MLF under multi-stressor conditions

The establishment of a continuous DE system using a sterilised bioreactor (Sartorius BBI system GmbH, Germany) was based on the method previously used in this laboratory (Betteridge et al. 2017) with a few modifications. DE began in RFCDGJM at pH 3.5 with 10.93% (v/v) ethanol at 22 °C. When bacteria were in the late-exponential phase, fresh RFCDGJM supplemented with extra ethanol was fed into the bioreactor vessel continuously at a rate of 12 mL/hour to maintain turbidity of 0.09-0.11, which is equivalent to OD_{600} of 0.4-0.6. Ethanol concentration was increased gradually to 13.5% (v/v) and pH was decreased to 3.3, over 350 days. DE was then moved to a second stage by gradually changing the medium to wine: 2015 Shiraz and RFCDGJM were supplied together to the culture, with the Shiraz feed gradually increasing over 100 days (MFCS/Win v3.0 config 2355 software; Sartorius BBI system GmbH, Germany) and according to a set culture turbidity as described. DE was terminated when the ethanol concentration reached 16.5% (v/v). Samples from the bioreactor were collected weekly for L-malic acid analysis and enumeration of bacteria.

2.7 Characterisation of *O. oeni* strains

Characterisation of SB3 and A90 was conducted in MRSAJ and RFCDGJM with 15% (v/v) ethanol. Preliminary characterisation of DE isolates was performed in micro-scale fermentations in micro-titre plates when *O. oeni* was evolved for

approximately 150 (*O. oeni* 1-1 to *O. oeni* 1-188), 220 (*O. oeni* 2-1 to *O. oeni* 2-110) and 350 (*O. oeni* 3-1 to 3-84) generations using the method described by Betteridge et al. (2017). Randomly picked colonies were inoculated into MRSAJ in deep-well plates, grown for 3 days at 30 ˚C before a 1 in 4 dilution in fresh RFCDGJM and introduction of 10 µL of each culture into 190 µL of the appropriate medium in a micro-titre plate. Replicate plates (10 copies) were used to allow sacrificial sampling at the required intervals. Plates sealed with titre tops were incubated at 22 ˚C for 12-20 days with 20% (v/v) CO_2. Strains that fermented more efficiently were then evaluated by laboratory-scale fermentations in 15 or 50 mL tubes in various media. The best performing strains were further characterised in red wines. To monitor laboratory-scale fermentations, 400 µL samples were collected aseptically every 24 to 48 hours. Half of the sample was immediately frozen at -20 ˚C for subsequent analysis of L-malic acid, while the remainder was used for microbial enumeration. All fermentations were conducted at 22 ˚C. The first and second micro-scale screening experiments were performed in duplicate while the final micro-scale screen was performed in quadruplicate. All larger laboratory-scale fermentations were performed in triplicate.

2.8 Data analysis

Data was processed using Microsoft Excel 2011. Plotting of graphs and statistical analysis was performed using Graphpad Prism 7.0 (GraphPad Software, Inc. USA). The student's t-test was used to compare the means of L-malic acid and CFUs of SB3 and A90 for MLF in MRSAJ and RFCDGJM. Two-way ANOVA – repeated measures and Fisher's least significant distance test was used to analyse L-malic acid consumption and viable cell numbers among each strain in laboratory-scale MLF. One-way ANOVA and Turkey's honest significant difference

tests were performed to compare concentrations of organic chemicals of wines after MLF among the tested strains. A confidence interval for each statistical analysis was set at 95%.

3. Results

3.1 Evolved strain A90 had similar MLF performance and growth to SB3 in a synthetic wine medium

To benchmark the MLF performance of SB3 *vs* A90, the strains were inoculated into MRSAJ (pH 5.5, 15% (v/v) EtOH) and RFCDGJM (pH 3.5, 15% (v/v) EtOH) at two cell densities (OD_{600} = 0.7 and 1.2). Both SB3 and A90 consumed malic acid faster at higher inoculation rates, as would be expected (Figure 1). L-malic acid degradation by A90 was significantly faster than SB3 in MRSAJ at both lower ($p = 0.0007$) and higher ($p = 0.0013$) inoculation rates (Figure 1A). Both strains completed fermentation of RFCDGJM, but only A90 completed MLF in MRSAJ, while its parent SB3 consumed just 60% of total L-malic acid in MRSAJ. In terms of growth in MRSAJ, viable cell numbers of both strains changed little during the course of fermentation, with the exception of the lower inoculation density of SB3, which decreased steadily throughout MLF (Figure 1B).

When inoculated into RFCDGJM (OD_{600} = 0.7), SB3 initially showed greater consumption of L-malic acid compared to A90; however, both strains ultimately completed MLF together on Day 15 (Figure 1C). No significant difference in L-malic acid consumption was detected between SB3 and A90 at the higher inoculum density ($p = 0.2585$). The trends in viable bacterial numbers (Figure 1D) were not significantly different between the 2 strains with either inoculation strategy ($p > 0.05$ in each situation).

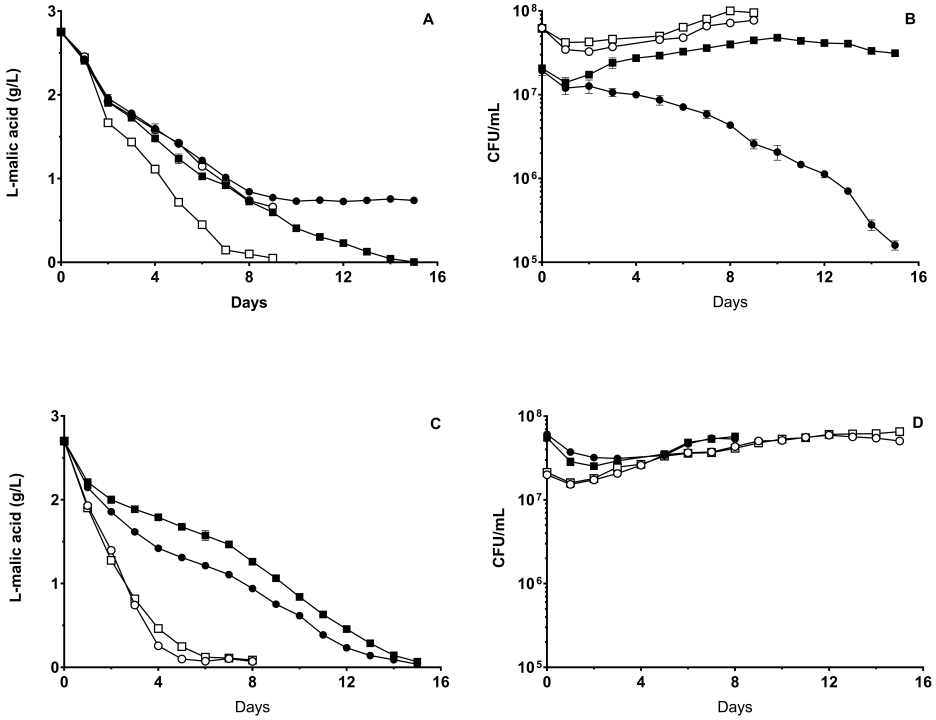

Figure 1. L-malic acid consumption (A, C) and bacterial population change (B, D) for MLF in MRSAJ (A, B) and RFCDGJM (C, D) fermented by SB3 (○: higher inoculum density; ●: lower inoculum density) and A90 (□: higher inoculum density; ■: lower inoculum density). Each point is the mean of triplicates ± SD.

3.2 Optimisation of A90 for multi-stress tolerance via directed evolution

To broaden the stress tolerance of A90, which had previously been evolved to be more ethanol tolerant, the strain was subjected to continuous culture under multi-stressor conditions (initially 10.9% (v/v) ethanol, pH 3.5, 5% (v/v) grape tannin extract, 22 °C). During the course of DE as the ethanol content increased toward 13.5% (v/v) and the pH decreased to 3.3, cell doubling time increased from 16 h to 72 h by the time the DE experiment was terminated (data not shown). The total number of elapsed generations was estimated based on doubling times calculated at different points during the DE experiment and the total time-frame of the experiment. Accordingly, approximately 350 generations were achieved by the

point when total SO_2 concentration, pH and the percentage of Shiraz in the medium reached 28 mg/L, 3.3 and 81% (v/v), respectively.

3.3 Evaluation and selection of individual clones in multi-stressor synthetic wine medium.

Samples were collected from the DE experiment at approximately 150, 220 and 350 generations, and individual clones were evaluated for their relative performance to the parent strain, A90, in micro-scale and laboratory-scale screening experiments. Fermentation performance ranged broadly amongst the isolates in each screen (Figure 2). After only 150 generations, evolved strains 1-143 and 1-161 with improved MLF efficiency were identified (Figure 2A). Strains 2-47 and 2-49 outperformed the other isolates and A90 in the second micro-scale screen with a fermentation duration of 8 days (Figure 2B). The basis for selection of candidate strains was an improved relative performance in comparison to A90, not absolute fermentation time. Differences in the latter were attributed to the non-standardised inoculation regimes (see Methods) used for convenience in the screening stage (data not shown). Larger-scale screens were conducted in RFCDGJM and in 50 mL tubes, which allowed standardisation of inoculation rates and conditions. In this way strains 1-143, 1-161, 2-47 and 2-49 confirmed their superiority as they completed MLF sooner than other selected isolates and strain A90 (S1 and S2, see Appendix 1). A mixed population of tested isolates was included in each micro-scale screening experiment to represent the average performance of the evolving culture. The mixed culture in each screen consumed more malic acid than the starting strain A90.

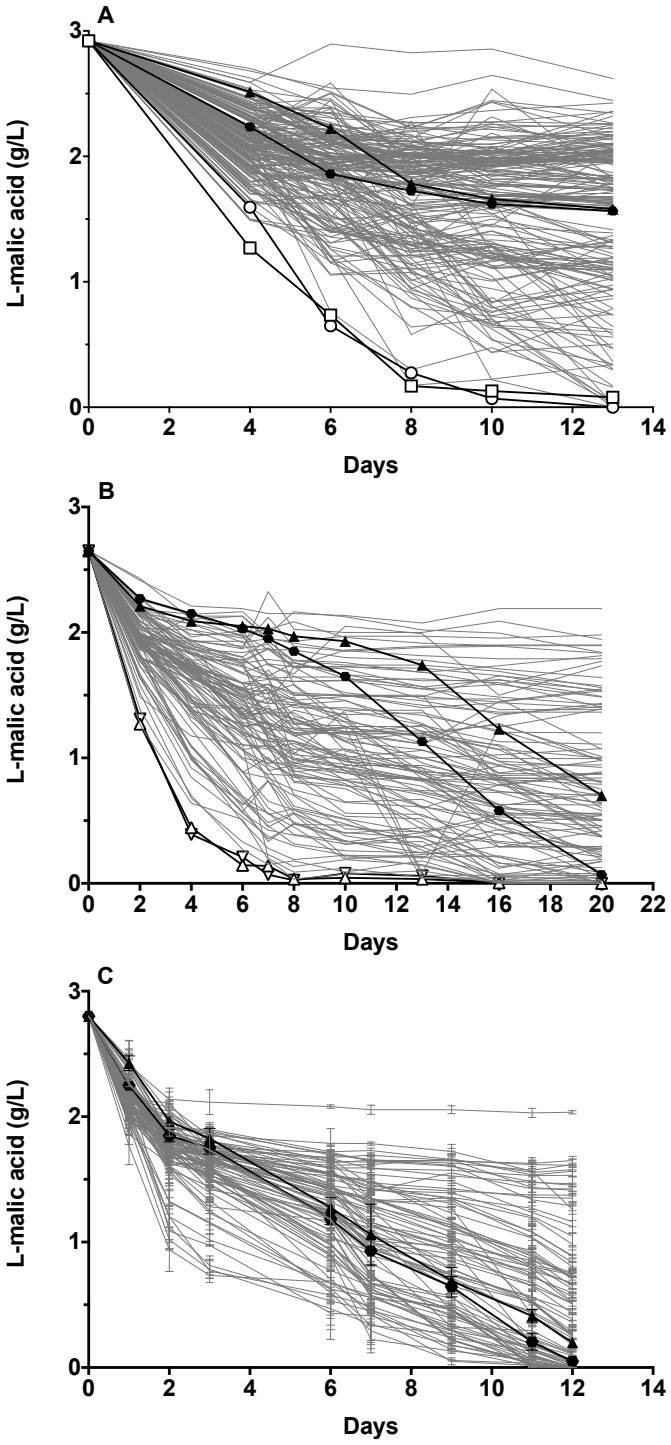

Figure 2. Evaluation of DE single isolates via three rounds of micro-scale screens at 150 generations (A), 220 generations (B) and 350 generations (C). A90 (▲); mixed population at each stage (●); strain 1-143 (□); strain 1-161 (○); strain 2-47 (△); strain 2-49 (▽). Selective media used in the first and second screens were RFCDGJM with 14% (v/v) ethanol at pH 3.3 with 2.92 and 2.65 g/L of L-malic acid, respectively. A mixture of 25% (v/v) RFCDGJM and 75% (v/v) Shiraz 2015 with 14.5% (v/v) ethanol, 2.8 g/L L-malic acid and 26 mg/L of total SO_2 at pH 3.4 was used to perform the third micro-scale screening experiment. Each point in A and B is the average of duplicates; each point in C is the mean ± SD of quadruplicates.

With a view to further improving the evolving population, the DE experiment continued until the increasing concentrations of ethanol and SO_2, and decreasing pH of the evolving media appeared to markedly reduce bacterial survival. At this point when the bioreactor was last sampled, only 84 individual clones were isolated from spread-plates of the undiluted culture ahead of the third micro-scale screen. Thirty-three out of 84 clones completed this micro-fermentation in selective media (Figure 2C).

These clones were then tested in a more stressful medium in 15 mL MLF cultures, along with the two best isolates from each of the previous screens. Fermentation performance differed drastically among the strains ($p<0.0001$). Nine strains, including six from the third screen consumed all L-malic acid in the medium within 6 days (Figure 3A). Degradation of L-malic acid by SB3 and A90 slowed gradually, leaving 0.82 and 0.47 g/L L-malic acid, respectively, when MLF stalled (Figure 3A). A further screening under harsher conditions resulted in no strain completing MLF, but still showed a more extensive utilisation of L-malic acid by e.g. strains 1-143, 1-161, 2-49 and 3-83 compared to SB3 and A90 (Figure 3B). Strains 1-161, 2-49 and 3-83 were further characterised in red wines.

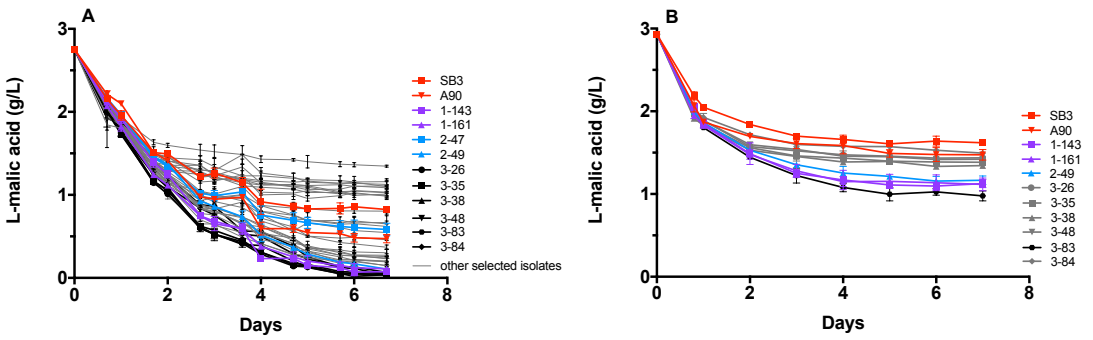

Figure 3. Strains 1-161, 2-49 and 3-83 were identified as superior in the 15 mL screen (A) and 50 mL screen (B). Selective medium used in were: (A) 25% (v/v) RFCDGJM, 75% (v/v) Shiraz 2015 with 15.1% (v/v) ethanol, 2.75 g/L L-malic acid, 26 mg/L total SO_2 at pH 3.35; (B) 20% (v/v) RFDGJM, 80% (v/v) Shiraz 2015 with 15.7% (v/v) ethanol, 2.93 g/L L-malic acid, 28 mg/L total SO_2 at pH 3.3. Each point is the mean of triplicates ± SD.

3.4 Superior DE candidates ferment faster than A90 and SB3 in red wines

The three DE isolates with the best MLF performance in synthetic wine-like media were tested in four red wines, each with differing chemical compositions, to test if they maintained improved MLF performance compared to the parent.

In the Mouvedre wine (Figure 4A), strains SB3, A90 and 2-49 had a similar MLF performance. They all completed fermentation by Day 13. Strains 1-161 and 3-83 outshone other strains in having fermentation durations of 8 and 9 days, respectively, being 38% and 31% quicker than A90. Throughout fermentation, the population of all strains was over 10^6 CFU/mL with minimal differences between these strains (Figure 4B). The Shiraz wine had an ethanol concentration of 16.7% (v/v) that was presumed to lead to a loss of viability (Figure 4D) and stuck MLF for all tested strains (Figure 4C). All DE derived strains consumed malic acid quicker than SB3 and A90 from Day 2 in the Shiraz wine and this advantage was extended. Eventually, 16%, 52% and 45% more L-malic acid was degraded by strains 1-161, 2-49 and 3-83 respectively, compared to A90. However, CFUs were not largely different (Figure 4D). Strains 1-161, 2-49 and 3-83 also completed MLF sooner in the Merlot wine, with corresponding fermentation durations of 11, 9 and 11 days. In comparison, strain SB3 utilised all malic acid within 18 days, whereas A90 appeared to arrest after about 10 days (Figure 4E). Trends in CFUs were an inverse reflection of the MLF behaviour of these strains (Figure 4F). In post-MLF (the nutrient deficient) Pinot Noir wine, even though fermentation slowed and eventually halted for all strains, DE-derived strains consumed more malic acid than SB3 and A90. Specifically, 19%, 57% and 47% more L-malic acid was utilised by strains 1-161, 2-49 and 3-83, compared to strain A90 (Figure 4G).

Apart from the utilisation of L-malic acid, the bacteria also metabolised glucose, fructose and other carbon sources (Table 2). Acetic acid can be formed as a secondary metabolite during MLF (Nehme et al. 2010). In the Mouvedre wine, strains A90, 1-161, 2-49 and 3-83 produced less acetic acid than the commercial strain SB3 during MLF. Acetic acid production of strains SB3, 1-161, 2-49 and 3-83 was similar to that in Merlot wine, but higher than that of A90 (Table 2).

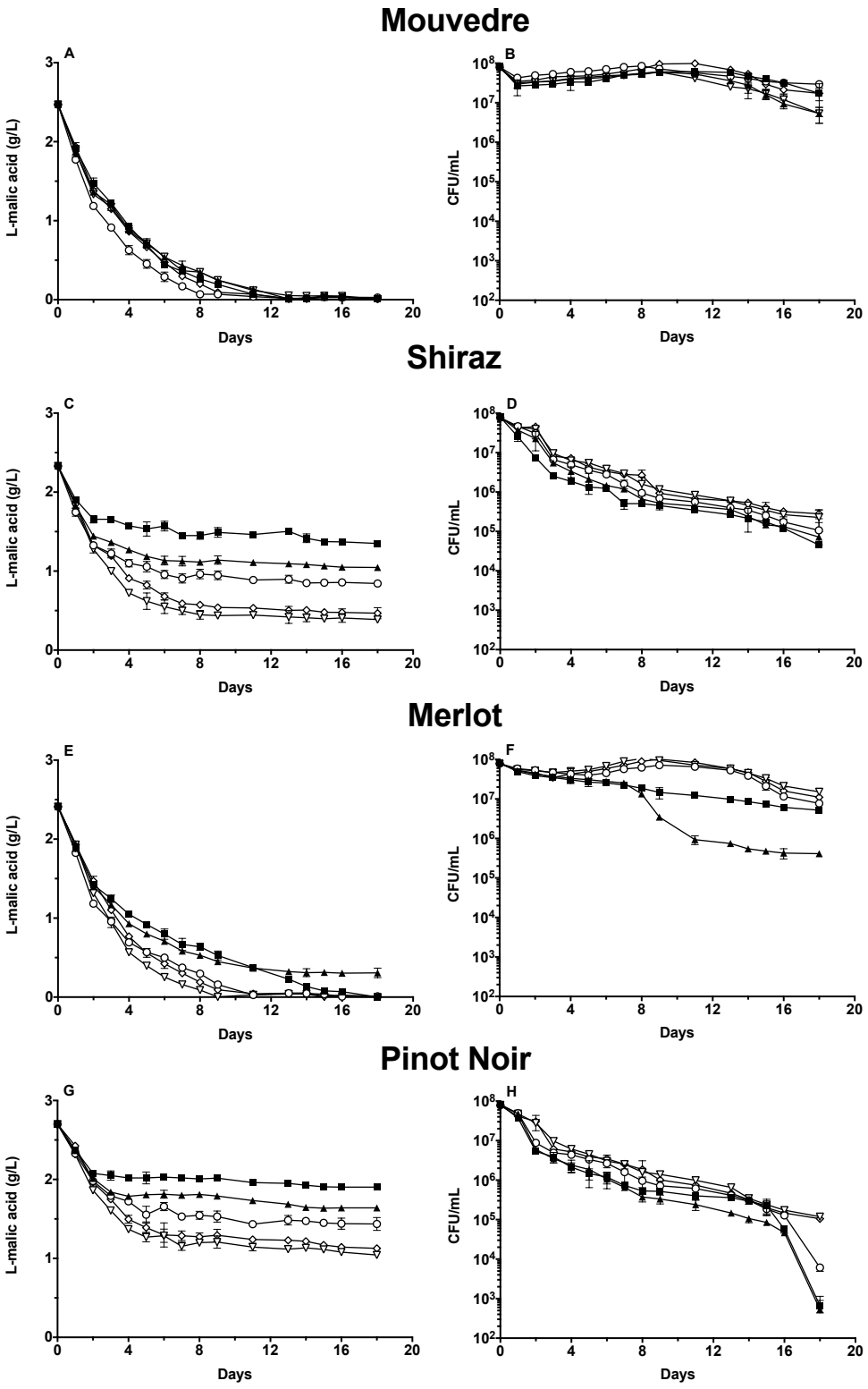

Figure 4. Malic acid utilisation and growth of DE derived strains 1-161(○), 2-49 (▽), and 3-83 (◇) and the parent strains SB3 (■)and A90 (▲)in four red wines. Each point is the mean of triplicates ± SD.

Table 2 Changes in consumption and production of sugars and acids during MLF in Mouvedre and Merlot wines

	Mouvedre					Merlot				
	SB3	**A90**	**1-161**	**2-49**	**3-83**	**SB3**	**A90**	**1-161**	**2-49**	**3-83**
Glucose consumed	0.92 ± 0.02^{a}	0.84 ± 0.01^{ab}	0.80 ± 0.01^{b}	0.80 ± 0.07^{b}	0.76 ± 0.06^{b}	0.82 ± 0.02^{a}	0.61 ± 0.01^{b}	0.63 ± 0.06^{b}	0.60 ± 0.06^{b}	0.68 ± 0.01^{b}
Fructose consumed	1.17	1.17	1.17	1.17	1.17	1.72	1.72	1.72	1.72	1.72
Malic acid consumed	2.46 ± 0.01^{a}	2.44^{b}	2.44 ± 0.02^{ab}	2.46 ± 0.01^{a}	2.46^{ab}	2.41^{a}	2.10 ± 0.06^{b}	2.40^{a}	2.41^{a}	2.40 ± 0.01^{a}
Lactic acid produced	1.09 ± 0.01^{a}	1.04 ± 0.04^{ab}	1.05 ± 0.02^{a}	0.98 ± 0.02^{bc}	0.97 ± 0.01^{c}	0.92^{a}	0.72 ± 0.03^{c}	0.91 ± 0.01^{a}	0.87 ± 0.01^{b}	0.87 ± 0.01^{b}
Acetic acid produced	0.49 ± 0.02^{a}	0.41 ± 0.02^{b}	0.41 ± 0.01^{b}	0.39 ± 0.02^{b}	0.39 ± 0.01^{b}	0.33 ± 0.04^{a}	0.23^{b}	0.31 ± 0.01^{a}	0.31^{a}	0.31 ± 0.01^{a}

Results were presented as mean of triplicates ± SD (g/L). Values in a given row with different letters are significantly different ($p<0.05$)

4. Discussion

In a previous study (Betteridge et al. 2017), commercial strain SB3 was evolved in the presence of increasing ethanol content in a laboratory medium, MRSAJ, to yield a more ethanol tolerant isolate, A90. A further comparison of these strains here confirmed the superior fermentation performance by A90 in MRSAJ but not in the complex, multi-stressor environment of FRCDGJM (Figure 1). This was the case at low inoculation rates and the higher rates analogous to those used to inoculating wines high in ethanol (Zapparoli et al. 2009). This result suggests that A90 has evolved to a particular niche and has limited abilities outside of this. Such trade-off phenomena occur commonly during evolution and can result in a mutant showing increased fitness in some environments but performing worse in others (reviewed by Kawecki et al. 2012).

RFCDGJM has a lower pH, more polyphenols and lower nutrition than MRSAJ. Such conditions are known to inhibit bacterial growth and fermentation ability (Henick-Kling 1993) and thereby should present a greater challenge to A90. Accordingly, further improvement of A90 was sought initially using RFCDGJM with the progressive transition to wine-relevant conditions using the Shiraz wine.

A cultivation strategy was used that was intended to favour greatest cell division and hence opportunities for mutations arising during DNA replication to increase the chance of variants arising, ideally fitter ones (Dragosits and Mattanovich 2013). A sequential batch fermentation was used with some success for dairy or probiotic LAB (Teusink et al. 2009; Bachmann et al. 2012; Douillard et al. 2016) and by our group to improve wine yeast (McBryde et al. 2006). The sequential batch approach has the potential advantage of exposing the culture to all of the compositionally different phases of the batch culture, thereby allowing

evolution in response to more diverse conditions. However, sequential batch fermentation was deemed inappropriate for this study of *O. oeni* given the slow growth and extended batch durations typical of this organism.

Instead, a continuous culture approach utilising a turbidostat was adopted to encourage the culture to divide as quickly as possible in a medium (Lane et al. 1999) relatively constant in composition in the shorter term but with increasing levels of stressors over the longer term. Work with *Escherichia coli* or *Saccharomyces cerevisiae* strains showed novel desired traits in some cases developed during the active growth phase (Ibarra et al. 2002; Wallace-Salinas and Gorwa-Grauslund 2013; Sandberg et al. 2014), and in other cases during the stationary phase (Zambrano et al. 1993; Zinser and Kolter 2000). The present study does not offer any specific insight into the importance of each phase. Nevertheless, it has been successful in generating at least three superior *O. oeni* strains from A90, whose MLF performance is significant better in various media and wines with multiple stressors.

Regardless of the experimental approach applied in DE, improved phenotypes are reported to appear across a wide range of generations (100 and 10,000 generations) (Dragosits and Mattanovich 2013). In agreement with this broad range, strains 1-161, 2-49 and 3-83 were selected as superior through screens conducted after 150, 220 and 350 generations, respectively. How long to perform a DE experiment is difficult to predict. Fitness gains are initially large in a new environment, but reduce over time due to accumulation of more deleterious mutations (Taddei et al. 1997; Elena and Lenski 2003; Sumby et al. 2014). Accordingly, the evolving population was monitored regularly to determine when clones with improved properties were apparent and the magnitude of any such

improvements relative to the parent. From the data derived from a direct comparison of isolates collected across the three time points (Figure 3), there is no clear trend to suggest that any of the latterly collected isolates were beginning to perform worse than isolates collected at earlier time points. Where comparisons were made of the single best isolated strains from each sampling time point in 4 different wines. (Figure 4), there is a trend whereby the isolates derived after longer evolution (i.e. 2-49 and 3-83) typically showed a more rapid MLF compared to strain 1-161 and, in turn, A90. It is therefore not clear whether further improvements may have been possible if the DE experiment had been continued.

The ability of the isolates to conduct MLF clearly varied according to the wine in question (Figure 4), which is highly consistent with many reports (e.g. Gockowiak and Henschke 2003) showing that performance varies according to the wine matrix. Nevertheless, in each wine one or more of the improved strains displayed a significant advantage over the parent strains in terms of malic acid degradation or viability. Better malic acid consumption can be due to a larger viable population of bacteria (Fahimi et al. 2014), but interestingly in the Shiraz fermentations, there were minimal differences between the strains at any one time (Figure 4D). Yet the extent of MLF varied markedly (Figure 4C). The basis for the improved performance remains to be determined.

The rate and extent of MLF were generally lowest in the Pinot Noir wine. Before use in this study, this wine had already undergone MLF and was supplemented with malic acid so as to mimic a more challenging, nutritionally depleted environment. Apart from the limited MLF that occurred, this condition also produced the greatest viability losses, although the cause and effect need to be determined for this. Nonetheless, the findings highlight the potential commercial

utility of some of the DE strains, particularly, 2-49 and 3-83, particularly under challenging conditions (e.g. high ethanol wine or nutritionally depleted wines).

Apart from the completion of MLF, the profile of metabolites produced is an important consideration for the suitability of strains for winemaking. All strains isolated from DE produced no more, if not less, acetic acid that the ultimate parent, SB3, in Mouvedre and Merlot wines (Table 2). In all cases the final concentrations remain well below detectable levels for this acid (Swiegers et al. 2005).

5. Conclusions

Our findings demonstrated the feasibility of using laboratory directed evolution to generate novel *O. oeni* strains that are more suitable under winemaking conditions. Screens of individual isolates taken at time points in the DE process identified improved strains, particularly strains 1-143, 1-161, 2-49 and 3-83, with better MLF performance than any other isolates and the parent. Subsequent trials confirmed the superior performance of these strains in an array of wines, indicating a broad applicability of these in wine fermentations. More in-depth characterisation of the evolved strains is on-going. To our knowledge, this study is the first successful application of DE to *O. oeni* to produce isolates better suited to an environment with multiple wine related stressors.

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References

Assad-García, J., Bonnin-Jusserand, M., Garmyn, D., Guzzo, J., Alexandre, H. and Grandvalet, C. (2008) An improved protocol for electroporation of *Oenococcus oeni* ATCC BAA-1163 using ethanol as immediate membrane fluidizing agent. *Letters in Applied Microbiology* **47**, 333-338.

Bachmann, H., Starrenburg, M.J., Molenaar, D., Kleerebezem, M. and van Hylckama Vlieg, J.E. (2012) Microbial domestication signatures of *Lactococcus lactis* can be reproduced by experimental evolution. *Genome Research* **22**, 115-124.

Berbegal, C., Peña, N., Russo, P., Grieco, F., Pardo, I., Ferrer, S., Spano, G. and Capozzi, V. (2016) Technological properties of *Lactobacillus plantarum* strains isolated from grape must fermentation. *Food Microbiology* **57**, 187-194.

Betteridge, A., Grbin, P. and Jiranek, V. (2015) Improving *Oenococcus oeni* to overcome challenges of wine malolactic fermentation. *Trends in Biotechnology* **33**, 547-553.

Betteridge, A.L., Sumby, K.M., Sundstrom, J.F., Grbin, P.R. and Jiranek, V. (2017) Ethanol tolerant *Oenococcus oeni* by directed evolution for more efficient malolactic fermentation. *Applied Microbiology and Biotechnology*, submitted on the 12th April 2017

Campos, F.M., Couto, J.A., Figueiredo, A.R., Tóth, I.V., Rangel, A.O. and Hogg, T.A. (2009) Cell membrane damage induced by phenolic acids on wine lactic acid bacteria. *International Journal of Food Microbiology* **135**, 144-151.

Capozzi, V., Russo, P., Beneduce, L., Weidmann, S., Grieco, F., Guzzo, J. and Spano, G. (2010) Technological properties of *Oenococcus oeni* strains isolated from typical southern Italian wines. *Letters in Applied Microbiology* **50**, 327-334.

Capucho, I. and San Romão, M.V. (1994) Effect of ethanol and fatty acids on malolactic activity of *Leuconostoc oenos*. *Applied Microbiology and Biotechnology* **42**, 391-395.

Carreté, R., Vidal, M.T., Bordons, A. and Constantí, M. (2002) Inhibitory effect of sulfur dioxide and other stress compounds in wine on the ATPase activity of *Oenococcus oeni*. *FEMS Microbiology Letters* **211**, 155-159.

Chu-Ky, S., Tourdot-Maréchal, R., Marechal, P.-A. and Guzzo, J. (2005) Combined cold, acid, ethanol shocks in *Oenococcus oeni*: effects on membrane fluidity and cell viability. *Biochimica et Biophysica Acta (BBA)-Biomembranes* **1717**, 118-124.

Da Silveira, M.G., Golovina, E.A., Hoekstra, F.A., Rombouts, F.M. and Abee, T. (2003) Membrane fluidity adjustments in ethanol-stressed *Oenococcus oeni* cells. *Applied and Environmental Microbiology* **69**, 5826-5832.

Da Silveira, M.G. and Abee, T. (2009) Activity of ethanol-stressed *Oenococcus oeni* cells: a flow cytometric approach. *Journal of Applied Microbiology* **106**, 1690-1696.

Darsonval, M., Alexandre, H. and Grandvalet, C. (2016a) Genetically engineered *Oenococcus oeni* strains to highlight the impact of *estA2* and *estA7* esterase genes on wine ester profile. *Food Microbiology* **60**, 21-28.

Darsonval, M., Msadek, T., Alexandre, H. and Grandvalet, C. (2016b) The antisense RNA approach: a new application for in vivo investigation of the stress response of *Oenococcus oeni*, a wine-associated lactic acid bacterium. *Applied and Environmental Microbiology* **82**, 18-26.

Davis, C.R., Wibowo, D., Eschenbruch, R., Lee, T.H. and Fleet, G.H. (1985) Practical implications of malolactic fermentation: A review. *American Journal of Enology and Viticulture* **36**, 290-301.

de Man, J., Rogosa, M. and Sharpe, M.E. (1960) A medium for the cultivation of lactobacilli. *Journal of Applied Microbiology* **23**, 130-135.

Dicks, L.M. (1994) Transformation of *Leuconostoc oenos* by electroporation. *Biotechnology Techniques* **8**, 901-904.

Dragosits, M. and Mattanovich, D. (2013) Adaptive laboratory evolution – principles and applications for biotechnology. *Microbial Cell Factories* **12**, 64.

Douillard, F.P., Ribbera, A., Xiao, K., Ritari, J., Rasinkangas, P., Paulin, L., Palva, A., Hao, Y. and de Vos, W.M. (2016) Polymorphisms, chromosomal rearrangements, and mutator phenotype development during experimental evolution of *Lactobacillus rhamnosus* GG. *Applied and Environmental Microbiology* **82**, 3783-3792.

Elena, S.F. and Lenski, R.E. (2003) Evolution experiments with microorganisms: the dynamics and genetic bases of adaptation. *Nature Reviews Genetics* **4**, 457-469.

Fahimi, N., Brandam, C. and Taillandier, P. (2014) A mathematical model of the link between growth and L-malic acid consumption for five strains of *Oenococcus oeni*. *World Journal of Microbiology and Biotechnology* **30**, 3163-3172.

Figueiredo, A.R., Campos, F., de Freitas, V., Hogg, T. and Couto, J.A. (2008) Effect of phenolic aldehydes and flavonoids on growth and inactivation of *Oenococcus oeni* and *Lactobacillus hilgardii*. *Food Microbiology* **25**, 105-112.

García-Ruiz, A., Moreno-Arribas, M.V., Martín-Álvarez, P.J. and Bartolomé, B. (2011) Comparative study of the inhibitory effects of wine polyphenols on the growth of enological lactic acid bacteria. *International Journal of Food Microbiology* **145**, 426-431.

Gockowiak, H. and Henschke, P.A. (2003) Interaction of pH, ethanol concentration and wine matrix on induction of malolactic fermentation with commercial 'direct inoculation' starter cultures. *Australian Journal of Grape and Wine Research* **9**, 200-209.

Henick-Kling, T. (1993) Malolactic fermentation, in: Fleet, G. T.(Ed.), *Wine Microbiology and Biotechnology*. Harwood Academic Publishers GmbH., United Kingdom. 289-326.

Hindré, T., Knibbe, C., Beslon, G. and Schneider, D. (2012) New insights into bacterial adaptation through *in vivo* and *in silico* experimental evolution. *Nature Reviews Microbiology* **10**, 352-365.

Ibarra, R.U., Edwards, J.S. and Palsson, B.O. (2002) *Escherichia coli* K-12 undergoes adaptive evolution to achieve *in silico* predicted optimal growth. *Nature* **420**, 186-189.

Iorizzo, M., Testa, B., Lombardi, S.J., García-Ruiz, A., Muñoz-González, C., Bartolomé, B. and Moreno-Arribas, M.V. (2016) Selection and technological potential of *Lactobacillus plantarum* bacteria suitable for wine malolactic fermentation and grape aroma release. *LWT-Food Science and Technology* **73**, 557-566.

Kawecki, T.J., Lenski, R.E., Ebert, D., Hollis, B., Olivieri, I. and Whitlock, M.C. (2012) Experimental evolution. *Trends in Ecology & Evolution* **27**, 547-560.

Lane, P., Oliver, S. and Butler, P. (1999) Analysis of a continuous-culture technique for the selection of mutants tolerant to extreme environmental stress. *Biotechnology and Bioengineering* **65**, 397-406.

Li, N., Duan, J., Gao, D., Luo, J., Zheng, R., Bian, Y., Zhang, X. and Ji, B. (2015) Mutation and selection of *Oenococcus oeni* for controlling wine malolactic fermentation. *European Food Research and Technology* **240**, 93-100.

McBryde, C., Gardner, J.M., de Barros Lopes, M. and Jiranek, V. (2006) Generation of novel wine yeast strains by adaptive evolution. *American Journal of Enology and Viticulture* **57**, 423-430.

Nehme, N., Mathieu, F. and Taillandier, P. (2010) Impact of the co-culture of *Saccharomyces cerevisiae – Oenococcus oeni* on malolactic fermentation and partial characterization of a yeast-derived inhibitory peptidic fraction. *Food Microbiology* **27**, 150-157.

Nielsen, J.C., Prahl, C. and Lonvaud-Funel, A. (1996) Malolactic fermentation in wine by direct inoculation with freeze-dried *Leuconostoc oenos* cultures. *American Journal of Enology and Viticulture* **47**, 42-48.

Ough, C. and Crowell, E. (1987) Use of sulfur dioxide in winemaking. *Journal of Food Science* **52**, 386-388.

Pretorius, I.S. and Bauer, F.F. (2002) Meeting the consumer challenge through genetically customized wine-yeast strains. *Trends in Biotechnology* **20**, 426-432.

Rankine, B. and Pocock, K. (1970) Alkalimetric determination of sulphur dioxide in wine. *Australian Wine, Brewing and Spirit Review* **88**, 40-44.

Saguir, F. and Manca de Nadra, M. (2002) Effect of L-malic and citric acids metabolism on the essential amino acid requirements for *Oenococcus oeni* growth. *Journal of Applied Microbiology* **93**, 295-301.

Sandberg, T.E., Pedersen, M., LaCroix, R.A., Ebrahim, A., Bonde, M., Herrgard, M.J., Palsson, B.O., Sommer, M. and Feist, A.M. (2014) Evolution of *Escherichia coli* to 42 ˚C and subsequent genetic engineering reveals adaptive mechanisms and novel mutations. *Molecular Biology and Evolution*, **31**, 2647-2662.

Sumby, K.M., Grbin, P.R. and Jiranek, V. (2014) Implications of new research and technologies for malolactic fermentation in wine. *Applied Microbiology and Biotechnology* **98**, 8111-8132.

Swiegers, J. H., Bartowsky, E. J., Henschke, P. A. and Pretorius, I. S. (2005) Yeast and bacterial modulation of wine aroma and flavour. *Australian Journal of Grape and Wine Research* **11**, 139-173.

Taddei, F., Radman, M., Maynard-Smith, J., Toupance, B., Gouyon, P. and Godelle, B. (1997) Role of mutator alleles in adaptive evolution. *Nature* **387**, 700-702.

Takahashi, M., Ohta, T., Masaki, K., Mizuno, A. and Goto-Yamamoto, N. (2014) Evaluation of microbial diversity in sulfite-added and sulfite-free wine by culture-dependent and-independent methods. *Journal of Bioscience and Bioengineering* **117**, 569-575.

Teusink, B., Wiersma, A., Jacobs, L., Notebaart, R.A. and Smid, E.J. (2009) Understanding the adaptive growth strategy of *Lactobacillus plantarum* by *in silico* optimisation. *PLoS Computational Biology* **5**, e1000410.

Torriani, S., Felis, G.E. and Fracchetti, F. (2010) Selection criteria and tools for malolactic starters development: an update. *Annals of Microbiology* **61**, 33-39.

Tourdot-Maréchal, R., Fortier, L.-C., Guzzo, J., Lee, B. and Diviès, C. (1999) Acid sensitivity of neomycin-resistant mutants of *Oenococcus oeni*: a relationship between reduction of ATPase activity and lack of malolactic activity. *FEMS Microbiology Letters* **178**, 319-326.

Vivas, N., Lonvaud-Funel, A. and Glories, Y. (1997) Effect of phenolic acids and anthocyanins on growth, viability and malolactic activity of a lactic acid bacterium. *Food Microbiology* **14**, 291-299.

Walker, M.E., Nguyen, T.D., Liccioli, T., Schmid, F., Kalatzis, N., Sundstrom, J.F., Gardner, J.M. and Jiranek, V. (2014) Genome-wide identification of the

Fermentome; genes required for successful and timely completion of wine-like fermentation by *Saccharomyces cerevisiae*. *BMC Genomics* **15**, 552.

Wallace-Salinas, V. and Gorwa-Grauslund, M.F. (2013) Adaptive evolution of an industrial strain of *Saccharomyces cerevisiae* for combined tolerance to inhibitors and temperature. *Biotechnology for Biofuels* **6**, 151.

Wibowo, D., Eschenbruch, R., Davis, C.R., Fleet, G.H. and Lee, T.H. (1985) Occurrence and growth of lactic acid bacteria in wine: a review. *American Journal of Enology and Viticulture* **36**, 302-313.

Zambrano, M., Siegele, D., Almirón, M., Tormo, A. and Kolter, R. (1993) Microbial competition: *Escherichia coli* mutants that take over stationary phase cultures. *Science* **259**, 1757-1760.

Zapparoli, G., Tosi, E., Azzolini, M., Vagnoli, P. and Krieger, S. (2009) Bacterial inoculation strategies for the achievement of malolactic fermentation in high-alcohol wines. *South African Journal of Enology and Viticulture* **30**, 49-55.

Zhang, J., Wu, C., Du, G. and Chen, J. (2012) Enhanced acid tolerance in *Lactobacillus casei* by adaptive evolution and compared stress response during acid stress. *Biotechnology and Bioprocess Engineering* **17**, 283-289.

Zinser, E.R. and Kolter, R. (2000) Prolonged stationary-phase incubation selects for *lrp* mutations in *Escherichia coli* K-12. *Journal of Bacteriology* **182**, 4361-4365.

Chapter 4. Physiological characterisation of experimentally evolved *Oenococcus oeni* strains

4.1 Introduction

In the previous chapter, generation of improved *O. oeni* strains via growth in a continuous culture for up to 350 generations was described. Three single isolates, strains 1-161, 2-49 and 3-83, were selected as being more efficient at consuming L-malic acid than the parent strain (A90) in wine/wine-like media. They were also able to ferment faster in various wines, indicating a wide applicability of these strains. These strains also need to be characterised with regard to other aspects, such as resistance to various stressors, which may restrict the progress of MLF in wine.

Henick-Kling (1993) emphasised that improved bacteria should have good resistance to common inhibitors in wine such as high ethanol, low pH and the presence of sulfur dioxide. Medium chain fatty acids (MCFA) can also be inhibitory to bacteria, even small amounts of which can reduce ATPase activity, leading to loss of cell viability (Carreté et al. 2002). Information on the stress tolerance of a strain would be beneficial to winemakers, knowledge of the minimum inhibitory concentration of different stressors would enable winemakers to predict whether a strain would be likely to survive and function in a particular juice or wine.

A good strain is also expected to have long-term stability of a desired phenotype. It is therefore important to investigate the stability of evolved *O. oeni* strains, as it has been reported that this microorganism has the ability to evolve rapidly (Marcobal et al. 2008). Genome sequencing revealed that *O. oeni* lacks the *mutS* and *mutL* genes (Makarova et al. 2006; Makarova and Koonin 2007) that

encode key enzymes involved in the methylated mismatch repair pathway, which corrects nucleotide base pair mismatches during DNA replication (Modrich 1991). Thus, the absence of these genes may cause undesirable mutations during cultivation. The ability to maintain the desired phenotype through growth for many generations in low stressful is therefore crucial for optimised strains.

Yeast-bacteria interaction is yet another factor to consider. The interaction of yeast and bacteria has been extensively studied, and may range from stimulatory to inhibitory (Nehme et al. 2008; Arnink et al. 2005; Remize et al. 2006; Muñoz et al. 2014; Rizk et al. 2016). The inhibition of bacterial growth by yeast mainly results from depletion of nutrients by yeast and the production of inhibitory products such as ethanol, decanoic acid, peptides and proteins. In contrast, it has also been suggested that some nitrogenous components, such as amino acids, released by yeast autolysis may stimulate bacterial growth (Alexandre et al. 2004). Considering the importance of yeast-bacteria interactions, it is therefore essential to trial evolved *O. oeni* strains in non-sterile wine to mimic a typical oenological environment.

Accordingly, this chapter reports the further characterisation of the evolved strains with regard to their stress tolerance, phenotype stability and fermentation ability in association with yeast. It was hoped that the evolved strains would have higher resistance to multiple stressors, stable phenotypes and fast fermentation.

4.2 Material and methods

4.2.1 Strains

Five *O. oeni* strains were used in this study; SB3, A90, 1-161, 2-49 and 3-83 (Table 4.1). A90 is an ethanol tolerant strain evolved from SB3 (Laffort) while

strains 1-161, 2-49 and 3-83 were evolved from A90 for more efficient MLF performance in a multi-stressor wine environment.

Table 4.1. *O. oeni* strains used in this study

Experiment	Strains
Tolerance assays	1-161, 2-49, 3-83, SB3, A90
Strain phenotypic stability	
Non-sterile MLF trials	2-49, 3-83, SB3, A90

4.2.2 Media/wine preparation

Multi-stress tolerance assays were conducted in Red Fermented Chemically Defined Grape Juice Medium (RFCDGJM) containing different levels of stressors (Table 4.2). RFCDGJM was prepared as described in Chapter 2 (Section 2.2.2). Analytical reagent grade (AR) ethanol and 10M NaOH or 37% (v/v) HCl were used to adjust ethanol concentration and pH to the indicated levels in Table 4.2. Final ethanol concentration and pH of the media were determined by a Wine ME/DMA 4500M alcolyser (Anton Paar) and a pH meter. Potassium metabisulfite (PMS) was used for SO_2 adjustment. A 40,000 mg/L SO_2 stock was made by dissolving 702 mg PMS into 10 mL sterile de-ionised water. To obtain final concentrations of 10, 20, 30 and 40 mg/L SO_2, 50, 100, 150 and 200 µL of the SO_2 stock was added into 200 mL RFCDGJM. As for the adjustment of medium chain fatty acids, a 4,000 mg/L stock was first prepared by dissolving 40 mg of the corresponding fatty acid into 10 mL ethanol. A dose of 50 µL fatty acid stock supplemented into 200 mL RFCDGJM would contribute 1 mg/L of the corresponding fatty acid. Based on this, an appropriate volume of the fatty acid stock was then diluted into 200 mL RFCDGJM to reach each tested level as shown in Table 4.1. Modified RFCDGJM was filter sterilised (0.22 µm) before inoculation.

Table 4.2. Levels of stressors in RFCDGJM for stress tolerance assays

Medium	Levels of stressors						
	Ethanol	**pH**	**SO_2**	**Octanoic acid (C8)**	**Decanoic acid (C10)**	**Dodecanoic acid (C12)**	**Mixed MCFAs**
RFCDGJM-1	14–18% (v/v), 2% (v/v) increments	3.5	0	0	0	0	0
RFCDGJM-2	12% (v/v)	2.8–3.4, 0.2 increments	0	0	0	0	0
RFCDGJM-3	15% (v/v)	2.8–3.4, 0.2 increments	0	0	0	0	0
RFCDGJM-4	12% (v/v)	3.5	10–40 mg/L, 10 mg/L increments	0	0	0	0
RFCDGJM-5	12% (v/v)	3.5	0	5–20 mg/L, 5 mg/L increments	0	0	0
RFCDGJM-6	12% (v/v)	3.5	0	0	5–20 mg/L, 5 mg/L increments	0	0
RFCDGJM-7	12% (v/v)	3.5	0	0	0	5–20 mg/L, 5 mg/L increments	0
RFCDGJM-8*	12% (v/v)	3.5	0	0	0	0	4.5–18 mg/L, 4.5 mg/L increments

*RFCDGJM-8 had equal concentrations of C8, C10 and C12 fatty acids, ranging from 1.5 – 6 mg/L (1.5 mg/L increments) of each fatty acid. The first condition contained 1.5 mg/L of each C8, C10 and C12, making 4.5 mg/L total MCFA; the second condition contained 3 mg/L of each C8, C10 and C12, making 9 mg/L total MCFA; the third condition contained 4.5 mg/L of each C8, C10 and C12, making 13.5 mg/L total MCFA; the fourth condition contained 6 mg/L of each C8, C10 and C12, making 18 mg/L total MCFA.

Phenotype stability of the evolved strains was evaluated by comparing performance of passaged strains (described in 4.2.3) to strains freshly grown from glycerol stocks in a 2017 Shiraz wine (Urrbrae, South Australia), with ethanol, SO_2 and pH adjusted to 14% (v/v), 20 mg/L and 3.6, respectively. An extra 200 mg/L of a commercial malolactic bacterial activator 'MALOSTART®' (Laffort) was supplemented to this medium before filtration (0.22 µm) and inoculation.

Optimised DE strains were also characterised in non-sterile 2017 Shiraz wine along with strains SB3 and A90. Approximately 1.5 tonnes of grapes were hand-picked from the Waite Campus vineyard, Urrbrae, South Australia. WIC Winemaking Services were then contracted to perform the large-scale alcoholic

fermentation and distribution to twelve 50 L stainless-steel kegs. In brief, the process involved the following: Grapes were de-stemmed, crushed, and supplemented with 30 mg/kg pectinase. The grape must was then inoculated with 250 mg/L of a commercial yeast strain, PDM (Mauri Yeast Australia Pty Ltd) and fermented on skins at 15–18 °C. The wine was pressed when the Baumé dropped below 2. After alcoholic fermentation (final ethanol 15.7% (v/v)), wine pH was adjusted to 3.45 by the addition of 1 g/L tartaric acid. The wine was settled at 15°C for four days. The supernatant was then aliquoted into twelve 50 L stainless-steel kegs. Twenty more litres of the same wine was collected and stored at 4 °C for subsequent MLF in Schott bottles. MLF in non-sterile Shiraz was carried out in both 50 L kegs and 250 mL Schott bottles.

4.2.3 Culture preparation and inoculation

All bacterial strains were first pre-cultured in 10 mL MRSAJ from glycerol stocks and were incubated for 4 days at 30 ˚C before any other treatments were performed.

Tolerance assay experiment

To standardise inoculation, 300 µL of each pre-cultured strain was sub-cultured into 13 mL of MRSAJ at 30 ˚C for 36 h. OD_{600} of each culture was adjusted to 0.5 prior to inoculation at a rate of 1:32 in 10 mL RFCDGJM containing different levels of stressors (Table 4.2). All cultures were incubated at 22 ˚C for a maximum of 10 days. Each assay type was performed in triplicate.

Strain phenotypic stability

Each pre-cultured strain was first diluted in fresh MRSAJ to an OD_{600} of 1.0. A volume of 250 µL diluted culture was then transferred into a 10 mL tube

containing 9.75 mL MRSAJ, and incubated at 30 ˚C until turbid (3–4 days). The sub-culturing procedure was repeated 9 times by which time the passaged culture reached 50 generations. In order to calculate the number of generations elapsed during each passage, a sample was taken for spot plating after each inoculation and post sub-culturing stage following the method described in Chapter 2 (Section 2.2.4). Following passaging, the cultures were inoculated into the 2017 Shiraz described in 4.2.2 along with freshly grown cultures of non-passaged strains revived from glycerol stocks. Fermentations were conducted in triplicate at 22 ˚C.

Strain performance in 250 mL and 50 L non-sterile Shiraz wine

Sufficient inocula for 250 mL fermentations were prepared through a 2-step method. Briefly, 5 mL pre-cultured bacteria of each strain (4.2.3) was transferred into 45 mL MRSAJ to make a 50 mL culture, which was harvested after 4 days incubation (2,000 × g for 5 min). The supernatants removed aseptically, and culture pellet re-suspended in 20 mL sterile (0.22 µm) wine-juice mix (1/3rd Shiraz 2017, 1/3rd tap water and 1/3rd apple juice). The suspension was then transferred into 230 mL of the sterile wine-juice mix and incubated at 22 ˚C for 4 days. Each culture was adjusted to an OD_{600} of approximately 1.0 before final inoculation with three biological triplicates at 2% (v/v) for each strain.

Three stages were involved to prepare inocula for the 50 L fermentation. For each strain, a 50 mL culture in MRSAJ was prepared in the same way as described for the 250 mL fermentation, which was then sub-cultured into 450 mL MRSAJ in Schott bottles, and similar incubation by which time the cultures became turbid. Cells were then harvested by centrifugation, washed and re-suspended in 100 mL sterile wine-juice mix and incubated at 22 ˚C for 4 days. The

OD_{600} reached 0.39 ± 0.05 before inoculation. The final inoculation of triplicate 50 L aliquots of non-sterile Shiraz was performed at a rate of 3% (v/v) for each strain.

4.2.4 Cell enumeration and chemical analysis for MLF trials

Samples were collected every 1–14 days to monitor viability and L-malic acid utilisation throughout each experiment. Plating of serially-diluted samples from the non-sterile fermentations used MRSAJ agar supplemented with 10 mg/L cycloheximide to prevent growth of *Saccharomyces* yeast. Colony forming units (CFUs) and L-malic acid were determined as described in Chapter 3 (Section 2.4 and 2.5).

4.2.5 Data analysis

Raw data was first processed with Microsoft Excel 2011. Graphing and statistical analysis were conducted using GraphPad Prism 7. Two-way ANOVA and Fisher's LSD tests were used to analyse the impact of time and strains on the viability and L-malic acid consumption. The confidence interval for each statistical analysis was set at 95%.

4.3 Results

4.3.1 Ethanol tolerance

Ethanol tolerance of DE strains at pH 3.5 (typical of red wine) was investigated by analysing L-malic acid consumption and bacterial viability in RFCDGJM with increasing concentrations of ethanol. For each strain, fermentation ability and viable cell numbers decreased when ethanol concentration increased (Fig. 4.1). At 14% ethanol, strain SB3 initially consumed L-malic acid faster than the evolved strains at Day 1 and Day 2; however all strains completed MLF by Day

4 (Fig. 4.1A). When ethanol concentration was increased to 16%, DE strains 1-161, 2-49 and 3-83 completed fermentation in 80% of the time of strains A90 and SB3 (Fig. 4.1C). Viable cell numbers of all strains were similarly high in RFCDGJM at 14% and 16% ethanol (Fig. 4.1B and D). Fermentation varied greatly when ethanol was at 18%: Strain SB3 consumed little L-malic acid whilst strains A90, 1-161, 2-49 and 3-83 consumed between 47% and 95% by Day 10 (Fig. 4.1E). Correspondingly, viability of strains was inversely related to MLF performance under this condition (Fig. 4.1F). No fermentation activity was detected at 20% ethanol for any of the strains (Fig. 4.1G) and viable cell numbers of SB3, A90 and 1-161 lost all viability whilst strains 2-49 and 3-83 retained about 10^3 CFU/mL ever after 10 days.

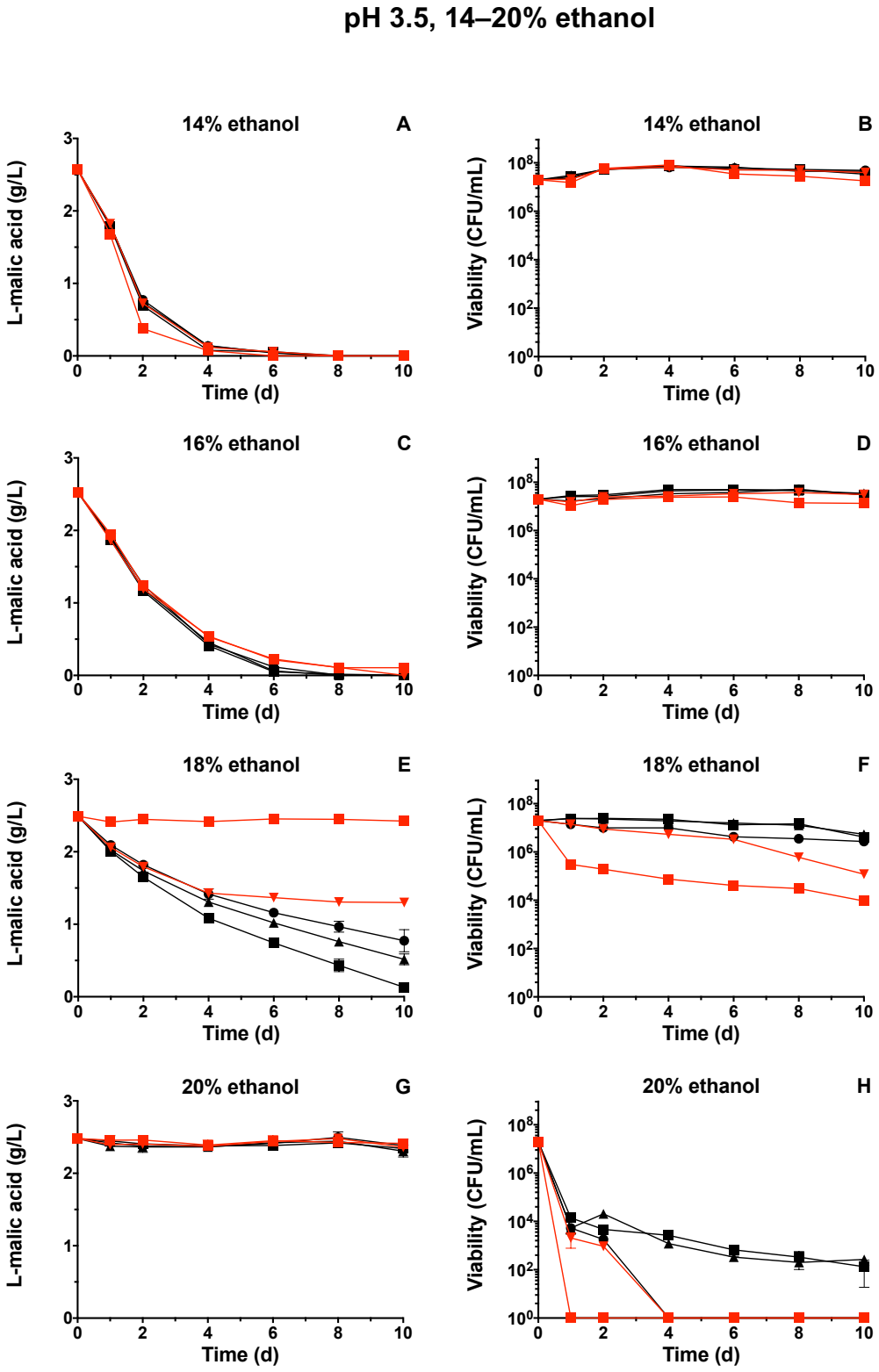


Fig. 4.1. L-malic acid consumption and CFUs of evolved strains 1-161 (●), 2-49 (■) and 3-83 (▲) compared to strains A90 (▼) and SB3 (■) in RFCDGJM. Values are the mean of three biological replicates ± SD.

4.3.2 pH tolerance at different ethanol concentrations

pH 2.8–3.4 with 12% ethanol

The influence of pH on fermentation and growth was first analysed in RFCDGJM at 12% (v/v) ethanol, typical of many wines. Fermentation duration was extended with decreasing pH within the range of 3.4 to 3.0 (Fig. 4.2A, C and E). At pH 3.4, strain SB3 outperformed other strains by completing fermentation in 2 days *vs* 4 days (Fig. 4.2A). However, the advantage of the optimised strains became more obvious at pH values starting at pH 3.2 when the DE strains completed MLF two days earlier than the parent strain A90 (8 days) (Fig. 4.2C). At pH 3.0, DE strains consumed all L-malic acid by Day 10 while the parent strains A90 and SB3 still had ~6% of the available L-malic acid to consume (Fig. 4.2E). Fermentation was not completed by any strain at pH 2.8; however, strains 1-161, 2-49 and 3-83 consumed approximately 8% more L-malic acid than strains A90 and SB3 by the end of this experiment. Viability of all strains was high and comparable in each case except at pH 2.8, a ~100 fold drop was seen after 4-6 days. In general, strains 2-49 and 3-83 performed much better than the other strains in this test.

pH 2.8–3.4, 12% ethanol

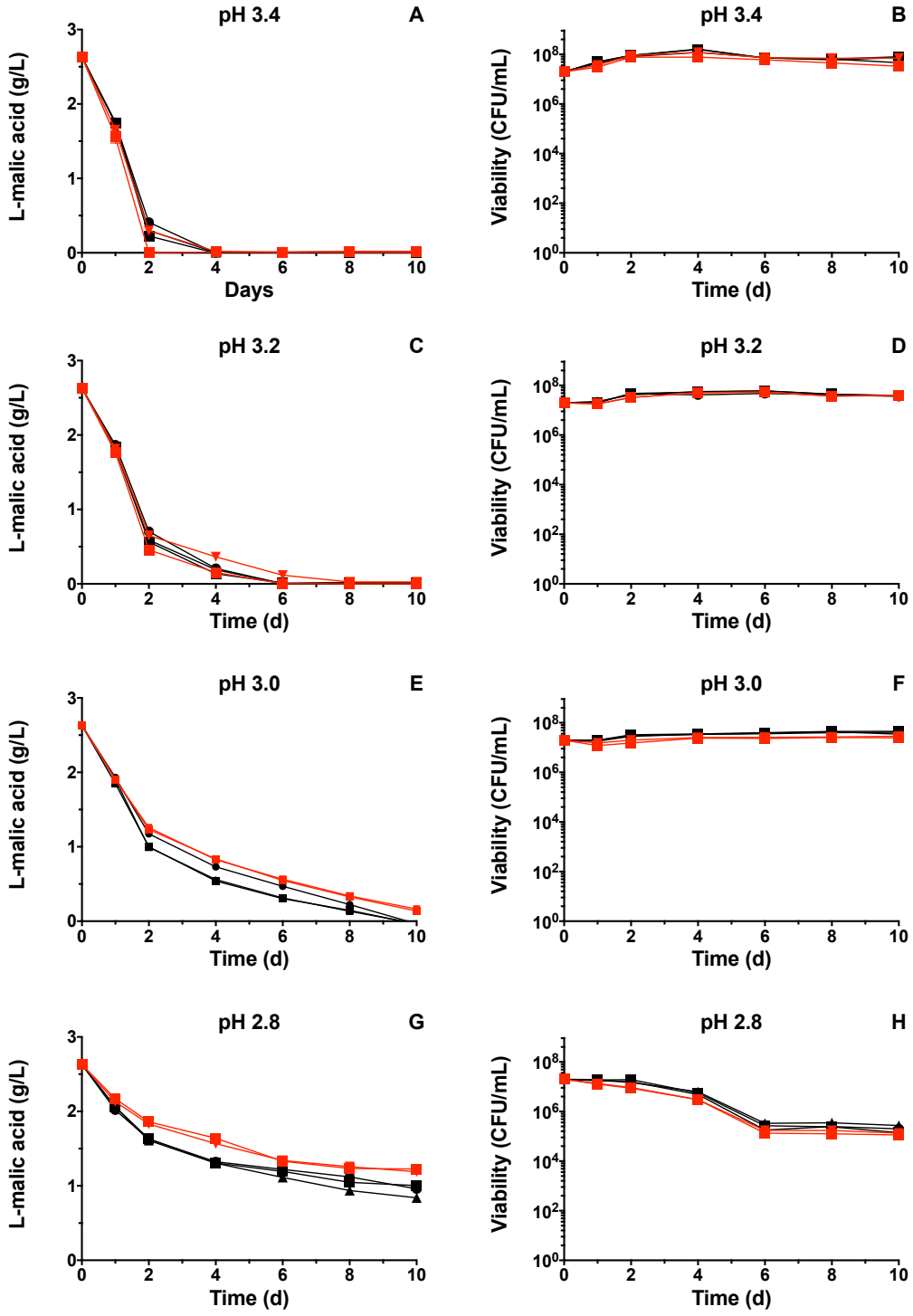


Fig. 4.2. L-malic acid consumption and CFUs of evolved strains 1-161 (●), 2-49 (■) and 3-83 (▲) compared to strains A90 (▼) and SB3 (■) in RFCDGJM. Values are the mean of three biological replicates ± SD.

pH 2.8–3.4 with 15% ethanol

The analysis of the effect of pH on bacterial MLF performance and viability was repeated at a higher ethanol concentration (15%). Under all tested pH levels, the new DE strains performed significantly better than strains A90 and SB3 in terms of both L-malic acid utilisation and growth ($p < 0.5$, Fig. 4.3). SB3 and A90 had similar fermentation kinetics at pH 3.4 and 3.2 while strains 1-161, 2-49 and 3-83 completed fermentation two and four days earlier (Fig. 4.3A and C). No strain was able to complete fermentation at pH 3.0 or pH 2.8; however, the three improved strains consumed a significantly larger amount of L-malic acid ($p < 0.05$) than the parent strains before fermentation became stuck (Fig. 4.3E and G). In terms of growth and viability, at pH 3.2 and pH 3.4, the population of all strains generally remained over 10^7 CFU/mL throughout the experiment (Fig. 4.3B and D). Viability declined gradually for all strains at pH 3.0, reducing to approximately 10^6 CFU/mL by day 10 (Fig. 4.3F). A more drastic decrease in viability was seen at pH 2.8 with viable cell numbers of strains A90 and SB3 dropping below 10^6 CFU/mL by Day 2, although viability of the three optimised strains maintained higher numbers throughout (Fig. 4.3H).

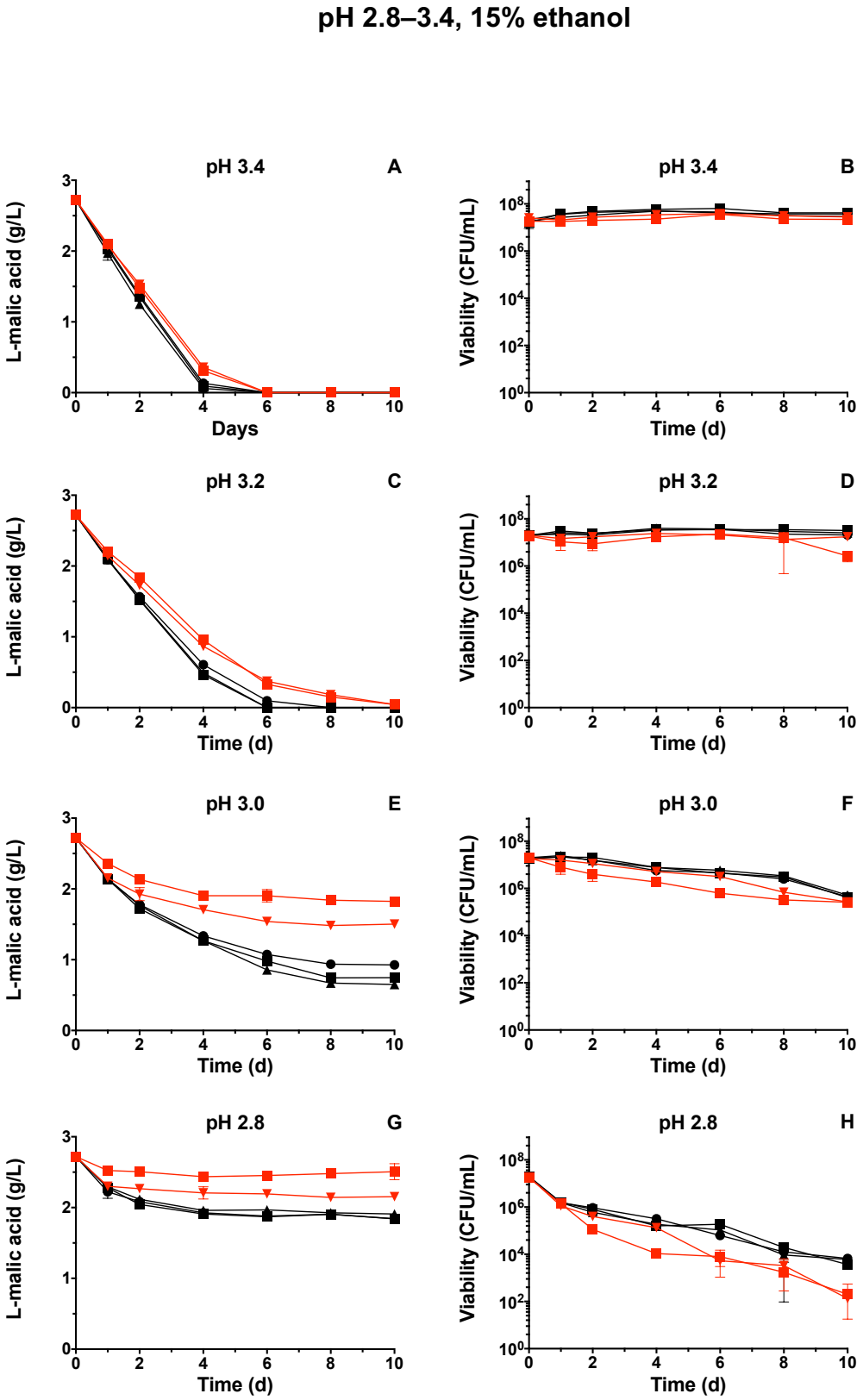


Fig. 4.3. L-malic acid consumption and CFUs of evolved strains 1-161 (●), 2-49 (■) and 3-83 (▲) compared to strains A90 (▼) and SB3 (■) in RFCDGJM. Values are the mean of three biological replicates ± SD.

4.3.3 SO_2 tolerance

The effect of SO_2 on the progress of MLF and viability was examined in RFCDGJM containing 12% (v/v) ethanol at pH 3.5. There was little impact on L-malic acid consumption and growth with the addition of 10 mg/L SO_2 (Fig. 4.4A and B) compared to the SO_2-free condition (see Fig. 4.2A and B). An increase of SO_2 to 20 mg/L showed that strains 1-161, 2-43 and 3-83 retained a significantly greater MLF activity and a larger viable population than strains SB3 and A90 by the end of fermentation (Fig. 4.4C and D). MLF activity and growth of all strains were strongly inhibited with the supplementation of 30 mg/L and 40 mg/L SO_2 (Fig. 4.4E–H).

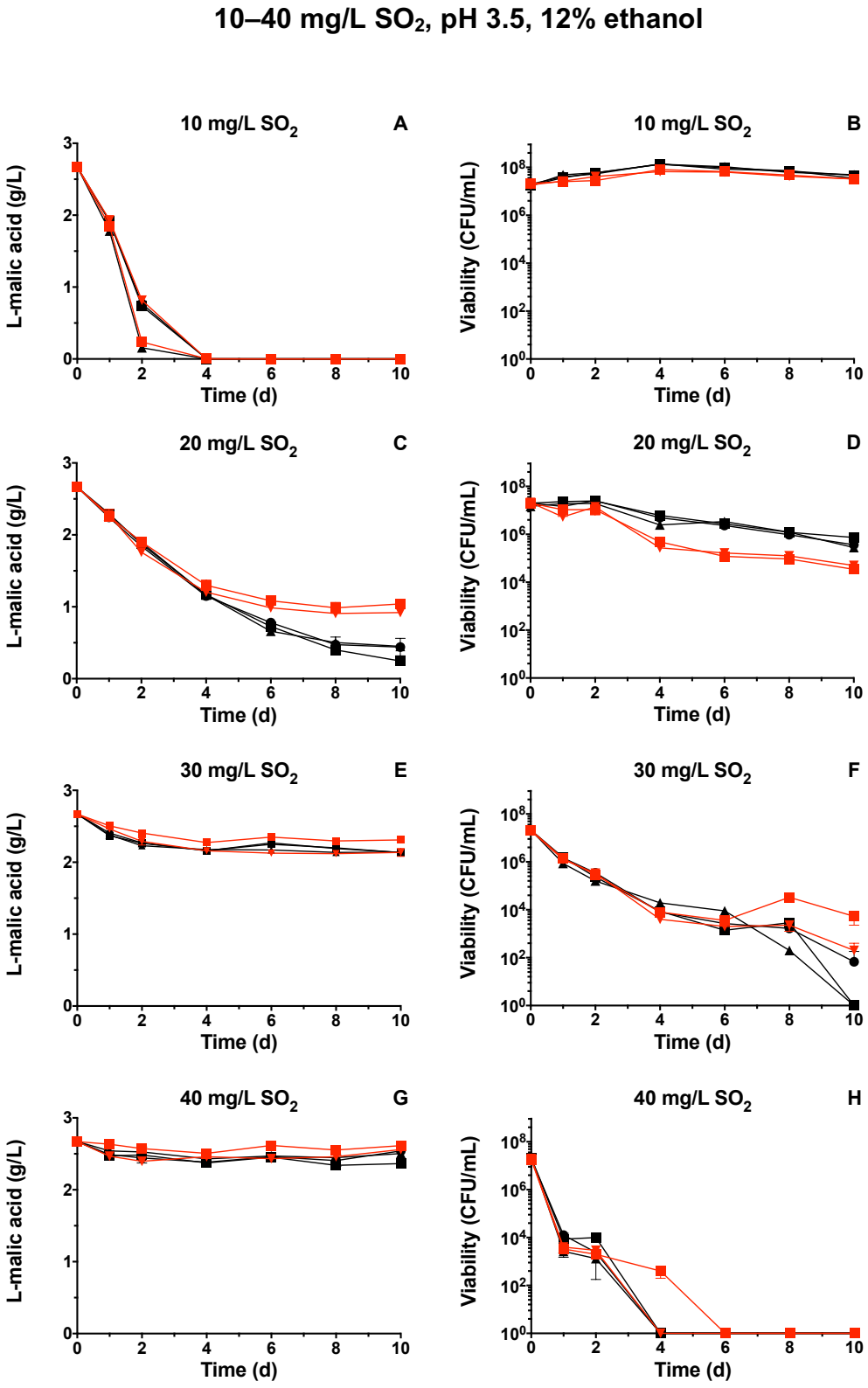


Fig. 4.4. L-malic acid consumption and CFUs of evolved strains 1-161 (●), 2-49 (■) and 3-83 (▲) compared to strains A90 (▼) and SB3 (■) in RFCDGJM. Values are the mean of three biological replicates ± SD.

4.3.4 Medium chain fatty acid (MCFA) tolerance

Octanoic acid (C8)

The addition of 5-20 mg/L C8 did not show any inhibitory effect on either MLF progress or growth for any of the five *O. oeni* strains assessed in RFCDGJM (Fig. 4.5); however, SB3 appeared to initially progress more quickly. A sampling at Day 3 may have revealed that SB3 had finished by this stage.

Decanoic acid (C10)

By comparison, decanoic acid did prove inhibitory at higher concentrations. Little effect was seen at 5 and 10 mg/L (Fig. 4.6A–D), while an intermediate degree of MLF inhibition was observed at 15 mg/L (Fig. 4.6E). SB3 was the most sensitive (Fig. 4.6E and F) whilst all DE strains showed greater robustness with 2-49 fermenting the fastest (Fig. 4.6E). No consumption of L-malic acid was observed for any strains in the presence of 20 mg/L C10 in RFCDGJM (Fig. 4.6G), and viability was reduced to ~10^3 CFU/mL in all cases (Fig. 4.6H).

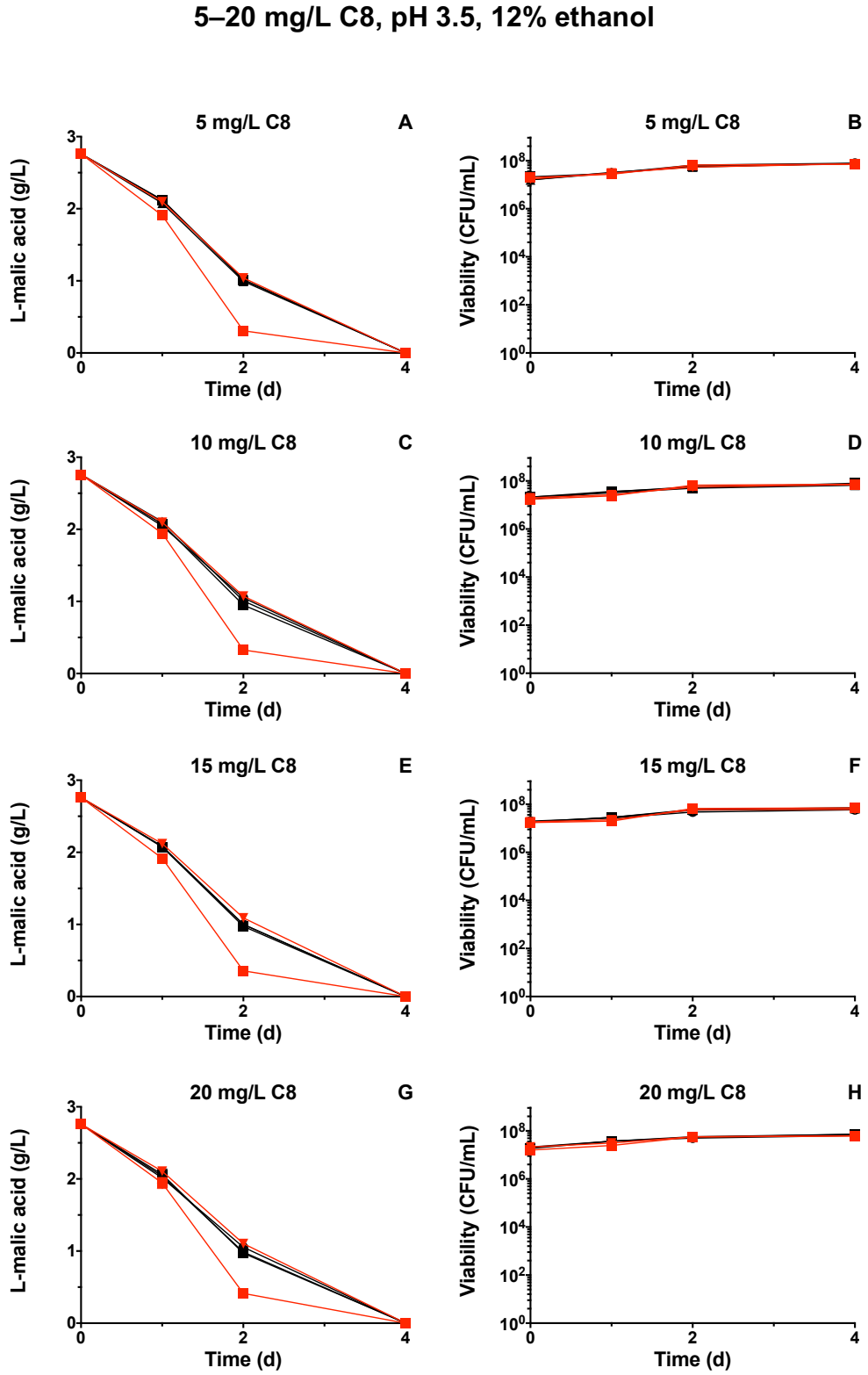


Fig. 4.5. L-malic acid consumption and CFUs of evolved strains 1-161 (●), 2-49 (■) and 3-83 (▲) compared to strains A90 (▼) and SB3 (■) in RFCDGJM. Values are the mean of three biological replicates ± SD.

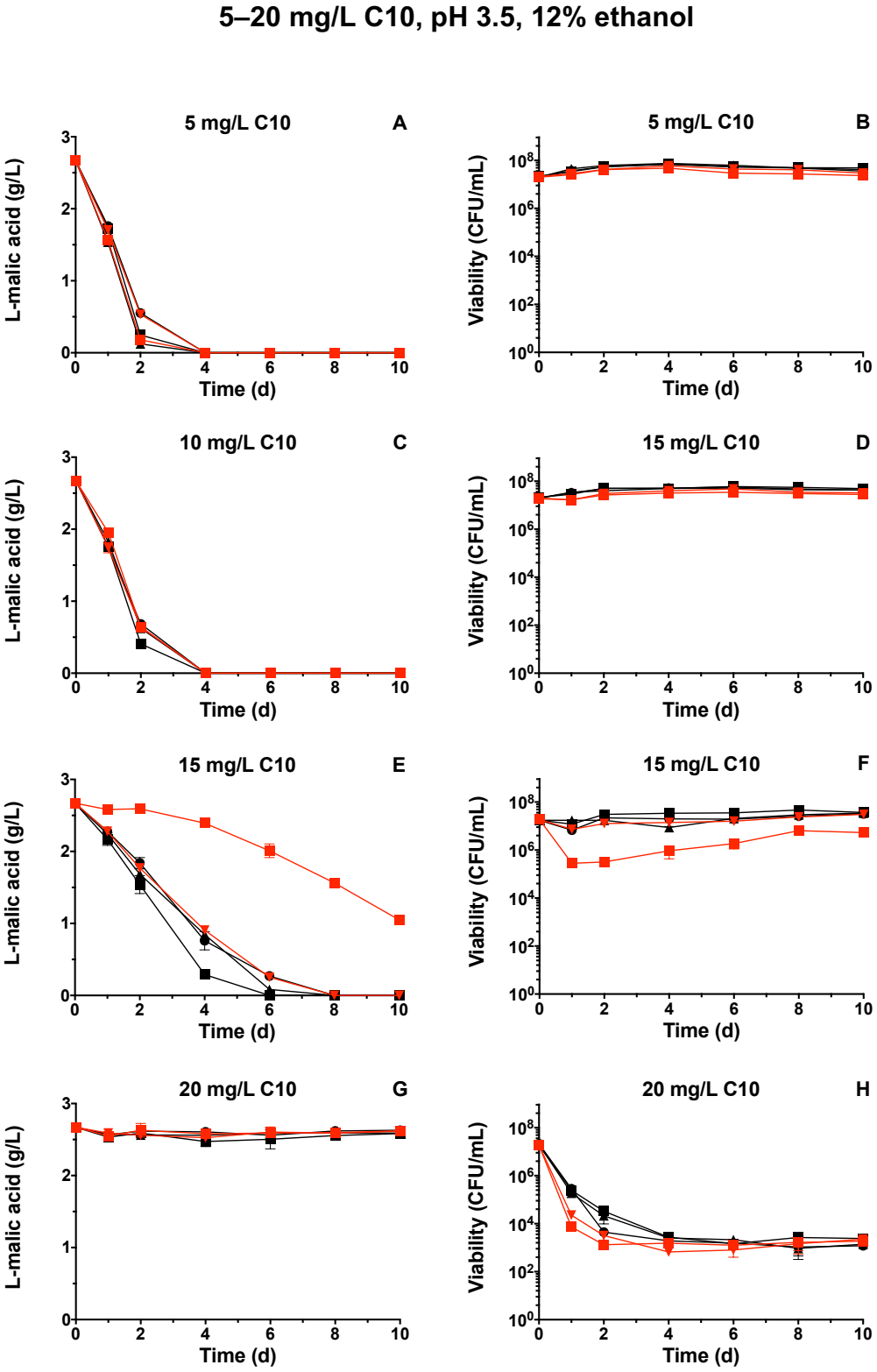


Fig. 4.6. L-malic acid consumption and CFUs of evolved strains 1-161 (●), 2-49 (■) and 3-83 (▲) compared to strains A90 (▼) and SB3 (■) in RFCDGJM. Values are the mean of three biological replicates ± SD.

Dodecanoic acid (C12)

Dodecanoic acid proved to be highly inhibitory at concentrations between 10 and 20 mg/L, at which no MLF occurred and viability dropped to between 10^3 and 10^4 CFU/mL (Fig. 4.7C–H). At the lowest concentration examined (5 mg/L) MLF was delayed with SB3 and in turn the DE strains finishing MLF sooner than A90 (Fig. 4.7A). Viability was unchanged during the experiment (Fig. 4.7B).

Mixed medium chain fatty acids

Given the likelihood that mixtures of MCFA occur in wine, a combination of the three used in this study was also examined. A total amount of 4.5 or 9 mg/L of mixed MCFA did not inhibit bacterial fermentation or growth (Fig. 4.8A–D). However, when the combined C8, C10 and C12 was increased to 13.5 mg/L, an obvious inhibition of MLF occurred (Fig. 4.8E). SB3 was the slowest, leaving 15% L-malic acid un-metabolised while strain 3-83 was the fastest with a fermentation of 8 days. (Fig. 4.8E). Bacterial viability was not affected at this concentration, except for strain SB3, whose population declined to 10^6 CFU/mL by Day 1 before recovering to 10^7 CFU/mL (Fig. 4.8F). Both malolactic activity and viability of all strains were strongly affected with the addition of 18 mg/L mixed MCFA (Fig. 4.8G and H).

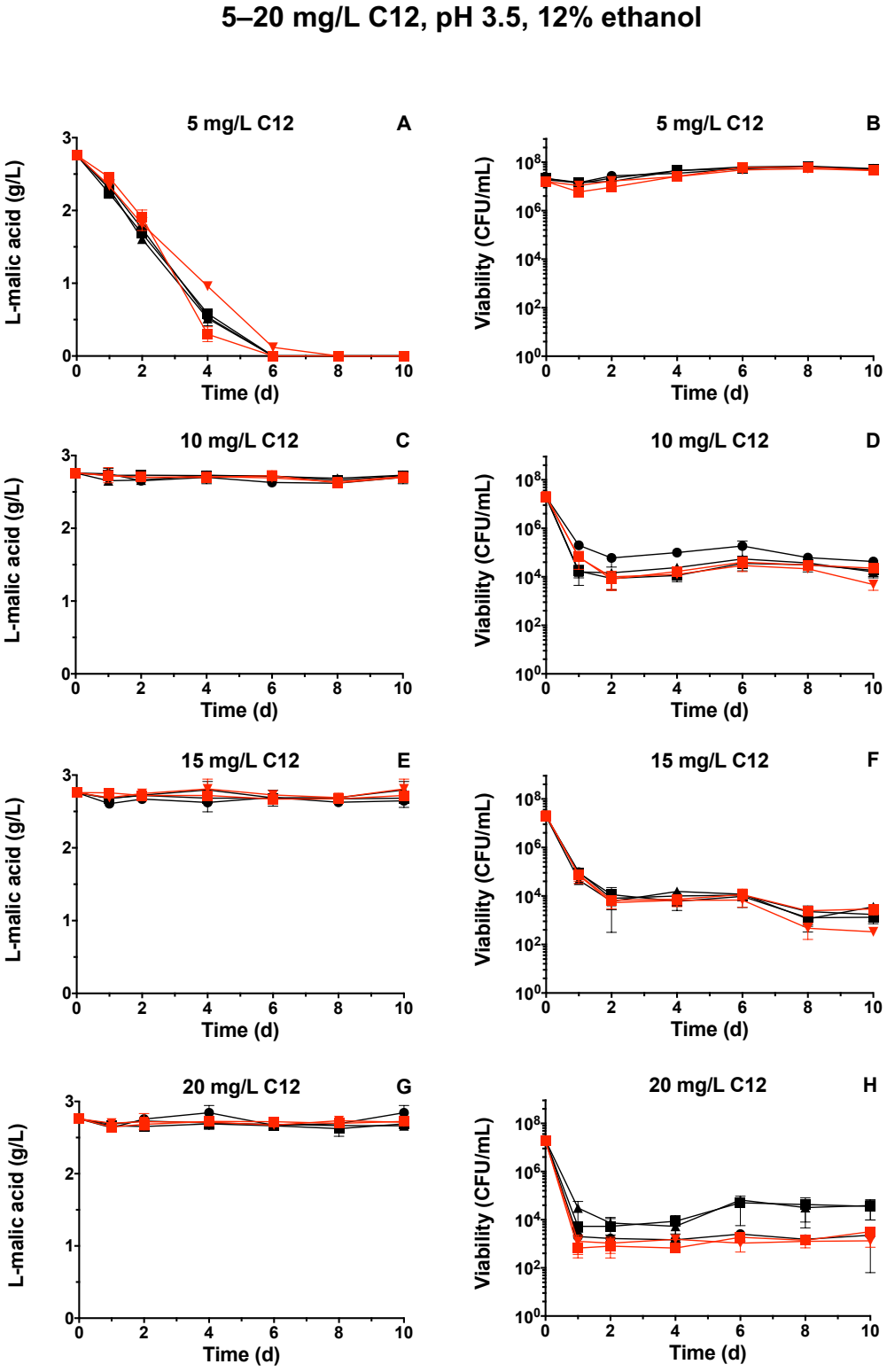


Fig. 4.7. L-malic acid consumption and CFUs of evolved strains 1-161 (●), 2-49 (■) and 3-83 (▲) compared to strains A90 (▼) and SB3 (■) in RFCDGJM. Values are the mean of three biological replicates ± SD.

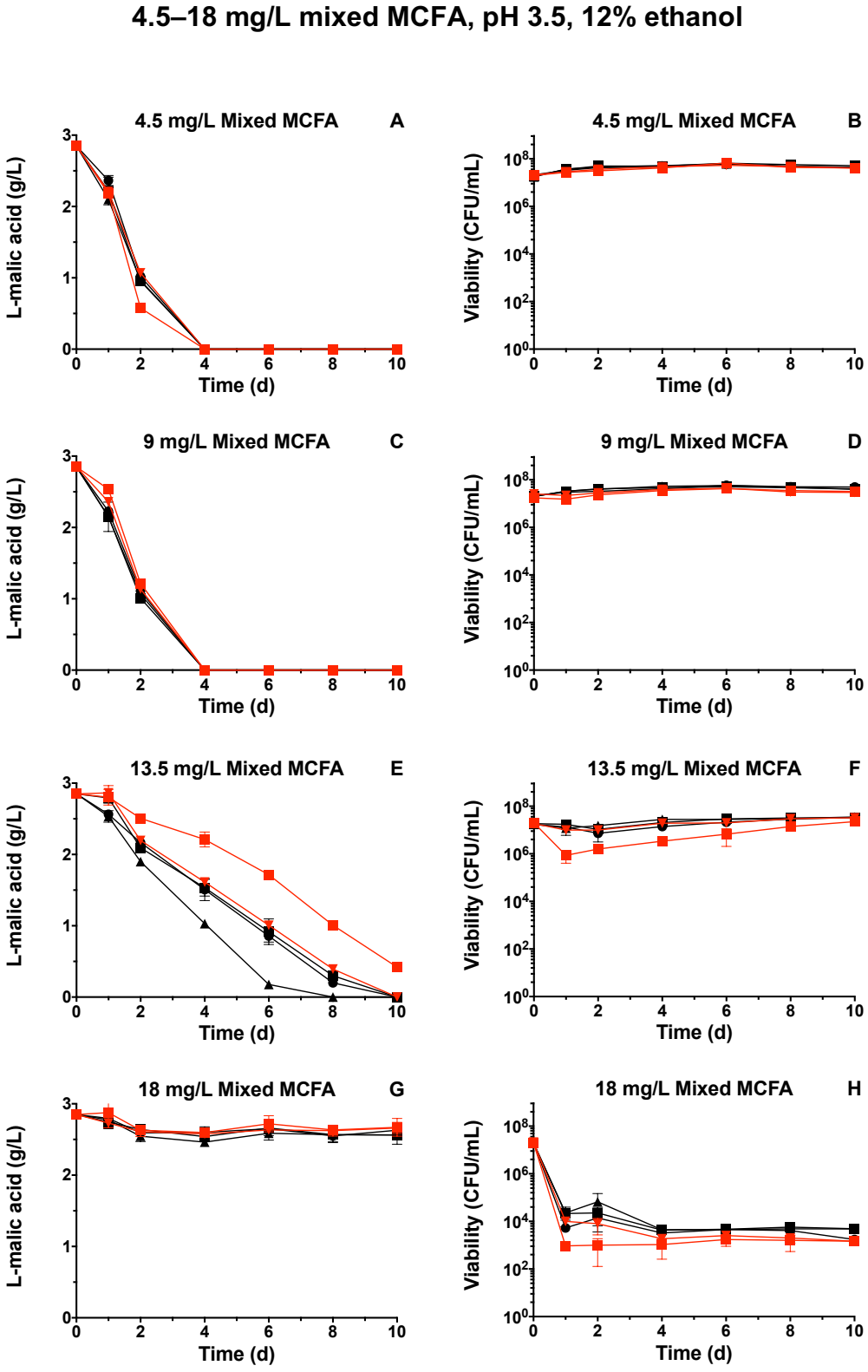


Fig. 4.8. L-malic acid consumption and CFUs of evolved strains 1-161 (●), 2-49 (■) and 3-83 (▲) compared to strains A90 (▼) and SB3 (■) in RFCDGJM. Values are the mean of three biological replicates ± SD.

4.3.5 Strain stability in Shiraz

DE strains 1-161, 2-49 and 3-83 were passaged in MRSAJ for at least 50 generations. Fermentation performance and cell viability were evaluated to test if they were able to maintain their robustness compared to SB3 and A90 in Shiraz containing multiple stressors. Both the evolved and the passaged evolved strains maintained their advantage over parental strains SB3 and A90 in terms of L-malic acid consumption and viability (Table 5.3). Passaged (p-) Strains p-1-161, p-2-49 and p-3-83 completed MLF in 12 days while the original strains SB3 and A90 only metabolised 60% and 68% L-malic acid, respectively before MLF became stuck (Table 5.3). In comparison to strains 1-161, 2-49 and 3-83, the passaged strains appeared marginally slower than non-passaged strains and potentially had reduced viability compared to their corresponding non-passaged strains (Table 5.3).

Table 5.3 L-malic acid consumption and viable populations of *O. oeni* in Shiraz

Strains	L-malic acid consumed[a]	Fermentation duration (d)	Viable population by Day 12[b] (CFU/mL)
SB3	60% (stuck MLF)	N/A	4.9×10^4
p-SB3	57% (stuck MLF)	N/A	2.1×10^4
A90	68% (stuck MLF)	N/A	4.4×10^4
p-A90	62% (stuck MLF)	N/A	2.9×10^4
1-161	100%	10	1.5×10^6
p-1-161	100%	12	1.1×10^6
2-49	100%	10	2.3×10^6
p-2-49	100%	12	1.9×10^6
3-83	100%	9	2.7×10^6
p-3-83	100%	11	2.3×10^6

[a] This Shiraz had 2.70 mg/L L-malic acid before MLF
[b] All strains were inoculated at approximately 5×10^7 CFU/mL

4.3.6 Malolactic fermentation in non-sterile Shiraz

In order to assess the impact of a wine matrix and potentially other organisms (e.g. yeast), a lab-scale MLF (250 mL) in a Shiraz was performed with all bacterial strains pre-cultured from glycerol stocks. Strain 2-49 finished MLF by Day 32 whilst 3-83, A90 and SB3 suffered from stuck fermentation from Day 24 (Fig. 4.9A).

Trials were also conducted in 50 L kegs to more closely mimic industrial fermentation conditions. Strains SB3, A90 and 3-83 were inoculated into the Shiraz at ~5 × 10^6 CFU/mL to commence MLF (Fig. 4.9D). Due to poor growth of the starter, strain 2-49 was inoculated at 2–4 × 10^6 CFU/mL. Even so, fermentation kinetics were similar to those of strain 3-83 (Fig. 4.9C and D). Fermentation by A90 became stuck when viable cell numbers dropped below 10^6 CFU/mL from Day 23 (Fig. 4.9 C and D). SB3 was highly viable and completed MLF by Day 78 while the evolved strains 2-49 and 3-83 took an additional 14 days to nearly complete. Microscopic observation of Day 56 samples showed that yeast was the dominant microbe in all samples even though alcoholic fermentation was completed approximately two months earlier (data not shown). It was difficult to find *O. oeni* cells in any of the samples, which is in accord with plating results (Fig. 4.9D).

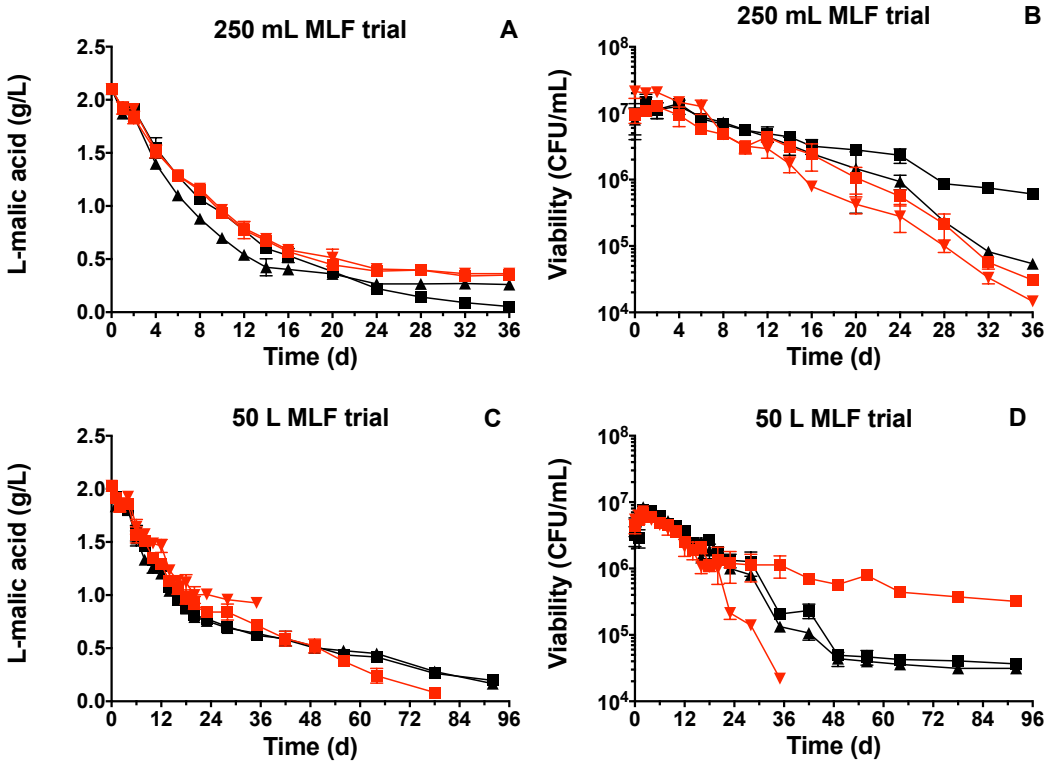


Fig. 4.9. L-malic acid consumption and population of strains SB3 (■), A90 (▼), 2-49 (■) and 3-83 (▲) in un-filtered Shiraz. Values are the mean of three biological replicates ± SD.

4.4 Discussion

Tolerance to common inhibitors in wines is of great importance to the wine bacterium *O. oeni*. This chapter documented viability and L-malic acid consumption of five *O. oeni* strains in RFCDGJM with different concentrations of multiple stressors. Combined stressors of ethanol, pH and SO_2 were introduced in the DE experiment, and levels of these stressors were increased over time (Chapter 3). As anticipated, the evolved strains had greater tolerance to combined stressors of ethanol and pH (Figs. 4.1, 4.2 and 4.3) and combined stressors of ethanol, pH and SO_2 (Fig. 4.4). Evolved strains 2-49 and 3-83 were able to survive in RFCDGJM with 20% (v/v) ethanol at pH 3.5. By comparison, this condition led to a complete loss of viability of parental strains SB3 and A90 (Fig. 4.1H). The evolved strains also fermented more L-malic acid and had higher viability than the

parent strain A90 in RFCDGJM with 15% (v/v) ethanol at pH 2.8 (Fig. 4.3G and H) and 20 mg/L SO_2, 12% (v/v) ethanol at pH 3.5 (Fig. 4.4C and D). These results, along with other similar studies involving DE of microorganisms (Teusink et al. 2009; Liccioli 2010; Bachmann et al. 2012; Betteridge et al. 2017) indicate that DE has the potential to generate strains with intended improvements.

Aside from ethanol, pH and SO_2, MCFA produced by yeast during alcoholic fermentation can also limit bacterial growth and reduce the ability of *O. oeni* to catabolise L-malic acid (Capucho and San Romão 1994). Thus, investigation of *O. oeni* tolerance of MCFA was considered in this study. Inhibition by MCFA was greater when chain length was greater (Figs. 4.5, 4.6, 4.7). Lonvaud-Funel et al. (1988) observed similar results, even though the minimum concentration of C10 and C12 to cause growth inhibition was lower than in this study, being 4 mg/L and 0.5 mg/L, respectively. This could be due to the different strains and medium used in the two studies.

A combination of MCFA caused greater inhibition to MLF than individual MCFAs (Lonvaud-Funel et al. 1988; Nehme et al. 2008). In this study, for strains 2-49 and 3-83, 4.5 mg/L of C12 coupled with 4.5 mg/L of C10 and C8 delayed MLF by 4 days. However, 5 mg/L of C8 and C10 did not affect MLF whilst 5 mg/L of C12 showed a delay of only 2 days in fermentation. Despite the inhibitory effect, both strains 2-49 and 3-83 were still quicker in utilising L-malic acid than A90 in RFCDGJM with 15 mg/L C10 (Fig. 4.6E), 5 mg/L C12 (Fig. 4.7A), or 13.5 mg/L mixed MCFAs (Fig. 4.8E), respectively, highlighting the improved tolerance to MCFAs developed during DE in the evolved strains. However, as MCFAs were not introduced during the DE experiment (Chapter 3), the superiority of the evolved strains over the parent under such conditions could be as a result of cross-

protection or cross-tolerance (Almario et al. 2013; Dragosits et al. 2013; Reyes et al. 2013; Oide et al. 2015). Genetic analysis has shown that SNPs in certain genes might be the basis for the cross-tolerance behaviour of *E. coli* (Dragosits et al. 2013).

The ethanol tolerant strain A90 grew more in MRSAJ with combined ethanol and pH stressors (Chapter 2). However, it should be noted that A90 also exhibited some fitness trade-offs since rates of L-malic acid consumption were slower than the parent SB3 in some cases, e.g. in RFCDGJM with C8 ranging from 5 to 20 mg/L (Fig. 4.5A, C, E and G) and in RFCDGJM containing C12 (Fig. 4.7A).

Strains 1-161, 2-49 and 3-83 also displayed fitness trade-off in SO_2 tolerance assays where viable populations of these evolved strains were less than that of A90 when inoculated in RFCDGJM with 30 mg/L of SO_2, even though all five strains showed no malolactic activity under this condition (Fig. 4.4 F). The fitness trade-off phenomenon is common during long-term laboratory evolution (Hong et al. 2011; Oakley et al. 2014; Hill et al. 2015; Schick et al. 2015). Dragosits et al. (2013) suggested that the upregulation of iron-related genes in osmotic and butanol evolved *E. coli* caused an excess of iron inside the cell thereby leading to the poor performance of these evolved bacteria under oxidative stress (Touati et al. 1995).

Since several physio-chemical factors inhibit *O. oeni* growth, many studies have sought to characterise growth of *O. oeni* strains under different conditions in laboratory media (Drici-Cachon et al. 1996; Capucho and San Romão 1994; Britz and Tracey 1990; G-Alegría et al. 2004; Chu-Ky et al. 2005; Olguín et al. 2009), or

wine/wine-like media (Davis et al. 1986; Knoll et al. 2011; Zhang et al. 2013). However, the differences in the level of stressors as well as strains used in each study make it difficult to compare results. In this study, the use of RFCDGJM eliminates variations of the components in this medium between trials while mimicking a wine-like environment. Results were reproducible when testing *O. oeni* growth in this medium, which can be demonstrated further by the similar growth of SB3 and A90 as evaluated in Chapter 2 in RFCDGJM under corresponding conditions.

Aside from stress tolerance, another requirement of the selected strains is the stability of the improved MLF phenotype that they exhibit. This is because *O. oeni* are considered hyper mutable as they lack the *mutS* and *mutL* genes (Makarova et al. 2006; Makarova and Koonin 2007). Both *mutS* and *mutL* encode key enzymes for the methylated mismatch repair pathway that corrects errors during DNA replication. After 50 generations, the efficient fermentation and higher viability phenotypes of evolved strains 1-161, 2-49 and 3-83 were still apparent in sterilised Shiraz with the combined stress of ethanol, SO_2 and pH (Table 5.3). This indicates that the improved stress tolerant phenotype developed during DE of these strains is stable for at least 50 generations of growth under low stress conditions. Nonetheless, the passaged strains did not perform as well as the non-passaged strains in regards to both fermentation and viability (Table 5.3), indicating some reversion may have occurred during sub-culturing.

Both the tolerance assay experiment and the evaluation of strain stability showed that the latterly isolated strains, 2-49 and 3-83, performed better than strain 1-161, which was isolated at an earlier stage. Perhaps the longer time in DE allowed more beneficial mutations to occur. As such, strains 2-49 and 3-83 were

therefore further characterised under more industrially relevant conditions. To this point, experiments were conducted in a sterile medium or wine. Fermentation in non-sterile wine is potentially more challenging them compared to MLF in sterile media or wine.

The advantage of the optimised DE strains compared to strain A90 still existed in both the 250 mL and 50 L fermentations; however, fermentation efficiency gradually decreased when scaling up fermentation (Fig. 4.9). The same effect was observed in a 220 L fermentation in Merlot conducted by SB3 and A90 where there was no significant difference between fermentation duration of these two strains (K. Sumby, personal communication). The reason why superiority of the optimised strains is not always transferred into larger scale fermentations is yet to be determined.

Prolonged MLF in non-sterile Shiraz might be a result of the yeast-bacteria interactions. It is possible that PDM used to ferment Shiraz in this study exerted an inhibitory effect on the four SB3 related strains. Both PDM and EC 1118 belong to the *Prise de Mousse* (PdM) clade with little overall genetic differences (Dunn et al. 2012; Borneman et al. 2016). In a recent study comparing the performance of wine-related bacteria in red chemically defined grape juice co-inoculated with commercial yeast strains, SB3 was not compatible with EC 1118 compared to other commercial bacterial strains (L. Bartle, personal communication). Additionally, competition between yeast and *O. oeni* for nutrients and carbon sources would make it difficult for *O. oeni* to grow in Shiraz leading to extended MLF duration.

Generally, CFUs of 10^6/mL are considered to be the threshold at which LAB can conduct MLF (Rosi and Canuti 2003; Remize et al. 2005). However, in the 50

L MLF trials, strains SB3, A90, 2-49 and 3-83 were still able to utilise L-malic acid when viable cell numbers dropped just below 10^6 CFU/mL (Fig. 4.9B). All strains only stopped fermenting when viable cell numbers dropped below 10^5 CFU/mL. Arnink and Henick-Kling (2005) found that MLF could occur even when cultures did not grow over 10^6 CFU/mL. While SB3, 2-49 and 3-83 still fermented at approximately 10^5 CFU/mL, fermentation was slow. Importantly, prolonged fermentation may increase the risk of oxidation and microbial spoilage in wine. Hence maintaining higher viable cell numbers in wine fermentations is crucial for industry.

Even though the OD_{600} of each culture was adjusted prior to inoculation, the inoculated viable cell numbers varied among strains in larger scale fermentations (Fig. 4.9B and D). For example, CFUs determined by plate counting of A90 at inoculation were much higher than the other strains. Some variability in CFU's could arise from different tendencies of the strains to form chains. However, a significant difference in CFUs might still indicate over inoculation of A90 compared to other strains in the 250 mL and 50 L fermentations (Fig. 4.9B and D). Differences in viability might have played a role in this. For some strains, the proportion of viable cells could have been larger than others at the same time. In future work, it would be helpful to compare growth rate and viability among strains in both laboratory media and the acclimation medium prior to inoculation. Culture preparation could then be adjusted based on this data to achieve a similar inoculation rate in the final media.

Several very recent studies have validated the use of flow cytometry and qPCR with DNA dyes for real time enumeration of wine-related microbes (Bouix and Ghorbal 2013; Vendrame et al. 2014; Rizzotti et al. 2015; Longin et al. 2017).

However, as *O. oeni* forms chains easily, it is difficult to enumerate with a flow cytometer accurately (L. Bartle, personal communication). If this problem is solved, it might be more valuable to employ a flow cytometer to monitor MLF.

4.5 Conclusion

Physiological characterisation of the DE optimised strains was performed with reference to three traits; tolerance to different stressors in RFCDGJM, strain stability in a sterile Shiraz, and fermentation ability in larger volumes of Shiraz with yeast present for interaction. The results show that DE improved the performance of *O. oeni* in these evolved strains to some extent. The evolved strains had faster MLF and more viability than commercial strain SB3 and the parent strain A90 under stressful conditions. However, the original strain SB3 had the fastest fermentation under less stressful conditions. The phenotypes of the evolved strains were also largely stable with fermentation by passaged strains being completed 2 days later than the corresponding original DE optimised strains. Overall, fermentation performance of the passaged mutants was still markedly better than SB3 and A90. Fermentation performance of the DE optimised strains was not desirable in non-sterile Shiraz, but it was still significantly better than parent strain A90 in both the 250 mL and 50 L scale fermentations.

The variation in phenotypes observed during tolerance assays of the DE strains and the parent A90 may be due to differences in genetic makeup of these strains. Therefore, the next chapter describes the genotypic characterisation of strains 2-49, 3-83 and A90 through whole genome sequencing to identify SNPs that might be responsible for improved stress tolerance, as well as to detect the presence of genes encoding core enzymes that could contribute to wine aroma and flavour profile.

Chapter 5 Whole genome sequencing and comparison of *O. oeni* genomes

5.1 Introduction

The previous chapters described the use of directed evolution to generate the strains, 2-49 and 3-83, which displayed faster consumption of L-malic acid and higher tolerance to combined low pH, high ethanol and SO_2 or decanoic acid stressors compared to their parent strain A90. These offered the opportunity to study the beneficial genetic mutations, in these conferring increased fitness under stressful winemaking conditions.

Previous studies have characterised a number of individual stress response genes, including *hsp18*, *clpX*, *ftsH*, *trxA*, *cfa*, *ggpps*, *rmlB*, *grpE*, *groES* and *atpB* in *O. oeni* under stressful conditions (Guzzo et al. 2000; Grandvalet et al. 2005, 2008; Cafaro et al. 2014; Da Silveira et al. 2004; Desroche et al. 2005; Jobin et al. 1999; Bourdineaud et al. 2003; Beltramo et al. 2006). These genes are involved in various cellular processes related to environmental stress adaptation.

One of the most widely studied genes, *hsp18* encodes a small heat shock protein Lo18, which has been shown to display a membrane-protective role in *O. oeni* (Maitre et al. 2014). A recent study targeting biological function of *O. oeni* Lo18 *in vivo* using an antisense RNA approach, confirmed the involvement of Lo18 in response to ethanol and acid shock, as well as its role in membrane stablisation during stress exposure (Darsonvol et al. 2016a). The cell membrane is generally considered the main target of environmental stressors and has been reported that *O. oeni* modifies membrane structure and composition to allow survival and growth in response to stress (Chu-Ky et al. 2005; Grandvalet et al.

2008; Bonomo et al. 2017). Cell membrane damage caused by stressors, for example ethanol, can lead to leakage of intracellular proteins. As a consequence, crucial cellular processes including synthesis of ATP and transport of amino acids are affected (Guzzo et al. 2000).

Cell wall biosynthesis can also protect *O. oeni* from cell damage in response to stressors. The protein MreB, which is associated with cell wall biogenesis, was downregulated in *O. oeni* strain PSU-1 after inoculation into a wine-like medium with 12% (v/v) ethanol at pH 3.4 (Margalef-Català et al. 2016). MreB1 and B2, which determine cell shape, were also found to be less abundant in acid-stressed cells of *Lb. plantarum* 423 (Heunis et al. 2014). These findings highlight the role of the cell envelope as a protective barrier for bacteria against oenologically related stressors in wine.

The uptake of nitrogen under stressful conditions might also assist *O. oeni* in coping with environmental stressors. Both Margalef-Català et al (2016) and Betteridge et al (2017) observed up-regulation of genes encoding amino acid transport in response to stress. Several genes associated with peptidases in PSU-1 are activated in response to 12% (v/v) ethanol at pH 3.4 (Margalef-Català et al. 2016), and peptides were suggested to be the dominant nitrogen compound in wine (Feuillat et al. 1998). These studies indicate the relevance of amino acids and peptides to stress adaptation in *O. oeni*.

In RNA-seq analysis, 15 amino acyl-transfer RNAs (tRNAs) were significantly downregulated in *O. oeni* strain AWRIB419 compared to AWRIB551 and AWRIB552 during MLF (Sternes et al. 2017). tRNAs are involved in protein synthesis and act as signalling molecules in several cellular processes, including

modification of phospholipids in cell membranes (Sternes et al. 2017). Accordingly, tRNAs may also play a role in response to stresses.

A whole genome sequencing (WGS) approach used to characterise previously generated strain (A90) highlighted the potential importance of SNP in several genes. Such an approach provides a way to identify all the mutations and has been widely used with success to study genetic evolution of various microbes produced via long-term evolution strategies (Deatherage et al. 2017; Tenaillon et al. 2016; Payen et al. 2016).

Genome comparison of the parent and improved evolved strains will expand current knowledge on *O. oeni* genomes as well as genes associated with stress resistance and improved MLF performance. In addition, the genetic makeup of *O. oeni* may offer important information to describe oenological traits of this wine bacterium. Apart from high tolerance to stressors, the improved LAB are also required to produce wines with desirable sensory attributes or at least no undesirable ones. Such a trait is often characterised by the expression of several genes involved in the formation of products that can impact the sensory profile of wine (Callejon et al. 2016; Cappello et al. 2014). Other than this approach, WGS is a straightforward means for determining the presence of genes potentially linked to specific traits in *O. oeni* genomes. For example, Mi (2016) observed the presence of genes encoding esterases and glycosidases in an indigenous *O. oeni* strain isolated from a Clare Valley winery through WGS. The presence of these genes suggested that the indigenous *O. oeni* strain had the potential to release desirable aromas during winemaking.

Using WGS and comparative genomics, this chapter describes the determination of genetic differences between the evolved strains and the parents,

including A90 and SB3. Additionally, genomic variations were also analysed between the SB3-related strains and the commercially available robust strain VP41 since the latter has been reported to conduct fast MLF (Van der Merwe 2008; L. Bartle, personal communication). Finally, genes relevant to wine quality were screened in the genomes of SB3, A90, 2-49 and 3-83 to detect their presence in these strains.

5.2 Material and methods

5.2.1 Bacterial strains

Four O*. oeni* strains had their whole genomes sequenced. They were SB3 (Laffort), A90 (Betteridge et al. 2017), 2-49 and 3-83 (Chapter 3).

5.2.2 Whole genome sequencing

O. oeni strains were grown in fresh MRSAJ medium for 3 days to reach exponential phase. Cultures in MRSAJ were then sent to the Australian Centre for Ecogenomics (ACE) at the University of Queensland for WGS. Both genomic DNA extraction and library preparation (using standard Nextera XT protocol, QC, quantification and pooling for sequencing) were performed by ACE. The genome was sequenced using the Illumina MiSeq (v2 2 × 150bp) platform. Raw sequencing data was received as short sequence reads of 35-150 bp as FASTQ files.

5.2.3 Genome assembly and annotation

Geneious R10 (Version 10.2.2, http://www.geneious.com) was used to analyse the sequence data (Fig. 5.1). Sequence reads were first paired and low-quality ends of the paired reads were trimmed using the default setting.

Two methods were then applied to assemble the genomes of *O. oeni* strains with various assemblers. Genome sequences were *de novo* assembled from paired reads using the SPAdes, MIRA, CAP3, Velvet (Version1.2.10), Geneious and Tadpole (Version 37.25) assemblers with default parameters, except for the Geneious assembler, where high sensitivity and medium speed was used. SPAdes, MIRA and CAP3 assemblers failed to assemble in this manner. Next, Paired reads were normalised using BBNorm (Version 37.25) to a target coverage of 40. The normalised reads were *de novo* assembled using SPAdes (Version 3.10.0), MIRA (Version 4.0) and CAP3 assemblers respectively but failed again (See 5.3.1 for more information). The SPAdes assembler was also used to assemble genomes from the raw data after trimming of low quality regions. All contigs generated from the above assemblers were quality trimmed; contigs with length less than 1000 bp were removed. Nucleotide BLAST was performed with each of the contigs produced by the Geneious assembler to analyse the presence of possible bacteriophage DNA, plasmids and genes of oenological traits.

Secondly, the paired reads were mapped to a reference genome, the PSU-1 sequence (Accession No. NC_008528, Table 5.1) using the Geneious assembler and Bowtie2 (Version 2.3.0). Finally, gene annotations were transferred from the PSU-1 sequence to the consensus sequences or contigs of all assembled genomes using Geneious R10 having a similarity of at least 95%.

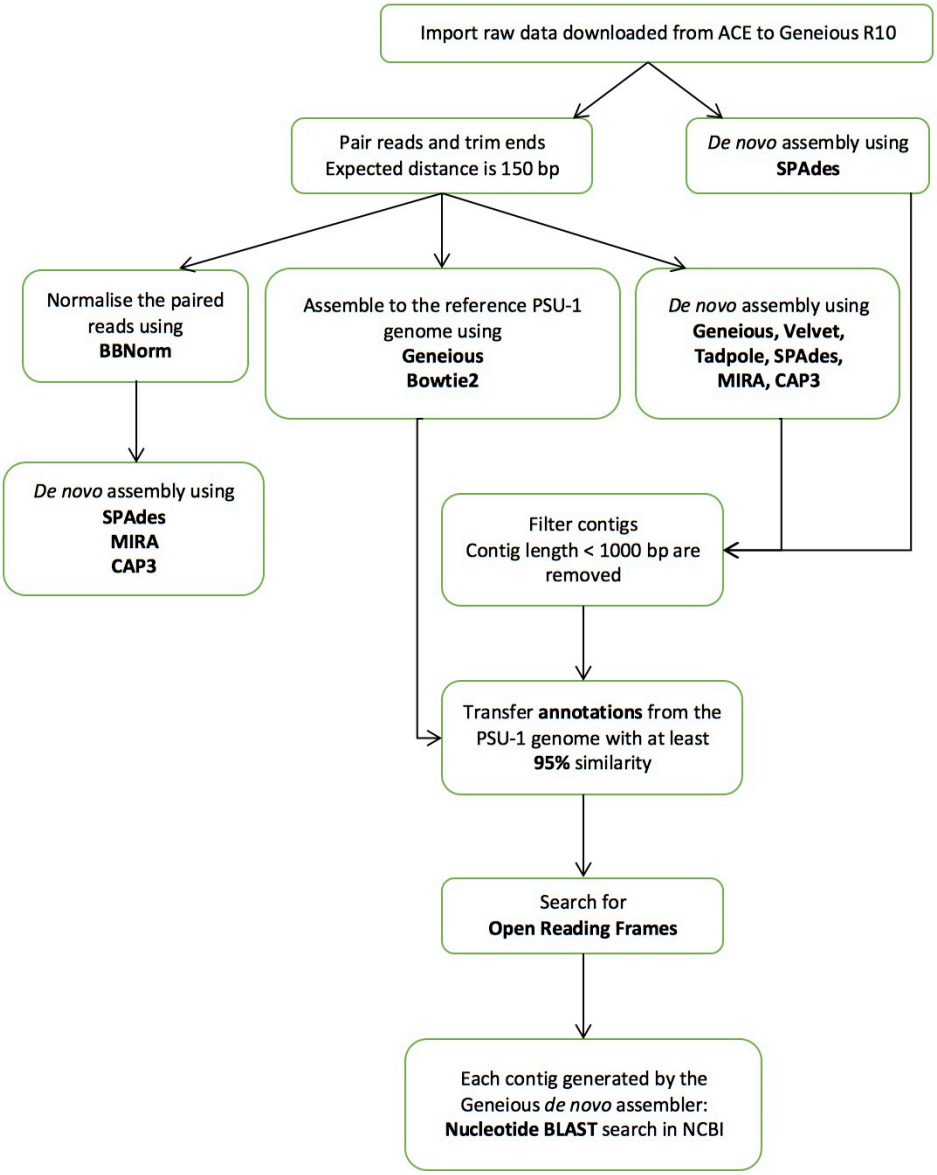


Fig. 5.1. Pipeline for *O. oeni de novo* genome assembly and annotation

5.2.4 Whole genome alignment and preliminary identification of SNPs

Genome alignments using LASTZ were performed 3 times to identify variations between genomes (Fig. 5.2). Specifically, the genomes of strains 2-49 and 3-83 were aligned against the genome of A90; the genomes of strains A90, 2-49 and 3-83 together were aligned against the genome of SB3; the genomes of strains SB3, A90, 2-49 and 3-83 together were aligned against the genome of

VP41 (Accession No. NZ_ACSE00000000.1; Table 5.1). SNPs were preliminarily identified using the “Find variation/SNPs” function of Geneious R10. Criteria for SNP identification were at least 50-fold coverage with 100% variant frequency.

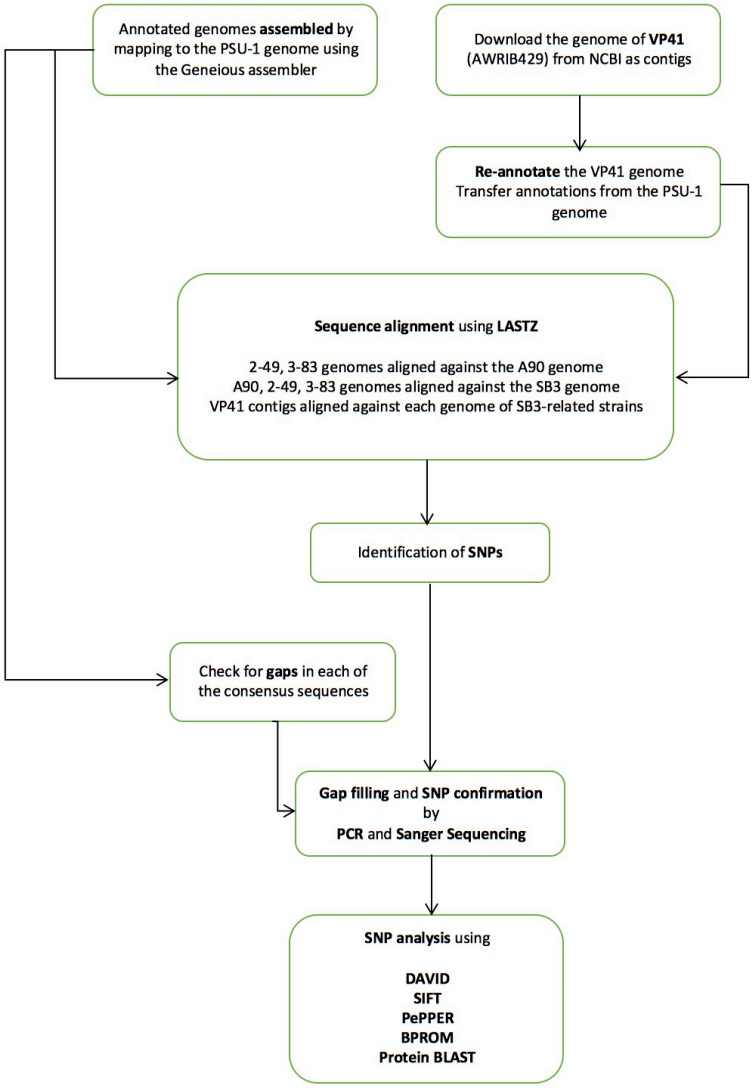


Fig. 5.2. Pipeline for SNP analysis of whole genome assemblies

Table 5.1. General features of VP41 and PSU-1 genomes

	Bio Projects	Size	Assembler	GC%	Contigs	N50	L50	Genes
VP41	PRJNA38663	1.93 Mb	Velvet	37.9%	58	85, 101 bp	8	2,028
PSU-1	PRJNA317	1.78 Mb	Jazz	37.9%	1	1.78 Mb	1	1,831

Information from NCBI

5.2.5 Genome gap filling and confirmation of SNPs between 2-49, 3-83 and A90

Primer walking was employed to fill the gaps (Appendix 3) within the sequencing of genomes of A90, 2-49 and 3-83. Primers complimentary to sequences 200–300 bp outside of the gaps were designed using Primer3 (http://bioinfo.ut.ee/primer3-0.4.0). PCR was conducted using Velocity DNA polymerase (BIO-21098, Bioline USA) or Ranger DNA polymerase (BIO-21121, Bioline USA) depending on the anticipated size of each gap. PCR reaction and setup (Appendix 4) was as per the manufacturer's instruction for each polymerase with modification only of extension time (2–10 min).

For confirmation of SNPs between strains 2-49, 3-83 and A90, primers for amplification of fragments containing each SNP identified by genome comparison were designed using Primer3. The primers were then used to amplify target regions of the DNA sequence using Velocity DNA polymerase.

All primers were blasted in NCBI (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) to check for nucleotide-specific binding. All primers (Appendix 2) were purchased from Sigma-Aldrich. Extraction of genomic DNA for PCR was performed as described in Appendix 5.

PCR products were visualised by agarose gel (0.7% (w/v)) electrophoresis in TAE (Tris-acetate-EDTA) buffer. PCR products were purified using a PCR clean-up kit (A9282, Promega USA) following the manufacturer's instructions and sent to the Australian Genome Research Facility (Adelaide) for Sanger sequencing.

Both the Sanger reads and paired reads were then re-assembled using the two methods described in 5.2.3 with the Geneious assembler. Sequences that might contain a SNP were also compared via pair-wise alignment using ClustalW alignment.

5.2.6 SNP analysis

SNPs of interest were analysed using various databases (Figure 5.2). Gene Ontology term enrichment and KEGG pathway mapping were analysed using DAVID (https://david.ncifcrf.gov/) (Huang et al. 2008; 2009a). SIFT (sorting intolerant from tolerant, http://sift.jcvi.org) analysis was performed on some genes of interest to predict the potential effect of non-synonymous SNPs on protein function (Kumar et al. 2009). BPROM (Solovyev and Salamov 2011) and PePPER (de Jong. et al. 2012) were used to predict if SNPs in non-coding regions were in promoter sequences. Genes that contained SNPs between strains 2-49 or 3-83 and A90 were also analysed using Protein BLAST to determine if they were similar to genes in other organisms.

5.3 Results

5.3.1 General genome features

De novo *genome assembly*

De novo assembly of the genome was performed with various assemblers described in 5.2.3. SPAdes failed to align paired reads because of erroneous κ-mers, but could be used to assemble raw data. Both MIRA and CAP3 failed to assemble genomes from paired reads as well as the normalised paired reads because of deep coverage. Based on the number of contigs generated, Geneious was considered the best assembler in this study, with 23–51 contigs generated for

each strain (Table 5.2). SPAdes, Velvet and Tadpole generated 43–70 scaffolds, 109–187 contigs and 263–298 contigs, respectively. General genome features are summarised in Table 5.2. The genome of these four strains was approximately 1.75 Mb in length with 38–40% GC content.

Table 5.2. Assembly and annotation statistics of *De novo* assembly and mapping with the Geneious assembler

Statistics	**SB3**[b]	**A90**		**2-49**		**3-83**	
		WGS[*]	**WGS+SS**[**]	**WGS**	**WGS+SS**	**WGS**	**WGS+SS**
***De novo* assembly using the Geneious assembler**							
Genome size (bp)	1,744, 213	1,750,653	1,751,442	1,748,544	1,752,895	1,746,786	1,752,820
GC content	38.6%	39.2%	39.2%	39.7%	37.9%	39.9%	39.9%
Contigs	51	23	19	31	25	35	26
Minimum contig length (bp)	1,049	1,479	1,762	1,049	1,168	1,111	1.080
Median contig length (bp)	12,617	60,316	71,615	28,808	27,780	18,122	33,041
Mean contig length (bp)	34,200	76,115	92,181	56,404	70,115	49,908	67,416
Max contig Length (bp)	154,772	434,861	281,729	367,469	367,468	309,855	358,187
N50 Length (bp)	87,176	143,331	195,050	102,701	207,845	129,505	129,506
Number of contigs >= N50	8	4	4	5	4	4	4
Number of genes	1,694	1,707	1,709	1,701	1,704	1,700	1,706
Number of ORFs[a]	18,176	18,219	18,266	18,191	18,241	18,198	18,250
Mapping to the PSU-1 genome using the Geneious assembler							
Genome size (bp)	1,763,797	1,751,945	1,754,796	1,744, 823	1,768,758	1,743,720	1,752,028
GC content	38.0%	38.0%	38.0%	38.0%	38.0%	38.0%	38.0%
Number of genes	1,725	1,728	1,729	1,727	1,729	1,708	1,729
Number of ORFs	18,042	18,019	18,021	18,018	18,026	18,031	18,031

[a] ORFs refers to open reading frames
[b] Gap filling was not performed for SB3
[*] Whole genome sequencing (MiSeq)
[**] Whole genome sequencing and Sanger sequencing

Reference guided assembly

A total 14 main gaps (> 100 bp) in SB3-related strains (SB3, A90, 2-49 and 3-83) were identified in draft genomes assembled to the PSU-1 sequence (Fig. 5.3). Three gaps were not covered after primer walking and Sanger Sequencing.

Regions of repetitive sequence close to Gaps 2 and 3 (Fig. 5.4A) resulted in poor Sanger reads, leading to a failure in creating a circular *O. oeni* genome. Gap 8, which was located downstream of a transposon (Fig. 5.4 B), also had poor Sanger reads. The remaining gaps were not located close to transposons and were filled with Sanger reads. As a consequence, the genome sizes increased by 1,500–6,000 bp after gap filling (Table 5.2).

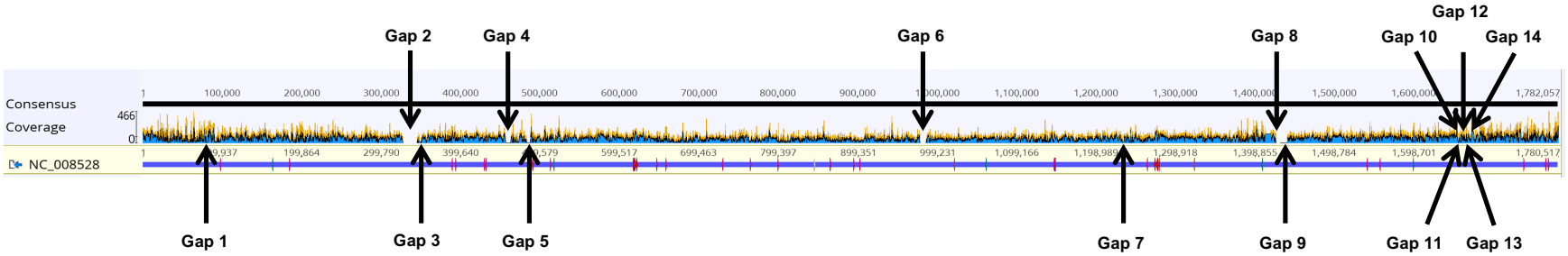


Fig. 5.3. Location of gaps (> 100 bp) when mapping the 2-49 genome to the reported PSU-1 genome using the Geneious assembler.

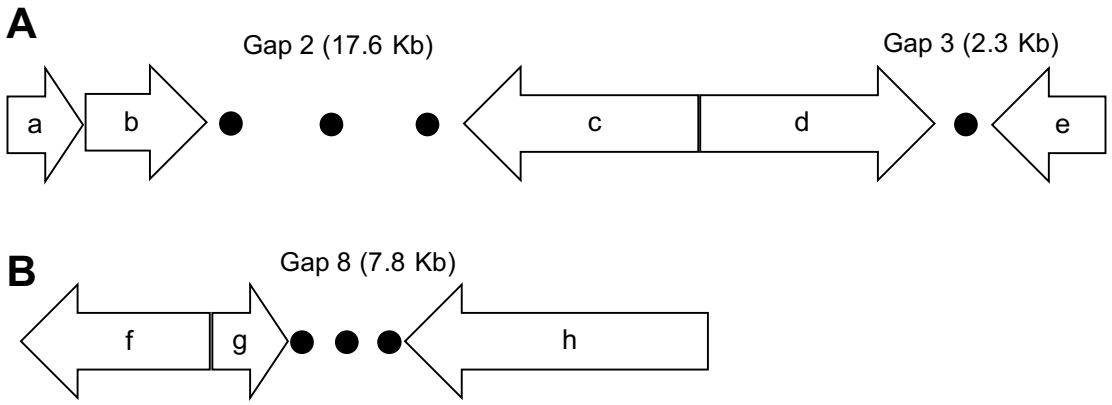


Figure 5.4. Location and sizes of Gaps 2, 3 and 8. The black circles indicate the location of the gaps with anticipated length based on PSU-1 indicated above. Genes labeled with letters are: a, OEOE_RS01600 encoding a transposon; b, OEOE_RS01605 encoding an IS5 family transposon; c, OEOE_RS01705 encoding an IS30 family transposon; d, OEOE_RS01710 encoding a metal transporter CorA; e, OEOE_RS01735 encoding a transposon; f, OEOE_RS07205 encoding a transposon; g, OEOE_RS07210 encoding a transposon; h, OEOE_RS07250 encoding a glycosyl transferase. Four genes, a, b, c and e located close to Gaps 2 and 3 were in repetitive sequence regions.

A nucleotide BLAST search across all of the contigs from *de novo* assemblies generated by the Geneious assembler, showed that the genomes of the four SB3 related strains contain DNA sequences near identical to two *O. oeni* phages; phiS13 (Accession No. KF183315.1) and phi9805 (Accession No. KF147927.1). Specifically, 303 bp located between OEOE_RS00475 and OEOE_RS00480 were 98% identical to sequences in phiS13, whilst 354 bp in gene OEOE_RS03290 were 99% identical to sequences in phi9805 (data not shown). In addition, there were sequences, ranging from several hundred base pairs to thousand base pairs, matching with plasmid sequences from other lactic acid bacteria, including *Lactobacillus* spp. and *P. damnosus*. (data not shown).

Searches of the genome sequences for genes encoding key enzymes of oenological relevance were performed after *de novo* assembly. All four strains

possessed 24 genes related to malic acid and citric acid metabolism, release of desirable aromas and formation of ethyl carbamate (Table 5.3). DNA sequences of these 24 genes were identical between strains. Genes involved in the formation of undesirable products histamine (*hdc*, Coton et al. 1998), tyramine (*tdc*, Arena et al. 2007) and putrescine (*odc*, Marcobal et al. 2004) were absent from all four strains.

Table 5.3. Genes of oenological relevance presented in strains SB3, A90, 2-49 and 3-83

Product/function	Gene	Accession number	Protein activity	locus tag	References[a]
Malic acid metabolism	*mleA*	X82326.2, CP000411.1	Malolactic enzyme	OEOE_RS07545	1, 2,
	mleP	X82326.2, CP000411.1	Malate permease	OEOE_RS07540	1, 2
	mleR	X82326.2, CP000411.1	MLF system transcription activator	OEOE_RS07550	1, 2
Citric acid metabolism	*citD*	CP000411.1	Citrate lyase γ-subunit	OEOE_RS02025	2
	citE	CP000411.1	Citrate lyase β-subunit	OEOE_RS02030	2
	citF	CP000411.1	Citrate lyase α-subunit	OEOE_RS02035	2
	maeP	CP000411.1	Putative citrate transporter	OEOE_RS02015	2
	alsD	CP000411.1	α-acetolactate decarboxylase	OEOE_RS08225	3
	alsS	CP000411.1	α-acetolactate synthase	OEOE_RS08220	3
Glycosidases	*bglD*	CP000411.1	β-glucosidase-related glycosidase	OEOE_RS01060	4
	celD	CP000411.1	β-glucosidase related glycosidase	OEOE_RS01045	5
Esterases	*estA2*	JX215243.1, KT454793.1	Predicted esterase	OEOE_RS07510	6, 7;
	estA7	JX215244.1, EAV39250.1	Predicted esterase	OEOE_RS03440	6, 7
	estB28	JX215242.1	Predicted esterase	OEOE_RS07590	6
	estCOo08	JX215240.1	Predicted esterase	OEOE_RS08470	8
Volatile sulfur compounds	*metC*	CP000411.1, JN819250.1	cystathionine γ -synthase	OEOE_RS03660	9
	metK	CP000411.1, KF887218.1	S-adenosylmethionine synthase	OEOE_RS04030	9
Exopolysachaaride	*it3*	CP000411.1	Glycosyltransferase	OEOE_RS08205	10
	dsrO	CP000411.1	Glycoside hydrolases	OEOE_RS02580	10
Ethyl Carbamate	*arcA*	CP000411.1; AF124851.1	Arginine deiminase	OEOE_RS05375	11
	arcB	AF124851.1	Ornithine transcarbamylase	N/A	11
	arcC	AF124851.1	Carbamate kinase	N/A	11
	arcD1	AF541253.1	Arginine/ornithine transporter	N/A	12
	arcD2	AF541253.1	Arginine/ornithine transporter	N/A	12

[a] (1) Labarre et al. 1996b; (2) Mills et al. 2005; (3) Garmyn et al. 1996; (4) Capaldo et al. 2011a; (5) Capaldo et al. 2011b; (6) Sumby et al. 2013a; (7) Darsonval et al. 2016b; (8) Sumby et al. 2013b; (9) Cappello et al. 2014; (10) Dimopoulou et al. 2014; (11) Tonon et al. 2001; (12) Divol et al. 2003

5.3.2 Comparison of genomes

A90 vs *2-49 and 3-83*

A total of 40 putative SNPs were initially identified in the two evolved strains 2-49 and 3-83 compared to A90. After PCR and Sanger sequencing for confirmation, 21 of them were determined to be false positives and were actually identical to A90. The remaining 19 putative SNPs included 10 SNPs located in 8 different genes (Table 5.4) and 9 SNPs in non-coding regions (Table 5.5).

Table 5.4. SNPs identified in coding regions in strains 2-49 and 3-83 when compared with A90 (A90→2-49/3-83).

Locus tag	Gene description	Codon change	Amino acid change	Strain(s)	Confirmed by Sanger sequencing?	Tolerated? (SIFT score[a])
OEOE_RS01465[b]	IS30 family transposase	C**G**G→C**A**G	K298Q	2-49	N	Y (0.26)
OEOE_RS01705[c]	IS30 family transposase	CG**C**→CG**A**	No change	2-49	N	Y (1.00)
OEOE_RS02820[d]	Acyltransferase	G**T**G→G**G**G	V215G	Both	N	N (0.02)
OEOE_RS04465[e]	Membrane protein	TT**T**→TT**A**	F193L	Both	N	Y (0.16)
OEOE_RS04995[b]	2-oxo-hepta-3-ene-1,7-dioate hydratase	**C**GC→**T**GC	R153C	2-49	N	N (0.00)
		T**G**T→T**T**T	C152F	3-83	N	N (0.00)
OEOE_RS05010[d]	Pseudo gene	TT**C**→TT**T**	No change	2-49	N	Y (1.00)
OEOE_RS05570	6-phosphogluconolactonase	**G**GG→**A**GG	G63R	2-49	Y	Y (0.88)
		T**C**G→T**G**G	S139W	3-83	Y	N (0.01)
OEOE_RS08000	UDP-N-acetylenolpyruvoylglucosamine reductase	TT**G**→TT**A**	No change	3-83	Y	Y (1.00)

[a] SIFT analysis was performed to determine whether the amino acid changes would be tolerated. A SIFT score less than 0.05 indicates that it is more likely to have an effect on protein function (Kumar et al. 2009)
[b] Incorrect PCR product based on the alignment of Sanger sequencing reads to WGS reads
[c] Located close to Gap 2, which contains repetitive sequences. Sanger read for Gap 2 is not successful
[d] The SNPs were identified in a more recent trial and were omitted for SNP confirmation by Sanger sequencing
[e] Failed to obtain PCR products

Of the 10 SNPs in coding regions, SIFT-predicted amino acid changes V215G in OEOE_RS02820, C152F, R153C in OEOE_RS04995 and S139W in OEOE_RS05570 would be deleterious with a score less than 0.05 (Table 5.4). Peptide search (www.uniprot.org) showed that OEOE_RS02820 was similar to AWRIB429_0542 (Accession: ACSE01000008.1), which is involved in cell

membrane and envelope biogenesis. OEOE_RS04995 appears to be a pseudo gene in PSU-1 as four stop codons are in the protein sequence whereas in A90, 2-49 and 3-83, there was only one stop codon at the end of the protein sequence, indicating OEOE_RS04995 might function in these strains. OEOE_RS04995, which encodes a 2-oxo-hepta-3-ene-1, 7-dioate hydratase, was similar to AWRIB429_1015 (Accession: ACSE010000016.1), which was not fully characterised (UniProt entry: D3L9I5). OEOE_RS05570 is involved in the 6-phospho-gluconolactone to 6-phospho-gluconate reaction of the pentose phosphate pathway (PPP), which generates pentose, NADPH and ribose-5-phosphate (KEGG ooe00030).

Both 2-49 and 3-83 had a point mutation in OEOE_RS04465, which is involved in potassium channel activity (UniProt entry: Q04FC8). Strain 2-49 also had a SNP in OEOE_RS05010, which encodes a 3-ketoacyl-acyl carrier protein (ACP)-reductase. However, there were 8 stop codons in the protein sequence in both A90 and 2-49, suggesting that this gene may not function in either strains. OEOE_RS08000 has a synonymous SNP in 3-83, a gene that catalysed the conversion of UDP-N-acetylglucosamine-enolpyruvate to UDP-N-acetylmuramic acid in chitin metabolism (KEGG ooe00550).

SNPs in non-coding regions are summarised in Table 5.5. BPROM was used to predict the -10 and -35 sequence elements that are contained in promoters, which provides a binding site for RNA polymerase and an associated sigma factor to initiate transcription (Souza et al. 2014). Both the -10 and -35 elements play an important role in the regulation of gene expression. The -10 element, which is essential for transcription initiation, is also known as the Pribnow box and has a consensus sequence of “TATAAT”. The -35 element, which is

located ~17 bp upstream of the -10 element, controls transcription rate and has a consensus sequence of “TTGACA”.

Table 5.5. SNPs identified in non-coding regions in strains 2-49 and 3-83 when compared with A90 (A90→2-49/3-83).

Sequence difference	SNP location	Promoter sequence?	Strain(s)	Confirmed by Sanger sequencing?
T→G[a]	66 bp upstream of OEOE_RS01465 and 230 bp upstream of OEOE_RS01470	N	3-83	N
T→C[b]	1742 bp downstream of OEOE_RS02350 and 5996 bp upstream of OEOE_RS02360	N	3-83	N
A→T[c]	1484 bp downstream of OEOE_RS03405 and 543 bp upstream of OEOE_RS03410	N	Both	N
A→T[c]	1492 bp downstream of OEOE_RS03405 and 536 bp upstream of OEOE_RS03410	Y	Both	N
C→G[c]	1502 bp downstream of OEOE_RS03405 and 526 bp upstream of OEOE_RS03410	Y	Both	N
G→C[c]	79 bp upstream of OEOE_RS03495 and 483 bp upstream of OEOE_RS03500	N	3-83	N
C→T	4 bp downstream of OEOE_RS03920 and 205 bp upstream of OEOE_RS03925	N	3-83	Y
A→C[d]	739 bp upstream of OEOE_RS05775 and 1813 bp upstream of OEOE_RS05790	N	Both	Y
A→G[e]	778 bp upstream of OEOE_RS08380	N	3-83	Y

[a] Incorrect PCR product based on the alignment of Sanger sequencing reads to WGS reads
[b] This SNP was identified in a more recent trial and was omitted for SNP confirmation by Sanger sequencing. Annotation of OEOE_RS02355 failed to transfer from PSU-1
[c] Only had PCR product of the parent A90
[d] Annotation of OEOE_RS05780 and OEOE_RS05785 failed to transfer from PSU-1
[e] Annotation of OEOE_RS08385 failed to transfer from PSU-1

Strain 3-83 had two SNPs in promoter regions predicted by BPROM (Fig. 5.5A). The first SNP, (A→T) and the second, (C→G) were located 536 bp and 526 bp upstream of OEOE_RS03410, respectively (Fig. 5.5A). OEOE_RS03410 encodes a membrane protein, which was related to potassium channel activity (UniProt entry: Q04XF5). Strain 2-49 also had these two SNPs.

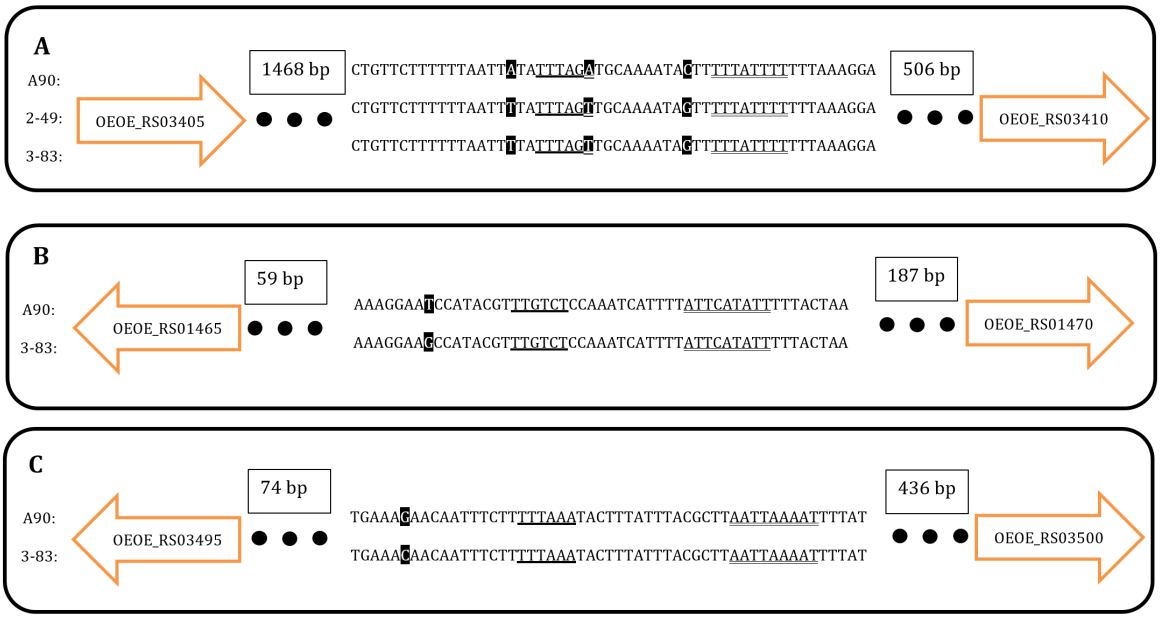


Fig. 5.5. SNPs in or close to -10 and -35 elements in 2-49/3-83 compared to A90. SNPs are highlighted in black boxes, -35 elements are underlined, -10 elements are double underlined. The black circles indicate sequence not shown of length indicated above.

There were also three SNPs between 3-83 and A90 in the non-coding regions located close to predicted -10 and -35 elements (Fig. 5.5). The first SNP (T→G) was located between OEOE_RS01465 and OEOE_RS01470 (Fig. 5.7B), 9 bp upstream of the -35 element predicted by BPROM. OEOE_RS01470 encodes an ATP-dependent helicase/deoxyribonuclease subunit B (UniProt entry: Q04GY8). The second (A→T) was 3 bp upstream of the predicted -35 element, and was located between OEOE_RS03405 and OEOE_RS03410 (Fig. 5.5A). This SNP was also present between 2-49 and A90. The final SNP (G→C) was located between OEOE_RS03495 and OEOE_RS03500, and 10 bp upstream of the -35 element predicted by BPROM (Fig. 5.5C). OEOE_RS03500 encodes a TetR family transcriptional regulator.

The remaining four SNPs (Table 5.5) in the non-coding regions were; T→C, located between OEOE_RS02350 and OEOE_RS02360 in 3-83; C→T, located

between OEOE_RS03920 and OEOE_RS03925 in strain 3-83; A→C, located between OEOE_RS05775 and OEOE_RS05790; A→G, located 778 bp upstream of OEOE_RS08380 in 3-83 (Table 5.5). When analysed with BLAST, the first, second and fourth SNPs seem to be located in coding regions, namely OEOE_RS02355, which encodes a polysaccharide transporter (RfbX); OEOE_RS05780, which encodes a peptidoglycan-binding protein; and OEOE_RS08385, which encodes a glycosyl transferase. Transfer of annotation of these genes failed probably due to similarity of them in A90, 2-49 and 3-83 was less than 95% compared to PSU-1 (5.2.3).

Two insertions were also identified between the evolved strains and A90 and have been further confirmed by PCR. The first insertion of “ACTA” was found between OEOE_RS05775 and OEOE_RS05790 (data not shown). When this region was analysed using Nucleotide BLAST, this insertion was in OEOE_RS05780 that encodes a peptidoglycan-binding protein. The second “TACG” was located between OEOE_RS08380 and OEOE_RS08390 (data not shown). Nucleotide BLAST showed this insertion to be in OEOE_RS08385, which encodes a glycosyl transferase.

SB3 vs *A90, 2-49 and 3-83*

In this study, a total of 12 putative SNPs were found between SB3 and A90. This includes 11 SNPs in coding regions (Table 5.6) and 1 SNP in a non-coding region (Table 5.7). These SNPs were also presented in DE strains 2-49 and 3-83. SNPs 1-10 in Table 5.6 and the non-coding SNP (Table 5.7) were also reported previously between A90 and SB3 (Betteridge 2015), and have been confirmed only between SB3 and A90 (Jiang and Betteridge, personal communication). Betteridge (2015) also reported the following point mutations in A90; V1018I in

OEOE_RS00870; C388G in OEOE_RS02580; Y421S in OEOE_RS01168; V72A in OEOE_RS05645; I331M in OEOE_RS07855. However, these SNPs were determined to be false positives by Sanger sequencing (Jiang and Betteridge, personal communication). Compared to SB3, A90 and the DE strains also had a SNP in OEOE_RS09200, whose protein sequence was identical to a few uncharacterised proteins in other *O. oeni* strains (data not shown). This SNP was not reported by Betteridge (2015).

Table 5.6. SNPs identified in coding regions in strains A90 when compared with SB3 (SB3→A90).

SNP	Locus tag	Gene description	Codon change	Amino acid change	Confirmed by Sanger sequencing	Tolerated? (SIFT score[a])
1	OEOE_RS00935	Transporter	TG**G**→TG**T**	W74C	N	Y (0.18)
2	OEOE_RS02085[d]	Translation initiation factor IF-2	C**C**G→C**A**G	P332Q	N	Y (1.00)
3	OEOE_RS02680[e]	HPr kinase/phosphorylase	C**A**C→C**G**C	H73R	N	Y (0.06)
4	OEOE_RS04175[f]	Membrane protein	**G**CT→**A**CT	A370T	N	Y (0.09)
5	OEOE_RS04370	SAM-dependent methyltransferase	CC**G**→CC**A**	No change	N	Y (1.00)
6	OEOE_RS05365[e]	Anaerobic ribonucleoside-triphosphate reductase	A**C**G→A**T**G	T339M	N	Y (1.00)
7	OEOE_RS06615	DNA-directed RNA polymerase subunit beta	**G**TT→**T**TT	V134F	N	Y (0.26)
8	OEOE_RS07625	Protein-(glutamine-N5) methyltransferase, release factor-specific	TG**G**→TG**T**	W273C	N	Y (0.13)
9	OEOE_RS08235	Hypothetical protein	C**T**C→C**C**C	L241P	N	Y (1.00)
10	OEOE_RS08915	tRNA	**T**GG→**C**GG	W6R	N	N/A[b]
11	OEOE_RS09200	Hypothetical protein	**C**AC→**T**AC	H12Y	N	N/A[b]

[a] SIFT analysis was performed to determine whether the amino acid changes were tolerated. A SIFT score less than 0.05 indicates that it is more likely to affect protein function (Kumar et al. 2009)
[b] No PSI (Position-specific Iterated) BLAST hits were identified with SIFT

Table 5.7. SNPs identified in non-coding regions in A90 compared with SB3 (SB3→A90).

Sequence difference	SNP location	Promoter sequence?	Confirmed by Sanger sequencing?
C→T	118 bp upstream of OEOE_RS01935 and 69 bp upstream of OEOE_RS01940	N	N

SB3-related strains vs *VP41 (AWRIB429)*

In order to investigate genetic basis of robust strain performance of VP41 (Borneman et al. 2010), genomics comparison was made between the available VP41 sequence and that of the SB3-related strains. The genome of VP41 downloaded from NCBI (Accession No. NZ_ACSE00000000.1) was re-annotated by transferring annotations from PSU-1 following the method described in 5.2.3 prior to genome comparisons.

Genetic variation between SB3-related strains and VP41 was much greater. A total 2,860 polymorphisms in 671 genes were identified. This includes 55 deletions, 50 insertions, 196 substitutions and 2559 SNPs (data not shown). Interestingly, of the 2559 SNPs, 16 were detected in 11 stress response genes (Table 5.8). Only V35I in *citF* and A258T in *ggpps* were predicted to be deleterious to the encoded protein via SIFT analysis.

Table 5.8. SNPs identified in stress response genes between SB3-related strains (SB3, A90, 2-49 and 3-83) and VP41(SB3 related strains→VP41).

Genes	Locus tag	Gene description	Codon change	Amino acid change	Tolerated? (SIFT score)
citF	OEOE_RS02035	Citrate lyase α-subunit	**G**TT→**A**TT	V35I	N (0.00)
mleA	OEOE_RS07545	Malolactic enzyme	G**C**C→G**G**C	A144G	Y (0.11)
mleR	OEOE_RS07550	MLF system transcription activator	**T**AC→**C**AC	Y8H	Y (0.19)
			GA**G**→GG**G**	E56G	Y (0.32)
			G**T**T→G**C**T	V242A	Y (1.00)
cfa	OEOE_RS05660	Cyclopropane-fatty-acyl phospholipid synthase	**G**CT→**T**CT	A81S	Y (0.16)
ggpps	OEOE_RS08860	Geranylgeranyl pyrophosphate synthase	C**G**A→C**A**A	R33Q	Y (0.49)
			TT**T**→TT**G**	F253L	Y (1.00)
			GCG→**A**CG	A258T	N (0.00)
			TCT→**G**CT	S306A	Y (1.00)
ftsH	OEOE_RS00895	Cell division protein	**A**TG→**G**TG	M138V	Y (0.42)
rmlB	OEOE_RS06990	dTDP-glucose 4,6-dehydratase	TT**G**→TT**A**	No change	N/A
Hsp18	OEOE_RS01385	Heat-shock protein Lo18	A**C**T→A**T**T	T133I	Y (0.06)
trxA	OEOE_RS08215	Thioredoxin	**C**CG→**T**CG	P47S	Y (0.19)
clpX	OEOE_RS03060	ATP-dependent Clp protease ATP-binding subunit ClpX	A**G**A→A**A**A	R681K	Y (0.91)
grpE	OEOE_RS06315	Protein GrpE	G**G**T→G**A**T	G171D	Y (1.00)

SIFT analysis was performed to determine whether the amino acid changes were tolerated in VP41. A SIFT score less than 0.05 indicates that it is more likely to affect protein function (Kumar et al. 2009).

Apart from point mutations in stress response genes, a SNP in VP41 (T→C) was located in the predicted promoter sequence, 26 bp upstream of the *mleA* gene (Fig. 5.6). There were a further four SNPs located close to the predicted promoter region. As all variations were identified with whole genome sequencing, these require additional confirmation.

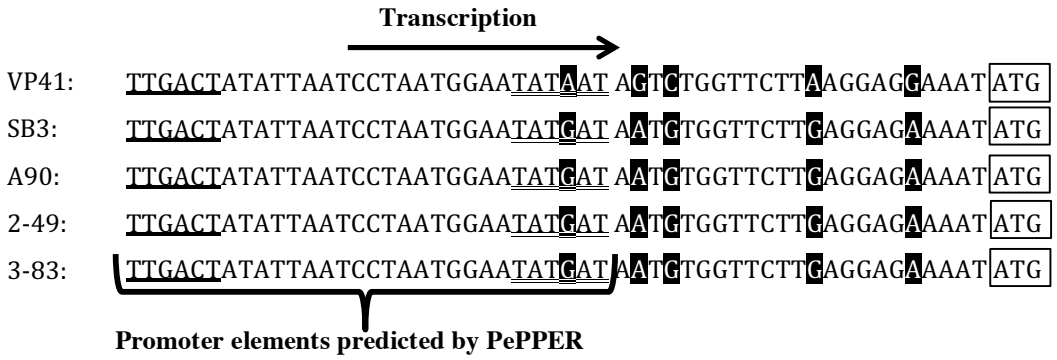


Fig. 5.6. SNPs in and close to the putative -10 and -30 elements upstream of the *mleA* gene in VP41 compared to SB3 related strains. SNPs are highlighted in the black box. Promoter elements predicted by PePPER was indicated in the brace. The -10 element (double-underlined) and -35 element (underlined) were identified manually. The start codon "ATG" of *mleA* was boxed. The arrow indicates transcription direction.

5.3 Discussion

A panel of four *O. oeni* strains were sequenced in order to give insight into their genetic changes following DE, as well as to determine the presence of genes important for wine quality. Strains SB3 and A90 had previously been sequenced using Roche 454 and Ion Torrent sequencing, respectively (Betteridge 2015). With the advancement of next-generation sequencing techniques, these strains were re-sequenced using Miseq (Illumina sequencing technology), which is considered to be more accurate.

The Illumina sequencing technology has been widely used to determine the genome sequences of many organisms (Tan et al. 2014; Capozzi et al. 2014;

Lamontanara et al. 2014). This approach produces shorter reads but at higher throughput. In this study, *de novo* assembly of the *O. oeni* genomes was performed using various assemblers. The Geneious assembler produced better results than Velvet and SPAdes. Genomes assembled using Velvet and SPAdes were repeated in Geneious R10, but results might ultimately be better if applied them directly.

When mapping all the paired reads to the reference genome PSU-1, some repetitive transposons were found to be close to the remaining large gaps, e.g. Gaps 2 and 3 (Fig. 5.4). Copies of the large repetitive sequence are a hindrance to assembly with shot-gun sequencing technologies when multiple copies of the sequences are collapsed to one location (Schatz et al. 2010) and may cause contig termination *de novo* assembly (Ricker et al. 2012). Despite difficulty in gaining quality Sanger sequencing reads across Gap 2 and 3, the genome size increased after filling other gaps (Table 5.1). Several large genomes, including the draft genomes of *Vitis vinifera* (Velasco et al. 2007) and *Cucumis sativus* var. *sativus* L. (Huang et al. 2009b) have been published also combined Sanger and WGS reads to produce high quality draft consensus sequences.

Other than primer walking by Sanger sequencing, long-read technologies such as the Oxford Nanopore or Pacific Bioscience (PacBio) that produce average reads over 10,000 bp would be an alternative for gap filling. This is because longer reads span more repeats, therefore, generally resulting in larger contig sizes (Schatz et al. 2010). However, as PacBio and Nanopore are associated with higher sequencing error rates (Giordano et al. 2017; Jain et al. 2016), a combination of the long-read sequencing and Illumina sequencing might make it possible to generate a complete *O. oeni* genome with high quality. Several studies

have reported using combined sequencing technologies for microbial genome assembly. One example is the assembly of an almost completed draft genome of *Dekkera bruxellensis* using both PacBio and Illuminina sequencing data. Olsen et al. (2015) found that the PacBio data could fill more than 5% of the *D. bruxellensis* genome not covered by the Illumina reads. With validation by multi-sequencing technologies to generate high quality microbial genomes, it would be worthwhile trying this approach with *O. oeni* to expand genomes currently available in NCBI since the PSU-1 genome was the only complete genome among 203 *O. oeni* strains that have been sequenced (https://www.ncbi.nlm.nih.gov/genome/genomes/541 accessed in August 2017).

Horizontal gene transfer (HGT) was observed in SB3 and related strains. Nucleotide BLAST reveals that SB3-related strains contain sequences highly similar to *O. oeni* phages and *Lactobacillus* plasmids. HGT can be common for *O. oeni* due to the absence of the mismatch repair genes *mutS* and *mutL* (Makarova et al. 2006). For example, HGT introduced the *odc* gene to *O. oeni* strain RM83, which is seldom present in *O. oeni* genomes, bringing about the ability to produce putrescine (Marcobal et al. 2006).

Bon et al. (2009) proposed six regions of genomic plasticity relevant to fitness in *O. oeni* genomes. The phi9805 homologue sequence was located in region 1 for SB3 related strains. It has been reported that bacteriophages in some cases may increase fitness of the host microbe (Mills et al. 2013) however, this has not been observed in *O. oeni* to date (Costantini et al. 2017). The function that the prophages display in the genomes of the four SB3-related strains needs further research. The existence of the *O. oeni* phage sequences in SB3-related strains may indicate an ancient infection of the phages in *O. oeni*. Oenococcal

prophages contain four types of integrase that was related to chromosomal integration site (Jaomanjaka et al. 2013). Part of the phage sequence may integrate into the *O. oeni* genome after many evolutionary stages.

Plasmids rarely integrate into bacterial chromosomes, but can be integrated into the chromosome via conjugation due to their mobile character (Ochman et al. 2000). However, gene replacement through conjugation can be problematic for *O. oeni*, as the transfer frequency is lower than the recombination frequency (Beltramo et al. 2004a; Sumby et al. 2014). Additionally, there have been no reports on integration of plasmids from *Lb*. spp. into *O. oeni* genomes, therefore it is unclear how these plasmids invaded and partially integrated into the SB3 genome.

The presence of genes associated with organic acid metabolism and organoleptic modifications in the four SB3 related strains were further analysed with WGS. These four strains have the potential to modify the sensory profile of wines, as they possess a number of genes that encode esterases and glycosidases (Table 5.3). For example, the esterase EstA2 encoded by *estA2* is able to synthesis ethyl butanoate and ethyl hexanoate (Sumby et al. 2013a), which are associated with fruity flavour, therefore these strains could have the ability to increase aroma complexity of the wine (Selli et al. 2004). Genes involved in the citrate metabolic pathway were also present in these four strains, suggesting they have the potential to metabolise citric acid. Diacetyl, which has a buttery flavour, is a major secondary metabolite of *O. oeni* in the citrate pathway. Production of diacetyl involves the participation of *alsS* and *alsD* (Garmyn et al. 1996), which were also detected in the strains (Table 5.3).

All strains have *mleA*, *mleP* and *mleR,* which are key enzymes for the metabolism of L-malic acid. No genetic differences in these genes were seen across the strains. Additionally, there were no differences between SB3-related strains in the predicted promoter regions of the *mle* cluster of genes. Furthermore, the movement of transposons can also activate downstream genes, resulting in improved phenotypes in DE strains (de Visser et al. 2004; Bongers et al. 2003; Lapierre et al. 2002). However, transposition of transposable elements was not observed across the four SB3-related strains. Thus, variations in physiological characters of these strains might be due to mutations in other genes.

OEOE_RS03410 located downstream of two putative SNPs in one of the predicted promoters also encodes a membrane protein. It has been reported that SNPs in the *fepA* promoter upregulated the *fepA* gene in osmotic stress-evolved *E. coli* strains (Dragosits et al. 2013). Accordingly, a similar change of expression may be occurring for OEOE_RS03410, but this needs to be confirmed experimentally.

OEOE_RS04465 had a putative SNP in both 2-49 and 3-83. Both OEOE_RS03410 and OEOE_RS04465 encode membrane proteins that have potassium channel activities. Potassium is involved in membrane adjustment (Follmann et al. 2009) and also acts as a second messenger for signalling during the osmotic stress response (Epstein 2003). Ochrombel et al. (2011a, b) have reported uptake of potassium lead to an improved homeostasis of the internal pH and improved the survival of *Corynebacterium glutamicum* (gram-positive soil bacterium) in response to acidic and osmotic stresses. These studies indicated the potential role of OEOE_RS03410 and OEOE_RS04465 in response to stressors. In addition, both OEOE_RS03410 and OEOE_RS04465 are functionally annotated

as integral components of the membrane. It has been widely reported that stressors act by perturbing the cell membrane (Cotter and Hill 2003; Da Silveira et al. 2003; Campos et al. 2009). Therefore, potentially the potassium channel activity of membrane proteins encoded by OEOE_RS04465 and OEOE_RS03410 could be affected by stressors, which may lead to a different stress response of *O. oeni*.

Strains 2-49 and 3-83 have SNPs in OEOE_RS05570, which is involved in the pentose phosphate pathway (PPP). NADPH generated during this process can be used in reductive biosynthesis reactions within cells, including fatty acid synthesis (Wakil 1989; Tehlivets et al. 2007). OEOE_RS05570 may be differentially expressed among strains in RFCDGJM and wines, leading to differences in cell membrane composition, which eventually affect growth and L-malic acid consumption of *O. oeni* under stressful conditions. Further, ribose-5-phosphate produced during PPP can be used in nucleic acid and nucleotide synthesis (Sprenger 1995), which is critical to numerous activities, including cell division.

Synonymous SNPs are thought to be of little significance since they result in no changes in amino acids. However, it has been reported certain synonymous mutations can alter protein translation efficiency and protein folding (Czech et al. 2010), mRNA structure and its stability (de Smit and Van Duin 1990), and are associated with the appearance of some phenotypes (Sauna and Kimchi-Sarfaty 2011). Strain 3-83 has a synonymous SNP in OEOE_RS08000, which is involved in chitin metabolism. UDP-N-acetylmuramic acid produced by OEOE_RS08000 is the precursor for peptidoglycan biosynthesis, which forms part of the cell envelope outside the cell plasma membrane (Typas et al. 2010), and therefore, might be the

first target for stressors. OEOE_ RS06975, encoding a peptidoglycan interpeptide bridge formation protein was overexpressed in response to 12% ethanol (Costantini et al. 2015) and 20% (v/v) ethanol (Betteridge et al. 2017), suggesting the role of peptidoglycan in response to stressors. There is also evidence that over-expression of a *Lb. plantarum* gene responsible for peptidoglycan biosynthesis in *E. coli* EC100 improves ethanol tolerance of the strain by 4.1-fold (Yuan et al. 2014).

In this study, the SNP (C→T) was found downstream of OEOE_RS03920 (tRNA), which is involved in L-cysteinyl-tRNA biosynthesis (KEGG ooe00970). Cysteine charged on tRNA can be delivered to ribosome for protein synthesis. Additionally, amino-acyl tRNAs are involved in a number of cellular processes such as cell wall formation (Lloyd et al. 2008), modification of membrane phospholipids (Roy and Ibba 2009) and labeling for protein degradation (Bachmair et al. 1986; Mogk et al. 2007). Given the importance of amino-acyl tRNAs, the expression of OEOE_RS03920 may have an impact on cell physiology and therefore stress tolerance, hence the role of this gene needs further investigation.

Some SNPs in A90, 2-49 and 3-83 compared to SB3 might be beneficial to bacteria under stress conditions. Both OEOE_RS07625 and OEOE_RS04370 encode a SAM-dependent methyltransferase. These proteins can interact with cyclopropane fatty acid (CFA) synthetase, which is associated with the production of CFAs (Ramos et al. 1997; Betteridge 2015). Transcriptional levels of both genes were significantly higher in A90 than SB3 under ethanol stress in MRSAJ (Betteridge et al. 2017). However, whether the altered expression of these genes could result in tolerance to stressors is unknown and needs further research.

VP41 is widely regarded as a robust MLF strain and its sequence is available (Borneman et al. 2010). Genomic analysis of SB3-related strains and VP41 indicated a high degree of genetic differences. Presumably the expression of *mleA*, which facilitates the conversion of L-malic acid to L-lactic acid in *O. oeni* (Labarre et al. 1996a), is higher in VP41 compared to SB3 related strains. Located upstream of *mleA*, *mleR* encodes a LysR-type regulatory protein, which should be involved in the regulation of *mleA* expression. The point mutation Y8H in *mleR* of VP41 was located in the DNA-binding helix-turn-helix motif (Labarre et al. 1996b). SIFT predicted this mutation would not result in a change of protein structure. However, it would be useful to reaffirm this to determine if this mutation could lead to a different expression of *mleA* between SB3-related strains and VP41.

A SNP was also identified in the -10 element of a putative promoter sequence upstream of *mleA* in VP41 compared to SB3-related strains. The -10 element of VP41 (TATAAT) is more typical in prokaryotes compared to the "TATGAT" in SB3-related strains. The -10 element can be recognised by sigma factors thereby assisting the attachment of RNA polymerase to promoter sequences (Souza et al. 2014). Both SB3-related strains and VP41 have the housekeeping sigma factor gene *rpoD* (OEOE_RS04785), which encodes the RNA polymerase sigma factor SigA belonging to the σ^{70} family. It is unknown whether transcription of *mleA* could also be regulated by SigA in addition to mleR. It is hypothesised that the point mutation in the -10 element may affect the binding of RNA polymerase and therefore affect the transcription of *mleA*, which is directly involved in L-malic acid metabolism. In order to confirm the function of the *mleA* promoter, it would be of interest to construct a vector containing this putative promoter sequence in *Lb.* spp. to determine if mRNA levels of *mleA* improve.

Future investigations should also focus on the role of SigA in regulation of *mleA* transcription. Over-expression or deletion of *rpoD* could be performed with *Lb.* spp. to examine whether expression levels of *mleA* are changed compared to the wild type strain.

A number of SNPs were identified in stress response genes between SB3-related strains and VP41 (Table 5.8). Among them the gene product of *Hsp18* (Lo18) is associated with the maintenance of membrane integrity and ethanol tolerance (Maitre et al. 2014). Four SNPs were identified in *ggpps*, one of which may result in a change of protein structure. Higher expression of *ggpps* was observed in ethanol stressed *O. oeni* cells (Cafaro et al. 2014). GGPPS, encoded by *ggpps*, is involved in biosynthesis of carotenoids, which can alter membrane fluidity in response to ethanol (Cafaro et al. 2014). Carotenoids also contribute to tolerance to oxidative stress and oleic acid in other microorganisms (Chamberlain et al. 1991; Clauditz et al. 2006; Yoshida et al. 2009). Future investigations should focus on whether the SNPs in *ggpps* lead to a change in protein structure and therefore function. The other genes listed in Table 5.8 are also responsible for response to different stressors and have been discussed in Chapter 1.

Apart from these genes, non-synonymous SNPs were also present in several ATP-binding cassette transporters (data not shown), which are often associated with stress tolerance and multidrug resistance (Bartowsky 2005). SNPs in known stress response genes only accounted for a small proportion of total mutations (2,860) seen between SB3-related strains and VP41. The genetic basis of the purported VP41 resistance to multi-stressors is likely to be complex, and may result from interaction of a number of genes. To study this, quantitative RT-PCR would not be a practical approach, as extensive effort and time are needed to

examine the 671 genes with 2,860 SNPs. Instead, RNA-seq approach to study expression of the whole genome in the upcoming future would be most suitable.

5.5 Conclusion

In order to understand the physiological behaviour of DE strains, genomes of SB3-related strains were characterised. Genome comparison was first made between 2-49/3-83 and A90 and generated a list of mutations. Some of these were in genes that may be associated with fatty acid and cell envelope biosynthesis, and therefore might be related to higher stress tolerance of the DE strains. Further comparisons were made between SB3 and DE strains A90, 2-49 and 3-83, which showed the beneficial mutations generated during previous DE in A90 were retained through further DE leading to strain 2-49 and 3-83. Lastly, genomes of SB3-related strains were compared with the VP41 genome where SNPs in known stress genes were identified. Aside from these SNPs, mutations in the putative promoter upstream of *mleA* may also contribute to the robust MLF performance of VP41.

For all mutations identified in this study, it would be interesting to study the expression of these genes in wine with multiple stressors. Those whose expression differs between the evolved strain and the parent may be important candidates, which can be further characterised by over-expressing in *Lb.* spp. to confirm their role in stress response, or a combination of both.

Higher tolerance of the evolved strains may also have resulted from interaction of multiple genes, which cannot be concluded simply from genome comparison. Therefore, RNA-seq could be an alternative approach to determine the basis of multi-stress resistance.

Genome features of SB3-related strains were also analysed in this study. All of them possessed genes associated with aroma modification and lacked genes responsible for biogenic amine formation, which might be beneficial during winemaking. This will contribute to future research of gene expression and quantification of aromatic compounds via GC-MS analysis to study their impact on wine quality.

In summary, genomic characterisation of SB3-related strains offers a better understanding of *O. oeni*. Mutations identified in this study will expand current knowledge on stress response genes and their function in *O. oeni*. Additional information on oenological related genes will also help guide bacterial selection.

Chapter 6 Discussion, future directions and conclusions

Oenococcus oeni starter cultures are widely used to conduct MLF during winemaking, however *O. oeni* growth and fermentation activity can be inhibited by stressors present in wine (Chapter 1). This project focused on using directed evolution (DE) to generate novel *O. oeni* starter cultures with improved multi-stressor tolerance and faster MLF compared to the parent, A90, and the commercial strain SB3. Compared to hundreds of commercial yeast on the market, there are only approximately 20 *O. oeni* starter cultures commercially available in Australia (Betteridge 2015). Thus, availability of additional and improved strains would give winemakers greater choice.

The first stage of this research involved the characterisation of multi-stress resistance of strain A90, previously improved for ethanol tolerance (Betteridge 2015) in both MRSAJ and RFCDGJM (Chapter 2). Viability of A90 was higher than its parent SB3 in MRSAJ but similar to SB3 in RFCDGJM, which has a composition more similar to wine. These findings suggest A90 is unlikely to have been satisfactorily improved for multi-stressor tolerance in wine. Therefore A90 required further optimisation to overcome challenges found in wines.

To further optimise A90 via DE, RFCDGJM with increasing ethanol and decreasing pH was used initially as the feed medium, before gradually shifting to Shiraz wine with SO_2. DE continued for 350 generations. After selection from a total of 378 single isolates based on MLF efficiency, three candidates, 1-161, 2-49 and 3-83, were found to conduct MLF faster than A90 and other DE isolates in a

wine-like medium. The fermentation phenotype of the three strains was further confirmed via screening of MLF in wine (Chapter 3).

In Chapter 4, in-depth phenotypical characterisation of the evolved strains was described. In comparison to the parents, SB3 and A90, the three strains displayed better fitness in RFCDGJM with particular wine relevant stressors, for instance 20 mg/L SO_2, 12 % (v/v) ethanol at pH 3.5. Moreover, the three optimised strains could also maintain the improved MLF phenotype in Shiraz wine with multiple stressors after at least 50 generations of growth in a low stress medium. Strains 2-49 and 3-83 showed a significantly faster fermentation than the earlier isolated strain 1-161, and were therefore chosen for further characterisation in larger scale fermentations under conditions more closely matching winemaking, and again confirmed their superiority compared to A90.

A genomic characterisation of SB3, A90, 2-49, 3-83 and the commercially available VP41 was undertaken in an attempt to explain why strains 2-49, 3-83 and VP41 were faster malolactic fermenters (Chapter 5). Whole genome sequencing was used as a tool to analyse the genetic features of strains 2-49, 3-83, SB3 and A90 and identify genetic differences between strains.

6.1 General discussion

6.1.1 Directed evolution

This assessment of evolved strain A90 in RFCDGJM (more comparable to wine) indicated growth or viability was similar to the parent SB3 in this medium, despite clearly superior performance when grown in MRSAJ. This apparent fitness trade-off highlighted one of the limitations of DE: evolution is closely tailored to the conditions of the feed medium.

The emergence of a fitness trade-off in non-selective environments has been widely reported in DE studies. For instance, *E. coli* strains with superior adaptive growth at low temperature were observed to have a relative fitness decrease at high temperature (Bennett and Lenski 2007), and vice versa (Rudolph et al. 2010; Rodríguez-Verdugo et al. 2014). The fitness trade-off at ancestral temperature was also reported with thermo-tolerant yeast strains (Caspeta and Nielsen 2015), or *S. cerevisae* strains evolved on galactose displayed attenuated glucose utilisation (Hong and Nielsen 2013).

In this study, in order to generate the most suitable *O. oeni* strain with the least fitness trade-offs under oenological conditions, some modifications were applied to establish an improved DE system. Firstly, *O. oeni* cells were exposed to a more wine-like medium to make them more adapted to the typical winemaking environment. RFCDGJM was used in the first stage of DE (0–220 generations), after which the feed medium was gradually changed to Shiraz. Secondly, a combination of ethanol, pH and SO_2 were introduced into the feed medium and with stress levels increasing over time. Thus, the evolved strains were required to tolerate multiple stressors. As anticipated, the selected DE strains, 2-49 and 3-83, consistently demonstrated faster or more L-malic acid consumption compared to A90 and SB3 in a number of fermentation trials (Chapters 3 and 4).

This study indicates that an appropriate DE system can be used to generate potential commercial *O. oeni* whose use ensures timely MLF. But consideration of sensory impacts is still required. For example, commercial strains may generate complex aroma profiles at the cost of longer fermentation duration. Commercial strains, PN4 and Alpha, required an extra one to three days to complete MLF in Tempranillo compared to strain C22L9 (Ruiz et al. 2010) whilst

producing more 2,3-butanedione (Izquierdo Cañas et al. 2013), which contributes to buttery notes of the wine (Bartowsky and Henschke 2004; Swiegers et al. 2005). In addition, some commercial strains may exhibit poor stress resistance. Commercial strain IOEB-SARCO 455 displayed the least tolerance to octanoic and decanoic acids among ten commercial strains, with the minimum inhibitory concentration of both acids being 25 mg/L on solid media compared to 400 mg/L for the more robust strains tested (Renouf and Favier 2010). Solieri et al. (2010) also found that commercial starter AGF115 displayed a much slower decarboxylation of L-malic acid compared to indigenous strains in synthetic wine at pH 3.2.

6.1.2 Phenotypic and genetic characterisation of DE strains

DE strains 1-161, 2-49 and 3-83 were further characterised in multiple MLF trials in RFCDGJM under a range of pH, ethanol, SO_2 and MCFA concentrations. When tested in RFCDGJM with combined ethanol and pH stress, it was obvious that DE increased the concentration of ethanol at which the evolved strains could still grow. Under other stressful conditions, even though both DE strains and the parent strain maintained some viable cells, DE strains displayed improved consumption of L-malic acid and/or greater viability compared to A90 (Chapter 4).

Such tolerance assays show the extent to which the evolved strains have been improved are important in a DE study, but have been valued differently by different researchers. In some DE studies, for example Sandberg et al. (2014), did not provide information on the upper and lower temperature boundaries of DE strains, but instead focused more on genome or transcriptome profiles of the evolved strains. In other DE studies, tolerance of stressors is generally checked singularly rather than combined with other stressors (Caspeta and Neilsen 2015;

Dhar et al. 2011; Oide et al. 2015; Rodríguez-Verdugo et al. 2014; Wang et al. 2011). Unlike previous work, in this study a more in-depth characterisation of the evolved strains was performed in RFCDGJM with several combined common stressors so as to indicate their applicability in wine, the ultimate goal being to generate starter cultures for industry.

From tolerance assays, strains 2-49 and 3-83 were chosen for further characterisation in larger-scale MLF trials. Even though strains 2-49 and 3-83 outperformed the parent A90 at laboratory-scales, they exhibited slow fermentation in 50 L trials. Reasons for the delay in L-malic acid decarboxylation were unclear, and may include inhibition through yeast-bacteria cell-cell interactions and insufficient inocula. Prolonged fermentation with selected *O. oeni* during scale up can be common (K. Sumby, personal communication; Solieri et al. 2010). However, in the case of other strains evolved by DE, no such issues were reported. Ekberg et al. (2013) even observed a faster fermentation of both evolved and parent *Saccharomyces pastorianus* strains in 10 L trials compared to 2 L, which may result from a higher fermentation temperature in the former.

Following phenotypic characterisation, the most promising strains 2-49 and 3-83, as well as SB3 and A90 were sequenced for identification of genetic mutations. A total of 12 SNPs were detected between SB3 and A90 and these SNPs were still present in strains 2-49 and 3-83 (Chapter 5). Among the 12 SNPs, 11 were the same as those reported by Betteridge (2015) for A90, and their role has been discussed by that author. Strains 2-49 and 3-83 had an additional 19 new SNPs that were identified in this study, some of which appear to be involved in cell envelope and fatty acids biosynthesis, DNA translation and internal pH homeostasis. Accordingly, such SNPs may lead to the observed higher tolerance

of the DE strains (Table 6.1). A further genetic comparison of SB3-related strains with the robust commercial strain VP41 suggested some new mechanisms that may be involved in the participation of *mleR*, putative *mleA* promoter and *rpoD*, and *ggpps* (Table 6.1).

Table 6.1. *O. oeni* genes and promoters bearing SNPs whose features might be beneficial for high multi-stress tolerance and faster MLF

Locus Tag/Genes	Protein function and its relevance to stress response	Genomic comparison	References
OEOE_RS03410	Has potassium channel activity to improve homeostasis of the internal pH	A90 *vs* 2-49 and 3-83	Ochrombel et al. 2011a, b
OEOE_RS03920	Involved in biosynthesis of L-cysteinyl -tRNA, which plays an important role in DNA translation, and might be involved in other cellular processes such as cell wall formation, modification of cell membrane and labelling proteins for degradation	A90 *vs* 3-83	Sternes et al. 2017
OEOE_RS04465	Has potassium channel activity to improve homeostasis of the internal pH	A90 *vs* 2-49 and 3-83	Ochrombel et al. 2011a, b
OEOE_RS05570	Involved in the pentose phosphate pathway. NADPH and ribose-5-phosphate generated in this pathway can be used for fatty acid biosynthesis and cell division, respectively	A90 *vs* 2-49 and 3-83	Wakil 1989; Tehlivets et al. 2007; Sprenger 1995
OEOE_RS08000	Catalyses the production of peptidoglycan, which forms part of the cell envelope	A90 *vs* 3-83	Typas et al. 2010
mleR	MLF system transcription activator	VP41 *vs* SB3-related strains	Labarre et al. 1996b
Putative *mleA* promoter	Binds with RNA polymerase and an associated sigma factor to initiate transcription.	VP41 *vs* SB3-related strains	Labarre et al. 1996a; Souza et al. 2014
ggpps	Encodes a geranylgeranyl pyrophosphate synthase (GGPPS) that is involved in biosynthesis of carotenoids that can alter membrane fluidity	VP41 *vs* SB3-related strains	Cafaro et al. 2014

6.2 Future research directions

6.2.1 Investigation on the genetic basis of multi-stress tolerance and fast MLF

Given the observed robustness of the DE strains from this study, commercial strains such as VP41, and the genomic differences identified between these strains, the genetic basis of multi-stress resistance and fast MLF should be investigated.

(1) Functional analysis of single genes

It would be interesting to characterise transcript abundance of genes in Table 6.1 using qPCR. Differential expression alone of these genes might be enough to explain the observed stress response. Following qPCR, candidate genes would ideally be cloned into an overexpression vector to conduct the stress tolerance of the host under a range of conditions. Unfortunately, it is difficult to perform genetic manipulations such as transformation in *O. oeni*. There have been several reports of the successful introduction of vectors into *O. oeni* (Dicks 1994; Assad‐García et al. 2008; Darsonval et al. 2016a; b), but none of them have been reported to be used on a regular successful basis. As such, the planned transformations might need to be made in *Lb.* spp., a close relative of *O. oeni* (Makarova et al. 2006).

A genome comparison with the robust commercial strain VP41 showed the -10 element upstream of *mleA* of VP41 is likely to be stronger than in the SB3-related strains. Both VP41 and SB3-related strains have *rpoD* encoding SigA that could potentially recognise the -10 element, thereby assisting the attachment of RNA polymerase to start transcription. Over-expression or deletion of *rpoD* in *Lb.* spp. followed by checking of *mleA* transcription abundance could be a way to confirm this hypothesis.

(2) Protein structure

SNPs in OEOE_RS02820, OEOE_RS05570 and *ggpps* were predicted to have a deleterious effect on protein structure with SIFT analysis (Chapter 5). The function of a protein is dependent on its 3-dimensional structure, thus it would be interesting to employ X-ray crystallography and nuclear magnetic resonance

techniques (Edwards et al. 2000) to confirm SIFT prediction and therefore determine if protein function has been changed. The same investigation could be applied to mleR in VP41 and that of SB3-related strains to confirm if the point mutation (Y8H) leads to a change in protein structure. This mutation was located in the DNA binding domain (Labarre et al. 1996b) so that any change in protein structure may have an effect on gene function.

(3) Gene interactions

SNPs identified in this study might not be the only contributor to the improved phenotype displayed in 2-49 and 3-83. Changes in one gene may affect expression of other genes. Whilst beyond the scope of this research due to time constraints, RNA-seq could be applied to study gene expression of the whole genome to check this. Such work would also characterise and quantify the transcriptional response of evolved strains *vs* the parent to combined stressors in wine. Novel interactions and associations of genes to stress tolerance might be disclosed.

6.2.2 Further Characterisation of the evolved strains in wines

Cabernet Sauvignon, Shiraz and Merlot are the dominant red varieties by volume in Australia, together accounting for 85% of the total red crush in 2016 (http://www.wfa.org.au/assets/vintage-reports/Vintage-Report-2016.pdf). In this study, evaluation of the evolved strains was only performed in Mouvedre, Merlot, Shiraz and Pinot Noir at a laboratory scale and Shiraz at a winery scale. Time did not permit characterisation of the evolved strains in additional wines made from other popular red varieties in Australia or in fact white varieties subjected to MLF. To fully characterise the applicability of the evolved strains, further comparisons

between 2-49, 3-83 and the parent strains SB3 and A90 as well as other commercial strains in different wines with different compositions, alcohol, pH and SO_2 levels is necessary.

Genetic characterisation (Chapter 5) revealed that all four strains have key genes encoding enzymes responsible for modification of wine aroma profile. They also contain the *arcA* and *arcB* genes that encodes enzymes to utilise L-arginine to form the undesirable compound ethyl carbamate. Production of citrulline (ethyl carbamate precursor) is correlated with the concentrations of glucose, fructose, citric and phenolic acids (Arena et al. 2013). Therefore, further work after MLF trials in wines should focus on biochemical characterisation and sensory analysis so as to confirm the production of positive aromas or at least minimal negative compounds.

6.2.3 Investigation of the genetic basis of the fitness trade-off in A90

The genetic basis of the fitness trade-off in A90 in RFCDGJM is unknown, and would be interesting to investigate further. Some researchers have proposed that evolutionary trade-off stems from antagonistic pleiotropy (AP) or mutation accumulation (MA). Specifically, the same mutation is related to both improved traits in one environment and reduced traits in alternative environments for AP, whereas different mutations are involved in either of the features for MA (Cooper and Lenski 2000; Elena and Lenske 2003). Genes with SNPs could be characterised individually by over-expression in *Lb.* spp. to examine if there is one gene that can favour its growth in MRSAJ but not in RFCDGJM to prove the AP mechanism. Otherwise, it may suggest that MA is the mechanism resulting in the fitness trade-off.

6.3 Conclusions

In summary, this project successfully used DE to generate two *O. oeni* strains 2-49 and 3-83 with improved multi-stress tolerance and MLF performance in wine. The strains developed in this study are a practical outcome for the industry since they have the potential to be used as new starter cultures to conduct faster MLF. The research described in this thesis not only expanded the current knowledge regarding *O. oeni* optimisation but also provides guidance for the improvement of food-related microbes via DE for enhanced microbial performance. Furthermore, genetic characterisation expands the knowledge of the *O. oeni* genome and also offers an opportunity to further explore the genetic basis of multi-stress tolerance, which would simplify *O. oeni* selection to a sequence-based approach: strains that contain beneficial mutations might be good candidates for industry use.

References

Adamo, G.M., Lotti, M., Tamás, M.J. and Brocca, S. (2012) Amplification of the *CUP1* gene is associated with evolution of copper tolerance in *Saccharomyces cerevisiae*. *Microbiology* **158**, 2325-2335.

Aleixandre-Tudó, J.L., Alvarez, I., Lizama, V., García, M.A.J., Aleixandre, J.L. and du Toit, W.J. (2013) Impact of caffeic acid addition on phenolic composition of Tempranillo wines from different winemaking techniques. *Journal of Agricultural and Food Chemistry* **61**, 11900-11912.

Alexandre, H., Costello, P.J., Remize, F., Guzzo, J. and Guilloux-Benatier, M. (2004) *Saccharomyces cerevisiae–Oenococcus oeni* interactions in wine: current knowledge and perspectives. *International Journal of Food Microbiology* **93**, 141-154.

Almario, M.P., Reyes, L.H. and Kao, K.C. (2013) Evolutionary engineering of *Saccharomyces cerevisiae* for enhanced tolerance to hydrolysates of lignocellulosic biomass. *Biotechnology and Bioengineering* **110**, 2616-2623.

Araya, C.L., Payen, C., Dunham, M.J. and Fields, S. (2010) Whole-genome sequencing of a laboratory-evolved yeast strain. *BMC Genomics* **11**, 88-98.

Arena, M.E. and Manca de Nadra, M.C. (2005) Influence of ethanol and low pH on arginine and citrulline metabolism in lactic acid bacteria from wine. *Research in Microbiology* **156**, 858-864.

Arena, M.E., Fiocco, D., de Nadra, M.C.M., Pardo, I. and Spano, G. (2007) Characterization of a *Lactobacillus plantarum* strain able to produce tyramine and partial cloning of a putative tyrosine decarboxylase gene. *Current Microbiology* **55**, 205-210.

Arena, M.E., Lisi, M.S., Manca de Nadra, M.C. and Alberto, M.R. (2013) Wine composition plays an important role in the control of carcinogenic precursor formation by *Lactobacillus hilgardii* X1B. *Journal of the Science of Food and Agriculture* **93**, 142-148.

Arnink, K. and Henick-Kling, T. (2005) Influence of *Saccharomyces cerevisiae* and *Oenococcus oeni* strains on successful malolactic conversion in wine. *American Journal of Enology and Viticulture* **56**, 228-237.

Asmundson, R.V. and Kelly, W.J. (1990) The effect of temperature and ethanol on the growth of *Leuconostoc oenos*. In *Proceedings of the 7th Australian Wine Industry Conference* eds. Williams, P.J., Davidson, D.M. and Lee, T.H. pp.251-252. The Australian Wine Research Institutute, Adelaide, South Australia.

Assad-García, J.S., Bonnin-Jusserand, M., Garmyn, D., Guzzo, J., Alexandre, H. and Grandvalet, C. (2008) An improved protocol for electroporation of *Oenococcus oeni* ATCC BAA-1163 using ethanol as immediate membrane fluidizing agent. *Letters in Applied Microbiology* **47**, 333-338.

Assani, A., Chabane, D., Haïcour, R., Bakry, F., Wenzel, G. and Foroughi-Wehr, B. (2005) Protoplast fusion in banana (*Musa* spp.): comparison of chemical (PEG: polyethylene glycol) and electrical procedure. *Plant Cell, Tissue and Organ Culture* **83**, 145-151.

Aukrust, T. and Blom, H. (1992). Transformation of *Lactobacillus* strains used in meat and vegetable fermentations. *Food Research International* **25**, 253-261.

Aukrust, T.W., Brurberg, M.B. and Nes, I.F. (1995) Transformation of *Lactobacillus* by electroporation. *Electroporation Protocols for Microorganisms*, 201-208.

Azevedo, L.C., Reis, M.M., Motta, L.F., da Rocha, G.O., Silva, L.A. and Andrade, J.B (2007) Evaluation of the formation of stability of hydroxyalkylsulfonic acids in wines. *Journal of Agriculture and Food Chemistry* **55**, 8670-8680.

Bachmair, A., Finley, D. and Varshavsky, A. (1986) *In vivo* half-life of a protein is a function of its amino-terminal residue. *Science* **234**, 179-186.

Bachmann, H., Fischlechner, M., Rabbers, I., Barfa, N., dos Santos, F.B., Molenaar, D., and Teusink, B. (2013) Availability of public goods shapes the evolution of competing metabolic strategies. *Proceedings of the National Academy of Sciences* **110**, 14302-14307.

Bachmann, H., Starrenburg, M.J., Molenaar, D., Kleerebezem, M. and van Hylckama Vlieg, J.E. (2012) Microbial domestication signatures of *Lactococcus lactis* can be reproduced by experimental evolution. *Genome Research* **22**, 115-124.

Bae, S., Fleet, G.H. and Heard, G.M. (2006) Lactic acid bacteria associated with wine grapes from several Australian vineyards. *Journal of Applied Microbiology* **100**, 712-727.

Barrick, J.E., Yu, D.S., Yoon, S.H., Jeong, H., Oh, T.K., Schneider, D., Lenski, R.E. and Kim, J.F. (2009) Genome evolution and adaptation in a long-term experiment with *Escherichia coli*. *Nature* **461**, 1243-1247.

Barry, J.A. and Gawrisch, K. (1994) Direct NMR evidence for ethanol binding to the lipid-water interface of phospholipid bilayers. *Biochemistry* **33**, 8082-8088.

Bartowsky, E. and Hensclike, P.A. (2004) The "buttery" attribute of wine- diacetyl - desirability, spoilage and beyond. *International Journal of Food Microbiology* **96**. 325- 352.

Bartowsky, E.J. (2005) *Oenococcus oeni* and malolactic fermentation – moving into the molecular arena. *Australian Journal of Grape and Wine Research* **11**, 174-187.

Bartowsky, E.J. and Borneman, A.R. (2011) Genomic variations of *Oenococcus oeni* strains and the potential to impact on malolactic fermentation and aroma compounds in wine. *Applied Microbiology and Biotechnology* **92**, 441-447.

Bauer, R. and Dicks, L.M.T. (2004) Control of malolactic fermentation in wine: a review. *South African Journal of Enology and Viticulture* **25**, 74-88.

Beltramo, C., Desroche, N., Tourdot-Maréchal, R., Grandvalet, C. and Guzzo, J. (2006) Real-time PCR for characterizing the stress response of *Oenococcus oeni* in a wine-like medium. *Research in Microbiology* **157**, 267-274.

Beltramo, C., Oraby, M., Bourel, G., Garmyn, D. and Guzzo, J. (2004a) A new vector, pGID052, for genetic transfer in *Oenococcus oeni*. *FEMS Microbiology Letters* **236**, 53-60.

Beltramo, C., Grandvalet, C., Pierre, F. and Guzzo, J. (2004b) Evidence for multiple levels of regulation of *Oenococcus oeni clpP-clpL* locus expression in response to stress. *Journal of Bacteriology* **186**, 2200-2205.

Bennett, A.F. and Hughes, B.S. (2009) Microbial experimental evolution. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology* **297**, R17-R25.

Bennett, A.F. and Lenski, R.E. (2007) An experimental test of evolutionary trade-offs during temperature adaptation. *Proceedings of the National Academy of Sciences* **104**, 8649-8654.

Bennett, A.F., Dao, K.M. and Lenski, R.E. (1990) Rapid evolution in response to high-temperature selection. *Nature* **346**, 79-81.

Betteridge A.L., Grbin, P.R. and Jiranek V. (2013) Enhanced winemaking efficiency: evolution of a superior lactic acid bacteria. Conference proceedings, 15th Australian wine industry technology conference, Sydeny NSW, 13-18th July.

Betteridge, A., Grbin, P. and Jiranek, V. (2015) Improving *Oenococcus oeni* to overcome challenges of wine malolactic fermentation. *Trends in Biotechnology* **33**, 547-553.

Betteridge, A.L. (2015) Enhanced wine-making efficiency through fool-proof malolactic fermentation: evolution of superior lactic acid bacteria: The University of Adelaide. PhD thesis.

Betteridge, A.L., Sumby, K. M., Sundstrom, J.F., Grbin, P. R. and Jiranek, V. (2017) Ethanol tolerant *Oenococcus oeni* by directed evolution for more efficient malolactic fermentation. *Applied Microbiology and Biotechnology*, submitted on the 12th April 2017

Bolhuis, H., van Veen, H.W., Poolman, B., Driessen, A.J. and Konings, W.N. (1997) Mechanisms of multidrug transporters. *FEMS Microbiology Reviews* **21**, 55-84.

Bolotin, A., Wincker, P., Mauger, S., Jaillon, O., Malarme, K., Weissenbach, J., Ehrlich, S.D. and Sorokin, A. (2001) The complete genome sequence of the lactic acid bacterium *Lactococcus lactis* ssp. lactis IL1403. *Genome Research* **11**, 731-753.

Bon, E., Delaherche, A., Bilhere, E., De Daruvar, A., Lonvaud-Funel, A. and Le Marrec, C. (2009) *Oenococcus oeni* genome plasticity is associated with fitness. *Applied and Environmental Microbiology* **75**, 2079-2090.

Bongers, R.S., Hoefnagel, M.H., Starrenburg, M.J., Siemerink, M.A., Arends, J.G., Hugenholtz, J. and Kleerebezem, M. (2003) IS*981*-mediated adaptive evolution recovers lactate production by *ldhB* transcription activation in a lactate dehydrogenase-deficient strain of *Lactococcus lactis*. *Journal of Bacteriology* **185**, 4499-4507.

Bonomo, M.G., Cafaro, C., Guerrieri, A., Crispo, F., Milella, L., Calabrone, L. and Salzano, G. (2017) Flow cytometry and capillary electrophoresis analyses in ethanol-stressed *Oenococcus oeni* strains and changes assessment of membrane fatty acid composition. *Journal of Applied Microbiology* **6**, 1615-1626.

Bordas, M., Araque, I., Alegret, J.O., El Khoury, M., Lucas, P., Reguant, C. and Bordons, A. (2013) Isolation, selection, and characterization of highly ethanol-tolerant strains of *Oenococcus oeni* from south Catalonia. *International Microbiology* **16**, 113-123.

Borneman, A.R., Bartowsky, E.J., McCarthy, J. and Chambers, P.J. (2010) Genotypic diversity in *Oenococcus oeni* by high-density microarray comparative genome hybridization and whole genome sequencing. *Applied Microbiology and Biotechnology* **86**, 681-691.

Borneman, A.R., Forgan, A.H., Kolouchova, R., Fraser, J. A. and Schmidt, S.A. (2016) Whole genome comparison reveals high levels of inbreeding and strain redundancy across the spectrum of commercial wine strains of *Saccharomyces cerevisiae*. *G3: Genes Genomes Genetics* **6**, 957-971.

Borneman, A.R., McCarthy, J.M., Chambers, P.J. and Bartowsky, E.J. (2012) Comparative analysis of the *Oenococcus oeni* pan genome reveals genetic diversity in industrially-relevant pathways. *BMC Genomics* **13**, 1-13.

Bouix, M. and Ghorbal, S. (2013) Rapid enumeration of *Oenococcus oeni* during malolactic fermentation by flow cytometry. *Journal of Applied Microbiology* **114**, 1075-1081.

Boumerdassi, H., Monnet, C., Desmazeaud, M. and Corrieu, G. (1997) Isolation and properties of *Lactococcus lactis* subsp. *lactis* biovar diacetylactis CNRZ 483 mutants producing diacetyl and acetoin from glucose. *Applied and Environmental Microbiology* **63**, 2293-2299.

Bourdineaud, J.P. (2006) Both arginine and fructose stimulate pH-independent resistance in the wine bacteria *Oenococcus oeni*. *International Journal of Food Microbiology* **107**, 274-280.

Bourdineaud, J.P., Nehme, B., Tesse, S. and Lonvaud-Funel, A. (2004) A bacterial gene homologous to ABC transporters protect *Oenococcus oeni* from ethanol and other stress factors in wine. *International Journal of Food Microbiology* **92**, 1-14.

Bourdineaud, J.P., Nehmé, B., Tesse, S. and Lonvaud-Funel, A. (2003) The *ftsH* gene of the wine bacterium *Oenococcus oeni* is involved in protection against environmental stress. *Applied and Environmental Microbiology* **69**, 2512-2520.

Bravo-Ferrada, B. M., Tymczyszyn, E. E., Gómez-Zavaglia, A. and Semorile, L. (2014) Effect of acclimation medium on cell viability, membrane integrity and ability to consume malic acid in synthetic wine by oenological *Lactobacillus plantarum* strains. *Journal of Applied Microbiology* **116**, 360-367.

Britz, T.J. and Tracey, R.P. (1990) The combination effect of pH, SO_2, ethanol and temperature on the growth of *Leuconostoc oenos*. *Journal of Applied Bacteriology* **68**, 23-31.

Burns, T.R. and Osborne, J.P. (2013) Impact of malolactic fermentation on the color and color stability of Pinot Noir and Merlot wine. *American Journal of Enology and Viticulture* **64**, 370-377.

Cabras, P., Meloni, M., Melis, M., Farris, G. A., Budroni, M. and Satta, T. (1994) Interactions between lactic bacteria and fungicides during lactic fermentation. *Journal of Wine Research* **5**, 53-59.

Cafaro, C., Bonomo, M.G. and Salzano, G. (2014) Adaptive changes in geranylgeranyl pyrophosphate synthase gene expression level under ethanol stress conditions in *Oenococcus oeni*. *Journal of Applied Microbiology* **116**, 71-80.

Çakar, Z.P., Alkim, C., Turanli, B., Tokman, N., Akman, S., Sarıkaya, M., Tamerler, C., Benbadis, L. and François, J.M. (2009) Isolation of cobalt hyper-resistant mutants of *Saccharomyces cerevisiae* by *in vivo* evolutionary engineering approach. *Journal of biotechnology* **143**, 130-138.

Callejon, S., Sendra, R., Ferrer, S., and Pardo, I. (2016) Cloning and characterization of a new laccase from *Lactobacillus plantarum* J16 CECT 8944 catalyzing biogenic amines degradation. *Applied Microbiology and Biotechnology* **100**, 3113-3124.

Campos, F.M., Couto, J.A., Figueiredo, A.R., Tóth, I.V., Rangel, A.O. and Hogg, T.A. (2009) Cell membrane damage induced by phenolic acids on wine lactic acid bacteria. *International Journal of Food Microbiology* **135**, 144-151.

Capaldo, A., Walker, M.E., Ford, C.M. and Jiranek, V. (2011a). β-Glucoside metabolism in *Oenococcus oeni*: cloning and characterisation of the phospho-β-glucosidase bglD. *Food chemistry* **125**, 476-482.

Capaldo, A., Walker, M.E., Ford, C.M. and Jiranek, V. (2011b) beta-Glucoside metabolism in *Oenococcus oeni*: cloning and characterization of the phospho- β-glucosidase CelD. *Journal of Molecular Catalysis B: Enzymatic* **69**, 27-34.

Capozzi, V., Russo, P., Beneduce, L., Weidmann, S., Grieco, F., Guzzo, J. and Spano, G. (2010) Technological properties of *Oenococcus oeni* strains isolated from typical southern Italian wines. *Letters in Applied Microbiology* **50**, 327-334.

Capozzi, V., Russo, P., Lamontanara, A., Orrù, L., Cattivelli, L. and Spano, G. (2014) Genome sequences of five *Oenococcus oeni* strains isolated from Nero Di Troia wine from the same Terroir in Apulia, Southern Italy. *Genome Announcements* **2**, e01077-14.

Cappello, M.S., De Domenico, S., Logrieco, A. and Zapparoli, G. (2014) Bio-molecular characterisation of indigenous *Oenococcus oeni* strains from Negroamaro wine. *Food microbiology* **42**, 142-148.

Capucho, I. and San Romão, M.V. (1994) Effect of ethanol and fatty acids on malolactic activity of *Leuconostoc oenos*. *Applied Microbiology and Biotechnology* **42**, 391-395.

Carr, J.G., Davies, P.A. and Sparks, A.H. (1976) The toxicity of sulphur dioxide towards certain lactic acid bacteria from fermented apple juice. *Journal of Applied Bacteriology* **40**, 201-212.

Carreté, R., Vidal, M.T., Bordons, A. and Constantí, M. (2002) Inhibitory effect of sulfur dioxide and other stress compounds in wine on the ATPase activity of *Oenococcus oeni*. *FEMS Microbiology Letters* **211**, 155-159.

Caspeta, L. and Nielsen, J. (2015) Thermotolerant yeast strains adapted by laboratory evolution show trade-off at ancestral temperatures and preadaptation to other stresses. *mBio* 6, e00431-15.

Caspritz, G. and Radler, F. (1983) Malolactic enzyme of *Lactobacillus plantarum*. Purification, properties, and distribution among bacteria. *Journal of Biological Chemistry* **258**, 4907-4910.

Cecconi, D., Milli, A., Rinalducci, S., Zolla, L. and Zapparoli, G. (2009) Proteomic analysis of *Oenococcus oeni* freeze-dried culture to assess the importance of cell acclimation to conduct malolactic fermentation in wine. *Electrophoresis* **30**, 2988-2995.

Chamberlain, N.R., Mehrtens, B.G., Xiong, Z.H.U.O., Kapral, F.A., Boardman, J.L. and Rearick, J.I. (1991) Correlation of carotenoid production, decreased membrane fluidity, and resistance to oleic acid killing in *Staphylococcus aureus* 18Z. *Infection and Immunity* **59**, 4332-4337.

Charusanti, P., Conrad, T.M., Knight, E.M., Venkataraman, K., Fong, N.L., Xie, B., Gao, Y. and Palsson, B. (2010) Genetic basis of growth adaptation of *Escherichia coli* after deletion of *pig*, a major metabolic gene. *PLoS Genetics* **6**, e1001186.

Chassy, B.M. and Flickinger, J.L. (1987) Transformation of *Lactobacillus casei* by electroporation. *FEMS Microbiology Letters* **44**, 173-177.

Cheynier, V., Dueñas-Paton, M., Salas, E., Maury, C., Souquet, J.M., Sarni-Manchado, P. and Fulcrand, H. (2006) Structure and properties of wine pigments and tannins. *American Journal of Enology and Viticulture* **57**, 298-305.

Chu-Ky, S., Tourdot-Marechal, R., Marechal, P.A. and Guzzo, J. (2005) Combined cold, acid, ethanol shocks in *Oenococcus oeni*: effects on membrane fluidity and cell viability. *Biochimica et Biophysica Acta (BBA)-Biomembranes* **1717**, 118-124.

Clauditz, A., Resch, A., Wieland, K.P., Peschel, A. and Götz, F. (2006) Staphyloxanthin plays a role in the fitness of *Staphylococcus aureus* and its ability to cope with oxidative stress. *Infection and Immunity* **74**, 4950-4953.

Cocconcelli, P., Morelli, L., Vescovo, M. and Bottazzi, V. (1986) Intergeneric protoplast fusion in lactic acid bacteria. *FEMS Microbiology Letters* **35**, 211-214.

Comitini, F., Ferretti, R., Clementi, F., Mannazzu, I. and Ciani, M. (2005) Interactions between *Saccharomyces cerevisiae* and malolactic bacteria: preliminary characterization of a yeast proteinaceous compound(s) active against *Oenococcus oeni*. *Journal of Applied Microbiology* **99**, 105-111.

Conrad, T.M., Frazier, M., Joyce, A.R., Cho, B.-K., Knight, E.M., Lewis, N.E., Landick, R. and Palsson, B.Ø. (2010) RNA polymerase mutants found through adaptive evolution reprogram *Escherichia coli* for optimal growth in minimal media. *Proceedings of the National Academy of Sciences* **107**, 20500-20505.

Conrad, T.M., Lewis, N.E. and Palsson, B.Ø. (2011) Microbial laboratory evolution in the era of genome-scale science. *Molecular Systems Biology* **7**, 509

Cooper, V.S. and Lenski, R.E. (2000) The population genetics of ecological specialization in evolving *Escherichia coli* populations. *Nature* **407**, 736-739.

Costantini, A., Doria, F., Saiz, J.C. and Garcia-Moruno, E. (2017) Phage-host interactions analysis of newly characterized *Oenococcus oeni* bacteriophages: Implications for malolactic fermentation in wine. *International Journal of Food Microbiology* **246**, 12-19.

Costantini, A., Rantsiou, K., Majumder, A.,Jacobsen, S.,Pessione, E. and Svensson, B. (2015) Complementing DIGE proteomics and DNA subarray analyses to shed light on *Oenococcus oeni* adaptation to ethanol in wine-simulated conditions. *Journal of Proteomics* **123**,114–127.

Costello, P., Francis, I. and Bartowsky, E. (2012) Variations in the effect of malolactic fermentation on the chemical and sensory properties of Cabernet Sauvignon wine: interactive influences of *Oenococcus oeni* strain and wine matrix composition. *Australian Journal of Grape and Wine Research* **18**, 287-301.

Costello, P.J., Morrison, G.J., Fleet, G.H. and Lee, T.H. (1983) Numbers and species of lactic acid bacteria in wines during vinification. *Food Technology in Australia* **35**, 14-18.

Coton, E., Rollan, G.C. and Lonvaud-Funel, A. (1998) Histidine carboxylase of *Leuconostoc oenos* 9204: purification, kinetic properties, cloning and nucleotide sequence of the *hdc* gene. *Journal of Applied Microbiology* **84**, 143-151.

Cotter, P.D. and Hill, C. (2003) Surviving the acid test: responses of gram-positive bacteria to low pH. *Microbiology and Molecular Biology Reviews* **67**, 429-453.

Couto, J.A., Rozes, N. and Hogg, T. (1996) Ethanol-induced changes in the fatty acid composition of *Lactobacillus hilgardii*, its effects on plasma membrane fluidity and relationship with ethanol tolerance. *Journal of Applied Microbiology* **81**, 126-132.

Cox, D.J. and Henick-Kling, T. (1995) Protonmotive force and ATP generation during malolactic fermentation. *American Journal of Enology and Viticulture* **46**, 319-323.

Curran, T.M., Lieou, J. and Marquis, R.E. (1995) Arginine deiminase system and acid adaptation of oral *streptococci*. *Applied and Environmental Microbiology* **61**, 4494-4496.

Czech, A., Fedyunin, I., Zhang, G. and Ignatova, Z. (2010) Silent mutations in sight: co-variations in tRNA abundance as a key to unravel consequences of silent mutations. *Molecular Biosystems* **6**, 1767-1772.

Da Silveira, M.G. and Abee, T. (2009) Activity of ethanol-stressed *Oenococcus oeni* cells: a flow cytometric approach. *Journal of Applied Microbiology* **106**, 1690-1696.

Da Silveira, M.G., Baumgärtner, M., Rombouts, F.M. and Abee, T. (2004) Effect of adaptation to ethanol on cytoplasmic and membrane protein profiles of *Oenococcus oeni*. *Applied and Environmental Microbiology* **70**, 2748-2755.

Da Silveira, M.G., Golovina, E.A., Hoekstra, F.A., Rombouts, F.M. and Abee, T. (2003) Membrane fluidity adjustments in ethanol-stressed *Oenococcus oeni* cells. *Applied and Environmental Microbiology* **69**, 5826-5832.

Da Silveira, M.G., San Romão, M.V., Loureiro-Dias, M.C., Rombouts, F.M. and Abee, T. (2002) Flow cytometric assessment of membrane integrity of ethanol-stressed *Oenococcus oeni* cells. *Applied and Environmental Microbiology* **68**, 6087-6093.

Darsonval, M., Msadek, T., Alexandre, H. and Grandvalet, C. (2016a) The antisense RNA approach: a new application for *in vivo* investigation of the stress response of *Oenococcus oeni*, a wine-associated lactic acid bacterium. *Applied and Environmental Microbiology* **82**, 18-26.

Darsonval, M., Alexandre, H. and Grandvalet, C. (2016b) Genetically engineered *Oenococcus oeni* strains to highlight the impact of *estA2* and *estA7* esterase genes on wine ester profile. *Food Microbiology* **60**, 21-28.

Davis, C.R., Wibowo, D., Eschenbruch, R., Lee, T.H. and Fleet, G.H. (1985) Practical implications of malolactic fermentation: a review. *American Journal of Enology and Viticulture* **36**, 290-301.

Davis, C.R., Wibowo, D., Fleet, G.H. and Lee, T.H. (1988) Properties of wine lactic acid bacteria: their potential enological significance. *American Journal of Enology and Viticulture* **39**, 137-142.

Davis, C.R., Wibowo, D.J., Lee, T.H. and Fleet, G.H. (1986) Growth and metabolism of lactic acid bacteria during and after malolactic fermentation of wines at different pH. *Applied and Environmental Microbiology* **51**, 539-545.

de Jong, A., Pietersma, H., Cordes, M., Kuipers, O.P. and Kok, J. (2012) PePPER: a webserver for prediction of prokaryote promoter elements and regulons. *BMC Genomics* **13**, 299.

De Orduna, R.M. (2010) Climate change associated effects on grape and wine quality and production. *Food Research International* **43**, 1844-1855.

de Smit, M.H. and Van Duin, J. (1990) Secondary structure of the ribosome binding site determines translational efficiency: a quantitative analysis. *Proceedings of the National Academy of Sciences* **87**, 7668-7672.

de Visser, J.A.G., Akkermans, A.D., Hoekstra, R.F. and De Vos, W.M. (2004). Insertion-sequence-mediated mutations isolated during adaptation to growth and starvation in *Lactococcus lactis*. *Genetics* ***168***, 1145-1157.

Deatherage, D.E., Kepner, J.L., Bennett, A.F., Lenski, R.E. and Barrick, J.E. (2017) Specificity of genome evolution in experimental populations of *Escherichia coli* evolved at different temperatures. *Proceedings of the National Academy of Sciences* **114**, E1904-E1912.

Delmas, F., Pierre, F., Coucheney, F., Divies, C. and Guzzo, J. (2001) Biochemical and physiological studies of the small heat shock protein Lo18 from the lactic acid bacterium *Oenococcus oeni*. *Journal of Molecular Microbiology and Biotechnology* **3**, 601-610.

Denayrolles, M., Aigle, M., and Lonvaud-Funel, A. (1994) Cloning and sequence analysis of the gene encoding *Lactococcus lactis* malolactic enzyme: relationships with malic enzymes. *FEMS Microbiology Letters* **116**, 79-86.

Desroche, N., Beltramo, C. and Guzzo, J. (2005) Determination of an internal control to apply reverse transcription quantitative PCR to study stress response in the lactic acid bacterium *Oenococcus oeni*. *Journal of Microbiological Methods* **60**, 325-333.

Deuerling, E., Paeslack, B. and Schumann, W. (1995) The ftsH gene of *Bacillus subtilis* is transiently induced after osmotic and temperature upshift. *Journal of Bacteriology* **177**, 4105-4112.

Dhar, R., Sägesser, R., Weikert, C., Yuan, J. and Wagner, A. (2011) Adaptation of *Saccharomyces cerevisiae* to saline stress through laboratory evolution. *Journal of Evolutionary Biology* **24**, 1135-1153.

Dicks, L., Dellaglio, F. and Collins, M. (1995) Proposal to reclassify *Leuconostoc oenos* as *Oenococcus oeni* [corrig.] gen. nov., comb. nov. *International Journal of Systematic Bacteriology* **45**, 395-397.

Dicks, L.M. (1994) Transformation of *Leuconostoc oenos* by electroporation. *Biotechnology Techniques* **8**, 901-904.

Dicks, L.M. and Endo, A. (2009) Taxonomic status of lactic acid bacteria in wine and key characteristics to differentiate species. *South African Journal of Enology and Viticulture* **30**, 72-90.

Díez, L., Rojo-Bezares, B., Zarazaga, M., Rodríguez, J.M., Torres, C. and Ruiz-Larrea, F. (2012) Antimicrobial activity of pediocin PA-1 against *Oenococcus oeni* and other wine bacteria. *Food Microbiology* **31**, 167-172.

Dimopoulou, M., Vuillemin, M., Campbell-Sills, H., Lucas, P.M., Ballestra, P., Miot-Sertier, C., Favier, M., Coulon, J., Moine, V. and Doco, T. (2014) Exopolysaccharide (EPS) synthesis by *Oenococcus oeni*: from genes to phenotypes. *PloS One* **9**, e98898.

Divol, B., Tonon, T., Morichon, S., Gindreau, E. and Lonvaud-Funel, A. (2003) Molecular characterization of *Oenococcus oeni* genes encoding proteins involved in arginine transport. *Journal of Applied Microbiology* **94**, 738-746.

Dott, W. and Trüper, H.G. (1976) Sulfite formation by wine yeasts. *Archives of Microbiology* **108**, 99-104.

Dragosits, M. and Mattanovich, D. (2013) Adaptive laboratory evolution–principles and applications for biotechnology. *Microbial Cell Factories* **12**, 64-81.

Dragosits, M., Mozhayskiy, V., Quinones-Soto, S., Park, J. and Tagkopoulos, I. (2013) Evolutionary potential, cross-stress behavior and the genetic basis of acquired stress resistance in *Escherichia coli*. *Molecular Systems Biology* **9**, 643-656.

Drici-Cachon, Z., Guzzo, J., Cavin, J.F. and Divies, C. (1996) Acid tolerance in *Leuconostoc oenos*: isolation and characterization of an acid-resistant mutant. *Applied Microbiology and Biotechnology* **44**, 785-789.

du Toit, M., Engelbrecht, L., Lerm, E. and Krieger-Weber, S. (2011) *Lactobacillus*: the next generation of malolactic fermentation starter cultures–an overview. *Food and Bioprocess Technology* **4**, 876-906.

Dunn, B., Richter, C., Kvitek, D.J., Pugh, T. and Sherlock, G. (2012) Analysis of the *Saccharomyces cerevisiae* pan-genome reveals a pool of copy number variants distributed in diverse yeast strains from differing industrial environments. *Genome Research* **22**, 908-924.

Edwards, A.M., Arrowsmith, C.H., Christendat, D., Dharamsi, A., Friesen, J.D., Greenblatt, J.F., and Vedadi, M. (2000) Protein production: feeding the crystallographers and NMR spectroscopists. *Nature Structural & Molecular Biology* **7**, 970-972.

Edwards, C.C. and Beelman, R.B. (1987) Inhibition of the malolactic bacterium *Leuconostoc oenos* (PSU-1) by decanoic acid and subsequent removal of the inhibition by yeast ghosts. *American Journal of Enology and Viticulture* **38**, 239-242.

Edwards, G.G., Beelman, R.B, Bartley, C.E. and McConnell, A.L. (1990) Production of decanoic acid and other volatile compounds and the growth of yeast and malolactic bacteria during vinification. *American Journal of Enology and Viticulture* **41**, 48-56.

Ekberg, J., Rautio, J., Mattinen, L., Vidgren, V., Londesborough, J. and Gibson, B. R. (2013) Adaptive evolution of the lager brewing yeast *Saccharomyces pastorianus* for improved growth under hyperosmotic conditions and its influence on fermentation performance. *FEMS Yeast Research* **13**, 335-349.

Elena, S.F. and Lenski, R.E. (2003) Evolution experiments with microorganisms: the dynamics and genetic bases of adaptation. *Nature Reviews Genetics* **4**, 457-470.

Eliasz, A.W., Chapman, D. and Ewing, D.F. (1976) Phospholipid phase transitions. Effects of n-alcohols, n-monocarboxylic acids, phenylalkyl alcohols and quatenary ammonium compounds. *Biochimica et Biophysica Acta (BBA)-Biomembranes* **448**, 220-233.

Eom, H.J., Cho, S.K., Park, M.S., Ji, G.E. and Han, N.S. (2010) Characterization of *Leuconostoc citreum* plasmid pCB18 and development of broad host range shuttle vector for lactic acid bacteria. *Biotechnology and Bioprocess Engineering* **15**, 946-952.

Epstein, W. (2003) The roles and regulation of potassium in bacteria. *Progress in Nucleic Acid Research and Molecular Biology* **75**, 293-320.

Favier, M., Bilhère, E., Lonvaud-Funel, A., Moine, V. and Lucas, P.M. (2012) Identification of pOENI-1 and related plasmids in *Oenococcus oeni* strains performing the malolactic fermentation in wine. *PLoS One* **7**, e49082.

Feuillat M., Charpentier C. and Maujean A. (1998) Les composés azotés. In *Oenologie: Fondements Scientifiques et Techniques*. pp.94–116. Paris, France: Lavoisier.

Fiocco, D., Capozzi, V., Goffin, P., Hols, P. and Spano, G. (2007) Improved adaptation to heat, cold, and solvent tolerance in *Lactobacillus plantarum*. *Applied Microbiology and Biotechnology* **77**, 909-915.

Fleet, G.H (2003) Yeast interactions and wine flavor. *International Journal of Food Microbiology* **86**, 11-22.

Fleet, G.H. and Heard, G. (1993) Yeasts: growth during fermentation. In *Wine Microbiology and Biotechnology* ed. Fleet, G.H. pp.27-54. Chur, Switzerland: Harwood Academic Publishers.

Follmann, M., Becker, M., Ochrombel, I., Ott, V., Krämer, R. and Marin, K. (2009) Potassium transport in *Corynebacterium glutamicum* is facilitated by the putative channel protein CglK, which is essential for pH homeostasis and growth at acidic pH. *Journal of Bacteriology* **191**, 2944-2952.

Franquès, J., Araque, I., Palahí, E., del Carmen Portillo, M., Reguant, C. and Bordons, A. (2017) Presence of *Oenococcus oeni* and other lactic acid bacteria in grapes and wines from Priorat (Catalonia, Spain). *LWT-Food Science and Technology* **81**, 326-334.

Fugelsang, K.C. and Edwards, C.G. (2007) *Wine Microbiology: Practical Applications and Procedures*. New York, USA: Springer.

G-Alegría, E., López, I., Ruiz, J.I., Sáenz, J., Fernández, E., Zarazaga, M., Dizy, M., Torres, C. and Ruiz-Larrea, F. (2004) High tolerance of wild *Lactobacillus plantarum* and *Oenococcus oeni* strains to lyophilisation and stress environmental conditions of acid pH and ethanol. *FEMS Microbiology Letters* **230**, 53-61.

García-Ruiz, A., Bartolomé, B., Martínez-Rodríguez, A.J., Pueyo, E., Martín-Álvarez, P.J. and Moreno-Arribas, M.V. (2008) Potential of phenolic compounds for controlling lactic acid bacteria growth in wine. *Food Control* **19**, 835-841.

García-Ruiz, A., Moreno-Arribas, M.V., Martín-Álvarez, P.J. and Bartolomé, B. (2011). Comparative study of the inhibitory effects of wine polyphenols on the growth of enological lactic acid bacteria. *International Journal of Food Microbiology* **145**, 426-431.

Garmyn, D., Monnet, C., Martineau, B., Guzzo, J., Cavin, J. F. and Diviès, C. (1996) Cloning and sequencing of the gene encoding α-acetolactate decarboxylase from *Leuconostoc oenos*. *FEMS Microbiology Letters* **145**, 445-450.

Giordano, F., Aigrain, L., Quail, M.A., Coupland, P., Bonfield, J.K., Davies, R.M. and Yue, J.X. (2017) *De novo* yeast genome assemblies from MinION, PacBio and MiSeq platforms. *Scientific Reports* **7**, 3935.

Gottesman, S., Clark, W.P., de Crecy-Lagard, V. and Maurizi, M.R. (1993) ClpX, an alternative subunit for the ATP-dependent Clp protease of *Escherichia coli*. Sequence and *in vivo* activities. *Journal of Biological Chemistry* **268**, 22618-22626.

Gottesman, S., Squires, C., Pichersky, E., Carrington, M., Hobbs, M., Mattick, J.S. and Foster, T. (1990) Conservation of the regulatory subunit for the Clp ATP-dependent protease in prokaryotes and eukaryotes. *Proceedings of the National Academy of Sciences* **87**, 3513-3517.

Grandvalet, C., Assad-García, J.S., Chu-Ky, S., Tollot, M., Guzzo, J., Gresti, J. and Tourdot-Maréchal, R. (2008) Changes in membrane lipid composition in ethanol-and acid-adapted *Oenococcus oeni* cells: characterization of the *cfa* gene by heterologous complementation. *Microbiology* **154**, 2611-2619.

Grandvalet, C., Coucheney, F., Beltramo, C. and Guzzo, J. (2005) CtsR is the master regulator of stress response gene expression in *Oenococcus oeni*. *Journal of Bacteriology* **187**, 5614-5623.

Guerrini, S., Mangani, S., Granchi, L. and Vincenzini, M. (2002) Biogenic amine production by *Oenococcus oeni*. *Current Microbiology* **44**, 374-378.

Guzzo, J., Jobin, M.P., Delmas, F., Fortier, L.C., Garmyn, D., Tourdot-Maréchal, R. and Diviès, C. (2000) Regulation of stress response in *Oenococcus oeni* as a function of environmental changes and growth phase. *International Journal of Food Microbiology* **55**, 27-31.

Guzzon, R., Poznanski, E., Conterno, L., Vagnoli, P., Krieger-Weber, S. and Cavazza, A. (2009) Selection of a new highly resistant strain for malolactic fermentation under difficult conditions. *South African Journal of Enology and Viticulture* **30**, 133-141.

Henick-Kling, T. (1986) Growth and metabolism of *Leuconostoc oenos* and *Lactobacillus plantarum* in wine. PhD Thesis: The University of Adelaide

Henick-Kling, T. (1988) Yeast and bacteria control in winemaking. In *Wine analysis* eds. Linskens, H.F. and Jackson, J.F. pp.276-315. Berlin, Germany: Springer.

Henick-Kling, T. (1993) Malolactic fermentation. In *Wine Microbiology and Biotechnology* ed. Fleet, G.H. pp. 289-326. Chur, Switzerland: Harwood Academic Publishers.

Henschke, P.A., Costello, P.J. and Bartowsky, E. (2002) Management of malolactic fermentation: wine flavour manipulation. *Australian and New Zealand Grapegrower and Winemaker*, 7-14.

Heunis, T., Deane, S., Smit, S. and Dicks, L.M. (2014) Proteomic profiling of the acid stress response in *Lactobacillus plantarum* 423. *Journal of Proteome Research* **13**, 4028-4039.

Hill, J.A., O'Meara, T.R. and Cowen, L.E. (2015) Fitness trade-offs associated with the evolution of resistance to antifungal drug combinations. *Cell Reports* **10**, 809-819.

Hong, K.K. and Nielsen, J. (2013) Adaptively evolved yeast mutants on galactose show trade-offs in carbon utilization on glucose. *Metabolic Engineering* **16**, 78-86.

Hong, K.K., Vongsangnak, W., Vemuri, G.N. and Nielsen, J. (2011) Unravelling evolutionary strategies of yeast for improving galactose utilization through integrated systems level analysis. *Proceedings of the National Academy of Sciences* **108**, 12179-12184

Hood,A. (1983) Inhibition of growth of wine lactic acid bacteria by acetaldehyde-bound sulphur dioxide. *Australian and New Zealand Grapegrower and Winemaker* **232**, 34-43.

Hornsey, I.S.(2007) Lactic acid bacteria and malolactic fermentation. In *The Chemistry and Biology of Winemaking* ed Hornsey,I.S. pp.203-241. Cambridge, UK: Royal Society of Chemistry.

Hua, Q., Joyce, A.R., Palsson, B.Ø. and Fong, S.S. (2007) Metabolic characterization of *Escherichia coli* strains adapted to growth on lactate. *Applied and Environmental Microbiology* **73**, 4639-4647.

Huang, D.W., Sherman, B.T. and Lempicki, R.A. (2008) Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nature Protocols* **4**, 44-57.

Huang, D.W., Sherman, B.T. and Lempick, R.A (2009a). Bioinformatics enrichment tools: paths towards comprehensive functional analysis of large gene lists. *Nucleic Acids Research* **37**, 1-13.

Huang, S., Li, R., Zhang, Z., Li, L., Gu, X., Fan, W. and Ren, Y. (2009b) The genome of the cucumber, *Cucumis sativus* L. *Nature Genetics* **41**, 1275-1281.

Ibarra, R., Edwards, J. and Palsson, B. (2002) *Escherichia coli* K-12 undergoes adaptive evolution to achieve *in silico* predicted optimal growth. *Nature* **420**, 186-189.

Ito, K. and Akiyama, Y. (2005) Cellular functions, mechanism of action, and regulation of FtsH protease. *Annual Review of Microbiology* **59**, 211-231.

Izquierdo Cañas, P.M., Romero García, E., Pérez Martin, F., Prieto Seseña, S., Manso Heras, J.M., and Herreros Palop, M.L. (2013) Behaviour during malolactic fermentation of three strains of *Oenococcus oeni* used as direct inoculation and acclimatisation cultures. *South African Journal of Enology and Viticulture* **34**, 1-9.

Jackowetz, J.N. and de Orduña, R.M. (2012) Metabolism of SO_2 binding compounds by *Oenococcus oeni* during and after malolactic fermentation in white wine. *International Journal of Food Microbiology* **155**, 153-157.

Jain, M., Olsen, H.E., Paten, B. and Akeson, M. (2016) The Oxford Nanopore MinION: delivery of nanopore sequencing to the genomics community. *Genome Biology* **1**, 1-11.

Jaomanjaka, F., Ballestra, P., Dols-lafargue, M. and Le Marrec, C. (2013) Expanding the diversity of oenococcal bacteriophages: insights into a novel group based on the integrase sequence. *International Journal of Food Microbiology* **166**, 331-340.

Jobin, M. P., Delmas, F., Garmyn, D., Divies, C. and Guzzo, J. (1997) Molecular characterization of the gene encoding an 18-kilodalton small heat shock protein associated with the membrane of *Leuconostoc oenos*. *Applied and Environmental Microbiology* **63**, 609-614.

Jobin, M.P., Garmyn, D., Divies, C. and Guzzo, J. (1999) Expression of the *Oenococcus oeni trxA* gene is induced by hydrogen peroxide and heat shock. *Microbiology* **145**, 1245-1251.

John, R.P., Gangadharan, D. and Madhavan Nampoothiri, K. (2008) Genome shuffling of *Lactobacillus delbrueckii* mutant and *Bacillus amyloliquefaciens* through protoplasmic fusion for L-lactic acid production from starchy wastes. *Bioresource Technology* **99**, 8008-8015.

Johnston, M.D., Hanlon, G.W., Denyer, S.P. and Lambert, R.J.W. (2003) Membrane damage to bacteria caused by single and combined biocides. *Journal of Applied Microbiology* **94**, 1015-1023.

Knoll, C., Divol, B. and du Toit, M. (2008) Genetic screening of lactic acid bacteria of oenological origin for bacteriocin-encoding genes. *Food Microbiology* **25**, 983-991.

Knoll, C., Fritsch, S., Schnell, S., Grossmann, M., Rauhut, D. and du Toit, M. (2011) Influence of pH and ethanol on malolactic fermentation and volatile aroma compound composition in white wines. *LWT-Food Science and Technology* **44**, 2077-2086.

Kouassi, Y. and Shelef, L.A. (1998) Inhibition of *Listeria monocytogenes* by cinnamic acid: possible interaction of the acid with cysteinyl residues. *Journal of Food Safety* **18**, 231-242.

Krieger-Weber, S. (2009) Application of yeast and bacteria as starter cultures. In *Biology of Microorganisms on Grapes, in Must and in Wine* eds. König, H., Unden, G. and Fröhlich, J. pp.489-511. Berlin, Germany: Springer.

Kumar, P., Henikoff, S. and Ng, P.C. (2009) Predicting the effects of coding non-synonymous variants on protein function using the SIFT algorithm. *Nature Protocols* **4**, 1073-1081.

Kunkee, R.E. (1967) Control of malo-lactic fermentation induced by *Leuconostoc citrovorum*. *American Journal of Enology and Viticulture* **18**, 71-77.

Kunkee, R.E. (1984) Selection and modification of yeasts and lactic acid bacteria for wine fermentation. *Food Microbiology* **1**, 315-332.

Labarre, C., Guzzo, J., Cavin, J.F. and Divies, C. (1996a) Cloning and characterization of the genes encoding the malolactic enzyme and the malate permease of *Leuconostoc oenos*. *Applied and Environmental Microbiology* **62**, 1274-1282.

Labarre, C., Divies, C. and Guzzo, J. (1996b) Genetic organization of the *mle* locus and identification of a mleR-like gene from *Leuconostoc oenos*. *Applied and Environmental Microbiology* **62**, 4493-4498.

Laffort Oenologie. Catalogue [Online]. Available at http://www.laffort.com/images/stories/telechargement/fiches%20commerciales/2%20-%20FC%20-%20ANGLAIS/FC_ANG_Lactoenos_SB3.pdf accessed in June 2017.

Lafon-Lafourcade, S., Carre, E. and Ribéreau-Gayon, P. (1983) Occurrence of lactic acid bacteria during the different stages of vinification and conservation of wines. *Applied and Environmental Microbiology* **46**, 874-880.

Lafon-Lafourcade, S., Geneix, C. and Ribéreau-Gayon, P. (1984) Inhibition of alcoholic fermentation of grape must by fatty acids produced by yeasts and their elimination by yeast ghosts. *Applied and Environmental Microbiology* **47**, 1246-1249.

Lamontanara, A., Orrù, L., Cattivelli, L., Russo, P., Spno, G. and Capozzi, V. (2014) Genome sequence of *Oenococcus oeni* OM27, the first fully assembled genome of a strain isolated from an Italian wine. *Genome Announcements* **2**, e00658-14.

Landete, J.M., Ferrer, S., Monedero, V. and Zúñiga, M. (2013) Malic enzyme and malolactic enzyme pathways are functionally linked but independently regulated in *Lactobacillus casei* BL23. *Applied and Environmental Microbiology* **79**, 5509-5518.

Landete, J.M., García-Haro, L., Blasco, A., Manzanares, P., Berbegal, C., Monedero, V. and Zúñiga, M. (2010) Requirement of the *Lactobacillus casei* MaeKR two-component system for L-malic acid utilization via a malic enzyme pathway. *Applied and Environmental Microbiology* **76**, 84-95.

Lapierre, L., Mollet, B. and Germond, J. E. (2002) Regulation and adaptive evolution of lactose operon expression in *Lactobacillus delbrueckii*. *Journal of Bacteriology* **184**, 928-935.

Larsen, J.T., Nielsen, J.C., Kramp, B., Richelieu, M., Bjerring, P., Riisager, M. J. and Edwards, C.G. (2003) Impact of different strains of *Saccharomyces cerevisiae* on malolactic fermentation by *Oenococcus oeni*. *American Journal of Enology and Viticulture* **54**, 246-251.

Lee, D.H. and Palsson, B.Ø. (2010) Adaptive evolution of *Escherichia coli* K-12 MG1655 during growth on a nonnative carbon source, L-1, 2-propanediol. *Applied and Environmental Microbiology* **76**, 4158-4168.

Lerm, E., Engelbrecht, L. and du Toit, M. (2011) Selection and characterisation of *Oenococcus oeni* and *Lactobacillus plantarum* South African wine isolates for use as malolactic fermentation starter cultures. *South African Journal of Enology and Viticulture* **32**, 280-295.

Liccioli, T. (2010) Improving fructose utilization in wine yeast using adaptive evolution. PhD thesis, the University of Adelaide.

Lloyd, A.J., Gilbey, A.M., Blewett, A.M., De Pascale, G., El Zoeiby, A., Levesque, R.C. and Dowson, C.G. (2008) Characterization of tRNA-dependent peptide bond formation by MurM in the synthesis of *Streptococcus pneumoniae* peptidoglycan. *Journal of Biological Chemistry* **283**, 6402-6417.

London, J., Meyer, E.Y. and Kulczyk, S.R. (1971) Detection of relationships between *Streptococcus faecalis* and *Lactobacillus casei* by immunological studies with two forms of malic enzyme. *Journal of Bacteriology* **108**, 196-201.

Longin, C., Petitgonnet, C., Guilloux-Benatier, M., Rousseaux, S. and Alexandre, H. (2017) Application of flow cytometry to wine microorganisms. *Food Microbiology*, **62**, 221-231.

Lonvaud-Funel, A. (1995) Microbiology of the malolactic fermentation: molecular aspects. *FEMS Microbiology Letters* **126**, 209-214.

Lonvaud-Funel, A. (2001) MiniReview Biogenic amines in wines: role of lactic acid bacteria. *FEMS Microbiology Letters* **199**, 9-13.

Lonvaud-Funel, A., Joyeux, A. and Desens, C. (1988) Inhibition of malolactic fermentation of wines by products of yeast metabolism. *Journal of the Science of Food and Agriculture* ***44***, 183-191.

López, I., Tenorio, C., Zarazaga, M., Dizy, M., Torres, C. and Ruiz-Larrea, F. (2007) Evidence of mixed wild populations of *Oenococcus oeni* strains during wine spontaneous malolactic fermentations. *European Food Research and Technology* **226**, 215-223.

Machielsen, R., Siezen, R.J., van Hijum, S.A. and van Hylckama Vlieg, J.E. (2011) Molecular description and industrial potential of Tn*6098* conjugative transfer

conferring alpha-galactoside metabolism in *Lactococcus lactis*. *Applied and Environmental Microbiology* **77**, 555-563.

Maitre, M., Weidmann, S., Dubois-Brissonnet, F., David, V., Covès, J. and Guzzo, J. (2014) Adaptation of the wine bacterium *Oenococcus oeni* to ethanol stress: role of the small heat shock protein Lo18 in membrane integrity. *Applied and Environmental Microbiology* **80**, 2973-2980.

Makarova, K., Slesarev, A., Wolf, Y., Sorokin, A., Mirkin, B., Koonin, E., Pavlov, A., Pavlova, N., Karamychev, V., Polouchine, N., Shakhova, V., Grigoriev, I., Lou, Y., Rohksar, D., Lucas, S., Huang, K., Goodstein, D.M., Hawkins, T., Plengvidhya, V., Welker, D., Hughes, J., Goh, Y., Benson, A., Baldwin, K., Lee, J.H., Diaz-Muniz, I., Dosti, B., Smeianov, V., Wechter, W., Barabote, R., Lorca, G., Altermann, E., Barrangou, R., Ganesan, B., Xie, Y., Rawsthorne, H., Tamir, D., Parker, C., Breidt, F., Broadbent, J., Hutkins, R., O'Sullivan, D., Steele, J., Unlu, G., Saier, M., Klaenhammer, T., Richardson, P., Kozyavkin, S., Weimer, B. and Mills, D. (2006) Comparative genomics of the lactic acid bacteria. *Proceedings of the National Academy of Sciences* **103**, 15611-15616.

Makarova, K.S. and Koonin, E.V. (2007) Evolutionary genomics of lactic acid bacteria. *Journal of Bacteriology* **189**, 1199-1208.

Marcobal, Á., de las Rivas, B., Moreno-Arribas, M.V. and Munoz, R. (2006) Evidence for horizontal gene transfer as origin of putrescine production in *Oenococcus oeni* RM83. *Applied and Environmental Microbiology* **72**, 7954-7958.

Marcobal, A., de las Rivas, B., Moreno-Arribas, M.V. and Munoz, R. (2004) Identification of the ornithine decarboxylase gene in the putrescine-producer *Oenococcus oeni* BIFI-83. *FEMS Microbiology Letters* **239**, 213-220.

Marcobal, A.M., Sela, D. A., Wolf, Y.I., Makarova, K.S. and Mills, D.A. (2008) Role of hypermutability in the evolution of the genus *Oenococcus*. *Journal of Bacteriology* **190**, 564-570.

Margalef-Català, M., Araque, I., Bordons, A., Reguant, C. and Bautista-Gallego, J. (2016) Transcriptomic and proteomic analysis of *Oenococcus oeni* adaptation to wine stress conditions. *Frontiers in Microbiology* **7**, 1554-1554.

Marquis, R.E., Burne, R.A., Parsons, D.T. and Casiano-Colon, A.E. (1993) Arginine deiminase and alkali generation in plaque. In *Cariology for the Nineties* eds. Bowen, W.H. and Tabak, L.A. pp.309-317. New York, USA: University of Rochester Press.

Martínez-Pinilla, O., Martínez-Lapuente, L., Guadalupe, Z. and Ayestarán, B. (2012) Sensory profiling and changes in colour and phenolic composition produced by malolactic fermentation in red minority varieties. *Food Research International* **46**, 286-293.

Maurizi, M.R., Clark, W.P., Katayama, Y., Rudikoff, S., Pumphrey, J., Bowers, B. and Gottesman, S. (1990) Sequence and structure of ClpP, the proteolytic component of the ATP-dependent Clp protease of *Escherichia coli*. *Journal of Biological Chemistry* **265**, 12536-12545.

McBryde, C., Gardner, J.M., de Barros Lopes, M. and Jiranek, V. (2006) Generation of novel wine yeast strains by adaptive evolution. *American Journal of Enology and Viticulture* **57**, 423-430.

Mendoza, L.M., de Nadra, M.C.M. and Farías, M.E. (2010) Antagonistic interaction between yeasts and lactic acid bacteria of oenological relevance: partial characterization of inhibitory compounds produced by yeasts. *Food Research International* **43**, 1990-1998.

Mi, X. (2016) Selecting new malolactic fermentation starter cultures. School of Agriculture, Food and Wine, University of Adelaide, Masters thesis.

Mills, D.A., Rawsthorne, H., Parker, C., Tamir, D. and Makarova, K. (2005) Genomic analysis of *Oenococcus oeni* PSU-1 and its relevance to winemaking. *FEMS Microbiology Reviews* **29**, 465-475.

Mills, S., O'Sullivan, O., Hill, C., Fitzgerald, G. and Ross, R.P. (2010) The changing face of dairy starter culture research: from genomics to economics. *International Journal of Dairy Technology* **63**, 149-170.

Mills, S., Shanahan, F., Stanton, C., Hill, C., Coffey, A., and Ross, R. P. (2013) Movers and shakers: influence of bacteriophages in shaping the mammalian gut microbiota. *Gut Microbes* **4**, 4-16.

Minty, J.J., Lesnefsky, A. A., Lin, F., Chen, Y., Zaroff, T. A., Veloso, A.B. and Rouillard, J. M. (2011) Evolution combined with genomic study elucidates genetic bases of isobutanol tolerance in *Escherichia coli*. *Microbial Cell Factories* **10**, 18.

Modrich, P. (1991) Mechanisms and biological effects of mismatch repair. *Annual Review of Genetics* **25**, 229-253.

Mogk, A., Schmidt, R. and Bukau, B. (2007) The N-end rule pathway for regulated proteolysis: prokaryotic and eukaryotic strategies. *Trends in Cell Biology* **17**, 165-172.

Morelli, L., Cocconcelli, P., Bottazzi, V., Damiani, G., Ferretti, L. and Sgaramella, V. (1987) *Lactobacillus* protoplast transformation. *Plasmid* **17**, 73.

Muñoz, R., Moreno-Arribas, M.V. and Rivas, B.D.l. (2011) Lactic Acid Bacteria. In *Molecular Wine Microbiology* eds. Santiago, A.V.C., Muñoz, R. and Garcia, R.G. pp.191-226. Academic Press, London, UK: Elsevier.

Muñoz, V., Beccaria, B. and Abreo, E. (2014) Simultaneous and successive inoculations of yeasts and lactic acid bacteria on the fermentation of an unsulfited Tannat grape must. *Brazilian Journal of Microbiology* **45**, 59-66.

Nehme, N., Mathieu, F. and Taillandier, P. (2008) Quantitative study of interactions between *Saccharomyces cerevisiae* and *Oenococcus oeni* strains. *Journal of Industrial Microbiology and Biotechnology* **35**, 685-693.

Nygaard, M. and Prahl, C. (1996) Compatibility between strains of *Saccharomyces cerevisiae* and *Leuconostoc oenos* as an important factor for successful malolactic

fermentation. In *Proceedings of the 4th International Symposium on Cool-Climate Viticulture and Oenology* **1620**

O'Sullivan, E. and Condon, S. (1997) Intracellular pH is a major factor in the induction of tolerance to acid and other stresses in *Lactococcus lactis*. *Applied and Environmental Microbiology* **63**, 4210-4215.

Oakley, C.G., Ågren, J., Atchison, R.A. and Schemske, D.W. (2014) QTL mapping of freezing tolerance: links to fitness and adaptive trade-offs. *Molecular Ecology* **23**, 4304-4315.

Ochman, H., Lawrence, J.G. and Groisman, E.A. (2000) Lateral gene transfer and the nature of bacterial innovation. *Nature* **405**, 299-304.

Ochrombel, I., Ott, L., Krämer, R., Burkovski, A. and Marin, K. (2011a) Impact of improved potassium accumulation on pH homeostasis, membrane potential adjustment and survival of *Corynebacterium glutamicum*. *Biochimica et Biophysica Acta (BBA)-Bioenergetics* **1807**, 444-450.

Ochrombel, I., Becker, M., Krämer, R. and Marin, K. (2011b) Osmotic stress response in *C. glutamicum*: impact of channel-and transporter-mediated potassium accumulation. *Archives of Microbiology* **193**, 787-796.

Oide, S., Gunji, W., Moteki, Y., Yamamoto, S., Suda, M., Jojima, T. and Inui, M. (2015) Thermal and solvent stress cross-tolerance conferred to *Corynebacterium glutamicum* by adaptive laboratory evolution. *Applied and Environmental Microbiology* **81**, 2284-2298.

Olguín, N., Bordons, A. and Reguant, C. (2009) Influence of ethanol and pH on the gene expression of the citrate pathway in *Oenococcus oeni*. *Food Microbiology* **26**, 197-203.

Olsen, E.B., Russell, J.B. and Henick-Kling, T. (1991) Electrogenic L-malate transport by *Lactobacillus plantarum*: a basis for energy derivation from malolactic fermentation. *Journal of Bacteriology* **173**, 6199-6206.

Olsen, R.A., Bunikis, I., Tiukova, I., Holmberg, K., Lötstedt, B., Pettersson, O.V. and Vezzi, F. (2015) *De novo* assembly of *Dekkera bruxellensis*: a multi technology approach using short and long-read sequencing and optical mapping. *GigaScience* **4**, 56.

Osborne, J.P. and Edwards, C.G. (2006) Inhibition of malolactic fermentation by *Saccharomyces* during alcoholic fermentation under low-and high-nitrogen conditions: a study in synthetic media. *Australian Journal of Grape and Wine Research* **12**, 69-78.

Osborne, J.P. and Edwards, C.G. (2007) Inhibition of malolactic fermentation by a peptide produced by *Saccharomyces cerevisiae* during alcoholic fermentation. *International Journal of Food Microbiology* **118**, 27-34.

Osborne, J.P., Dubé Morneau, A. and Mira de Orduna, R. (2006) Degradation of free and sulfur-dioxide-bound acetaldehyde by malolactic lactic acid bacteria in white wine. *Journal of Applied Microbiology* **101**, 474-479.

Ough, C.S., Crowell, E.A. and Gutlove, B.R. (1988) Carbamyl compound reactions with ethanol. *American Journal of Enology and Viticulture* **39**, 239-242.

Paquin, C.E. and Adams, J. (1983) Relative fitness can decrease in evolving asexual populations of *S. cerevisiae*. *Nature* **302**, 495-500

Parekh, S., Vinci, V.A. and Strobel, R.J. (2000) Improvement of microbial strains and fermentation processes. *Applied Microbiology and Biotechnology* **54**, 287-301.

Patnaik, R., Louie, S., Gavrilovic, V., Perry, K., Stemmer, W.P., Ryan, C.M. and del Cardayré, S. (2002) Genome shuffling of *Lactobacillus* for improved acid tolerance. *Nature Biotechnology* **20**, 707-712.

Payen, C., Sunshine, A.B., Ong, G.T., Pogachar, J.L., Zhao, W. and Dunham, M.J. (2016) High-throughput identification of adaptive mutations in experimentally evolved yeast populations. *PLoS Genetics* **12**, e1006339.

Petri, R. and Schmidt-Dannert, C. (2004) Dealing with complexity: evolutionary engineering and genome shuffling. *Current Opinion in Biotechnology* **15**, 298-304.

Poolman, B., Molenaar, D., Smid, E.J., Ubbink, T., Abee, T., Renault, P.P. and Konings, W.N. (1991) Malolactic fermentation: electrogenic malate uptake and malate/lactate antiport generate metabolic energy. *Journal of Bacteriology* **173**, 6030-6037.

Pozo-Bayón, M., G-Alegría, E., Polo, M., Tenorio, C., Martín-Álvarez, P., Calvo De La Banda, M., Ruiz-Larrea, F. and Moreno-Arribas, M. (2005) Wine volatile and amino acid composition after malolactic fermentation: effect of *Oenococcus oeni* and *Lactobacillus plantarum* starter cultures. *Journal of Agricultural and Food Chemistry* **53**, 8729-8735.

Pozo-Bayón, M., Pardo, I., Ferrer, S. and Moreno-Arribas, M. (2009) Molecular approaches for the identification and characterisation of oenological lactic acid bacteria. *African Journal of Biotechnology* **8**. 3995-4001

Radler, F. (1990) Possible use of nisin in winemaking. II. Experiments to control lactic acid bacteria in the production of wine. *American Journal of Enology and Viticulture* **41**, 7-11.

Ramos, J.L., Duque, E., Rodríguez-Herva, J.J., Godoy, P., Haïdour, A., Reyes, F. and Fernández-Barrero, A. (1997) Mechanisms for solvent tolerance in bacteria. *Journal of Biological Chemistry* **272**, 3887-3890.

Rankine, B.C. and Pocock, K.F. (1969) Influence of yeast strain on binding of SO_2 in wines on its formation duing fermentation. *Journal of the Science of Food and Agriculture* **20**, 22-49.

Reguant, C., Bordons, A., Arola, L. and Rozes, N. (2000) Influence of phenolic compounds on the physiology of *Oenococcus oeni* from wine. *Journal of Applied Microbiology* **88**, 1065-1071.

Remize, F., Augagneur, Y., Guilloux-Benatier, M. and Guzzo, J. (2005) Effect of nitrogen limitation and nature of the feed upon *Oenococcus oeni* metabolism and extracellular protein production. *Journal of Applied Microbiology* **98**, 652-661.

Remize, F., Gaudin, A., Kong, Y., Guzzo, J., Alexandre, H., Krieger, S. and Guilloux-Benatier, M. (2006) *Oenococcus oeni* preference for peptides: qualitative and quantitative analysis of nitrogen assimilation. *Archives of Microbiology* **185**, 459-469.

Renouf, V. and Favier, M. (2010) Genetic and physiological characterisation of *Oenococcus oeni* strains to perform malolactic fermentation in wines. *South African Journal of Enology and Viticulture* **31**, 75-81.

Reyes, L.H., Abdelaal, A.S. and Kao, K.C. (2013) Genetic determinants for n-butanol tolerance in evolved *Escherichia coli* mutants: cross adaptation and antagonistic pleiotropy between n-butanol and other stressors. *Applied and Environmental Microbiology* **79**, 5313-5320.

Ribéreau-Gayon, P., Dubourdieu, D., Donèche, B. and Lonvaud, A. (2006a) Lactic acid bacteria. In *Handbook of Enology, The Microbiology of Wine and Vinifications*. pp.115-137. Chichester, UK: John Wiley and Sons.

Ribéreau-Gayon, P., Dubourdieu, D., Donèche, B. and Lonvaud, A. (2006b) Metabolism of lactic acid bacteria. In *Handbook of Enology, The Microbiology of Wine and Vinifications*. pp.139-159. Chichester, UK: John Wiley and Sons.

Ricker, N., Qian, H. and Fulthorpe, R.R. (2012) The limitations of draft assemblies for understanding prokaryotic adaptation and evolution. *Genomics* **100**, 167-175.

Rigomier, D., Bohin, J.-P. and Lubochinsky, B. (1980) Effects of ethanol and methanol on lipid metabolism in *Bacillus subtilis*. *Journal of General Microbiology* **121**, 139-149.

Rizk, Z., El Rayess, Y., Ghanem, C., Mathieu, F., Taillandier, P. and Nehme, N. (2016) Impact of inhibitory peptides released by *Saccharomyces cerevisiae* BDX on the malolactic fermentation performed by *Oenococcus oeni* Vitilactic F. *International Journal of Food Microbiology* **233**, 90-96.

Rizzotti, L., Levav, N., Fracchetti, F., Felis, G.E. and Torriani, S. (2015) Effect of UV-C treatment on the microbial population of white and red wines, as revealed by conventional plating and PMA-qPCR methods. *Food Control* **47**, 407-412.

Rochat, T., Boudebbouze, S., Gratadoux, J.J., Blugeon, S., Gaudu, P., Langella, P. and Maguin, E. (2012) Proteomic analysis of spontaneous mutants of *Lactococcus lactis*: involvement of GAPDH and arginine deiminase pathway in H_2O_2 resistance. *Proteomics* **12**, 1792-1805.

Rochat, T., Gratadoux, J.J., Corthier, G., Coqueran, B., Nader-Macias, M.E., Gruss, A. and Langella, P. (2005) *Lactococcus lactis* SpOx spontaneous mutants: a family of oxidative-stress-resistant dairy strains. *Applied and Environmental Microbiology* **71**, 2782-2788.

Rodríguez-Verdugo, A., Carrillo-Cisneros, D., González-González, A., Gaut, B.S. and Bennett, A.F. (2014) Different tradeoffs result from alternate genetic adaptations to a common environment. *Proceedings of the National Academy of Sciences* **111**, 12121-12126.

Romano, P. and Suzzi, G. (1993) Sulfur dioxide and wine microorganisms. In *Wine Microbiology and Biotechnology* ed. Fleet, G.H. pp.373-393. Chur, Switzerland: Harwood Academic Publisher.

Rosenberg, S.M. and Hastings, P.J. (2003) Microbiology and evolution: modulating mutation rates in the wild. *Science* **300**, 1382-1383.

Rosi, I. and Canuti, V. (2003) Influence of different pH values and inoculation time on the growth and malolactic activity of a strain of *Oenococcus oeni*. *Australian Journal of Grape and Wine Research* **9**, 194-199.

Roy, H. and Ibba, M. (2009) Broad range amino acid specificity of RNA-dependent lipid remodeling by multiple peptide resistance factors. *Journal of Biological Chemistry* **284**, 29677-29683.

Rudolph, B., Gebendorfer, K.M., Buchner, J. and Winter, J. (2010) Evolution of *Escherichia coli* for growth at high temperatures. *Journal of Biological Chemistry* **285**, 19029-19034.

Ruiz, P., Izquierdo, P.M., Sesena, S. and Palop, M.L. (2008) Intraspecific genetic diversity of lactic acid bacteria from malolactic fermentation of Cencibel wines as derived from combined analysis of RAPD-PCR and PFGE patterns. *Food Microbiology* **25**, 942-948.

Ruiz, P., Izquierdo, P.M., Sesena, S. and Palop, M.L. (2010) Selection of autochthonous *Oenococcus oeni* strains according to their oenological properties and vinification results. *International Journal of Food Microbiology* **137**, 230-235.

Ruiz, P., Izquierdo, P.M., Sesena, S., Garcia, E. and Palop, M.L. (2012) Malolactic fermentation and secondary metabolite production by *Oenoccocus oeni* strains in low pH wines. *Journal of Food Science* **77**, M579-585.

Sahl, H.G. (1991). Pore formation in bacterial membranes by cationic. *Nisin and Novel Antibiotics*, 347-358.

Sakamoto, K., Margolles, A., Van Veen, H.W. and Konings, W.N. (2001) Hop resistance in the beer spoilage bacterium *Lactobacillus brevis* is mediated by the ATP-binding cassette multidrug transporter HorA. *Journal of Bacteriology* **183**, 5371-5375.

Salema, M., Lolkema, J.S., San Romão, M.V. and Dias, M.L. (1996) The proton motive force generated in *Leuconostoc oenos* by L-malate fermentation. *Journal of Bacteriology* **178**, 3127-3132.

Salou, P., Leroy, M.J., Goma, G. and Pareilleux, A. (1991) Influence of pH and malate-glucose ratio on the growth kinetics of *Leuconostoc oenos*. *Applied Microbiology and Biotechnology* **36**, 87-91.

Sandberg, T.E., Pedersen, M., LaCroix, R. A., Ebrahim, A., Bonde, M., Herrgard, M.J. and Feist, A.M. (2014) Evolution of *Escherichia coli* to 42 ˚C and subsequent genetic engineering reveals adaptive mechanisms and novel mutations. *Molecular Biology and Evolution* **31**, 2647-2662.

Sauna, Z.E. and Kimchi-Sarfaty, C. (2011) Understanding the contribution of synonymous mutations to human disease. *Nature Reviews Genetics* **12**, 683-691.

Schatz, M.C., Delcher, A.L. and Salzberg, S.L. (2010) Assembly of large genomes using second-generation sequencing. *Genome Research* **20**, 1165-1173.

Schick, A., Bailey, S.F. and Kassen, R. (2015) Evolution of fitness trade-offs in locally adapted populations of *Pseudomonas fluorescens*. *The American Naturalist* **186**, S48-S59.

Selli, S., Cabaroglu, T., Canbas, A., Erten, H., Nurgel, C., Lepoutre, J.P. and Gunata, Z. (2004) Volatile composition of red wine from *cv.* Kalecik Karasi grown in central Anatolia. *Food Chemistry* **85**, 207-213.

Sico, M.A., Bonomo, M.G., D'Adamo, A., Bochicchio, S. and Salzano, G. (2009) Fingerprinting analysis of *Oenococcus oeni* strains under stress conditions. *FEMS Microbiology Letters* **296**, 11-17.

Siegumfeldt, H., Rechinger, K.B. and Jakobsen, M. (2000) Dynamic changes of intracellular pH in individual lactic acid bacterium cells in response to a rapid drop in extracellular pH. *Applied and Environmental Microbiology* **66**, 2330-2335.

Sleight, S.C. and Lenski, R.E. (2007) Evolutionary adaptation to freeze-thaw-growth cycles in *Escherichia coli*. *Physiological and Biochemical Zoology* **80**, 370-385.

Sleight, S.C., Orlic, C., Schneider, D. and Lenski, R.E. (2008) Genetic basis of evolutionary adaptation by *Escherichia coli* to stressful cycles of freezing, thawing and growth. *Genetics* **180**, 431-443.

Smith, W.M., Pham, T.H., Lei, L., Dou, J., Soomro, A.H., Beatson, S.A. and Turner, M. S. (2012) Heat resistance and salt hypersensitivity in *Lactococcus lactis* due to spontaneous mutation of *llmg_1816* (gdpP) induced by high-temperature growth. *Applied and Environmental Microbiology* **78**, 7753-7759.

Solieri, L., Genova, F., De Paola, M. and Giudici, P. (2010) Characterization and technological properties of *Oenococcus oeni* strains from wine spontaneous malolactic fermentations: a framework for selection of new starter cultures. *Journal of Applied Microbiology* **108**, 285-298.

Solopova, A., Bachmann, H., Teusink, B., Kok, J., Neves, A.R. and Kuipers, O.P. (2012) A specific mutation in the promoter region of the silent *cel* cluster accounts

for the appearance of lactose-utilizing *Lactococcus lactis* MG1363. *Applied and Environmental Microbiology* ***78***, 5612-5621.

Solovyev, V. and Salamov, A. (2011) Automatic annotation of microbial genomes and metagenomic sequences. *Metagenomics and its Applications in Agriculture, Biomedicine and Environmental Studies*, 61-78.

Souza, B.M., Castro, T.L.D.P., Carvalho, R.D.D.O., Seyffert, N., Silva, A., Miyoshi, A. and Azevedo, V. (2014) σ^{ECF} factors of gram-positive bacteria: a focus on *Bacillus subtilis* and the CMNR group. *Virulence* **5**, 587-600.

Spath, K., Heinl, S. and Grabherr, R. (2012) Direct cloning in *Lactobacillus plantarum*: electroporation with non-methylated plasmid DNA enhances transformation efficiency and makes shuttle vectors obsolete. *Microbial Cell Factories* **11**, 141-149.

Spettoli, P., Nuti, M.P. and Zamorani, A. (1984) Properties of malolactic activity purified from *Leuconostoc oenos* ML34 by affinity chromatography. *Applied and Environmental Microbiology* **48**, 900-901.

Sprenger, G.A. (1995) Genetics of pentose-phosphate pathway enzymes of *Escherichia coli* K-12. *Archives of Microbiology* **164**, 324-330.

Stephanopoulos, G. (2002) Metabolic engineering by genome shuffling. *Nature Biotechnology* **20**, 666-668.

Sternes, P.R., Costello, P.J., Chambers, P.J., Bartowsky, E.J. and Borneman, A.R. (2017) Whole transcriptome RNAseq analysis of *Oenococcus oeni* reveals distinct intra-specific expression patterns during malolactic fermentation, including genes involved in diacetyl metabolism. *International Journal of Food Microbiology* **257**, 216-224.

Stuart, M.R., Chou, L.S. and Weimer, B.C. (1999) Influence of carbohydrate starvation and arginine on culturability and amino acid utilization of *Lactococcus lactis* subsp. *lactis*. *Applied and Environmental Microbiology* **65**, 665-673.

Sumby, K.M., Grbin, P.R. and Jiranek, V. (2010) Microbial modulation of aromatic esters in wine: Current knowledge and future prospects. *Food Chemistry* **121**, 1-16.

Sumby, K.M., Jiranek, V. and Grbin, P.R. (2013a) Ester synthesis and hydrolysis in an aqueous environment, and strain specific changes during malolactic fermentation in wine with *Oenococcus oeni*. *Food Chemistry* **141**, 1673-1680.

Sumby, K.M., Grbin, P.R. and Jiranek, V. (2013b) Characterization of EstCOo8 and EstC34, intracellular esterases, from the wine-associated lactic acid bacteria *Oenococcus oeni* and *Lactobacillus hilgardii*. *Journal of Applied Microbiology* **114**, 413-422.

Sumby, K.M., Grbin, P.R. and Jiranek, V. (2014). Implications of new research and technologies for malolactic fermentation in wine. *Applied Microbiology and Biotechnology* **98**, 8111-8132.

Swiegers, J.H., Bartowsky, E.J., Henschke, P.A. and Pretorius, I.S. (2005) Yeast and bacterial modulation of wine aroma and flavour. *Australian Journal of Grape and Wine Research* **11**, 139-173.

Tan, B., e Silva, R.D.A., Rozycki, T., Nesbø, C. and Foght, J. (2014) Draft genome sequences of three *Smithella* spp. obtained from a methanogenic alkane-degrading culture and oil field produced water. *Genome Announcements* **2**, e01085-14.

Tehlivets, O., Scheuringer, K., and Kohlwein, S.D. (2007) Fatty acid synthesis and elongation in yeast. *Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids* **1771**, 255-270.

Teixeira, H., Goncalves, M., Rozes, N., Ramos, A. and San Romão, M. (2002) Lactobacillic acid accumulation in the plasma membrane of *Oenococcus oeni*: a response to ethanol stress? *Microbial ecology* **43**, 146-153.

Tenaillon, O., Barrick, J.E., Ribeck, N., Deatherage, D.E., Blanchard, J.L., Dasgupta, A. and Schneider, D. (2016) Tempo and mode of genome evolution in a 50,000-generation experiment. *Nature* **536**, 165-170.

Terrade, N. and Mira de Orduña, R. (2009) Determination of the essential nutrient requirements of wine-related bacteria from the genera *Oenococcus* and *Lactobacillus*. *International Journal of Food Microbiology* **133**, 8-13.

Teusink, B., Wiersma, A., Jacobs, L., Notebaart, R.A. and Smid, E.J. (2009) Understanding the adaptive growth strategy of *Lactobacillus plantarum* by *in silico* optimisation. *PLoS Computational Biology* **5**, e1000410.

Tonon, T., Bourdineaud, J.P. and Lonvaud-Funel, A. (2001) The *arcABC* gene cluster encoding the arginine deiminase pathway of *Oenococcus oeni*, and arginine induction of a CRP-like gene. *Research in Microbiology* **152**, 653-661.

Touati, D., Jacques, M., Tardat, B., Bouchard, L. and Despied, S. (1995) Lethal oxidative damage and mutagenesis are generated by iron in delta fur mutants of *Escherichia coli*: protective role of superoxide dismutase. *Journal of Bacteriology* **177**, 2305-2314.

Tourdot-Maréchal, R., Fortier, L.C., Guzzo, J., Lee, B. and Diviès, C. (1999) Acid sensitivity of neomycin-resistant mutants of *Oenococcus oeni*: a relationship between reduction of ATPase activity and lack of malolactic activity. *FEMS Microbiology Letters* **178**, 319-326.

Typas, A., Banzhaf, M., van Saparoea, B.V.D.B., Verheul, J., Biboy, J., Nichols, R.J. and Breukink, E. (2010) Regulation of peptidoglycan synthesis by outer-membrane proteins. *Cell* **143**, 1097-1109.

van Bokhorst-van de Veen, H., Smelt, M.J., Wels, M., van Hijum, S.A., de Vos, P., Kleerebezem, M. and Bron, P.A. (2013) Genotypic adaptations associated with prolonged persistence of *Lactobacillus plantarum* in the murine digestive tract. *Biotechnology Journal* **8**, 895-904.

Van der Merwe, H. (2008) The evaluation of malolactic fermentation starter cultures under South African winemaking conditions. Stellenbosch University, PhD thesis.

Velasco, R., Zharkikh, A., Troggio, M., Cartwright, D.A., Cestaro, A., Pruss, D. and Malacarne, G. (2007) A high quality draft consensus sequence of the genome of a heterozygous grapevine variety. *PloS One* **2**, e1326.

Vendrame, M., Manzano, M., Comi, G., Bertrand, J. and Iacumin, L. (2014). Use of propidium monoazide for the enumeration of viable *Brettanomyces bruxellensis* in wine and beer by quantitative PCR. *Food Microbiology*, *42*, 196-204.

Vidal, M.T., Poblet, M., Constantí, M. and Bordons, A. (2001) Inhibitory effect of copper and dichlofluanid on *Oenococcus oeni* and malolactic fermentation. *American Journal of Enology and Viticulture* **52**, 223-229.

Vivas, N., Lonvaud-Funel, A. and Glories, Y. (1997) Effect of phenolic acids and anthocyanins on growth, viability and malolactic activity of a lactic acid bacterium. *Food Microbiology* **14**, 291-300.

Volschenk, H., van Vuuren, H.J.J. and Viljoen-Bloom, M. (2006) Malic acid in wine: origin, function and metabolism during vinification. *South African Journal of Enology and Viticulture* **27**, 123-136.

Wagner, P.L. and Waldor, M.K. (2002) Bacteriophage control of bacterial virulence. *Infection and Immunity* **70**, 3985-3993.

Wakil, S.J. (1989) Fatty acid synthase, a proficient multifunctional enzyme. *Biochemistry* ***28***, 4523-4530.

Wang, Y., Li, Y., Pei, X., Yu, L. and Feng, Y. (2007) Genome-shuffling improved acid tolerance and L-lactic acid volumetric productivity in *Lactobacillus rhamnosus*. *Journal of Biotechnology* **129**, 510-515.

Wang, Y., Manow, R., Finan, C., Wang, J., Garza, E. and Zhou, S. (2011) Adaptive evolution of nontransgenic *Escherichia coli* KC01 for improved ethanol tolerance and homoethanol fermentation from xylose. *Journal of Industrial Microbiology and Biotechnology* **38**, 1371-1377.

Warth, A.D. (1988) Effect of benzoic acid on growth yield of yeasts differing in their resistance to preservatives. *Applied and Environmental Microbiology* **54**, 2091-2095.

Weber, F.J. and de Bont, J.A. (1996) Adaptation mechanisms of microorganisms to the toxic effects of organic solvents on membranes. *Biochimica et Biophysica Acta (BBA)-Reviews on Biomembranes* **1286**, 225-245.

Wehrl, W., Niederweis, M. and Schumann, W. (2000) The FtsH protein accumulates at the septum of *Bacillus subtilis* during cell division and sporulation. *Journal of Bacteriology* **182**, 3870-3873.

Wells A. and Osborne J.P. (2011) Production of SO_2 binding compounds and SO_2 by *Saccharomyces* during alcoholic fermentation and the impact on malolactic fermentation. *South African Journal of Enology and Viticulture* **32**, 267-279

Wen, A.M., Delaquis, P., Stanich, K. and Toivonen, P. (2003) Antilisterial activity of selected phenolic acids. *Food Microbiology* **20**, 305-311.

Wisselink, H.W., Toirkens, M.J., Wu, Q., Pronk, J.T. and van Maris, A.J. (2009) Novel evolutionary engineering approach for accelerated utilization of glucose, xylose, and arabinose mixtures by engineered *Saccharomyces cerevisiae* strains. *Applied and Environmental Microbiology* **75**, 907-914.

Woo, K.M., Chung, W.J., Ha, D.B., Goldberg, A.L. and Chung, C.H. (1989) Protease Ti from *Escherichia coli* requires ATP hydrolysis for protein breakdown but not for hydrolysis of small peptides. *Journal of Biological Chemistry* **264**, 2088-2091.

Yang, D. and Woese, C.R. (1989) Phylogenetic structure of the "*leuconostocs*": an interesting case of a rapidly evolving organism. *Systematic and Applied Microbiology* **12**, 145-149.

Yoshida, K., Ueda, S. and Maeda, I. (2009) Carotenoid production in *Bacillus subtilis* achieved by metabolic engineering. *Biotechnology Letters* **31**, 1789-1793.

Yu, L., Pei, X., Lei, T., Wang, Y. and Feng, Y. (2008) Genome shuffling enhanced L-lactic acid production by improving glucose tolerance of *Lactobacillus rhamnosus*. *Journal of Biotechnology* **134**, 154-159.

Yuan, Y., Bi, C., Nicolaou, S.A., Zingaro, K.A., Ralston, M. and Papoutsakis, E.T. (2014) Overexpression of the *Lactobacillus plantarum* peptidoglycan biosynthesis *murA2* gene increases the tolerance of *Escherichia coli* to alcohols and enhances ethanol production. *Applied Microbiology and Biotechnology* **19**, 8399-8411.

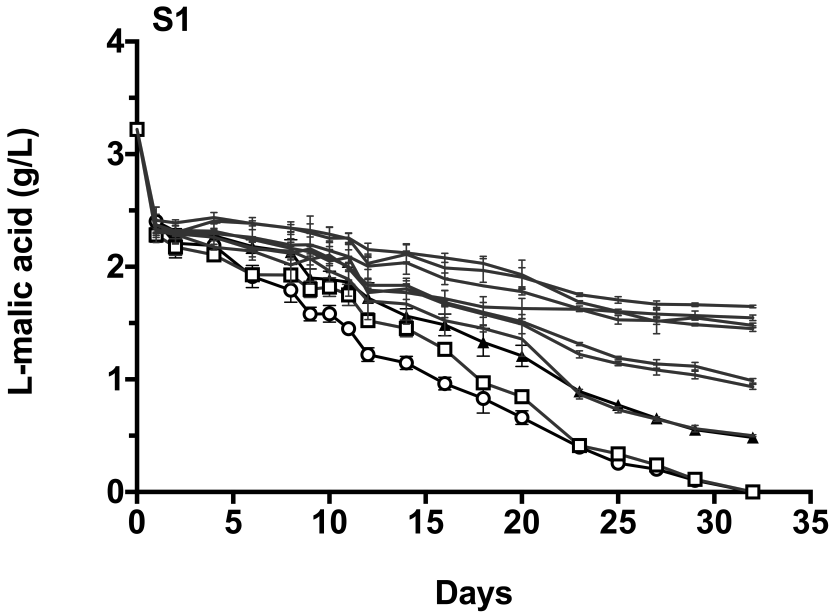
Zhang, G., Fan, M., Lv, Q., Li, Y., Liu, Y., Zhang, S. and Zhang, H. (2013) The effect of cold, acid and ethanol shocks on synthesis of membrane fatty acid, freeze-drying survival and malolactic activity of *Oenococcus oeni*. *Annals of Microbiology* **63**, 477-485.

Zhang, J., Wu, C., Du, G. and Chen, J. (2012) Enhanced acid tolerance in *Lactobacillus casei* by adaptive evolution and compared stress response during acid stress. *Biotechnology and Bioprocess Engineering* **17**, 283-289.

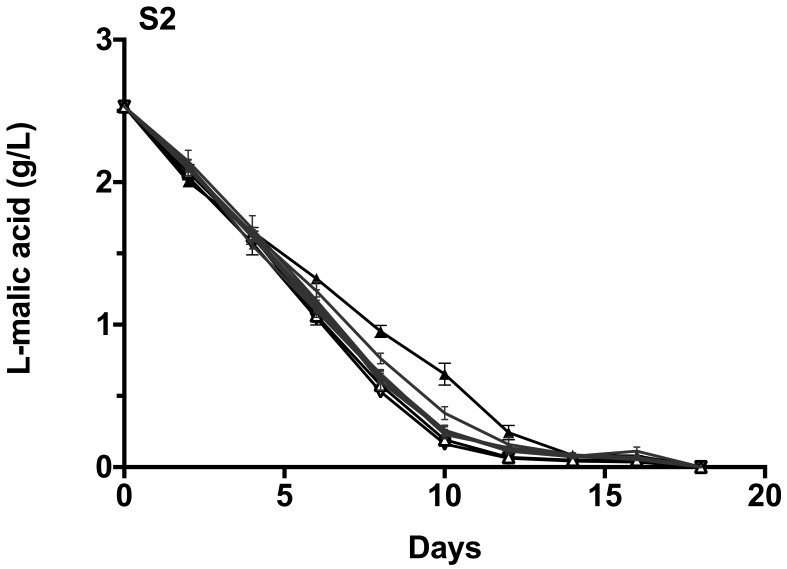
Zhang, Y.X., Perry, K., Vinci, V.A., Powell, K., Stemmer, W.P.C. and Cardayre, S.B.D. (2002) Genome shuffling leads to rapid phenotypic improvement in bacteria. *Nature* **415**, 644-646.

Zúñiga, M., Pardo, I. and Ferrer, S. (2003) Conjugative plasmid pIP501 undergoes specific deletions after transfer from *Lactococcus lactis* to *Oenococcus oeni*. *Archives of Microbiology* **180**, 367-373.

Appendix 1: Supplementary figures in Chapter 3



S1. Fermentation performance of selected strains from the 150 generation micro-scale screening experiment in RFCDGJM containing 15% (v/v) ethanol, 2.92 g/L L-malic acid at pH 3.3. strains 1-143 (□) and 1-161 (○) consumed more malic acid than A90 (▲). Each point is the mean of triplicates ± SD.



S2. Fermentation performance of selected strains from the 220 generation micro-scale screening experiment in RFCDGJM containing 15% (v/v) ethanol, 2.53 g/L L-malic acid at pH 3.35. Strains 2-47 (△) and 2-49 (▽) fermented faster than A90 (▲). Each point is the mean of triplicates ± SD.

Appendix 2: Primers used in this study

1) Primers used to confirm SNPs between A90 and SB3 (primers designed by Betteridge, A.L.)

Locus Tag	Forward Primer (5'−3')	Reverse Primer (5'−3')
OEOE_RS00380NG	GGGATTTTAAACGGTATCTGGTTAG	CGGCTAGTACTAATGACCAAACTGTA
OEOE_RS00870	CCGATTCCTGAGGTGGTAGAT	CTCCGGCATCATTCTTTTTAAC
OEOE_RS00935	CTTTTACGGCTGTCTCTTTTACTC	AGCGAAATCGCAGGAATGATA
OEOE_RS01940NG	ATCGAACCCTCATCTCAGGAA	GCAAGTAAGCAAGCATTGAAG
OEOE_RS02085	CGTGCTCACCCACATAATC	GCCAGGTGTATCAAGGAAAGTAA
OEOE_RS02355NG	GGTCCACGGAATCACATAAATAG	GCCGCAAGAAACTGGTATGA
OEOE_RS02680	TGTTTGGGGTGAAGACCATT	AAGCAGGCGGGAAGAAGT
OEOE_RS04175	CGCTTTGCTGGTTGATTTTA	ATAACGCCACTTCTTGTCCAT
OEOE_RS04370	TGTGGAGTGAAGCAAAGACG	CGGCAAAACAACTAATCAAAGG
OEOE_RS04445	GCCTTGTATGGATTGGAGTGA	CAACGAATGTTTAGCGGAAA
OEOE_RS04845	GAGTTGCTGATTTGTTGGATGA	GATTGCGTCCCATTTATTGA
OEOE_RS04930	TATGCGTCCTTTGGTTGAGG	GTGCCATCGTTTCCGTTTACT
OEOE_1040NG	AAGAAGTTGTCACCGCCACT	CGTTTGAGAGGATTGGTTCATT
OEOE_RS05365	CATTTTCATCCCACCATCCTT	GCCAGACGCCATTTACGAC
OEOE_RS05625	TCGGTGCTATTTTGGGTATGA	CGTTATCCGCTTGGGAAGA
OEOE_RS05645	GCAGGAATCGGTGGAATG	ATTGTTGGGGTTAGTGGTGCT
OEOE_RS06920	CTCGTGCTTTGTTCTTTTGGA	GCTGCTTTTAGAGGGCAATGA
OEOE_RS07625	AGTCGGGAAAGCAACAACC	GCTGGAACTGAAAGCGAAAA
OEOE_RS07855	TTGCCATCGTTTGGTCATC	GGTCAGGTAATCCGTTTGGTAG
OEOE_RS08235	AGAACGAAGGGATTGATTGA	AGCCCACAGTTTAGGAATGA
OEOE_RS08915	CGCTCTCGCAATCCTCATC	ACCGCTGCCTTACCGACTT

2) Primers used to confirm SNPs between 2-49/3-83 and A90 (primers designed by Jiang, J.)

Locus Tag	Forward Primer (5'−3')	Reverse Primer (5'−3')
OEOE_RS00155	ATCATTAACCGGGCAAGAGGA	CCTGCCGTTTTAAGGGTATTCC
OEOE_RS00320_pair 1	ATGGTATTATCGCGTTCCG	CTAATCAAACTCTTCGTAAGTATTAGG
OEOE_RS00320_pair 2	TTTAGTCAGAGGCGGCCAAG	CTGACAATGCCGCGTGATTT
OEOE_RS00570	TTTTACCTTCGGTCGAGCCC	CTTTCTCTTGGCGCAACTGG
OEOE_RS00960NG	TCGAAAATTGAAAGCGGGCAG	CGACCGTAATAGTATCGCCGT
OEOE_RS00975NG	TTTGCTCCTATGCCAACGCT	TCGATGCAGAGGTCAAAGCC
OEOE_RS01340NG	GAGCAAACTGCCCGATCAAG	TTGGGAATCTTCGACTGGGC

OEOE_RS01460NG_pair 1	AGAATTTCGTCACGCTGGGT	AATCACGGTTCCTTTGGGCA
OEOE_RS01460NG_pair 2	CAAGAATTTCGTCACGCTGGG	TCAGCAAAACCCCTGACGATT
OEOE_RS01465	CAAGAATTTCGTCACGCTGGG	CGATCGCAAATCACGGTTCC
OEOE_RS01465NG	CGTGATCGCTTGTATTCGGC	CGTCCACTTCGGCAGAGAAC
OEOE_RS01735NG	TGGTAAGTTCGCCGAGCAATG	CATCGCAGCAATCACAGCAAG
OEOE_RS02275NG	TGATGTTTCAGCACCGGGAG	TGGCAAATTTCCTGGTTGGT
OEOE_RS02280	CAGCAAGCGGAACAGTGGTA	TTGGGAATGGGAATCTCGGC
OEOE_RS02410NG	TTGGCTCCATGGTCAAGTGG	TAGTACTGATGCCTCCAAGTCA
OEOE_RS02820	TGGTCGCTTTCTGTCGAAGC	TCGGACAGTAAGGAGAAGCG
OEOE_RS03920NG	ATGTTCCGTCGATGTCGCTG	CCATCGCGTTCCTGTAAATCG
OEOE_RS04160	TGTTGTTGAAGGATTGCGCC	TAAGATGTCTCGCCTTCGGC
OEOE_RS04465	CTGTCAGCACAGCTTGGTTG	TGGGCAAGTGGAATAGACCAG
OEOE_RS04995_pair 1	AGTTGCTCTCTCACTCGGATTAC	TCCCGATTCACGCTTCAAGG
OEOE_RS04995_pair 2	GGTTTATTCGGGCAGCTTTCC	GGATCCCTTGCTTGAAGTCGA
OEOE_RS05000NG_pair 1	CGCGATGAGATATGTTGCGC	TCGATAGTGTTGTTTGGACATTACG
OEOE_RS05000NG_pair 2	GGCCACGTTTGCTAAGTCATC	GAGTTACTCCAGTTGGCACGA
OEOE_RS05000NG_pair 3	GTATAGACAACACGGCCACCA	TACCGAAGCAGCAGTCGATTT
OEOE_RS05235	CGAATGGCAGACCGACAAGT	CAGCCTCCCACAGAAACTCAC
OEOE_RS05425	ACAAGAGCTCTCCGTGCTTC	CGATGGCAGATAAACTTGGCG
OEOE_RS05570	CCAACGTTCCAGCCAAGTTT	TACTGAAGCGTATGTGCCGA
OEOE_RS05780	TCCAGAAGCGACGGTATAGC	TTGATTGGCGAAACGGATGC
OEOE_RS05780NG_pair 1	CTGGCTGCAGAACTAGCACT	CCAAGGGCAGACTGAAGAGG
OEOE_RS05780NG_pair 2	AGTTGTTGCCATAATCGCAGC	CCAATGATAACTTTATGTGCACGTG
OEOE_RS05780NG_pair 3	CACGTGCACATAAAGTTATCATTGG	TCCGCTTGTTATCAAAGCGC
OEOE_RS05780NG_pair 4	TGAAATCAACGGTCAGCTCCA	TTATCAAAGCGCACGATGCTG
OEOE_RS05840_pair 1	TGCAGATCTTATAGAGCAAACCGA	GTCGTTGGTTCAAATCCAGCC
OEOE_RS05840_pair 2	CGCTATGATAAGGGAAATATGGTAG	TGATAACGCAGAGGTCCCAG
OEOE_RS05940	ATTGTCGAAGCAGCTGACGA	TAATCACCTGCAGGCTGGTC
OEOE_RS06240	GGCCGCTTTAGTCGGTATGT	CCAAGCGTTGCAGGAACTTT
OEOE_RS06415NG_piar 1	ATAATTACGGAGCCGCGATGT	CTTCTTGTCCTCCTTGCGACT
OEOE_RS06415NG_pair 2	TCCGCCCAGTCTTTCTAACT	ATGTTCACACGCGTCTACCA
OEOE_RS06420	GCGTTCTGTTCGGTCGAGTA	GACTATCGCCAGTCTCACCG
OEOE_RS06425	CTAGAATTACGGGCCGGTCC	ACGATTCTGGATGCCACGAA
OEOE_RS06425NG	TCGTGGCATCCAGAATCGTC	TCCAAGAAGCTTACGATGCA
OEOE_RS07200NG_pair 1	TCCCAAAAGAGTTAACGGCCA	TGATAACGGTCCGCTCGAAG
OEOE_RS07200NG_pair 2	TCCTTTTGCATCGGTGTGGTA	CGCTTTCAACATTGGCAAGGA
OEOE_RS08000	ATTTGGAGCAGGCTTACCGG	TCTATATGAATGCCGGCGCC
OEOE_RS08070	TATCACCGGCGTTACTCTGC	ACCCTACTCCATGTCGAGCA
OEOE_RS08380NG	AAGGCCGACATAACCTGTACC	AGACTGGTCTTTTGCCGAATAC
OEOE_RS08450	GCGTTCTGTTCGGTCGAGTA	CGCCAGTCTCACCGGTATTT
OEOE_RS08465	CTTGTCCCAGCTAACGTAATCG	TGCTGCACGAATTGGAAGAG
OEOE_RS08465NG	ATCGAAATCTCGCTTGCAGC	TCCGTGTCCTCTCAGATCGT

OEOE_RS08880	GGAACGTTTTGCCATCCACC	CTGTACTTGTGACTTCAATAGCTGC

3) Primers used to fill small gaps (gaps<100 bp) for A90, 2-49 and 3-83 (primers designed by Jiang J.)

Locus Tag	Forward Primer (5'−3')	Reverse Primer (5'−3')
OEOE_RS00290NG	TGCCCGTGATGAAGATTACAAAC	TTCCTTAAAGTTAGCAATAGCCACC
OEOE_RS00295NG	CCGAATCTTGTCCTGCAAAC	CAATTTACGTTCTAGTTCCTGTACC
OEOE_RS00330NG	TGGTGCACTCACGTCAATCA	TGTGCCTCGGGGAAATAGTTTG
OEOE_RS00420NG	TGGTGGGCTTAGATCCAACG	CCCACCTTTCCATCCTGTGT
OEOE_RS00455NG	AAGAGGAGGCGTTTAAGTATCGA	CAAGATAGTGATTCCTTGATAATGCCC
OEOE_RS00960_pair 1	CCTATTGGCGAGAAACTTCATTTGA	TCTGACTGAATTTCCGAACGC
OEOE_RS00960_pair 2	TCGAACTGCTAATGCTGAAGGA	TGAATCTTGCTGGATCTCTTTCCA
OEOE_RS00970	TTGTTAGCTACGGCAACATTGG	CGCAACTTTACTGCCGTAGC
OEOE_RS01705NG	TGTTGACGGTGATCGGTTCA	TGCCCACCTTCATGTATCTCG
OEOE_RS01935NG	GGATCATCCAGAATTGTATTGAACG	TTGCTGAAACATCGGCGTTC
OEOE_RS02285NG	ACTTGTGCTTATATGGCTGGAAC	CGGGTCGAGTTGGCTAGAAC
OEOE_RS02310NG_pair 1	TCGTTTCAGCAGTCATAGGTGA	AAGTTAGAAGTTTCCGAAGATCCAG
OEOE_RS02310NG_pair 2	ACGTCTTGAACTGGGGAACTC	ACTCGGAAAAACTGCGCAAAG
OEOE_RS02600	CAAGGTTTGCAGCTGTTTCCA	CCGCTTGCTTCCTGAAATTCT
OEOE_RS03310NG	CCCTCGGGAATAATCACCATATGT	CACATTGATCCGCTGATTCTTCC
OEOE_RS03405NG_pair 1	ATTCGAACCGATGCCCCAG	TGGAAATACGCTCTGGCTGC
OEOE_RS03405NG_pair 2	ATCAGCAATACATTGATCCTGTAGC	TCTGTCAAATGATTGTTAGGCACC
OEOE_RS03420	TCGAGCGATACCAATTCCAAGA	CGGATAAAACTCCAGGCAGTG
OEOE_RS03495NG_pair 1	TGAGCCAACGAAACCAGTTG	CCATTGAATGGTAGTCCGATATGC
OEOE_RS03495NG_pair 2	AAAGTTATTCAGCCGCCCAAC	GCGACCAAGAACACCTAATGC
OEOE_RS03790_pair 1	TGAGTGGATTGGTGTCGATGC	AGCCATAACATTTCCTGCATCA
OEOE_RS03790_pair 2	TGAGTGGATTGGTGTCGATGC	CTTCAGGAGCGGCATCAATTG
OEOE_RS04035NG	TGCTACCTGGCCAACTTACG	CTATCACAAGCGCTAAAGCCG
OEOE_RS04040NG	CAAGGCGAGAATTGCCGATG	TCCCTGTTGGACATTGGCTG
OEOE_RS04150NG_pair 1	CCTTTGAAAGATAGTTCTGGCGAC	CGTTGTTTCAAGCGCCAAGT
OEOE_RS04150NG_pair 2	GCCGCTACCTTTATTGGTGG	CCTGTCTGATTAACAAAATTCGCTC
OEOE_RS04170NG	AGGCAGGTAAGTAGAAGGCC	TTGCCGATTCAACAGTAATCCTAG
OEOE_RS04245NG	TTGGTGCCATTATCGTGGGC	GCGATGTTAACAATGCTGGCA
OEOE_RS04450NG	TCATCGATTATTGTCACGTCGGA	CGCAGCTACGATTATTGGCG
OEOE_RS04760NG_pair 1	TTACCTTTGCCGGTCCAACC	CATCAGGACGCTTTACACGC
OEOE_RS04760NG_pair 2	CCTGAATTACCTTTGCCGGTC	CCTTGTTCGGCCCAATACTCT
OEOE_RS06495NG	TTGTCTACTGATACCGGCTTCA	ACCTCTACCGAAGTTGGAAG
OEOE_RS06865_pair 1	GGTCCCCCTCGGTTTCTCTA	ACGCCAGCATCTTTCACTTG
OEOE_RS06865_pair 2	AGATCATCGACTTTGCGCTCA	TTTGACGAGTGGAAGAGCGAA
OEOE_RS06905	TGGAAGAAACTCCGCTATTGCT	TTCCGGCGATACCCTTTACG
OEOE_RS07080NG	TGCGTCCCTCCAAAATGACG	TGCACTGTCGATCTTGCTGT
OEOE_RS07740NG	TGCAGATTAGAACCATTGGCATC	AGCCCACACTTTCAGAGAGTC

OEOE_RS07785NG	TCGTTGGTGGAGCCGTTATC	CAAAGAAGCCGCTTATGCTCC
OEOE_RS07810NG	TGAAGGCCGTGCTAACATCC	TGTCTATTTGTCCCACGCGG
OEOE_RS08065NG	TTACACTGCCAGCAAACACG	TGTCGTTTCGGCAACTGTTG

4) Primers used to fill in large gaps (gaps>100 bp) for A90, 2-49 and 3-83 (primers designed by Jiang J.)

Gap	Forward Primer (5'−3')	Reverse Primer (5'−3')
Gap 1	GCGTCTGCTTTGCGAAACC	TTCGATTCTAATGCCTGCCGG
Gap 2_pair 1	ACGGTAGCTAAACGCAACGA	GGCGATCAATTGAACAGCGT
Gap 2_pair 2	GGTGAAAATGATGCCGCTACC	TGCCCACCTTCATGTATCTCG
Gap 2_pair 3	TTCCTATGCTGCTTCGCTTGA	CCCACCTTCATGTATCTCGGG
Gap 3_pair 1	TTTGGATGCGACGCAAAACA	TGGCTAGAGAATTGTCGGCG
Gap 3_pair 2	TGCTGTGCTGATTATCTCTGT	ACATAAGGGAAAAGCAGGCGT
Gap 4	TCTTCGTATGGTGCGGAAGT	TGTTCAGTGTGTAGGACTTGTGA
Gap 5	GTATCATACCAGTTTCTTGCGGC	ATCGGCCCTGTCAACAAACT
Gap 6	TTAAGCCAGTTCATGTTGGGC	CGTGCACTGGAACTATTGAAGC
Gap 7	TGGTCTGGTAAGGAGCAAACA	TCAGCAATACCATCCCCACC
Gap 8_pair 1	GCCCGCTTGATCCAATGAC	CTGCTCAGATTAATCAGACTGAGAC
Gap 8_pair 2	ACCGCCGCTGCTTTTAAAATT	CTTTGAAGCGGGTCGGAATTG
Gap 8_pair 3	TTCTGTAGTTGCTTGAAGGCTTC	CTTTGAAGCGGGTCGGAATTG
Gap 9	TGACGCCTTCCAAATATCGTC	TTCAAGCAGCAAAAGGTCGC
Gap 10	GTTGCATATCCGTCGCTGAC	TCTGCTGTGTTTGAAACGAGC
Gap 11	CCGGCATATTGTCCGATTGC	ATTAATGTTGGCGCAGTCGG
Gap 12	AAGGCCGACATAACCTGTACC	ATTGGGATCGCAGCAAAGAAG
Gap 13	TTCTGTAGCAAGATATCACTTTGGG	TTGGAAAGATGGGACCTGGG
Gap 14	AATTCTTACAAAGGGTTGATCGCC	TGCTGATAATGACACGCCAAC

Appendix 3: Detailed information about the main gaps in genomes of SB3-related strains assembled to the PSU-1 genome

Gaps	Location (PSU-1 genome)	Size (bp)	Genes in the gap (based on PSU-1)
1	80,321—81,007	686	OEOE_RS09000—OEOE_RS09005
2	327,554—345,196	17,642	OEOE_RS01610—OEOE_RS01700
3	347,709—350,047	2,338	OEOE_RS01715—OEOE_RS01730
4	456,846—462,676	5,830	OEOE_RS02250—OEOE_RS02265
5	483,346—487,320	3,974	OEOE_RS02365—OEOE_RS02400
6	977,730—985,462	7,732	OEOE_RS05020—OEOE_RS05070
7	1,232,803—1,233,864	1,061	OEOE_RS06380
8	1,411,439—1,419,200	7,761	OEOE_RS07215—OEOE_RS07245
9	1,425,650—1,439,945	14,295	OEOE_RS07275—OEOE_RS07320
10	1,654,982—1,655,260	278	OEOE_RS08365
11	1,659,318—1,659,534	216	OEOE_RS08385
12	1,660,159—1,663,434	3,275	OEOE_RS08385—OEOE_RS08395
13	1,663,779—1,663,969	190	OEOE_RS08405
14	1,664,408—1,664,743	335	OEOE_RS08405

Appendix 4: PCR cycling steps

1) PCR using Velocity DNA polymerase

Step	Temperature	Time	Repeat
Initial denaturation	98 ˚C	2 min	1
Denaturation	98 ˚C	30 sec	29
Annealing[a]	55–65˚C	30 sec	
Extension[b]	72 ˚C	30 sec–10 min	
Final extension	72 ˚C	10 min	1

[a]A temperature gradient between 55–65˚C was first tested in order to determine the optimal annealing temperature.

[b]Extension time was dependent on the sizes of PCR products: 15 sec/Kb for amplicons < 5Kb and 60 sec/Kb for larger amplicons.

2) PCR using Ranger DNA polymerase

Step	Temperature	Time	Repeat
Initial denaturation	95 ˚C	1 min	1
Denaturation	98 ˚C	10 sec	29
Annealing/extension[a]	55–65˚C	8–10 min	

[a]A temperature gradient between 55–65˚C was first tested in order to determine the optimal annealing/extension temperature. Annealing/extension time used for each PCR cycle was 30–60 sec/Kb.

Appendix 5: Protocol for DNA extraction from *O. oeni* cells

1. Grow cultures in 10 mL fresh MRSAJ aseptically
2. Spin 10 mL of bacterial cultures at 8,000 ×g for 5 min, discard the supernatant wash with 1 mL TE solution (10 mM Tris-HCl at pH 8.0, 1mM EDTA), transfer liquid culture into a 2 mL screw cap tube, centrifuge at 8,000g for 2 min, and discard the supernatant. Repeat this step again.
3. Re-suspend the cell pellet with 600 µL of TE solution containing 20 µL of 50 mg/mL lysozyme, vortex and incubate at 37˚C for 1 h
4. Add 30 uL 10% SDS and 4 µL proteinase K (14-22 mg/mL) to the cell suspension, vortex and incubate at 37˚C for 2 h
5. Add 100 µL 5 mol/L NaCl and 80 µL CTAB (hexadecylytrimethyl ammonium bromide) – NaCl to the cell suspension, vortex and incubate at 65˚C for 30 min
6. Purify the crude DNA: add an equal volume of phenol/chloroform/isoamyl alcohol (25: 24:1), vortex and centrifuge at 10,000 g for 5 min, carefully transfer the supernatant into a clean 2 mL screw cap tube, and repeat this procedure 1-2 times. Add an equal volume of chloroform/isoamyl alcohol (24:1), vortex and centrifuge at 10,000g for 5 min and carefully transfer the supernatant into a clean 2 mL screw cap tube. Repeat this procedure 1 time. (Use 1 mL wide bore tips to avoid cutting of genomic DNA)
7. Precipitate genomic DNA by adding 2 volumes of cold ethanol and 0.1 volume of sodium acetate (3M, pH 5.2). Leave the reagents overnight at -20˚C to allow DNA precipitation. Gently mix and centrifuge at 10,000 ×g for 5 min and discard the supernatant.
8. Wash the DNA pellet 2 times with 70% cold ethanol to remove residual CTAB. Re-spin 5 min at room temperature to re-pellet DNA.
9. Leave the DNA pellet to air-dry at room temperature
10. Dissolve the DNA pellet with 60 µL of TE buffer containing 200 µg/mL RNase (1.2 µL of 10 mg/L RNase A). Incubate at 37˚C for 15 min. Store DNA samples at -80˚C for subsequent use.
11. Qualify and quantify DNA spectrophotometrically using Nanodrop (Infinite 200 PRO, Tecan, Männedorf, Switzerland).