Appl Microbiol Biotechnol (2011) 91:603–612
DOI 10.1007/s00253-011-3294-3

Newly generated interspecific wine yeast hybrids introduce flavour and aroma diversity to wines

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Received: 12 January 2011 / Revised: 16 March 2011 / Accepted: 27 March 2011 / Published online: 3 May 2011

Abstract Increasingly, winemakers are looking for ways to introduce aroma and flavour diversity to their wines as a means of improving style and increasing product differentiation. While currently available commercial yeast strains produce consistently sound fermentations, there are indications that sensory complexity and improved palate structure are obtained when other species of yeast are active during fermentation. In this study, we explore a strategy to increase the impact of non-*Saccharomyces cerevisiae* inputs without the risks associated with spontaneous fermentations, through generating interspecific hybrids between a *S. cerevisiae* wine strain and a second species. For our experiments, we used rare mating to produce hybrids between *S. cerevisiae* and other closely related yeast of the *Saccharomyces* sensu stricto complex. These hybrid yeast strains display desirable properties of both parents and produce wines with concentrations of aromatic fermentation products that are different to what is found in wine made using the commercial wine yeast parent. Our results demonstrate, for the first time, that the introduction of genetic material from a non-*S. cerevisiae* parent into a wine yeast background can impact favourably on the wine flavour and aroma profile of a commercial *S. cerevisiae* wine yeast.

Keywords *Saccharomyces* sensu stricto · Interspecific hybrids · Metabolites · Fermentation products · Wine yeast

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Introduction

The *Saccharomyces* sensu stricto complex consists of a number of closely related species (Naumov 1987; Vaughan-Martini and Martini 1987; Naumov et al. 2010). Of these, *Saccharomyces cerevisiae* has been utilised by humans down through the ages, culminating in recent decades in a large number of industrial *S. cerevisiae* wine yeast strains being available to commercial winemaking. These strains show robust growth characteristics in grape juice, tolerating both the initial high sugar concentration at the onset of fermentation and high ethanol concentrations towards the end. In contrast, other *Saccharomyces* species generally ferment more slowly than *S. cerevisiae* and are often unable to tolerate the high alcohol concentrations encountered. However, there are indications that sensory complexity and more rounded palate structure is obtained when other species of yeast are active during fermentation, as in the case of traditional, spontaneous fermentations. Spontaneous fermentations allow the many different species of indigenous microorganisms that populate the vineyard, grape-picking equipment and winery to contribute to vinification. Studies on spontaneous ferments have identified a number

of non-*Saccharomyces* species present at the early stages of fermentation (Fleet and Heard 1993), and products of the metabolism of these species are thought to contribute to more complex aroma and flavour profiles in the wine. Nonetheless, because of their unpredictable nature, the desirability of spontaneous fermentations is a source of debate, many winemakers preferring to inoculate with a proven *S. cerevisiae* industrial strain.

Experiments using inoculations of mixtures of *S. cerevisiae* strains in a grape juice show dynamic population fluctuations between strains (Howell et al. 2004; King et al. 2008), with unpredictable fermentation outcomes. The situation is even worse when less robust non-*S. cerevisiae* strains are used. Fermentations using co-inocula of *S. cerevisiae* and non-*Saccharomyces* strains typically have limited success, with the non-*Saccharomyces* strain having only a minor impact on wine aroma and composition (Soden et al. 2000). The dominance of *S. cerevisiae* over other species in spontaneous fermentations is due mainly to their tolerance of high sugar and high ethanol concentrations (Pretorius 2000) and perhaps for some *S. cerevisiae* strains the capacity to produce 'killer' compounds that trigger cellular death of non-*Saccharomyces* strains (Heard and Fleet 1987; Perez-Navzdo et al. 2006).

An alternative to co-ferment that avoids growth competition between species is to use an interspecific hybrid strain, where the genomes of different species are contained within the one cell. Species of the *Saccharomyces* sensu stricto clade are able to mate with each other to form interspecific hybrids, but the hybrids formed are sterile, having non-viable ascospores (Naumov et al. 2000). This occurs in nature and, in fact, one member of the *Saccharomyces* sensu stricto complex, *Saccharomyces pastorianus*, the lager making yeast, has been identified as a stable, natural hybrid from an evolutionary timeframe (Groth et al. 1999) resulting from a cross between *S. cerevisiae* and a *Saccharomyces bayanus*-type yeast (Masneuf et al. 1998; Marinomi et al. 1999; Dunn and Sherlock 2008).

We have used a rare-mating strategy (Spencer and Spencer 1996) to generate interspecific hybrids between a robust diploid *S. cerevisiae* commercial wine strain, AWRI 838, and strains of either *Saccharomyces paradoxus* or *Saccharomyces kudriavzevii*. AWRI 838 is an isolate of EC 1118 and genomic sequencing has revealed that it is a diploid (Novo et al. 2009).

In nature, yeast mating is activated by the presence of pheromones produced by haploid yeast, but sporulation of the wine yeast parent to generate haploid spores might lead to the loss of important wine fermentation traits. Rare mating relies upon an infrequent event (1×10^{-6} cells) whereby mating type switching within the diploid genome leads to a cell homozygous at the mating type loci, either **a**/**a** or α/α (Gunge and Nakatomi 1972). These homozygotes are able to enter the mating pathway and can conjugate with a cell of the opposite mating type, leading to an interspecific hybrid.

In order to establish an experimental precedent and for ease of selection, the diploid wine yeast strain was first mated with a haploid, auxotrophic *S. paradoxus* strain. Metabolite analysis was performed on the resultant interspecific hybrid to confirm that the addition of the *S. paradoxus* genome had an impact on the parental wine yeast metabolome. Additional interspecific hybrids were then generated using random spores of wild-type strains of either *S. paradoxus* or *S. kudriavzevii*. Hybrids resulting from each of the wild-type crosses were chosen for grape juice fermentation and the wine analysed for important wine fermentation compounds.

Materials and methods

Yeast strains and media

Parental strains are *S. cerevisiae* AWRI 838 (an isolate of the commercial wine yeast strain EC 1118), *S. paradoxus* strains N17–78–*Mat**a** ho ura3 lys2 met13* provided by Rhona Borts (Hunter et al. 1996) and 52–153 (Herman J. Phaff Yeast Culture Collection, University of California Davis) and *S. kudriavzevii* type strain NCYC 2889. Strains generated from this study are listed in Table 1. All yeasts were grown in YEPD medium (1% (*w*/*v*) yeast extract, 2% (*w*/*v*) peptone, 2% (*w*/*v*) glucose) with shaking (100 rpm) at 25°C. Mitochondrial mutants of AWRI 838 were isolated by treating cells for 8 h in synthetic complete medium containing 10 μg mL^{-1} ethidium bromide. Cells were then diluted in water, and due to their inability to utilise glycerol as a carbohydrate source, the mitochondrial mutants were revealed by their petite colony growth on YPDG (1% (*w*/*v*) yeast extract, 2% (*w*/*v*) peptone, 3% (*w*/*v*) glycerol and 0.1% (*w*/*v*) glucose; Sherman et al. 1986).

Generation of interspecific hybrid yeast

Rare-mating, essentially as described by Spencer and Spencer (1996), was used throughout. Strains were grown to stationary

Table 1 Hybrid strains generated in this study

Cross	Hybrids	AWRI number
AWRI 838 × N17–78	A1–A5	A2 = AWRI 1519
AWRI 838 × 52–153	B1–B5	B2 = AWRI 1501
AWRI 838 × NCYC 2889	C1–C7	C1 = AWRI 1503

phase in YEPD at 27°C. Spores of strains 52–153 and NCYC 2889 were generated by inoculating the equivalent of 2 mL of a washed YEPD culture into 5 mL of sporulation medium (1%, *w*/*v* potassium acetate). After sporulation, cells were washed and re-suspended in sterile water. In a 250-mL conical flask, 1 mL of each parent strain was added to 20 mL of fresh YEPD and incubated for 7 days at 27°C. Appropriate numbers of cells were washed in sterile water and plated onto selective plates. Wild-type strains were assayed under several phenotypic conditions to determine selection criteria for hybridisation. Selection in mating experiments was performed on YNB–glycerol–ethanol plates (0.67% (*w*/*v*) yeast nitrogen base without amino acids, 3% (*w*/*v*) glycerol, 3% (*v*/*v*) ethanol, 2% (*w*/*v*) agar) for the auxotrophic strain cross and YEP–glycerol–ethanol plates (1% (*w*/*v*) yeast extract, 2% (*w*/*v*) peptone, 3% (*w*/*v*) glycerol, 14% (*v*/*v*) ethanol, 2% (*w*/*v*) agar) for wild-type strain crosses.

PCR confirmation of hybrids

For all strains, DNA was purified using mechanical breakage with glass beads (Ausubel et al. 1994). Yeast cells were disrupted using a Mini-Beadbeater® (BioSpec) for 3 min with glass beads. Genomic DNA was used as template for PCR analyses, with amplification using the δ transposon primer set MLD1 5′-CAAAATTCACCTAAA/TTCTCA-3′ and MLD2 5′-GTGGATTTTTATTCCAACA-3′ (Ness et al. 1993) and the intron primer set EI1 5′-CTGGCTTGGTGTATGT-3′ and LA2 5′-CGTGCAGGTGTTAGTA-3′ (de Barros Lopes et al. 1996). PCR fragments were resolved on a 1.5% (*w*/*v*) agarose gel. rDNA PCR-RFLP was conducted using the rDNA Internal Transcribed Spacer unit primer pair ITS1 5′-TCCGTAGGTGAACCTGCGG-3′ and ITS4 5′-TCCTCCGCTTATTGATATGC-3′ and the Restriction Enzyme *Hae*III and fragments were resolved on a 2% (*w*/*v*) agarose gel (Esteve-Zarzosa et al. 1999).

Fermentation stress assay plates

Assay plates containing 14% (*w*/*v*) ethanol were produced by addition of a requisite volume of 99% (*w*/*v*) ethanol to cooled YEPD. The plates were wrapped in parafilm during storage and after plating were incubated at 22°C. Glucose assay plates of YEP plus 25% (*w*/*v*) glucose were also incubated after plating at 22°C. Strains were grown to stationary in liquid YPD (2 days), and 5 μL of 10-fold serial dilutions was spotted to plates.

Chemical profiling of volatile metabolite products

Hybrid strains generated from the *S. cerevisiae* × *S. paradoxus* N17–78 cross were screened for robust growth in YEPD, and a single strain, AWRI 1519, was selected for further study. Parent and hybrid strains were inoculated in triplicate at 1×10^6 cells from a pre-culture (2 days growth in YEPD) into 50 mL of Synthetic Complete medium with 4× amino acid mix (Sambrook and Russel 2001) and 8% (*w*/*v*) glucose. On completion of fermentation (<0.25% residual sugar as determined with Clinitest® tablets, Bayer, Switzerland), duplicate samples were analysed for volatile metabolites using gas chromatography–mass spectroscopy (GC-MS; Eglinton et al. 2002).

Fermentation product analysis of hybrid-generated wines compared to their commercial yeast parent

Small-scale industrial ferments were carried out at a commercial winery in 240 L barrels. Chardonnay grapes were machine harvested with no sulphur dioxide added. Fruit was tank-pressed, homogenised and transferred to barrels. Triplicate fermentations were conducted at 11–17° C using either the commercial wine yeast parent AWRI 838, the *S. cerevisiae* × *S. paradoxus* hybrid strain, AWRI 1501 or the *S. cerevisiae* × *S. kudriavzevii* hybrid strain, AWRI 1503. Wines were not produced by the non-*S. cerevisiae* parents, as neither was able to grow in the Chardonnay grape juice. At completion of fermentation (determined with Clinitest® tablets), wines were settled with sulphur dioxide and ascorbic acid added and treated with 250 μg/L copper. Triplicate wines were then pooled, filtered and bottled. Chemical analysis of target compounds, previously identified as important for wine flavour and aroma, was undertaken from duplicate samples of the resultant wines using GC-MS preceded by a headspace solid-phase micro-extraction (HS-SPME), with polydeuterated internal standards for stable isotope dilution analysis (Siebert et al. 2005).

Statistical analysis

A one-way analysis of variance and Student's *t* test (<0.05) were used to determine significant differences of compound concentrations between media and wines fermented by each yeast strain.

Results

Rare matings

Colonies formed on selection plates following interspecific matings were scored (Table 2) and subsequently picked onto new selection plates. Mating efficiency in the AWRI 838 × N17–78 cross was 30-fold greater than the AWRI 838×52–153 cross, while the less closely related *S.*

Table 2 Frequency of interspecific hybridisation

Strain cross	Frequency of hybridisation
AWRI 838 × N17–78	2×10^{-6}
AWRI 838 × 52–153	6×10^{-8}
AWRI 838 × NCYC 2889	2×10^{-8}

kudriavzevii strain had the lowest mating frequency of the crosses at 100-fold less than the *S. cerevisiae* × *S. paradoxus* haploid cross. The differences in mating efficiency between the crosses could be due to a number of factors; for example, *S. paradoxus* 52–153 spores may have mated with each other reducing the pool of spores available to mate with *S. cerevisiae* whereas N17–78 is a stable haploid; there may be inherent differences in sporulation efficiency between the two non-*S. cerevisiae* species; and the greater evolutionary distance between *S. cerevisiae* and *S. kudriavzevii*.

Confirmation of hybrid status of mating products

The hybrid nature of colonies from the *S. cerevisiae* × *S. paradoxus* crosses was confirmed by PCR analysis of genomic DNA utilising amplification with δ transposon primers and intron primers (Figs. 1a, b and 2a, b). A control PCR using DNA from both parents revealed that the transposon primers showed a bias towards *S. cerevisiae* targets, while the intron primers showed a bias towards *S. paradoxus* targets.

The AWRI 838 × N17–78 hybrids showed a transposon PCR pattern with specific bands from both parents, however, not all of the parent-specific bands were observed in all hybrids. For instance, hybrid strains A1, A2 A3 and A4 contain all five major bands amplified from the *S. cerevisiae* parent, while hybrid strain A5 is missing the lowest *S. cerevisiae* specific band (Fig. 1a). The intron PCR pattern for this cross showed a bias towards the *S. paradoxus* genome, with all hybrids having the complete set of *S. paradoxus* bands but only faint *S. cerevisiae* specific bands (Fig. 1b).

The AWRI 838×52–153 hybrids showed a transposon PCR pattern with mainly *S. cerevisiae* specific bands, but, again, not all bands were amplified in each hybrid (Fig. 2a), as hybrid strains B1 and B3 are missing both of the two lowest *S. cerevisiae* specific bands whereas B5 is missing only the lowest band. Three *S. paradoxus* specific bands were amplified strongly in the intron PCR (Fig. 2b), with all five hybrid strains amplifying the middle band, but not the top band. Hybrid B1 alone amplified the lowest *S. paradoxus* specific band. Collectively, PCR analyses confirmed the hybrid nature of the putative hybrid strains.

The hybrid nature of products from the *S. cerevisiae* × *S. kudriavzevii* cross was unable to be confirmed by transposon or intron PCR, as both analyses showed a fragment pattern attributed to the AWRI 838 parent only (Fig. 3a, b). ITS PCR-RFLP targeting the rDNA tandem repeat loci, however, revealed the existence of rDNA from both species within these hybrid strains (Fig. 3c).

Interspecific hybrids inherited wine-relevant traits from the wine yeast parent

Two confirmed interspecific hybrids were chosen for grape juice fermentation studies: AWRI 1501 from the *S. cerevisiae* × *S. paradoxus* (wild-type) cross and AWRI 1503 from the *S. cerevisiae* × *S. kudriavzevii* cross. Assay plates were designed to test the tolerance of hybrids to two major stresses encountered during fermentations: high sugar and high ethanol concentrations. Medium incorporat-

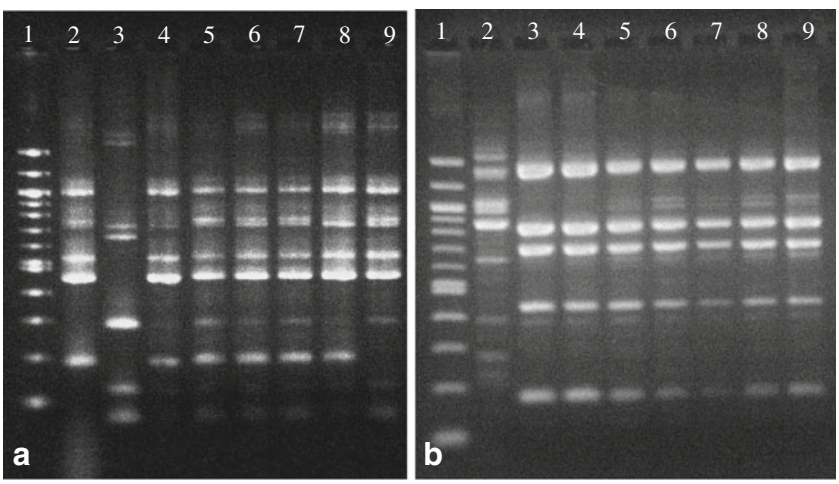

Fig. 1 Transposon PCR (**a**) and intron PCR (**b**) of AWRI 838 × N17–78 interspecific hybrid strains. *Lanes*: *1* 100 bp ladder, *2* AWRI 838 (*S. cerevisiae* parent), *3* N17–78 (*S. paradoxus* parent), *4* DNA from both AWRI 838 and N17–78, *5* to *9* hybrids A1 to A5

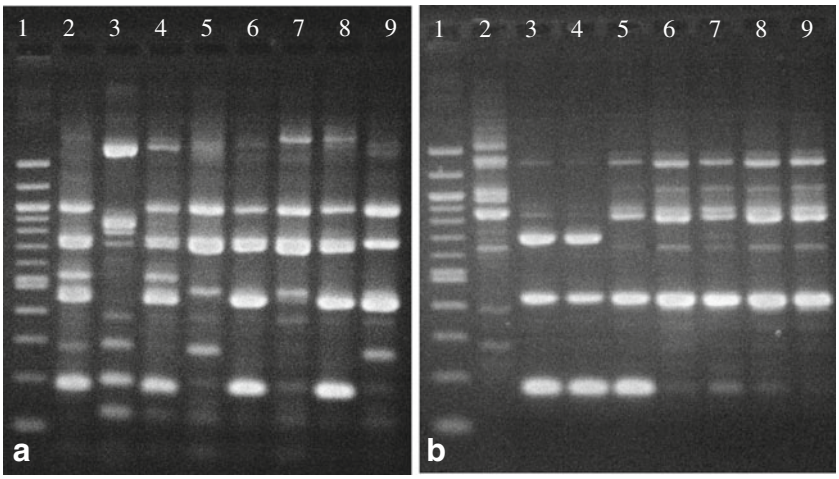

Fig. 2 Transposon PCR (**a**) and intron PCR (**b**) of AWRI 838× 52–153 interspecific hybrid strains. *Lanes*: *1* 100 bp ladder, *2* AWRI 838 (*S. cerevisiae* parent), *3* 52–153 (*S. paradoxus* parent), *4* DNA from both AWRI 838 and 52–153, *5* to *9* hybrids B1 to B5

ing a high concentration of glucose (25%, *w*/*v*) allowed robust growth of both hybrids and the *S. cerevisiae* parent, while the *S. kudriavzevii* parent showed less robust growth and the *S. paradoxus* parent no growth at all (Fig. 4). Neither the *S. paradoxus* nor the *S. kudriavzevii* parent was able to grow on high ethanol (14%, *v*/*v*) plates, but both hybrid strains grew well, although AWRI 1503 showed slightly weaker growth than the *S. cerevisiae* parent.

Chemical analysis of volatile metabolites from hybrid AWRI 1519 and parent strains in defined medium

After the completion of fermentation (<0.25% residual sugar), GC-MS analysis of defined medium fermented by AWRI 1519 and its parent strains identified 32 compounds (Table 3), 13 of which showed a significant changed concentration for the hybrid relative to the *S. cerevisiae* wine yeast parent. The chemical concentration profile of the hybrid volatile metabolites followed the gamut of all possible outcomes. In some cases, the hybrid strain produced a compound at the higher-producing parent level, but on other occasions produced a compound at the lower-producing parent level. For example, in the case of benzaldehyde, the hybrid generated 13.0 μg/L, an amount equivalent to 85% of the *S. paradoxus* parent (14.9 μg/L), while the *S. cerevisiae* parent generated considerably less (2.44 μg/L). Conversely, for dodecalactone, the hybrid generated 3.51 μg/L, a level similar to the *S. cerevisiae* parent (6.5 μg/L), whereas the *S. paradoxus* parent generated a far greater amount (30.9 μg/L). Some compounds were produced by the hybrid at an intermediate level between the two parental levels (e.g. 2-phenylethyl acetate), while two compounds were produced by the hybrid at remarkably lower levels than for either parent (*cis*-4-hydroxymethyl-2-methyl-1,3-dioxolane and *cis*-5-hydroxy-2-methyl-1,3-dioxane). A third compound, ethyl hexanoate, was produced by the hybrid at a concentration much higher than the cumulative total of the parents.

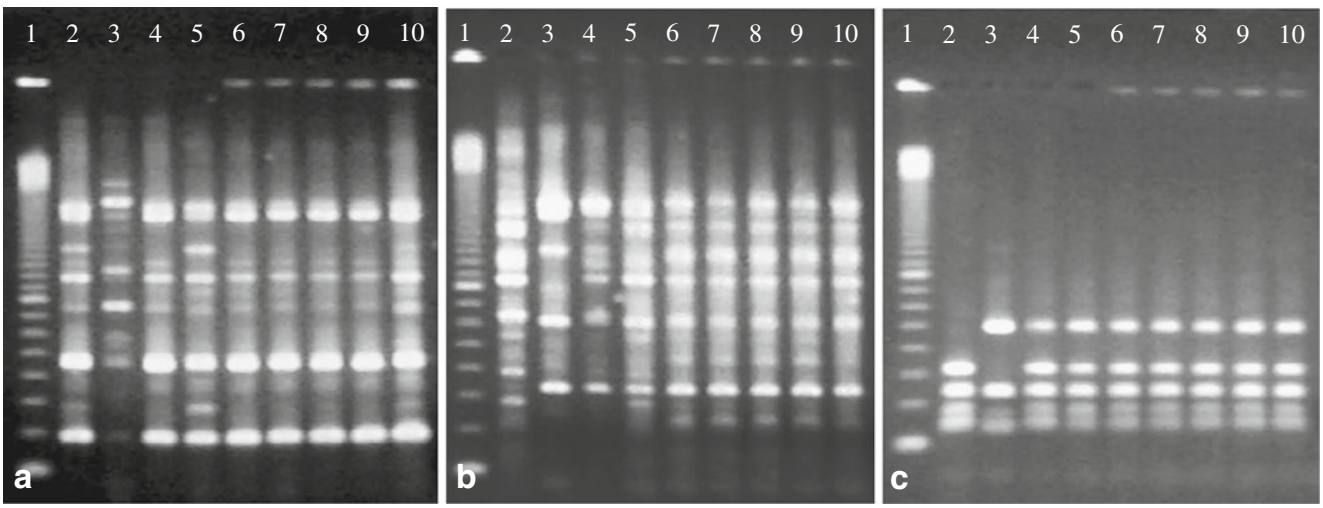

Fig. 3 Transposon PCR (**a**), intron PCR (**b**) and ITS PCR-RFLP (**c**) of AWRI 838 × NCYC 2889 interspecific hybrid strains. *Lanes*: *1* 100 bp ladder, *2* AWRI 838 (*S. cerevisiae* parent), *3* NCYC 2889 (*S. kudriavzevii* parent), *4* to *10* hybrids C1 to C7

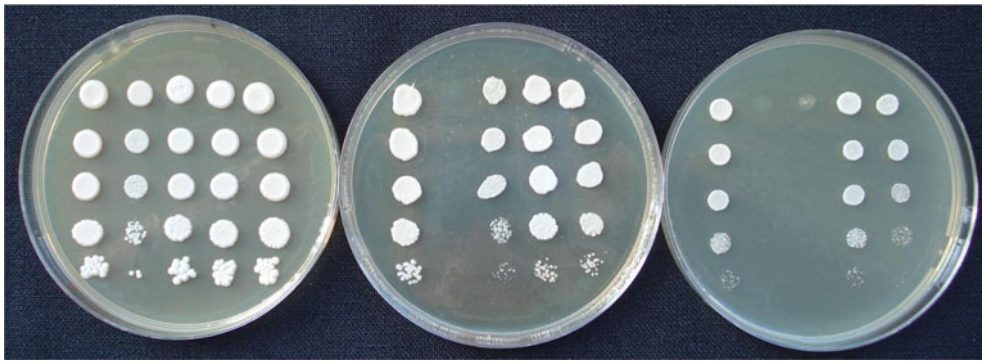

Fig. 4 Fermentation stress assay plates. Assay plates *left* to *right*: YEPD control, YEP-25% glucose, YEPD-14% ethanol. Strains, *left* to *right*, are AWRI 838 (*S. cerevisiae* parent), 52–153 (*S. paradoxus* parent), NCYC 2889 (*S. kudriavzevii* parent), AWRI 1501 (*S. cerevisiae* × *S. paradoxus* interspecific hybrid) and AWRI 1503 (*S. cerevisiae* × *S. kudriavzevii* interspecific hybrid). Spotted cultures are in 10-fold serial dilutions from *top* to *bottom*

Chemical analysis of fermentation products of hybrids AWRI 1501 and AWRI 1503 from small-scale grape juice industrial ferments

Small-scale (240 L) industrial ferments of Chardonnay juice were carried out using interspecific hybrid strains AWRI 1501 and AWRI 1503 and the wine yeast parent AWRI 838 alone, as the non-*S. cerevisiae* parents were unable to grow in the juice. The resultant wines (all having fermented to completion with <0.25% residual sugar) were analysed for volatile compounds using HS-SPME-GC-MS targeting 31 compounds (Table 4) previously established as important flavour and aroma compounds in wine (Siebert et al. 2005).

Relative to the *S. cerevisiae* parent strain, AWRI 1501 showed noteworthy differences in the concentration levels of 17 of the compounds analysed, with six compounds increasing and 11 compounds decreasing. Similarly, AWRI 1503 produced considerable differences in 20 compounds relative to the *S. cerevisiae* parent; seven showed an increase and 13 showed a decrease in concentration. A number of compounds that can have a negative effect on wine aroma and flavour were produced at much lower concentrations by the hybrid yeasts: acetic acid (vinegar), 3-methylbutanoic acid (blue cheese) and ethyl acetate (nail polish) decreased to 35%, 50% and 60%, respectively, of the concentrations produced by the *S. cerevisiae* parent. On the other hand, a number of compounds that contribute to fruity aromas had increased levels in the hybrid yeast wines. Ethyl hexanoate (green apple) levels increased to 120% for both hybrid yeast wines while the fruity aroma compounds, ethyl butanoate and ethyl propanoate, also showed increases, 117% and 160% (AWRI 1501) and 123% and 124% (AWRI 1503), respectively. 2-Methylpropyl acetate (banana) was produced in higher amounts by hybrid strain AWRI 1503. Compounds associated with more complex characters were also produced at increased levels to the parent wine yeast: Hexanoic acid (sweaty) levels were 137% (AWRI 1501) and 135% (AWRI 1503) and butanol (fusel) were 122% and 110%, respectively.

Discussion

Rapid and consistent fermentations are essential in large-scale, commercial wine production, and the majority of wineries worldwide rely upon inoculating their ferments with active dried yeast products from a yeast-manufacturing company. These ADY products are commonly strains of *S. cerevisiae*, although a small number have been identified as natural hybrids between members of the *Saccharomyces* sensu stricto species (Masneuf et al. 1998; Groth et al. 1999; Gonzalez et al. 2006; Bradbury et al. 2006). Different wine yeasts vary in their efficiency and reliability when fermenting grape juice (Pretorius 2000) and can impart different sensory properties to wine (King et al. 2008). This variation in yeast strain performance and delivery of product quality gives winemakers options when attempting to tailor their products to the preferences of different market segments. Development of new yeast strains with improved and/or desirable novel flavours is of growing importance for winemakers needing to produce wines that are differentiated from others in a competitive, over-crowded market.

Traditional breeding techniques are commonly used for yeast strain improvement (Winge and Lausten 1938; Pretorius 2000), and typically, these strain development programmes involve hybridising yeast of the same *Saccharomyces* species (i.e. *S. cerevisiae*), to produce intraspecific hybrids. This manuscript describes, for the first time, laboratory-based interspecific matings between a *S. cerevisiae* wine yeast strain and strains from two other *Saccha-*

Table 3 Chemical analysis of volatile metabolites from hybrid AWRI 1519 and parent strains in defined medium

Compound	RT (min)	AWRI 838 (μg/L)	N17–78 (μg/L)	AWRI 1519 (μg/L)
cis-4-Hydroxymethyl-2-methyl-1,3-dioxolane	6.06	397 (±51) b	1,620 (±593) a	37.8 (±27.0) c
Ethyl hexanoate	6.20	7.6 (±3.4) b	1.78 (±0.8) c	25.4 (±3.4) a
trans-4-Hydroxymethyl-2-methyl-1,3-dioxolane	6.47	39.0 (±15) b	1,421 (±376) a	58.0 (±10) b
Benzaldehyde	6.57	2.44 (±0.2) b	14.9 (±5.4) a	13.0 (±0.2) a
Dihydro-2-methyl-thiophenone	6.95	3.56 (±1.5) b	62.4 (±34.9) a	6.4 (±1.9) b
3-Methylthiopropanol	7.28	981 (±137) a	134 (±67) b	1,121 (±686) a
Hexanoic acid	7.39	47.1 (±21) a	44.5 (±36) a	50.5 (±23) a
Ethyl heptanoate	7.47	31.8 (±8.1) a	39.4 (±8.7) a	29.8 (±8.3) a
cis-5-Hydroxy-2-methyl-1,3-dioxane	7.55	95.0 (±13) b	604 (±247) a	5.3 (±1.3) c
Phenylacetaldehyde	7.81	30.7 (±5.9) b	44.4 (±4.2) a	47.5 (±4.3) a
Ethyl octanoate	8.69	37.1 (±3.1) b	31.6 (±3.5) b	45.6 (±3.8) a
2-Phenylethanol	8.95	49,487 (±6,254) a	13,842 (±2,971) b	46,883 (±28,682) a
2-Hydroxy-3,3-dimethyl γ butyrolactone	9.10	45.9 (±4.9) a	64.6 (±24.0) a	22.0 (±7.9) b
Succinic anhydride	9.72	1.74 (±0.4) b	110 (±83) a	41.0 (±37.4) a
4-Ethyl benzaldehyde	9.91	60.8 (±8.0) a	58.4 (±23) a	69.2 (±13.2) a
Benzothiazole	9.96	78.0 (±33.0) a	89.3 (±10.2) a	49.7 (±27.3) a
2-Phenylethyl acetate	10.08	60.3 (±6.0) a	7.8 (±5.3) c	30.3 (±4.4) b
4-Hydroxy-5-oxohexanoic acid lactone	10.43	14.5 (±3.0) b	1,183 (±247) a	7.1 (±7.0) b
Ethyl decanoate	10.92	18.6 (±2.0) ab	23.9 (±6.6) a	12.2 (±7.5) b
5-Hydroxymethyl furfural	11.64	56.2 (±9.0) a	36.9 (±20.0) a	48.5 (±28.8) a
4-(1-Hydroxyethyl) γ butanolactone	11.85	178 (±23) b	709 (±306) a	484 (±283) ab
3-Hydroxy-4-phenyl-2-butanone	12.06	1,145 (±200) a	254 (±88) b	294 (±108) b
Nerolidol	12.94	17.7 (±4.9) a	24.7 (±7.7) a	25.5 (±12.7) a
Di-*tert*-butylphenol	13.14	28.2 (±5.2) b	40.0 (±12.7) b	86.1 (±15.2) a
2-Propylphenol	13.48	28.6 (±4.2) a	10.7 (±4.1) b	43.6 (±9.7) a
4-Hydroxyphenyl ethanol	13.94	15,823 (±1,997) a	5,483 (±1,304) b	13,629 (±3,696) a
Tyrosol acetate	14.85	16.0 (±4.2) a	2.45 (±1.8) b	3.49 (±1.6) b
Dodecalactone	15.58	6.5 (±1.4) b	30.9 (±7.3) a	3.51 (±0.6) b
2-Hydroxybenzothiazole	16.50	64.4 (±20.9) b	298 (±63) a	23.9 (±7.4) b
Tryptophanol	16.95	2,073 (±279) a	615 (±306) b	984 (±178) b
1-Acetyl-β-carboline	17.98	32.2 (±4.5) a	28.9 (± 0.9) a	27.6 (±5.1) a
3-Formyl indole	18.41	33.6 (±8.5) b	60.0 (±21) a	20.3 (±9.1) b

Levels not connected by same letter are significantly different

romyces sensu stricto species—*S. paradoxus* and *S. kudriavzevii*—in order to generate wine yeast that produce novel wine and flavour aroma profiles.

Initially, for ease of selection, to optimise mating conditions, and for proof of concept experiments, *S. cerevisiae* wine yeast hybrids were generated using a genetically modified (GM) laboratory *S. paradoxus* haploid strain carrying auxotrophic markers. However, as only non-GM yeast are used by the Australian wine industry, interspecific hybrids were subsequently generated using non-genetically modified, natural isolates of *S. paradoxus* and *S. kudriavzevii*. Yeast mating is activated by the presence of pheromones normally produced by haploid yeast, and so the parent *S. paradoxus* and *S. kudriavzevii* strains were sporulated to generate haploid spores. To minimise the risk of potential loss of important wine yeast fermentation properties, the wine yeast parent was not sporulated; rare matings (Spencer and Spencer 1996) were used to form presumptive triploid interspecific hybrids.

Strain-specific and species-specific banding patterns generated using primers that target δ-transposon regions, introns and rDNA regions were used as markers to confirm the presence of each parental input in the resultant hybrid strains. Although a degree of preferential amplification of the *S. cerevisiae* parent genome was observed with the transposon primers, the intron primers showed a preference for *S. paradoxus* genomic sequences in the *S. cerevisiae* × *S. paradoxus* hybrids. However, both δ-transposon and intron primer sets showed a preference for *S. cerevisiae* DNA sequences in the *S. cerevisiae* × *S. kudriavzevii*

Table 4 Fermentation products in wines made using hybrids (AWRI 1501 and AWRI 1503) or parent (AWRI 838) yeast strains

Compound	Descriptor	AWRI 838	AWRI 1501	AWRI 1503
Acetic acid, mg/L	Vinegar	386 (±7) a	141 (±4) b	128 (±4) b
Ethyl acetate, mg/L	Nail polish	73.4 (±0.1) a	41.2 (±0.3) c	45.4 (±0.3) b
Ethyl butanoate, μg/L	Acid fruit	504 (±3) c	592 (±8) b	624 (±6) a
Ethyl-2-methylbutanoate, μg/L	Sweet fruit	10.4 (±0.1) a	8.3 (±0.3) b	6.7 (±0.1) c
Ethyl-3-methylbutanoate, μg/L	Berry	9.2 (±0.1) a	9.2 (±2.0) a	7.9 (±0.5) c
Ethyl-2-methylpropanoate, μg/L	Fruity	71.9 (±1) a	49.0 (±0.5) b	47.2 (±0.1) b
Ethyl propanoate, μg/L	Fruity	190 (±1) c	307 (±6) a	237 (±5) b
2-Methylbutyl acetate, μg/L	Banana, fruity	97.0 (±3) a	52.8 (±4.9) c	72.5 (±5.8) b
2-Methylpropyl acetate, μg/L	Banana, fruity	56.9 (±0.5) b	55.4 (±1.0) b	66.0 (±0.1) a
2-Methylbutanol, mg/L	Nail polish	26.5 (±1) a	30.0 (±0.9) a	25.1 (±2.8) a
Propanoic acid, μg/L	Vinegar	1,368 (±44) a	965 (±300) b	373 (±195) c
2-Methylpropanoic acid, μg/L	Cheese, rancid	493 (±31) a	583 (±11) a	523 (±108) a
2-Methylbutanoic acid, μg/L	Cheese, sweaty	236 (±80) a	239 (±14) a	189 (±12) a
2-Phenylethanol, mg/L	Roses	45.5[a]	48.0[a]	45.5[a]
2-Phenylethyl acetate, μg/L	Flowery	404 (±1) a	239 (±10) c	290 (±1) b
Hexanoic acid, mg/L	Cheese, sweaty	5.1 (±0.1) b	7.0 (±0.3) a	6.9 (±0.2) a
Decanoic acid, mg/L	Fatty	3.46 (±0.3) a	3.21 (±0.4) a	2.77 (±0.3) a
Octanoic acid, mg/L	Rancid, harsh	9.3 (±0.4) a	5.2 (±0.4) b	8.9 (±0.6) a
Hexyl acetate, μg/L	Sweet, perfume	142 (±1) a	41.6 (±0.2) c	60.5 (±0.2) b
Ethyl lactate, mg/L	Strawberry	34.7 (±3.5) a	27.7 (±2.3) a	16.5 (±0.8) b
3-Methylbutanoic acid, μg/L	Blue cheese	492 (±18) a	266 (±26) b	235 (±15) b
2-Methylpropanol, mg/L	Fusel, spirituous	28.0 (±0.3) a	28.0 (±0.2) a	24.8 (±0.1) b
3-Methylbutyl acetate, mg/L	Banana	2.14 (±0.01) a	2.02 (±0.01) a	2.06 (±0.04) a
Butanol, μg/L	Fusel, spirituous	810 (±10) c	990 (±30) a	890 (±5) b
Hexanol, mg/L	Green, grass	2.43 (±0.01) a	2.31 (±0.01) b	2.19 (±0.03) c
Ethyl octanoate, mg/L	Sweet, soap	1.52 (±0.01) a	1.55 (±0.08) a	1.39 (±0.03) a
Ethyl decanoate, μg/L	Pleasant, soap	677[a]	600[a]	551[a]
Ethyl dodecanoate, μg/L	Soapy, estery	150[a]	256[a]	287[a]
3-Methylbutanol, mg/L	Harsh, nail polish	140[a]	165[a]	171[a]
Ethyl hexanoate, mg/L	Green apple	1.00 (±0.01) b	1.24 (±0.02) a	1.24 (0.03) a
Butanoic acid, mg/L	Cheese, rancid	2.24 (±0.04) b	3.73 (±0.10) a	3.37 (±0.14) a

Levels not connected by same letter are significantly different

[a] Data based on a single determination

hybrid. Nonetheless, the hybrid status of all progeny used in fermentative work was confirmed using the above primer sets, or by targeting the rDNA ITS region that, upon restriction digestion, generated species-specific banding patterns.

Interestingly, different hybrids generated from the same cross did not give identical banding patterns. This may be due to genome loss or rearrangement during the incipient stages of interspecific hybrid evolution. In previous work, genomic analyses of natural interspecific yeast hybrids have identified loss of varying portions of parental genomes (Dunn and Sherlock 2008; Belloch et al. 2009), and plant studies have shown that changes within newly formed interspecific hybrid genomes occur rapidly leading to extensive inter- and intra-genome rearrangements and gene loss (Song et al. 1995; Kashkush et al. 2002).

Genetic stability analysis (using the same PCR approach as for confirmation of hybridisation) was carried out on each hybrid strain generated in this study. Twenty individual isolates from each hybrid strain were assessed after 50 generations in YEPD and at the end of model medium and grape juice fermentations. No further change in fingerprint profile was identified (results not shown).

Genome loss and rearrangement in newly formed wine yeast hybrids might lead to loss of industrially important traits, such as stress tolerance. Two stresses common to grape juice fermentations are high sugar concentration experienced at the beginning of fermentation and high ethanol concentration that builds towards the end of fermentation. Hence, assay plates designed to select for tolerances to these stresses were used to confirm that

hybrids chosen for further investigation at least retained these traits.

All hybrids generated for this work clearly produce different volatile fermentation product profiles to the wine strain parent. Chemical analysis of volatile metabolites in spent minimal medium for AWRI 1519 showed this yeast to be very different to its parental strains. In some cases, levels of metabolites for the hybrid followed closely that of the 'highest-producing' parent, but, on other occasions, a compound was produced at the level of the 'lower-producing' parent. Moderating effects (where hybrid levels are midway between parents) were also noted. Intriguingly, a small number of compounds were produced by the hybrid in a considerably reduced concentration relative to either parent, or at a level much higher than a cumulative amount. It is possible that flavour-active metabolites of interspecific hybrids, at concentrations not predicted by their parental metabolite profiles, could lead the generation of new yeast strains capable of creating unique wine styles from conventional grape varieties.

Chardonnay wines produced by hybrids AWRI 1501 and AWRI 1503 again showed compound concentrations that were greater or less than produced by the wine yeast parent. Interestingly, the magnitude of differences varied between the two interspecific hybrids, highlighting the potential for different hybrid strains to tailor wines towards different consumer groups (Lattey et al. 2007). The specific contribution of the non-*S. cerevisiae* parents was not assessed, as neither was able to grow in the Chardonnay grape juice. The compounds that were present at altered levels in the hybrid-made wines contribute flavours such as fruits (banana, strawberry and green apple), perfumes and flowers, and compounds with the more pungent attributes of blue cheese, rancid cheese and fusel. High concentrations of flavour or aroma compounds in wine result in a greater sensory impact but may also lead to the masking of less obvious flavours and aromas. Conversely, lowering the level of a particular compound may result in the unmasking of other flavours and aromas within the wine (Saison et al. 2009).

It is important to note that the number of differences in fermentation products between hybrid-made wine and *S. cerevisiae*-made wine will be a conservative estimate, as the fermentation product analysis targeted only compounds that have previously been identified as important contributors to flavour and aroma in wines, wines typically produced by a single industrial *S. cerevisiae* strain (Siebert et al. 2005). Thus, there may be other important flavour and aroma compounds produced by the input of the non-*S. cerevisiae* genome component of the hybrid strains that were not considered in this study.

Metabolite differences between hybrid and parental strain(s) were identified in both model medium and grape juice fermentations. These differences in metabolite levels may be the direct result of polyploidy (Hull-Sanders et al. 2009); the additive effect of an extra genome; synergistic genetic interactions (Mani et al. 2008); heterosis, whereby the hybrid displays superior growth and yield over both parents (Lippman and Zamir 2006); or differences in gene expression. Differences in gene expression could be explained by the observations that divergence of transcription factor binding sites across the *Saccharomyces* species far exceeds the interspecies variation in orthologous genes (Borneman et al. 2007); alterations in transcription factor binding within the hybrid genome could lead to differences in gene regulation effecting metabolite production. All, or any, of the above genomic effects would potentially contribute to the novel wine flavour and aroma profiles produced by interspecific wine yeast hybrids.

Performance of interspecific wine yeast hybrids in an industrial setting

Informal blind tastings on wines made using interspecific wine yeast hybrids described in this manuscript concluded that the hybrid yeast wines were more complex, with a wider range of flavour and aroma attributes (results not shown). The above hybrids have since been used to produce award winning wines and are now available commercially having been adopted by winemakers internationally.

In conclusion, this manuscript describes a new strategy for developing wines with greater complexity. By combining the genomes of a commercial *S. cerevisiae* wine yeast strain and other *Saccharomyces* sensu stricto yeast, we have successfully bred new commercial wine yeast strains capable of producing novel wine aroma and flavour profiles. These new hybrid yeasts can assist winemakers in their search for tools that introduce flavour and aroma diversity to their wines.

Acknowledgements This work was financially supported by Australia's grapegrowers and winemakers through their investment body the Grape and Wine Research Development Corporation, with matching funds from the Australian Government. The AWRI is part of the Wine Innovation Cluster.

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