A breeding strategy to harness flavor diversity of Saccharomyces interspecific hybrids and minimize hydrogen sulfide production

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Abstract
Industrial food-grade yeast strains are selected for traits that enhance their application in quality production processes. Wine yeasts are required to survive in the harsh environment of fermenting grape must, while at the same time contributing to wine quality by producing desirable aromas and flavors. For this reason, there are hundreds of wine yeasts available, exhibiting characteristics that make them suitable for different fermentation conditions and winemaking practices. As wine styles evolve and technical winemaking requirements change, however, it becomes necessary to improve existing strains. This becomes a laborious and costly process when the targets for improvement involve flavor compound production. Here, we demonstrate a new approach harnessing preexisting industrial yeast strains that carry desirable flavor phenotypes – low hydrogen sulfide (H₂S) production and high ester production. A low-H₂S Saccharomyces cerevisiae strain previously generated by chemical mutagenesis was hybridized independently with two ester-producing natural interspecies hybrids of S. cerevisiae and Saccharomyces kudriavzevii. Deficiencies in sporulation frequency and spore viability were overcome through use of complementary selectable traits, allowing successful isolation of several novel hybrids exhibiting both desired traits in a single round of selection.

Introduction
Wine yeast improvement is a continuous process of fulfilling winemaking needs (Pretorius, 2000; Giudici et al., 2005). Strain improvement strategies are numerous – often complementary to each other – and the choice among them is based on three factors: (1) the genetic nature of traits (monogenic or polygenic); (2) the knowledge of the genes involved (rational or blind approaches); and (3) the phenotypic trait requirement (Giudici et al., 2005). Because of the lack of acceptance for the use of recombinant yeasts, only classical techniques such as clonal selection of variants, mutation and selection, and mating/hybridization are currently used to produce food-grade starter cultures (Pretorius, 2000; Schilter & Constable, 2002). Improvement of wine yeast properties means not only enhancing fermentation performance, but also optimizing the production of yeast secondary metabolites such as volatile aroma compounds. Some compounds are detrimental to wine quality, and therefore, their production should be minimized. A well-studied example is hydrogen sulfide (H₂S), a yeast metabolite that imparts rotten-egg off-flavor in wine (Zambonelli et al., 1984; Tezuka et al., 1992; Omura et al., 1995; Hansen & Kielland-Brandt, 1996; Mendes-Ferreira et al., 2002; Nowak et al., 2004). On the other hand, enhancing the formation of positive aroma compounds belonging to chemical families such as aldehydes, ketones, alcohols, acids, and esters by wine yeasts has also been a priority (Pretorius, 2000; Pretorius & Bauer, 2002; Fleet, 2003).

A recent approach to the improvement of wine yeast strains involved generation of interspecies hybrids within the Saccharomyces sensu stricto group (Bellon et al., 2011). Natural interspecies hybrids were previously isolated from
wine fermentation (Gonzalez et al., 2006), while the lager yeast *S. pastorianus* is in fact a natural hybrid of *Saccharomyces cerevisiae* and *S. bayanus* (Sipiczki, 2008; Libkind et al., 2011). Generally considered sterile and a reproductive ‘dead-end,’ *Saccharomyces* interspecies hybrids can potentially undergo further hybridization (de Barros Lopes et al., 2002). Indeed, through application of molecular identification methods, natural double (Sipiczki, 2008) and triple hybrids (*S. cerevisiae × Saccharomyces uvarum × Saccharomyces kudriavzevii*) (Masneuf et al., 2002; de Barros Lopes et al., 2002; Gonzalez et al., 2006) were identified.

Our aim, therefore, was to demonstrate that existing *Saccharomyces* interspecies hybrids can be used in breeding programs with characterized *S. cerevisiae* mutants, to develop novel industrial wine yeast strains with improved aroma traits.

**Materials and methods**

**Yeast strains**

All the yeast strains were obtained from The Australian Wine Research Institute (AWRI) culture collection. Yeast cultures were maintained on solid yeast peptone dextrose (YPD) agar plates.

**Media and grape musts**

YPD was composed of 2% D-glucose, 1% yeast extract, and 2% peptone and solidified with 2% agar. Sporulation media (SM) were composed of 1% potassium acetate, 0.05% D-glucose, 0.1% yeast extract, and 2% agar. BiGGY agar (Oxoid, Australia) was prepared using manufacturer’s instructions. YP-Galactose was prepared with 2% D-galactose, 1% yeast extract, and 1% peptone and solidified with 2% agar at different pH values. Two filter-sterilized Chardonnay grape juices were used for wine fermentation (CHS and CH07). Both juices were filter-sterilized using a 0.65-µm/0.22-µm cartridge (Sartorius, Germany).

**Screening for parental strains**

The screening of candidate parental strains was performed in two stages. First, the strains were grown on BiGGY agar, which provides a qualitative indication of H2S production potential (Giudici & Kunkee, 1994). The chemically mutagenized low-H2S strains included in this study were isolated by Cordente et al. (2009) using this medium.

The second stage was a laboratory fermentation trial with sterile-filtered Chardonnay juice (CHS). Yeast pre-cultured sequentially in YPD and CHS were inoculated at 10⁶ cells mL⁻¹ in 200 mL CHS, with alcoholic fermentation conducted in 250-mL flasks at 17 °C. Fermentation progress was followed by CO₂ weight loss, measured every 24 h. Ferments were considered complete when CO₂ release was lower than 1 g L⁻¹ day⁻¹, and the concentration of reducible sugars was lower than 2 g L⁻¹ (Clini-test, Bayer, Germany).

**Sporulation, tetrad dissection, and viability of spores**

Strains were sporulated on solid SM media for 5 days at 28 °C. Spores were obtained using a standard zymolyase protocol (Burke et al., 2000). Using a micromanipulator microscope (Singer, UK), the spores were separated and distributed on an YPD plate. Viability of spores was evaluated after incubation of spores for 3 days, at 28 °C.

**Screening for complementary phenotypic markers of parental strains**

To develop discriminating methodologies for selection of hybrids, we performed a range of tests to determine complementary phenotypic markers. We tested the parental strains for ability to use different carbon sources in liquid media (glucose, fructose, saccharose, mannose, and galactose) at 6% w/v, except for raffinose at 12% w/v, with 1% yeast extract and 1% peptone (Kreger-van Rij, 1984). We also tested their ability to grow under high concentrations (0, 150, 200, and 250 mg L⁻¹) of SO₂ and ethanol (8, 10, 11, 12, and 13% v/v) in YPD broth at pH 3.5, after 72 and 48 h, respectively. Finally, the ability for growth under low pH (from 2.5 to 2.9) conditions was estimated in YPD broth after 24 h. All the liquid media were sterilized prior to use, by membrane filtration (0.22 µm, Sartorius, Germany), and media were inoculated at a concentration of 10⁶ cells mL⁻¹. The strains were also tested for growth at 37 °C on YPD solid agar media after 24 h. Strains were spotted on solid media after overnight growth in YPD broth at a concentration of 3 × 10³ cells per spot. All the tests were performed in triplicate.

**Mass-mating and selection of potential hybrids**

Yeast strains AWRI 1116 (interspecies hybrid 1, strain A), 1539 (interspecies hybrid 2, strain B), and 1640 (low-H₂S strain, strain C) were inoculated on solid SM for 5 days at 28 °C, and cells were incubated with zymolyase for 1 h at 30 °C on a rotor incubator. Then, 0.1 mL of sterile 0.5-mm glass beads was added and incubated on a rotor at 30 °C for 1 h. Asci were then disrupted by addition of 1 mL sterile water and vortex for 1–2 min (Burke et al.,
2000). Spores were collected, washed in sterile H2O, mixed, and inoculated in YPD broth. After 7 days at 20 °C with rotary shaking (120 r.p.m.), the media were spread onto selective plates (200 µL per plate).

For hybrids of AWRI 1116 × 1640 (A × C), YP-Galactose solid media at pH 3.1 were used, and hybrids were isolated after 4 days at 28 °C. Hybrids of AWRI 1539 × 1640 (B × C) were selected on YP-Galactose solid media after overnight growth at 37 °C. All the putative hybrids were analyzed (in duplicate) for H2S production on BiGGY plates (Giudici & Kunkee, 1994). Hybrids of AWRI 1539 × 1640 and of AWRI 1116 × 1640 showing lower H2S production than AWRI 1539 and AWRI 1116, respectively, were submitted for further genotypic identification.

**Molecular analysis of hybrids**

Genomic DNA extraction of yeast strains was performed using glass beads for cell wall breakage (Burke et al., 2000). ITS-PCR-RFLP (McCullogh et al., 1998) was performed to detect inheritance of *S. kudriavzevii* genomic regions containing ribosomal DNA regions, while transposon-PCR (Ness et al., 1993) was to differentiate the *S. cerevisiae* component of parental strains, and their progeny. A 1.5-kb fragment of the *S. kudriavzevii* MET10 gene was amplified by PCR with specific primers, and a 1.4-kb fragment of the *S. cerevisiae* MET10 gene was amplified by PCR with specific primers. All MET10 PCR products were cleaned using the QIAquick PCR Purification kit (QIAGEN, Australia) and used as templates for the sequencing reaction. Sequencing was carried out on an ABI PRISM 3730xl DNA Analyzer (Applied Biosystems Australia) by the Australian Genome Research, an ABI PRISM 3730xl DNA Analyzer (Applied Biosystems, Japan). The sequencing reaction. Sequencing was carried out on an ABI PRISM 3730xl DNA Analyzer (Applied Biosystems Australia) by the Australian Genome Research Facility Ltd sequencing service (Adelaide). All analyses of genotypic fingerprints were performed on the screening cartridge of the QIAxcel capillary electrophoresis system (QIAGEN).

**Alcoholic fermentations of Chardonnay grape juice with H2S detection**

Laboratory-scale fermentations in filter-sterilized Chardonnay juice CH07 were carried out in the same conditions used for selection of parental strains. H2S liberated during fermentation was quantitated by a trap-based method, using precision gas detector tubes (Kitagawa, Japan).

**Analysis of principal nonvolatile compounds**

The wines produced from Chardonnay CH07 were analyzed for glucose, fructose, ethanol, glycerol, and acetic, citric, malic, succinic, lactic, and tartaric acid by HPLC using a Bio-Rad HPX-87H column as described previously (Nissen et al., 1997). Sulfite (SO2) measurements were taken using the Flow Injection analysis using the Lachat Instrument Quick Chem(r) FIA + 8000 series (Cordente et al., 2009).

**Gas chromatography-mass spectrometry (GC-MS) analysis**

All analyses were performed on an Agilent 7890 gas chromatograph equipped with Gerstel MPS2 multipurpose sampler and coupled to an Agilent 5975C VL mass selective detector. Instrument control and data analysis were performed with Agilent G1701A Revision E.02.00 ChemStation software.

**Nonchromatographic volatile fingerprinting**

Nonchromatographic volatile fingerprinting of wine samples was performed by Metabolomics Australia (Adelaide) as follows. Each sample was prepared in duplicate by diluting 1 : 4 with 10% potassium hydrogen tartrate, pH adjusted 3.5. The gas chromatograph was fitted with a deactivated guard column (Restek 5 m × 180 µm × 0.18 µm). Helium (Ultra High Purity) was used as the carrier gas at a flow rate of 1.3 mL min⁻¹ in constant flow mode. The oven temperature held constant at 200 °C for the duration of the analysis.

The sample was heated to 40 °C for 5 min with agitation. A volume of 2.5 mL of the headspace was removed using a heated (55 °C) syringe. The content of the syringe was then injected into a Gerstel PVT (CIS 4) inlet fitted with a Tenax TA inlet liner cooled to 15 °C using solvent vent mode (pressure 2 psi for 2 min). Following capture of analytes on the Tenax liner, the injector is heated to 330 °C at 12 °C s⁻¹ (pressure 12.104 psi).

The mass spectrometer quadrupole temperature is set at 150 °C, the source was set at 250 °C, and the transfer line is held at 280 °C. Positive ion electron impact spectra at 70 eV are recorded in scan mode with a mass range of 50–400 and 4 scans s⁻¹.

Raw data were exported from ChemStation (Agilent G1701A Revision E.02.00 ChemStation Software). Mass spectra were summed (scan 173–scan 391) and binned (0.5 mass units) using R (version 2.9.2).

**Targeted analyses of volatile compounds**

Targeted analyses of fermentation-derived higher alcohols, acetate-, and ethyl esters were performed by Metabolomics Australia (Adelaide) on wine samples by GC-MS using a stable isotope dilution assay as previously...
described (Siebert et al., 2005), with modifications to sample concentration and introduction. Briefly, the SPME (solid-phase microextraction) sample concentration and introduction technique was replaced with a large volume headspace using a cooled Tenax liner, as described for the nonchromatographic volatile fingerprinting method mentioned earlier.

Further modifications to the method of Siebert et al. (2005) included the following: Wine samples were prepared in two dilutions 1/20 and 3/10 with model wine (13.8% ethanol, 10% potassium hydrogen tartrate, pH 3.5). The gas chromatograph was fitted with a 30 m Resteck Stabilwax-DA that has a 5 m × 0.18 mm Resteck Stabilwax-DA that has a 5 m × 0.18 mm retention gap. Helium was used as the carrier gas with linear velocity of 24.6 cm s⁻¹ and flow rate of 0.78 mL min⁻¹ in constant flow mode. The oven temperature started at 33 °C was held at this temperature for 4 min, then increased to 60 °C at 4 °C min⁻¹, then heated at 8 °C min⁻¹ to 230 °C, and held at this temperature for 5 min.

Prior to injection, the inlet was cooled to 0 °C with liquid nitrogen. While maintaining 0 °C, the sample was introduced to the inlet at 25.0 μL s⁻¹ (penetration, 22.0 mm) using split mode (split ratio, 33 : 1; split flow, 25.78 mL min⁻¹). Following capture of analytes on the Tenax liner, the injector was heated to 330 °C with a constant flow mode. The oven temperature started at 33 °C, held at this temperature for 4 min, then increased to 60 °C at 4 °C min⁻¹, then heated at 8 °C min⁻¹ to 230 °C, and held at this temperature for 5 min.

The mass spectrometer quadrupole temperature was set at 150 °C, the source was set at 250 °C, and the transfer line was held at 280 °C. All data processing was performed on Agilent G1701A Revision E.02.00 ChemStation software.

### Statistical analysis

The analyzed data from alcoholic fermentation trials were studied by one-way ANOVA (Microsoft Office Excel 2003). The statistical level of significance was set at P ≤ 0.05. The means were compared with the Tukey’s honestly significant different test. Volatile fingerprinting ASCII data matrices were imported into The Unscrambler (Camo, Version 9.8), and principal component analysis (PCA) was performed.

### Results

#### Selection of parental strains and screening for complementary phenotypic markers

The first industrial, food-grade, low-H₂S-producing wine yeast strains were obtained by chemical mutagenesis of the widely used commercial strain Maurivin PDM (Cor-dente et al., 2009). To develop strains with improved traits for wine production, we hybridized the strain displaying the lowest H₂S production (AWRI 1640, strain C) from that study, with yeast strains known to produce relatively high concentrations of positive flavor compounds.

These strains, AWRI 1116 and 1539 (strains A and B, respectively), both natural interspecific hybrids of S. cerevisiae and S. kudriavzevi, were used to ferment Chardonnay juice CHS. Chemical analysis of the wines showed that the overall production of volatile fermentation products was 37% and 22% higher for AWRI 1116 and 1539, respectively, in comparison with the low-H₂S strain AWRI 1640 (Table 1). Both AWRI 1116 and 1539 produced increased levels of acetate esters (excluding ethyl acetate) and higher alcohols than AWRI 1640. Of note, both AWRI 1116 and 1539 produced significant higher concentrations of 2-methylpropyl acetate (banana, fruity aroma) and 2- and 3-methylbutyl acetate (banana aroma), while producing less ethyl acetate (nail polish/solvent aroma). In the case of AWRI 1539, this strain also produced significant higher levels of 2-phenylethyl acetate (rose aroma) than AWRI 1640.

Qualitative assessment of H₂S production on BiGGY agar confirmed the higher potential for H₂S production by AWRI 1116 and 1539. Both strains displayed a dark

### Table 1. Volatile compounds (µg L⁻¹) produced by parental strains AWRI 1116, 1539, and 1640 at the end of fermentation in a Chardonnay juice (CHS)

<table>
<thead>
<tr>
<th>Volatile compound</th>
<th>Strain</th>
<th>1116 (A)</th>
<th>1539 (B)</th>
<th>1640 (C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethyl propanoate</td>
<td>280ab</td>
<td>129b</td>
<td>1074a</td>
<td></td>
</tr>
<tr>
<td>Ethyl 2-methylpropanoate</td>
<td>92ab</td>
<td>100a</td>
<td>50b</td>
<td></td>
</tr>
<tr>
<td>Ethyl butanoate</td>
<td>88a</td>
<td>150b</td>
<td>189c</td>
<td></td>
</tr>
<tr>
<td>Ethyl 2-methylbutanoate</td>
<td>23a</td>
<td>21a</td>
<td>39c</td>
<td></td>
</tr>
<tr>
<td>Ethyl 3-methylbutanoate</td>
<td>21a</td>
<td>20a</td>
<td>19c</td>
<td></td>
</tr>
<tr>
<td>Ethyl hexanoate</td>
<td>193b</td>
<td>261a</td>
<td>235ab</td>
<td></td>
</tr>
<tr>
<td>Total ethyl esters</td>
<td>697</td>
<td>681</td>
<td>1606</td>
<td></td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>73008c</td>
<td>42143b</td>
<td>99556c</td>
<td></td>
</tr>
<tr>
<td>2-methylpropyl acetate</td>
<td>53a</td>
<td>55a</td>
<td>36b</td>
<td></td>
</tr>
<tr>
<td>2-methylbutyl acetate</td>
<td>99a</td>
<td>74b</td>
<td>49c</td>
<td></td>
</tr>
<tr>
<td>3-methylbutyl acetate</td>
<td>783a</td>
<td>651a</td>
<td>312b</td>
<td></td>
</tr>
<tr>
<td>2-phenylethyl acetate</td>
<td>189ab</td>
<td>316a</td>
<td>98b</td>
<td></td>
</tr>
<tr>
<td>Hexyl acetate</td>
<td>27ab</td>
<td>32a</td>
<td>19b</td>
<td></td>
</tr>
<tr>
<td>Total esters*</td>
<td>1151</td>
<td>1128</td>
<td>514</td>
<td></td>
</tr>
<tr>
<td>2-methylpropanol</td>
<td>75219a</td>
<td>72771a</td>
<td>34282b</td>
<td></td>
</tr>
<tr>
<td>Butanol</td>
<td>243a</td>
<td>179a</td>
<td>1115a</td>
<td></td>
</tr>
<tr>
<td>2-methylbutanol</td>
<td>86938a</td>
<td>57774b</td>
<td>36123b</td>
<td></td>
</tr>
<tr>
<td>3-methylbutanol</td>
<td>220304a</td>
<td>191513a</td>
<td>112810b</td>
<td></td>
</tr>
<tr>
<td>Hexanol</td>
<td>1569a</td>
<td>1545a</td>
<td>1680a</td>
<td></td>
</tr>
<tr>
<td>Total alcohols</td>
<td>384273</td>
<td>323752</td>
<td>186010</td>
<td></td>
</tr>
</tbody>
</table>

Results are the mean of two independent replicates. Standard deviations were typically about 10% and never exceeded 20%. Means with the same letter are not significantly different from each other (Tukey’s test, P < 0.05).

*Excluding ethyl acetate.
brown phenotype in this medium, while strain AWRI 1640 had a white phenotype (data not shown).

Spore viability was evaluated for each of the three parental strains, and we were unable to isolate a viable spore among 18 tetrads; thus, we were reliant upon mass-mating.

To discriminate hybrids from the parental strains, complementary phenotypic markers were sought (Table 2). Carbon source utilization screening revealed that the parental low-H2S-producing strain AWRI 1640 was not able to grow on galactose. The interspecies hybrid AWRI 1539 could not grow at 37 °C, while AWRI 1116 grew slowly at low pH in comparison with AWRI 1640. Therefore, we designed media and growth conditions for selective isolation of hybrids by mass-mating (see Materials and methods).

A total of 31 potential hybrids of AWRI 1539 × 1640 (B × C) and 65 potential hybrids of AWRI 1116 × 1640 (A × C) were isolated from selective plates. The hybrids were screened for H2S production on BiGGY agar plates. A total of 20 B × C and 24 A × C potential hybrid colonies were lighter in color compared to AWRI 1539 and AWRI 1116, respectively (data not shown), and were chosen for further molecular characterization.

Table 2. Identification of complementary phenotypes for parental strains AWRI 1116, 1539, and 1640

<table>
<thead>
<tr>
<th>Strain</th>
<th>Temperature 37 °C</th>
<th>Carbon source</th>
<th>pH 2.5</th>
<th>pH 2.6</th>
<th>pH 2.7</th>
<th>pH 2.8</th>
<th>pH 2.9</th>
<th>Ethanol (v/v%) 8</th>
<th>Ethanol (v/v%) 10</th>
<th>Ethanol (v/v%) 11</th>
<th>Ethanol (v/v%) 12</th>
<th>Ethanol (v/v%) 13</th>
<th>SO2 (mg L⁻¹) 0</th>
<th>SO2 (mg L⁻¹) 150</th>
<th>SO2 (mg L⁻¹) 200</th>
<th>SO2 (mg L⁻¹) 250</th>
</tr>
</thead>
<tbody>
<tr>
<td>1116 (A)</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>1539 (B)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>1640 (C)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>

Growth (+) and absence of growth (−) are shown.

Confirmation of hybrid genotypes

Two techniques were applied to identify and discriminate between parental strains and hybrids. ITS-PCR-RFLP analysis (Fig. 1a) confirmed the presence of S. cerevisiae and S. kudriavzevii ribosomal DNA regions within the genomes of three putative B × C hybrids (AWRI 1808, 1809, and 1810). By transposon-PCR (Fig. 1b), the three previously identified B × C hybrids and 2 A × C hybrids (AWRI 1811 and 1812) displayed differential fingerprints that were intermediate to their relevant parental strains.

Further molecular characterization was applied to the five selected hybrids by sequencing the S. cerevisiae MET10 gene, which encodes for the α-subunit of the sulfite reductase enzyme. AWRI 1640 has a heterozygous point mutation (G176A) in the MET10 gene, which encodes for the low-H2S phenotype of the strain (Cordente et al., 2009). All five hybrids were found to contain this mutation, along with extra copies of wild-type S. cerevisiae MET10 alleles. All B × C hybrids contained three extra MET10 alleles, one of them coming from the AWRI 1640 parental strain and the other two from the S. cerevisiae genome of AWRI 1539. In the A × C hybrids, two extra MET10 alleles were found, one from AWRI 1640 and the other from AWRI 1116. In addition, the S. kudriavzevii MET10 gene was found to be present in all five novel low-H2S-producing hybrids (Fig. 2).

Laboratory-scale fermentations of novel hybrids in Chardonnay grape juice

The five hybrid strains were further characterized in a 200 mL fermentation experiment in Chardonnay juice CH07. The A × C hybrids AWRI 1811 and 1812 fermented at a similar rate to AWRI 1640, which was slightly faster than AWRI 1116 (Fig. 3). On the other hand, the B × C hybrids AWRI 1808, 1809, and 1810 (Fig. 4) showed intermediate fermentation rates between their parental strains AWRI 1640 and 1539, the latter being the fastest.

The major nonvolatile compounds were analyzed at the end of fermentation, and some differences were observed between strains (Table 3). All the hybrids produced significant lower concentrations of acetic acid (< 0.1 g L⁻¹) than their parental strains. AWRI 1640 produced significantly higher concentrations of glycerol than AWRI 1116 and 1539 parental strains, and this was also the case for all of the novel hybrids.

Cumulative production of H2S was measured throughout fermentation, and SO2 production was analyzed at
the end of fermentation. Both AWRI 1539 and 1116 were found to be relatively high H$_2$S producers (975 and 850 mg L$^{-1}$, respectively), while producing minimal concentrations of SO$_2$ (Table 3). On the other hand, no H$_2$S production was observed for AWRI 1640; however, high levels of total SO$_2$ (146 mg L$^{-1}$) were accumulated, in accordance with Cordente et al. (2009). A $\times$ C hybrids AWRI 1811 and 1812 behaved similarly to AWRI 1640, with low or even nondetectable levels of H$_2$S, respectively, and a high SO$_2$ production phenotype. On the other hand, B $\times$ C hybrids AWRI 1808, 1809, and 1810 showed an intermediate H$_2$S and SO$_2$ production phenotype when compared to their parental strains.

Volatile fingerprints of wines made with novel hybrids

Utilizing a nonchromatographic analytical method, volatile fingerprints were obtained for each of the three parental strains and the five novel hybrids. PCA of the binned mass spectroscopy data reflects the fermentation products profiles (Table 1) in that wines made with AWRI 1116 and AWRI 1539 cluster more closely together, while AWRI 1640 is clearly separated from them (Fig. 5). Wines made with each of the novel hybrids clustered intermediate to their respective parental strains. Interestingly, the A $\times$ C hybrids exhibit a volatile fingerprint
that clusters closer with that of the AWRI 1640 parental strain, while the B × C hybrids clustered more closely to the AWRI 1539 parental strain.

**Discussion**

Although natural and laboratory-bred interspecies hybrids exhibit interesting oenological traits such as intense and complex flavor production (Swiegers et al., 2009; Bellon et al., 2011), they have a low breeding potential and are generally considered as an end point for further breeding (Hawthorne & Philippson, 1994; Marinoni et al., 1999; Greig et al., 2002; Sebastiani et al., 2002).

In this study, we demonstrate that it is possible to breed interspecies hybrids to develop novel wine yeast strains with desired oenological traits. This was possible through the combination of a mass-mating strategy, the use of complementary phenotypic markers for the selection of hybrids, and the simplicity of the method used for screening of H₂S production. It is important to note that through use of a phenotype such as thermotolerance for selection, shown to be polygenic in industrial strains of *S. cerevisiae* (Marullo et al., 2009), we may have excluded some hybrids with desirable properties.

Characterization of the five selected hybrid strains using molecular biology techniques suggested a mixed inheritance of parental genomes. All hybrids contain at least a part of the genome of each of the parental strains: *S. kudriavzevii* and *S. cerevisiae* from the flavor-active parents (either AWRI 1539 or AWRI 1116), and *S. cerevisiae* from the low-H₂S-producing strain (AWRI 1640). All five selected hybrids contained both MET10 alleles from the low-H₂S-producing strain and at least one *S. cerevisiae* allele from the flavor-active parent, in addition to the *S. kudriavzevii MET10* allele. Genome stabilization in Saccharomyces interspecies hybrids has been shown to involve extensive translocation, segmental duplication, and even chromosomal loss (Antunovics et al., 2005); thus, it is reasonable to expect our novel hybrids to display extensive aneuploidy.

The presence of MET10 G176A allele in all five hybrids strongly indicated that this mutation was responsible for the low-H₂S phenotype of the strains, as demonstrated for the AWRI 1640 parental strain (Cordente et al., 2009). From these results, it is also clear that the G176A allele has a strong dominant effect, not only over other *S. cerevisiae* alleles, but also over those from *S. kudriavzevii*. Therefore, AWRI 1640 could be used in the future as a tool for production of new hybrids, with low H₂S production combined with other important industrial traits.

The main concern of the low-H₂S-producing strains is their high SO₂ production during fermentation (Cordente et al., 2009), which can cause the inhibition of malolactic fermentation by lactic acid bacteria in wines (Rankine & Bridson, 1971). With our strategy, the risk of producing high amounts of SO₂ could be reduced. Three of the five selected strains showed a significant reduction in SO₂ levels when compared with the low-H₂S parental strain. In this regard, the most oenologically suitable strain was found to be the B × C hybrid AWRI 1810, which produced the lowest amount of SO₂ (17 mg L⁻¹) of all novel hybrids and only produced 15% as much H₂S as AWRI 1539. In all wines, SO₂ was only present in the bound form, and no free SO₂ could be detected, the latter being the most antimicrobially active. This suggests that both B × C hybrids AWRI 1808 and 1809 could also be utilized.
in commercial wine production in spite of their relatively high SO2 production (80 and 67 mg L\(^{-1}\), respectively), if malolactic fermentation is not practiced (Ribereau-Gayon et al., 2006).

On the basis that fermentation kinetics and SO2 and H\(_2\)S production by the two A\(_9\)C hybrids were more similar to AWRI 1640 than to the AWRI 1116 strain, we can speculate that there has been a major contribution of the genetic background from AWRI 1640. This was consistent with the fermentation product ‘fingerprints’ as PCA shows that AWRI 1811 and 1812 wines clustered closer to the low-H\(_2\)S S. cerevisiae parental strain.

As for the B\(_9\)C hybrids, AWRI 1808 and 1809 showed intermediate fermentation kinetics and SO2/H\(_2\)S production between both parental strains; on the other hand, AWRI 1810 had faster kinetics and produced a similar amount of SO2 as the AWRI 1539 parental strain, perhaps reflecting a major contribution of the genetic material from this strain. The PCA of volatile fingerprints was consistent with the other observations for AWRI 1808 and 1809, as they clustered intermediate to the two parental strains. The volatile fingerprint for AWRI 1810, on the other hand, was least similar to either parental strain.

The genome sequence for the commercial wine yeast strain VIN7 (of which AWRI 1539 is an isolate) was recently shown to be comprised of diploid S. cerevisiae chromosomes and an almost complete complement of S. kudriavzevii chromosomes (Borneman et al., 2012). The genomic composition of AWRI 1116 remains to be determined; however, preliminary data infer an incomplete complement of S. kudriavzevii chromosomes (authors’ own unpublished data). Our observations are, therefore, in accordance with other studies, where the presence of the S. kudriavzevii genome affected aroma
compound production during alcoholic fermentation (Swiegers et al., 2009; Bellon et al., 2011). An unexpected outcome was that all novel hybrids generated in this study produced very low levels of acetic acid during fermentation, a desirable trait in wine production (Ribeiru-Gayon et al., 2006).

In conclusion, we demonstrate that wine flavor diversity associated with Saccharomyces interspecific hybrids can be combined with desirable oenological traits of S. cerevisiae mutants, through application of a mass-mating approach taking advantage of their differential phenotypic traits as selectable markers.

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References


