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Application of directed evolution to develop ethanol tolerant *Oenococcus oeni* for more efficient malolactic fermentation

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Abstract Malolactic fermentation (MLF) is an important step in winemaking, which can be notoriously unreliable due to the fastidious nature of *Oenococcus oeni*. This study aimed to use directed evolution (DE) to produce a more robust strain of *O. oeni* having the ability to withstand high ethanol concentrations. DE involves an organism mutating and potentially adapting to a high stress environment over the course of extended cultivation. A continuous culture of *O. oeni* was established and exposed to progressively increasing ethanol content such that after approximately 330 generations, an isolate from this culture was able to complete MLF in high ethanol content medium earlier than its parent. The ethanol tolerance of a single isolate, A90, was tested to confirm the phenotype and its fermentation performance in wine. In order to investigate the genotypic differences in the evolved strain that led to the ethanol tolerance phenotype, the relative expression of a number of known stress response genes was compared between SB3 and A90. Notably, there was increase in *hsp18* expression in 20% (*v*/*v*) ethanol by both strains with A90 exhibiting a higher degree of expression. This study is the first to use directed evolution for *O. oeni* strain improvement and confirms that this technique can be used successfully for the development of new candidate strains for the wine industry. This study also adds to the current knowledge on the genetic basis of ethanol tolerance in this bacterium.

Keywords *Oenococcus* · Wine · Ethanol · Stress response · Directed evolution · Malolactic fermentation

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Introduction

Oenococcus oeni is a vital microorganism for the wine industry; the stabilisation, deacidification and organoleptic changes that it facilitates in wine are an integral part of the production of many wine styles. More specifically in most red wines and some white wines, a secondary or malolactic fermentation (MLF) is carried out following alcoholic fermentation by lactic acid bacteria (LAB), most commonly *O. oeni*. MLF involves the conversion of L-malic acid to L-lactic acid and carbon dioxide, resulting in deacidification and improvement of microbial stability of the wine. Wine is typically a nutritionally poor environment and has physicochemical properties that can hinder the growth and malolactic activity of *O. oeni*. Common inhibitors in wine include, high ethanol content, low pH, low temperature, low nutrient availability, the presence of sulfur dioxide (SO_2), phenolic compounds and acetic acid (Bauer and Dicks 2004). Furthermore, the alcohol concentration in wines has been rising in recent decades (Godden et al. 2015) and MLF in wines with a high ethanol content commonly stalls (Zapparoli et al. 2009). Consequently increased LAB ethanol tolerance is a desirable trait. To alleviate ethanol stress, a number of strain improvement methods are available that encompass both recombinant and non-recombinant protocols (Betteridge et al. 2015). To produce strains suitable for use in the wine industry, non-recombinant methods are

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preferred since recombinant techniques are still not favoured by some consumers. Even if the use of recombinant strains was favoured by the wine industry, there are still no reliable methods of genomic manipulation for use in *O. oeni*.

Directed evolution (DE) is a process based on Darwin's theory of natural selection and is also known as adaptive evolution, stationary phase mutation, directed mutation or stress response mutation (Rosenberg and Hastings 2003). The process involves adaptation to a high stress environment through spontaneous DNA mutations. Those organisms that have gained advantageous mutations prosper, proliferate and dominate under the specific stress (Foster 1999). DE has been successfully used with several organisms. *Lactobacillus plantarum* growth on glycerol under anaerobic conditions is too slow to be accurately measured; however, after approximately 500 generations under continuous selection using glycerol as a limiting factor, growth rate was improved by over 10-fold (Teusink et al. 2009). Another example is the replication of the natural adaptation of *Lactococcus lactis* from a plant niche to growth in milk in 1000 generations under laboratory conditions (Bachmann et al. 2012).

There are as yet no reports of the successful application of this method to *O. oeni*. Previous studies have shown that *O. oeni* is a rapidly evolving organism (Yang and Woese 1989). This is most likely due to the absence of the genes *mutS* and *mutL*, which encode two key enzymes in the methylated mismatch repair (MMR) pathway (Makarova et al. 2006; Makarova and Koonin 2007). The MMR pathway is a DNA excision-repair system, which corrects nucleotide base pair mismatches during DNA synthesis. The presence of *mutS* and *mutL* homologues is required for MMR to function (Miller 1996; Oliver et al. 2002). The correction of mismatches by MutS and MutL decreases the spontaneous mutation rate of a species; therefore, a defect or absence of the MMR system leads to an increase in mutation frequency (Miller 1996). A recent study comparing spontaneous mutation rates of *O. oeni* to those of closely related LAB species *Leuconostoc mesenteroides* and *Pediococcus pentosaceus*, which contain the relevant mismatch repair genes, showed a 100-fold greater rate of spontaneous mutation of *O. oeni* in response to antibiotics rifampin and erythromycin (Marcobal et al. 2008). One possible reason for the loss of MMR in *O. oeni* is that a high mutation rate generates beneficial mutations during adaptation to a restrictive environment such as wine (Marcobal et al. 2008). The high rate of mutation combined with the inhibitory properties of wine, therefore make *O. oeni* a perfect candidate for DE.

Stress response in *O. oeni* is controlled by a number of genes that alter several cellular processes. Genes reported to be involved in the cellular response to ethanol stress and contributing to biostability of wine include *ggpps*, *clpX*, *clcP*, *trxA*, *hsp18*, *ftsH*, *omrA*, *groESL*, *dnaK*, *clpA*, *ctrS* and *rmlB* (Sumby et al. 2014). The physiological response of *O. oeni* to ethanol stress has been well studied (Guzzo 2011; Wen-Ying and Zhen-Kui 2013). Ethanol perturbs the bacterial cell membrane leading to leakage of intracellular proteins, which affects the proton gradient and subsequently influences cellular processes, including ATP synthesis and the transport of amino acids (Guzzo et al. 2000). The physiological response of the cell to ethanol stress consists firstly of an increase in the proportion of cyclopropane fatty acids (CFAs) in the membrane, through conversion of *cis* vaccenic acid to lactobacillic acid (Teixeira et al. 2002), to counteract the effect of ethanol on membrane fluidity (Grandvalet et al. 2008), followed by an increase in membrane protein/phospholipid ratio. The precise mechanism by which *O. oeni* might be changed through a DE approach to improving its robustness during MLF might not be predictable, but once isolated, such strains offer a subject for further investigation of enhanced tolerance.

The aim of this study was to investigate the suitability of DE as a method for improving the robustness and hence MLF efficiency of an *O. oeni* strain that might subsequently be applied to winemaking. Specifically, the focus was on improving ethanol tolerance, a common wine stress known to inhibit MLF.

Materials and methods

Bacterial strains and maintenance

The strain used in this study, *Oenococcus oeni* SB3, was isolated from a commercial packet of this readily available wine starter culture (Laffort Oenology, Bordeaux, France). All bacterial isolates were cultured in de Man, Rogosa and Sharpe media (Cat. # AM103, Amyl Media, Victoria, Australia), supplemented with 20% apple juice (MRSAJ). When solid media was required, 2% agar was added to MRSAJ. Unless otherwise stated, all isolates were incubated at 30 °C for 5 days. Strains were stored at − 80 °C as glycerol stocks (culture grown for 5 days in MRSAJ and then supplemented with glycerol (final concentration 25% *v*/*v*)).

Directed evolution of *O. oeni* strain SB3

A continuous culture was established in a Biostat A plus bioreactor (Sartorius BBI System GmbH, Goettingen, Germany) and controlled using the MFCS/DA A plus 2.1 software (Sartorius BBI System GmbH). The empty bioreactor was autoclaved at 121 °C for 30 min and filled with 1100 mL of MRSAJ with 5% (*v*/*v*) ethanol. The medium was inoculated with a 10 mL culture of SB3 at an optical density measured at 600 nm to be greater than one ($OD_{600} > 1.0$). The volume in the bioreactor remained stable via a level sensor, feed/effluent pumps and control software. The culture temperature was maintained at 30 °C and stirred constantly at 100 rpm. The feed medium was MRSAJ via one pump starting at 54 mL h^{-1}.

Ethanol was added via a second pump throughout the course of the experiment at a constant rate of 2.4 mL h^{-1}. As the maximum specific growth rate (μ) of the population slowed in response to the ethanol addition, the feed rate of MRSAJ was also slowed over the course of the experiment to a final rate of 13.5 mL h^{-1}. In this way, ethanol concentration gradually increased to 15% (*v*/*v*) over the course of 290 days.

The culture was sampled weekly to measure optical density (OD_{600}) and determine viability (colony forming units) by plating on to MRSAJ agar. Once the bioreactor culture had surpassed 13% (*v*/*v*) ethanol, the *O. oeni* population was sampled and screened for improvement in L-malic acid consumption in the presence of ethanol compared to the parent SB3 strain, in a sacrificial microplate experiment described below.

Evaluation of the bioreactor population compared to the parent strain SB3

A replicated 96–well microtitre plate experiment was designed to measure growth and L-malic acid consumption of the evolved, potentially non-uniform population in MRSAJ with 15% (*v*/*v*) ethanol supplemented with 1 g L^{-1} L-malic acid. Screening was performed in quadruplicate 200 μL fermentations. Eight identical plates were generated, from an individual source plate, in order to enable sacrificial sampling over an 8–10-day period. This allowed a fresh plate to be analysed at each time point for monitoring of the depletion of L-malic acid, without influencing the performance of the small volume fermentations. Each plate was sealed with a membrane (Excel Scientific, 100-SEAL-PLT) and incubated at 30 °C in an anaerobic vessel (BD Diagnostic Systems, 260671, 260001). At each time point, the L-malic acid concentration was determined using the enzymatic method described below.

L-malic acid assay

L-Malic acid concentration was determined using a modified version of the Boehringer-Mannheim UV method (Boehringer-Mannheim 1995). Each well of a 96-well microtitre plate was filled with 150 μL of solution 1 (0.1 M glycylglycine, 0.1 M L-glutamate (adjusted to pH 10.0 with 5 M NaOH)) and 30 μL of solution 2 (0.0164 g mL^{-1} NAD^+) using a liquid-handling robot (Corbett Robotics CAS3800). The undiluted sample (4 μL) was then pipetted into each well except for the final column, which contained eight standards of L-malic acid solutions ranging from 0 to 3.5 g L^{-1} in 0.5 g L^{-1} increments. The plate was inserted into a plate reader (Infinite 200 PRO, Tecan, Männedorf, Switzerland), and 10 μL of glutamate oxaloacetate transaminase (GOT) solution (0.0269 U μL^{-1}) was added to each well of the plate using the injectors. The plate was shaken for 3 min before measurement at 340 nm (A0). Finally, 10 μL of the enzyme L-malate dehydrogenase (L-MDH) (0.0261 U μL^{-1}) were added to each well. The plate was shaken for 25 min and then read again at 340 nm (A1). The change in absorbance (ΔA) was calculated (A1–A0) for each of the standards and a standard curve generated for each individual plate. This equation was then applied to the ΔA for each of the samples within the plate to calculate L-malic acid concentration.

Characterisation of single isolates from the evolved bioreactor population

A replicated 96-well microtitre plate experiment, allowing sacrificial sampling, was designed to measure growth and L-malic acid consumption of single isolates from the evolved bioreactor population in MRSAJ with 15% (*v*/*v*) ethanol supplemented with 2 g L^{-1} L-malic acid. Each isolate was analysed in quadruplicate. A sample of the DE culture taken at 332 days (15% (*v*/*v*) ethanol) was plated onto MRSAJ agar and 180 individual colonies were isolated and inoculated into individual wells of two deep-well plates (Cat. # P-DW-20-C-S, Axygen, NY, USA) each containing 1 mL of MRSAJ. Deep-well plates were sealed with a breathable membrane (Cat. # AXY BF-400, Axygen, NY, USA) and incubated statically at 30 °C for 3 days. These liquid cultures were then diluted 1:5 in MRSAJ containing 15% (*v*/*v*) ethanol to generate source plates (Fig. 1). The source plates were arranged in quadrants, with each sample replicated once per quadrant, so that for each quadruplicate fermentation, the influence of the position of the sample in the plate was minimised. From the source plates, samples were diluted 1:20 into a 96-well plate (each well containing 190 μL of sterile MRSAJ with 15% (*v*/*v*) ethanol), using a liquid-handling robot (Corbett Robotics, Model # CAS3800). These six identical cell culture plates (Cat. # CLS3596, Corning, NY, USA) were prepared from each source plate and incubated statically and anaerobically in anaerobic vessels (Cat. # 260671, 260001, BD Diagnostic Systems) at 30 °C. One unique replicated set of the six identical plates was sampled at each of six time points. The plates were sampled regularly and monitored by determining L-malic acid concentration (as above) and therefore consumption over time.

Based on this analysis to determine the fastest L-malic acid consumption, a subset of isolates (27) were selected from the above screening to be evaluated in small-scale laboratory fermentations. Fermentations (10 and 50 mL) were performed in triplicate in MRSAJ with either 15% (*v*/*v*) ethanol at 22 °C or 13% (*v*/*v*) ethanol at 30 °C, respectively. Strains were grown in MRSAJ for 7 days to an $OD_{600} > 1.0$ and inoculated to fresh MRSAJ, with either 13 or 15% (*v*/*v*) ethanol, at a dilution of 1 in 50. Samples were taken regularly and L-malic acid concentration was determined enzymatically (as above). The strain that completed fermentation first, A90, was selected for further characterisation.

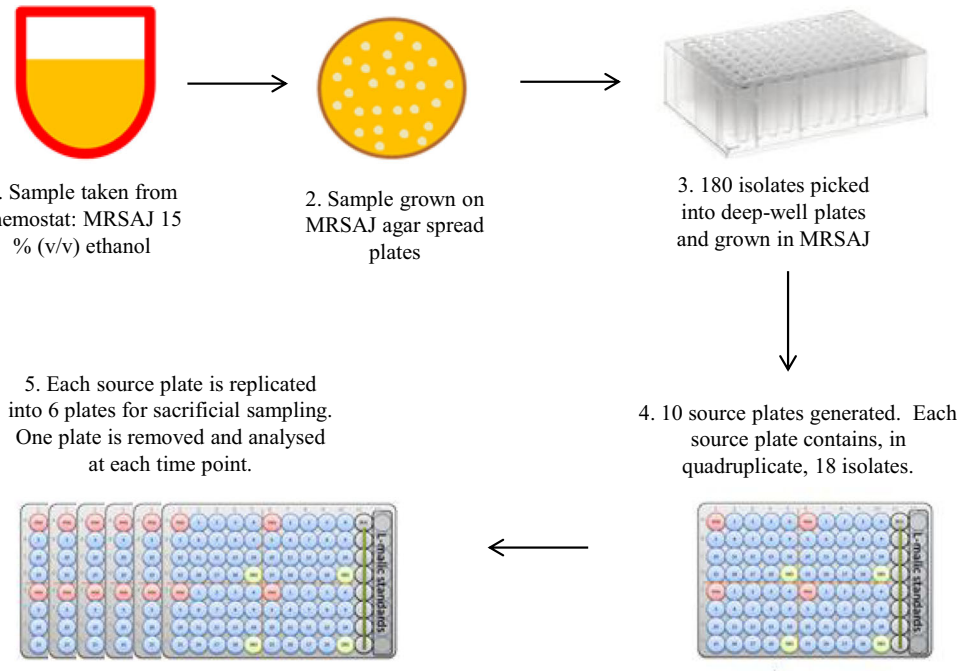

Fig. 1 Flow diagram representing the method for isolating 180 clones

Determination of survival from exposure to high ethanol

DE strain A90 and the parent SB3 were grown in MRSAJ to an OD_{600} > 1.0. An aliquot (200 μL) of each was then inoculated into 10 mL of MRSAJ with 0 or 20% (*v*/*v*) ethanol and incubated at 22 °C. Fermentations were sampled at 1, 12, 24, 36 and 48 h after inoculation. A 10-fold dilution series was performed (to 10^{-5}) and 2 μL samples were spotted onto MRSAJ agar. Plates were incubated in anaerobic vessels at 30 °C for 7 days prior to visualisation.

Malolactic fermentation performance of an evolved strain in wine

The MLF performance of A90 and parental strain SB3 was evaluated in a fermentation of a Pinot Noir wine (Tasmania, 13% (*v*/*v*) ethanol) to confirm that the ethanol tolerance phenotype of the isolate was not restricted to MRSAJ fermentations. A90 and SB3 were grown to an OD_{600} > 1.0 and inoculated into 10 mL of 0.22 μm filtered Pinot Noir wine. In addition, A90 and SB3 were also inoculated into the same Pinot Noir wine supplemented with ethanol to 15% (*v*/*v*). Samples (200 μL) were taken regularly and analysed for L-malic acid consumption (as above).

Strain passaging and ethanol tolerance

Single colonies of A90 and SB3 were isolated from ethanol-containing MRSAJ agar (20% *v*/*v* ethanol, 48 h) prepared during the generation of RNA for QPCR analysis (see below). Strains were cultured in 10 mL of MRSAJ in a CO_2 incubator at 30 °C until turbid. Optical density (OD_{600}) was adjusted to 1.2 and 250 μL of culture were used to inoculate fresh 10 mL MRSAJ. Strains were cultured for between 3 and 5 days, before re-adjusting culture OD_{600} to 1.2 and subsequently inoculating fresh 10 mL MRSAJ media. This process was repeated ten times for both A90 and SB3. To determine the number of generations that had occurred during each culture, a sample was taken immediately after each inoculation and at the end of each culture, prior to optical density adjustment. A 10-fold dilution series was performed (to 10^{-6}) and spot-plated onto MRSAJ agar to determine colony forming units. A minimum of 50 generations were achieved for both A90 and SB3. Passaged A90 and SB3 populations were inoculated (as above) into MRSAJ with 0, 18 and 21% (*v*/*v*) ethanol in triplicate and incubated at 22 °C. A 10-fold dilution series was performed (to 10^{-6}) on samples taken at 1, 24 and 48 h and spot-plated onto MRSAJ agar.

Transcriptional analysis

RNA extraction

Total RNA was extracted from six biological replicates of 50 mL cultures of A90 and SB3 under 4 conditions: (1) 1 h after inoculation into MRSAJ, (2) 1 h after inoculation into MRSAJ + 20% ethanol, (3) 24 h after inoculation into MRSAJ and (4) 24 h after inoculation into MRSAJ + 20% ethanol. *O. oeni* cells were harvested by centrifugation (5000 × *g*, 15 min), and the cell pellet was resuspended in 1 ml of cold 100% ethanol (− 30 °C) and transferred to a fresh 2 ml tube. The cells were then centrifuged (3800 × *g*, 30 s), the supernatant removed and the cell pellet

resuspended in 1 ml of TRIzol (Cat. # 15596026, Invitrogen, Australia) and frozen in liquid nitrogen. These were then stored at − 80 °C prior to RNA extraction.

RNA was extracted as follows: glass beads (425–600 μm, Cat. # G8772S, Sigma-Aldrich, Australia) were added to the cell suspension to 50% of the 1 ml volume. Cells were lysed by a bead-beater for 2 min and then incubated at 65 °C for 3 min. Chloroform (200 μL) was added and the tubes were shaken vigorously for 15 s and incubated at 20 °C for 5 min. Samples were centrifuged (12,800 × *g*, 10 min) at 4 °C, and the aqueous phase transferred to a fresh tube along with 500 μL of isopropanol. Samples were inverted six times and incubated at 20 °C for 10 min, followed by centrifugation (12,800 × *g*, 10 min, 4 °C). The supernatant was discarded and the RNA pellet washed in 1 ml of 75% cold ethanol (− 30 °C), all ethanol was then removed and the pellet air dried for 5–10 min. Purified RNA were dissolved in 50 μL of 0.1% dimethyl pyrocarbonate-treated water. RNA concentrations and purity were calculated by measuring absorbance at 260 and 280 nm using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific).

Reverse transcription and real-time qPCR

Prior to reverse transcription, total RNA was treated with 2 U of Turbo DNase (Cat. # AM1907, Invitrogen) as described by the manufacturer. Then, cDNA was synthesised using the iScript cDNA Synthesis Kit (Cat. # 1708891, Bio-Rad) as recommended. The absence of chromosomal DNA contamination was checked by real-time PCR. Initial experiments evaluated reference genes from previous studies (Sumby et al. 2012). The performance of these was analysed using qbase_plus software (Biogazelle) with *gmk*, *recA*, *gapA* and *ldhD* chosen as the most stable reference genes under the conditions of this study.

Primers for real-time PCR were designed with a length of about 20–25 bases, a G/C content of over 50% and a T_m of approximately 60 °C. Primer design software (Primer3) was used to select primer sequences. The length of the PCR products ranged between 107 and 200 bp. Potential primers were checked for gene-specific binding using NCBI primer design tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Primers were purchased from Sigma-Aldrich. All real-time PCR was carried out on a Bio-Rad CFX96 real-time PCR system with the SsoFast EvaGreen Supermix (Cat. # 1725203, Bio-Rad) in 96-well plates. After 1:5 dilution of cDNA, 1 μL was added to 14 μL of PCR mixture (7.5 μL EvaGreen Supermix, 1 μL each primer at 10 pmol μL^{-1} and 4.5 μL RNase-free water). cDNA was amplified by real-time PCR with specified primers (Table 1). In each run, a no RT-PCR and a non-template control were included. Thermal cycling conditions were as follows: initial denaturation at 95 °C for 30 s, followed by 38 cycles of denaturation at 95 °C for 5 s and annealing/extension at 60 °C for 5 s. A melt curve was included in the end of the run from 65 to 95 °C to verify the specificity of the real-time PCR reaction for each primer pair. Efficiency of amplifications was determined by running a standard curve for each tested primer set with serial dilutions of cDNA. qPCR efficiency and normalisation was carried out using qbase_plus software (Biogazelle). For each gene, the fold changes between the two samples were generated by calculating the ratio of the average of the normalised signals for all 20% ethanol replicates to the average of the normalised signals for all 0% ethanol replicates and are represented as log2-fold-changes.

Statistical analysis

Data analysis was performed using Graphpad Prism 6. The qPCR results are presented as the mean of six replicates ± SEM (standard error of the mean). Significant differences between the two strains were calculated using paired *t* tests, and the significance level was set at a *p* value of 0.05.

Results

Directed evolution of an industrially relevant *O. oeni* strain

The commercially available *O. oeni* strain SB3 was cultivated over 290 days in a bioreactor having progressively increased ethanol concentration in the feed from 0 to 15% (*v*/*v*) decreasing the growth rate from 0.10 to 0.05 h^{-1}. The bioreactor contents were sampled at appropriate intervals and screened for improvement in L-malic acid consumption compared to the parent. For samples taken from the evolving population at 290 days, there was little difference to the fermentation profile of the parent (SB3) in the absence of ethanol (Fig. 2). However, in the presence of 15% (*v*/*v*) ethanol the evolved population consumed all available L-malic acid at least 69 h earlier than SB3 (89.5 vs 158.5 h, respectively) a 56% improvement in fermentation time, thereby showing an improved ability to perform MLF under higher ethanol conditions.

Isolation and characterisation of evolved clones with improved MLF performance

Individual isolates were obtained after 332 days from the mixed population that showed improved fermentation performance when grown in MRSAJ with 15% (*v*/*v*) ethanol. Through microscale fermentations of 180 individual isolates, 27 were selected as being significantly improved performers. These were reassessed in larger 10 and 50 mL fermentations at 13 and 15% (*v*/*v*) ethanol and at 22 °C and 30 °C, respectively (Fig. 3). Strain A90 showed an ethanol tolerant phenotype and completed MLF in the shortest time.

Table 1 Primer sequences for the putative reference and target genes used in this study

Target gene	Description	Forward primer (5′ → 3′)	Reverse primer (5′ → 3′)	Reference
ldhD	d-Lactate dehydrogenase	GCCGCAGTAAAGAACTTGATG	TGCCGACAACACCAACTGTT	(Beltramo et al. 2006)
rpoA	Sigma α factor	GATGGAACTTGGCATTGGTG	AAGACTCGCTTGGACTAACAGAAC	(Sumby et al. 2012)
gyrB	Gyrase subunit B	CGTCCGGCTATGGAAGCTGT	ACAACCGAAGCGCCAACAC	(Sumby et al. 2012)
gapA	d-Glyceraldehyde-3-phosphate dehydrogenase (GA3PDH)	TCCACGCTTACACATCGACTCA	CGCTGAGCATGACCATTCAAC	(Sumby et al. 2012)
gmk	Guanylate kinase	CGCAGGAATGAGGTTGATGGT	CTGGCGCTGCTCGTCTATCC	(Sumby et al. 2012)
recA	Recombinase A	ACTTGCCGGTACACTGAATAGAAC	GAGCAGATCGACGCACTTCA	(Sumby et al. 2012)
rpoB	Subunit beta of DNA-directed RNA polymerase	GCTTGTTGACCGATGATGAG	ACCGTTAGGAGCAGCCATT	(Sumby et al. 2012)
ftsZ	Filamenting temperature-sensitive mutant Z—GTPase	TGCCGGATCGACACCTGA	CGGACGAGTAACAACGCCAAC	(Sumby et al. 2012)
pta	Phosphotransacetylase	CATGGCTGAGATTGCCGTTC	TCTCCTGCGCCAGCTTAGT	(Sumby et al. 2012)
ggpps	Geranylgeranyl pyrophosphate synthase	AACAGTTCGGACAAAGTACAG	CGACCATCTGTGTCATTTCA	(Cafaro et al. 2014)
citE	Citrate lyase	CCGCACGATGATGTTTGTTCC	GCTCAAAGAAACGGCATCTTCC	(Olguín et al. 2009)
hsp18	Stress protein Lo18	CGGTATCAGGAGTTTTGAGTTC	CGTAGTAACTGCGGGAGTAATTC	(Beltramo et al. 2006)
cfa	Cyclopropane fatty acid synthase	TGGTATTACATTGAGCGAGGAG	CGTCTTTGAGATCACGATAATCC	(Beltramo et al. 2006)
clpP	ClpP protease	CGGTACCAAAGGCAAGCGTTTTAT	CTCTTCCGAGTCTTCAAAAGTTGAT	(Beltramo et al. 2006)
atpB	OEOE_0665 F0F1-type ATP synthase, beta subunit	ATACTGATCCGGCTCCGGC	CAGCGGGATAAATACCTTG	(Olguín et al. 2015)
RS07535	S-ribosylhomocysteine lyase	AATTCCATCAAGCCGGTCCC	CCCAGCCAAACCAAACTTCG	This study
mleP	Malate permease	TCACGAAGAGCACCGTTGAA	GCGGATCAATGCCAAAGCTT	This study
mleR	Malolactic regulator	TGTCCTGATCGAAGACGCTG	ACCCTGGAATTGAGCAACGA	This study
mleA	Malolactic enzyme	CCGACAATTGCTGATACAATTGAA	GGCATCAGAAACGACCAGCAG	(Beltramo et al. 2006)
DDCarb	D-alanyl-D-alanine carboxypeptidase EAV39051.1	TTCAACATAGCAGTTGCGTC	AGGAACTTCACCCGATACGG	This study
RS06010	(OEOE_1247) glucosaminidase	AACCAATGCTTTAGCGGCAG	GCTGGCGCATGCTAAATTCA	This study
RS06975	(OEOE_1444) peptidoglycan interpeptide bridge formation protein	AAGGCTGTCAACAAGCTCGA	GAAAGCGGCCAGATTGATGC	This study
RS05625	(OEOE_1168) amino acid transport	CTAACCGGAAAGGGAGCGTT	TTCAGCGTAACAGAGACCGG	This study
ppk	(OEOE_1629) ppk	CTTAGCGCAGGCAAACGATC	TGATGAATCGAGGCCAGACG	This study
RS07625	(OEOE_1581) SAM-dependent methyltransferase	ACAAAGTCCAAGCCGGTGAA	GGCTGTCTCAACTTCCGGAA	This study
RS04370	(OEOE_0911) SAM-dependent methyltransferase	GTTCTGGCAAACTGGCTGTT	CGTTGCTTTGCCAGAGATAACA	This study
RS03525	(OEOE_0738) ABC-type multidrug transport system, ATPase component	TTGATCGGTCCATCAGGCAC	GACTTCTCGGTTTGGCATGC	This study
ddl	d:alanine–d:alanine ligase	CCTAAGCGAGCCTAATACAC	CCTGTTGGCTTCGATTCATTCC	(Olguín et al. 2010)
rmlB	dtdP-glucose-4,6-dehydratase	TATCCGCAATGCGCAATTGG	GAACGGTCAACATGCGATTCAG	(Olguín et al. 2010)
ctsR	Master regulator of stress response	GGGCCATGGCAGAAGCTAATATTTCAG	AAACGGGTGTTGATTACATAATT	(Beltramo et al. 2006)
clpX	Clp ATPase protein	GAAGCGTGTTAACGAGTCC	CAGCGACTGAGCCAAATAAG	(Olguín et al. 2010)
trxA	Thioredoxin	GCCACTTGGTGTACCCCTTGT	TCCATTTGCCGTTTCCTGGTTT	(Beltramo et al. 2006)
groES	GroES, heat shock chaperone class I	GCCACAACAGAACCCATCACTGGTT	GGCGATCGAATTGTTCTTAGTAT	(Beltramo et al. 2006)
grpE	GrpE, heat shock chaperone class II	CGCAGGCAGAAAAGAACAATC	GCTGAAGACGAAGCAGTTGC	(Beltramo et al. 2006)
gsh	Glutathione reductase	GGCATTATCACCGAGCTGTT	TCCCGAAGAAGCAAAGAAGA	(Bordas et al. 2015)
pyrB	OEOE_0258 pyrB aspartate carbamoyltransferase	GGCAGGTTGTTGCCAATC	TTGTTGCCGAGGACTTGTTGGG	(Olguín et al. 2015)
trhD	OEOE_0394 threonine dehydrogenase	AGAGTTCTTGCGCGAGAC	CCGGTGCCACTCATATTCTTAG	(Olguín et al. 2015)
amt	OEOE_0411 aminotransferase	TTGGACAGCGAAGGAAGAGT	GTTTATCTTCGGCCGTCAAC	(Olguín et al. 2015)

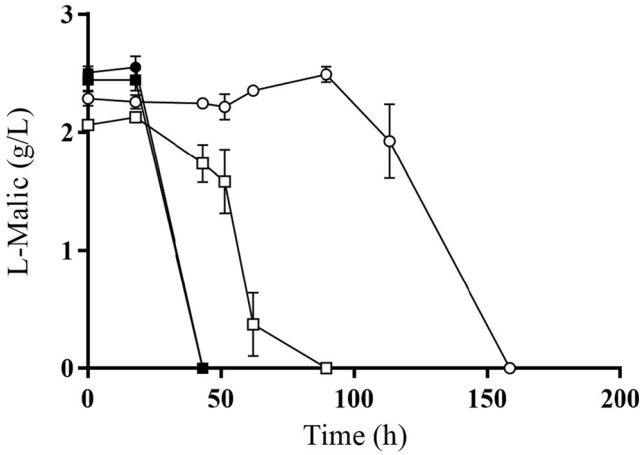

Fig. 2 Evaluation of MLF performance of an evolved population and the parent strain SB3 at 30 °C in MRSAJ supplemented with 1 g L^{-1} L-malic acid. Samples were screened in 200 μL microplate fermentations. L-malic acid consumption of the parent SB3 (●,○) and the evolved population (■,□) in 0% (*v*/*v*) (●,■) and 15% (*v*/*v*) (○,□) ethanol. The evolved population completed the consumption of available L-malic acid 69 h earlier than the parent in the presence of 15% (*v*/*v*) ethanol

Enhanced ethanol tolerance of evolved strain A90

The relative ethanol tolerance of strain A90 and the parent (SB3) was investigated by analysing growth and viability in the presence of high concentrations of ethanol. As a control, both strains were incubated in MRSAJ without ethanol and sampled at set time points for the duration of the experiment. No difference in viability between SB3 and strain A90 was observed at any of these times (Fig. 4). However, when exposed to 20% (*v*/*v*) ethanol for 48 h, the viability of parent (SB3) was reduced by at least four orders of magnitude when compared to A90.

Improved MLF performance of evolved strain A90 in Pinot Noir

MRSAJ is a highly nutritious medium especially when compared to wine. As the isolates may have been evolved specifically for the MRSAJ medium, the ability to conduct MLF in wine may have been lost or reduced. To test this, the MLF performance of A90 was monitored in Pinot Noir wine alongside the parent (SB3). Both strains were inoculated into Pinot Noir wine with an ethanol concentration of 13% (*v*/*v*) as well as the same wine with ethanol supplementation to 15% (*v*/*v*). At 13% (*v*/*v*) ethanol, A90 completed MLF of the wine in 75% of the time required by the parent (Fig. 5). This difference was more pronounced at 15% (*v*/*v*) ethanol with A90 finishing in 40% of the time of the parent (Fig. 5).

Enhanced ethanol tolerance of evolved strain A90 persists after 50 generations of passaging

Evolved strain A90 and the parent (SB3) were passaged in MRSAJ ten times, resulting in a minimum of 50 generations for each strain (data not shown). Ethanol tolerance of the resulting

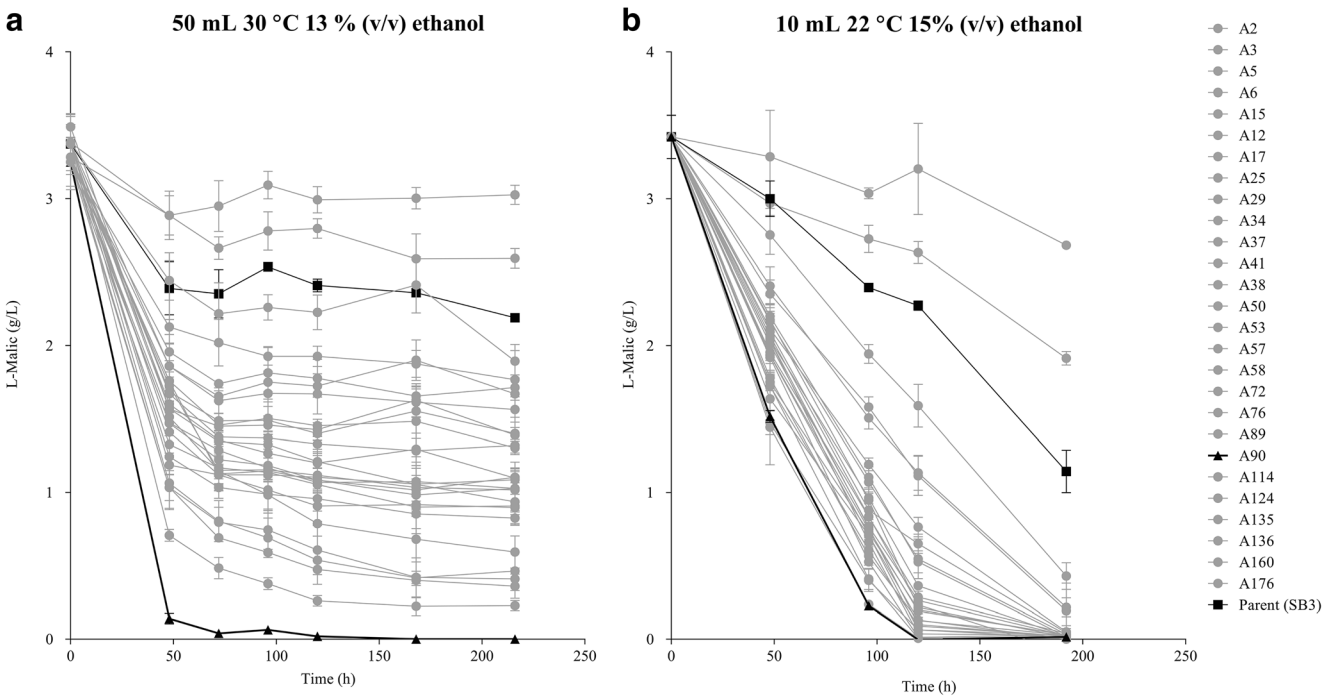

Fig. 3 Malic acid utilisation of 27 clonal isolates selected through the microplate screening and the parent (SB3). Samples were screened in either 50 mL MRSAJ at 30 °C (**a**) or 10 mL MRSAJ at 22 °C (**b**) supplemented with 2 g L^{-1} L-malic acid and 13 or 15% (*v*/*v*) ethanol, respectively. Values are the average of three biological replicates and error bars indicate the standard deviation. Strain A90 was selected as the most improved strain for further study

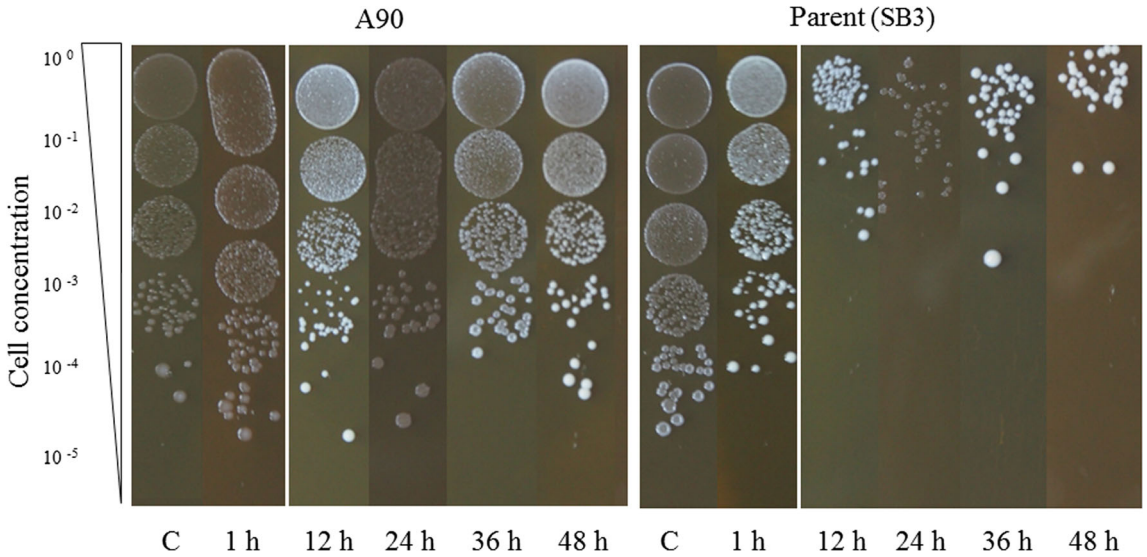

Fig. 4 Ethanol tolerance of evolved strain A90 and the parent SB3 exposed to 20% (*v*/*v*) ethanol at 20 °C. The impact of exposure to 20% (*v*/*v*) ethanol, for up to 48 h, on culturability of strains A90 and the SB3 was assessed by spot-plating on MRSAJ. Strains were sampled at 1, 12, 24, 36 and 48 h after inoculation in MRSAJ containing 20% (*v*/*v*) ethanol. Images are a composite of MRSAJ agar made at each time point of a 10-fold dilution series with the top sample an undiluted 2 μL sample. C is the inoculation control sampled after 1 h in 0% (*v*/*v*) ethanol, indicating a viable population

populations was re-evaluated to determine if the passaged A90 (p-A90) was still more ethanol tolerant than the passaged SB3 (p-SB3). After exposure to 21% (*v*/*v*) ethanol for either 24 or 48 h, p-A90 retained a higher percentage of viable cells, indicating a greater ethanol tolerance, compared to p-SB3 (Fig. S1). No difference in ethanol tolerance was observed at 18% (*v*/*v*) ethanol after either exposure duration. This data suggests a degree of stability of the ethanol tolerance attribute.

The addition of ethanol altered the expression of several targeted genes

In an effort to begin to understand the basis for the superior performance of A90, the expression of a selection of genes was examined. Several of the targeted genes changed expression due to ethanol exposure in both SB3 and A90. Genes that increased in expression after 1 h of ethanol exposure in both strains were *hsp18*, *trxA*, *grpE*, *groES*, *trhD*, *amt*, DDCarb, RS03525 and *clpP* (Table S1). Genes that decreased in expression following 1 h of ethanol exposure in both SB3 and A90 were *ddl*, *ppk*, RS06010, *atpB* and RS07625 (Table S1). Genes that displayed increased expression after 24 h of ethanol exposure in both SB3 and A90 were *rmlb*, *hsp18*, *trxA*, *gshR*, *ctsR*, *clpX*, *grpE*, *groES* and *clpP* (Table S1), while RS06975, *pyrB*, *mleP*, *mleR*, *ppk*, RS06010, *ggpps*, *DDCarb*, RS07625 and RS04370 showed decreased expression in both strains under the same conditions (Table S1). There were several genes showing significant difference between strains A90 and SB3 at both time points (Table 2).

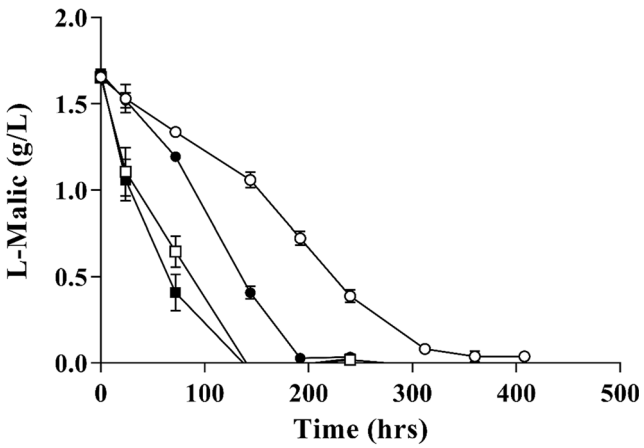

Fig. 5 Evaluation of MLF performance of evolved strain A90 and its parent, SB3, in Pinot Noir wine at 22 °C. Residual L-malic acid content for fermentations conducted by the parent SB3 (●,○) and evolved strain A90 (■,□) in Pinot Noir wine with 13% (●,■) and 15% (○,□) (*v*/*v*) ethanol

Discussion

Directed evolution was performed on the commercial *O. oeni* wine strain SB3, and preliminary characterisation of the mixed population (grown for 290 days, reaching 15% (*v*/*v*) ethanol) revealed a culture that fermented L-malic acid more rapidly than the parent in MRSAJ supplemented with 15% (*v*/*v*) ethanol and L-malic acid. The faster MLF phenotype of the DE strain A90 was only observed when conducted in the presence of ethanol. This is the first study using DE to target *O. oeni* and ethanol tolerance and shows that DE can be used to generate a population with improved MLF performance. A90 clearly outperformed its parent in terms of tolerance to high ethanol concentrations and MLF performance in the presence

Table 2 Relative expression of genes that were differentially expressed 1 and 24 h after ethanol addition to SB3 (parent strain) and A90 (DE strain) MRSAJ cultures

Target gene[a]	Description[b]	Relative expression[c]			
		1 h		24 h	
		SB3	A90	SB3	A90
Cell envelope biogenesis, outer membrane					
RS06975	Peptidoglycan interpeptide bridge formation protein	0.74 ± 0.19	− 1.10 ± 0.20*	− 2.51 ± 0.44	− 0.60 ± 0.15*
ddl	D-alanine D-alanine ligase	− 3.18 ± 0.24	− 2.06 ± 0.20*	0.20 ± 0.28	0.85 ± 0.20
Stress response					
trxA	Thioredoxin	2.58 ± 0.16	3.47 ± 0.27*	3.20 ± 0.42	4.00 ± 0.42
hsp18	Heat shock protein Lo18	3.43 ± 0.27	4.34 ± 0.34*	3.57 ± 0.45	5.40 ± 0.55*
Posttranslational modification, protein turnover, chaperones					
clpX	ATP-binding subunit of Clp protease and DnaK/DnaJ chaperones	0.98 ± 0.16	1.83 ± 0.21*	1.59 ± 0.35	3.20 ± 0.37*
groES	GroES, heat shock chaperone class I	2.23 ± 0.24	2.88 ± 0.20	4.39 ± 0.61	2.78 ± 0.33*
Amino acid transport and metabolism					
trhD	Threonine dehydrogenase-like Zn-dependent dehydrogenase	3.28 ± 0.27	2.71 ± 0.15	−2.54 ± 0.55	1.01 ± 0.21*
pyrB	Aspartate carbamoyltransferase	− 0.40 ± 0.18	1.38 ± 0.18*	− 3.19 ± 0.69	− 1.80 ± 0.24
RS05625	Amino acid permease	1.87 ± 0.17	0.46 ± 0.14*	− 0.64 ± 0.28	1.25 ± 0.19*
MLF/citrate metabolism					
mleR	MLF system transcription activator	− 0.35 ± 0.19	0.87 ± 0.20*	− 3.35 ± 0.71	− 2.26 ± 0.43
mleA	Malolactic enzyme	0.74 ± 0.20	− 1.41 ± 0.28*	− 0.71 ± 0.24	0.04 ± 0.18
citE	Citrate lyase beta subunit	1.49 ± 0.17	0.60 ± 0.21*	0.12 ± 0.31	− 1.09 ± 0.19*
Cellular biosynthetic process, secondary metabolism, energy metabolism					
ppk	Polyphosphate kinase	− 0.71 ± 0.15	− 0.72 ± 0.27	− 5.25 ± 1.06	− 2.38 ± 0.19*
RS06010	Glucosaminidase	− 1.53 ± 0.18	− 0.87 ± 0.21*	− 5.39 ± 1.60	− 2.29 ± 0.36*
ggpps	Geranylgeranyl pyrophosphate synthase	− 0.12 ± 0.18	0.17 ± 0.25	− 5.75 ± 1.67	− 2.73 ± 0.23*
amt	Aspartate aminotransferase	1.56 ± 0.22	2.44 ± 0.18*	−1.08 ± 0.40	1.51 ± 0.22*
Nucleotide binding					
atpB	F0F1-type ATP synthase, beta subunit	− 2.04 ± 0.22	− 0.86 ± 0.21*	− 1.38 ± 0.25	− 0.39 ± 0.15
DDCarb	D-alanyl-D-alanine carboxypeptidase EAV39051.1	1.37 ± 0.26	1.90 ± 0.19	− 4.98 ± 1.09	− 2.58 ± 0.52*
RS03525	ABC-type multidrug transport system, ATPase component	2.95 ± 0.18	2.57 ± 0.17	− 2.86 ± 0.58	0.07 ± 0.20*
Transferase					
cfa	Cyclopropane-fatty-acyl-phospholipid synthase	0.01 ± 0.16	0.18 ± 0.20	− 0.43 ± 0.28	1.71 ± 0.21*
RS07625	SAM-dependent methyltransferase	− 1.40 ± 0.17	− 0.86 ± 0.19	− 5.31 ± 1.15	− 2.75 ± 0.24*
RS04370	SAM-dependent methyltransferase	− 0.65 ± 0.21	− 0.59 ± 0.28	− 3.19 ± 0.58	− 1.17 ± 0.18*

[a] The gene names are taken from the NCBI database for *Oenococcus oeni* PSU-1 complete genome (http://www.ncbi.nlm.nih.gov)

[b] Gene descriptions were taken from NCBI database (http://www.ncbi.nlm.nih.gov) and UniProt database (http://www.uniprot.org)

[c] Relative expression of gene is described as the log2 of the fold change after ethanol addition with respect to 0% ethanol. A positive value for fold change indicates a higher expression while a negative value indicates a lower expression in 20% ethanol when compared with 0% ethanol in each strain

*Significant difference between the strains ($p < 0.05$)

of ethanol, additionally A90 formed a greater number of CFUs when plated on MRSAJ agar after 48 h exposure to 20% (*v*/*v*) ethanol compared to its parent (SB3). Importantly, during the DE process A90 did not lose its ability to conduct MLF. In a Pinot Noir wine containing 15% (*v*/*v*) ethanol, strain A90 completed MLF in 40% of the time required by the parent. This result is potentially significant to the wine industry. The alcohol content in wines has been increasing (Godden and Muhlack 2010) resulting in sluggish or failed MLF. More effective strains of *O. oeni* will reduce production time costs to the wine industry.

Stability of the phenotypic and presumably genetic changes were evaluated by passaging under non-ethanol selective conditions (MRSAJ). After 50 generations, the stability of the high ethanol tolerance phenotype of A90 was still apparent at 21% (*v*/*v*).

To determine the basis for the observed high ethanol tolerance phenotype, expression of several genes from both strains was investigated by qPCR in high (20% *v*/*v*) and 0% (*v*/*v*) ethanol media. Previous studies have suggested that *O. oeni* increases expression of many stress response genes in order to survive the harsh conditions found in wine. Olguín et al. (2010) showed that strains with higher relative expression of *hsp18*, *clpP*, *ctsR* and *rmlB* in high ethanol conditions showed better MLF performance in a modified FT80 medium. Bordas et al. (2015) also reported that *citE*, *hsp18* and *clpP* showed higher levels of expression, in ethanol tolerant strains compared to a low ethanol tolerant strain, in a 'wine-like' media. In this study, A90 showed higher expression of *hsp18* compared to SB3, but this was not the case for any other of the above genes. Maitre et al. (2014) proposed a model whereby *O. oeni* can adapt to ethanol stress through the synthesis of the small heat shock protein Lo18 encoded by *hsp18* and the subsequent modification of phospholipid content of the cell membrane. The existence of such unique features can be viewed as an evolutionary adaptation to the wine environment (Beltramo et al. 2006). The response of LAB to stress relies on the coordinated expression of genes that alter specific cellular processes (membrane biogenesis, transport, DNA metabolism, etc.). A network of regulators allows the cell to react to complex environmental changes and achieve a coordinated response.

When relative expression changes are compared between the two strains after 1 h of ethanol exposure, A90 showed an increased expression of eight of the tested genes compared to SB3: *ddl*, *trxA*, *hsp18*, *clpX*, *pyrB*, *mleR*, *amt* and *atpB* (Table 2). A90 also showed decreased expression of four of the tested genes compared to SB3: RS06975, RS05625, *mleA* and *citE*. When relative expression changes are compared between the two strains after 24 h of ethanol exposure, A90 showed higher expression of 15 of the tested genes compared to SB3: RS06975, *hsp18*, *clpX*, *trhD*, RS05625, *ppk*, RS06010, *ggpps*, *amt*, atpB, DDCarb, RS03525, *cfa*, RS07625 and RS04370 (Table 2). A90 also showed lower expression of two of the tested genes compared to SB3: *groES* and *citE*.

Our results show that in A90, *hsp18*, *amt*, *clpX* and *trxA* expression was increased after 24 with 20% (*v*/*v*) ethanol addition, and not only as a transient shock response. After growth in MRSAJ 20% (*v*/*v*) ethanol, the mRNA levels of these genes were higher than in the control (MRSAJ 0% ethanol). This result suggests that an improved basal level of gene expression followed the initial increase. This was only true in SB3 for *hsp18* and *trxA*, where the increases were not as great as A90. As these are two important stress response genes, they could be an important indicator of the reason for the improved phenotype of A90. The final gene showing consistent response to ethanol independent of time was *atpB*, where expression decreased relative to the control at both 1 h and 24 h. *atpB* encodes the beta subunit of a F0F1-type ATP synthase in which the primary function in most organisms is ATP synthesis. It is important to control ATP-driven proton pumping activity of ATP synthase in order to avoid wasteful ATP hydrolysis under conditions when no proton motive force can be generated (e.g. leaky damaged membrane). To avoid exhaustion of the intercellular ATP pool, expression of ATP synthases may be down-regulated to suppress ATPase activity. Additionally, a recent study of stress response in a wine-like medium reported that expression of enzymes encoding ATPase activity were down-regulated in *O. oeni*; however, the expression of *atpB* was not reported (Margalef-Catalá et al. 2016).

The genes that were down-regulated were dependent on time. For example both *mleA* and RS06975 initially decreased (1 h), but then increased slightly to levels that were not significantly different. Additionally, RS05625 expression was down-regulated in SB3 only after 24 h and A90 expression went up. RS05625 encodes an amino acid putative amino acid transporter, and the reason for its differential expression is currently unclear and warrants further investigation. A decrease in *citE* expression over time was also seen by Olguín et al. (2009); they however used media supplemented with citrate so it is hard to make a direct comparison. SB3 showed the same upregulation of *citE* as reported by Margalef-Catalá et al. (2016), whereby expression initially increased and then decreased slightly over time; they however only measured up to 8 h after inoculation. Even though A90 showed much lower initial expression of *citE* the general trend was the same as SB3 expression. Although *ddl* showed an initial down regulation after 1 h, expression was at similar levels to the no ethanol control at 24 h. Expression of *pyrB* decreased over time, in both strains; down regulation of *pyrB* over time was also observed by Margalef-Catalá et al. (2016). pyrB encodes an aspartate carbamoyltransferase which catalyses the first step in the pyrimidine biosynthetic pathway. The patterns of expression described above suggest a complex response to ethanol during the first 24 h of exposure.

Olguín et al. (2015) reported that genes involved in amino acid transport and metabolism, cell envelope biogenesis and transcription were most affected by ethanol addition (12% (*v*/

v)) in a modified MRS medium. That study, like many others, uses PSU-1, which has been shown to be a poor performer under stress conditions and during MLF. Our results showed that for both SB3 and A90, expression of genes involved in stress response, protein turnover proteolysis, chaperones, amino acid transport and metabolism, and nucleotide binding increased after 1 h exposure to ethanol. However, after 24 h, only genes involved in stress response, cell envelope biogenesis, protein turnover and chaperones, and proteolysis had higher relative expression in 20% (*v*/*v*) ethanol relative to the 0% (*v*/*v*) ethanol control. Additionally, Cafaro et al. (2014) reported that the gene encoding geranylgeranyl pyrophosphate synthase (ggpps), an enzyme involved in carotenoid biosynthesis, is overexpressed in *O. oeni* in response to ethanol. Carotenoid production has been shown to increase multistress tolerance when carotenoid biosynthesis genes are overexpressed in *Lactococcus lactis* (Hagi et al. 2013). However Cafaro et al. (2014) used late-exponential phase cells and an increase in *ggpps* expression was not observed in this study.

To investigate this further, future work would include knockout of individual genes of interest to show cause and effect relating to the ethanol tolerance phenotype. However, systems to easily make targeted genetic modifications to the *O. oeni* genome are not yet readily available. As discussed above, there are several studies reporting regulation of genes under stress conditions in *O. oeni*. However, it would appear that apart from a few generalisations, these changes are highly strain specific and warrant a more detailed investigation. This is outside the scope of the current project, but there is a need for analysis of multiple strains using RNA seq to provide a more holistic overview of *O. oeni* stress response.

This study is the first of its kind to use DE as a tool for the non-recombinant modification of *O. oeni*. We have successfully used DE to modify wine strain SB3, to have a higher ethanol tolerance in MRSAJ. Importantly, the ability to ferment L-malic acid was not lost during DE. Under high ethanol stress conditions the evolved strain showed superior L-malic acid consumption compared to the parent SB3, in both MRSAJ media and Pinot Noir. The DE strain A90 completed MLF in Pinot Noir in 40% of the time of SB3, suggesting this strain could be of value to the wine industry. Additionally, there were clear differences in the expression of several stress response genes. Most notably, the relative expression of *hsp18*, which encodes the small heat shock protein Lo18, was significantly higher in A90 compared to SB3 in 20% (*v*/*v*) ethanol. This result is in keeping with the enhanced ethanol tolerance phenotype of A90. The findings also highlight the opportunities for the use of DE as a method of non-recombinant strain improvement for the wine industry.

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Compliance with ethical standards

Conflict of interest ALB was supported by scholarships from AGWA (UA1101/P3102/GWR Ph 0901) and the University of Adelaide. KMS and JFS are supported by Wine Australia project funding (UA1302). The authors declare that they have no conflicts of interest.

Human and animal rights and informed consent This article does not contain any studies with human participants or animals performed by any of the authors.

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