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Genetic characterization of strains of *Saccharomyces uvarum* from New Zealand wineries

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ABSTRACT

We present a genetic characterization of 65 isolates of *Saccharomyces uvarum* isolated from wineries in New Zealand, along with the complete nucleotide sequence of a single sulfite-tolerant isolate. The genome of the New Zealand isolate averaged 99.85% nucleotide identity to CBS7001, the previously sequenced strain of *S. uvarum*. However, three genomic segments (37–87 kb) showed 10% nucleotide divergence from CBS7001 but 99% identity to *Saccharomyces eubayanus*. We conclude that these three segments appear to have been introgressed from that species. The nucleotide sequence of the internal transcribed spacer (ITS) region from other New Zealand isolates were also very similar to that of CBS7001, and hybrids showed complete genetic compatibility for some strains, with tetrads giving four viable progeny that showed 2:2 segregations of marker genes. Some strains showed high tolerance to sulfite, with genetic analysis indicating linkage of this trait to the transcription factor *FZF1*, but not to *SSU1*, the sulfite efflux pump that it regulates in order to confer sulfite tolerance in *Saccharomyces cerevisiae*. The fermentation characteristics of selected strains of *S. uvarum* showed exceptionally good cold fermentation characteristics, superior to the best commercially available strains of *S. cerevisiae*.

1. Introduction

Wine quality is strongly influenced by the levels of secondary compounds, which are determined in part by the yeast species or strains involved in the fermentation process (Romano et al., 1997). Hence, discovering new strains and new species of yeast is the focus of much research (Fleet, 2008).

Saccharomyces uvarum (also referred to as *S. bayanus* var. *uvarum*) is closely related to the main wine yeast, *Saccharomyces cerevisiae*. Both *S. cerevisiae* and *S. uvarum* are capable of efficient fermentation and both contain diverse strains, with different genetic and metabolic characteristics. While it occurs quite commonly in certain grape-growing areas in Europe, *S. uvarum* is currently a minor species in winemaking, although it is used for cider fermentation.

The taxonomy of the species has been the subject of much confusion, with the terms “bayanus” and “uvarum” both widely used. We will follow the nomenclature of Nguyen and Gaillardin (2005) and Libkind et al. (2011), and use *S. uvarum* to refer to the diploid species represented by the strain CBS7001, whose complete genome sequence has been determined (Cliften et al., 2003). *Saccharomyces eubayanus* is used to refer to a recently discovered diploid species that is the parent of lager yeasts (Libkind et al., 2011), with *S. bayanus* and *Saccharomyces pastorianus* retained for industrial hybrids.

Strains of *S. uvarum* or their hybrids have been isolated from vineyards and wineries in Europe (Rainieri et al., 1999; Naumov, 2000; Naumov et al., 2000, 2001, 2002; Rementeria et al., 2003; Naumova et al., 2004; Le Jeune et al., 2007; Magyar and Tóth, 2011), and also recently from New Zealand (Zhang et al., 2010a). In addition, the species was recently identified from beech trees in Argentina (Libkind et al., 2011) and a range of species in Tibet (Bing et al., 2014).

S. uvarum and its hybrids are known to be cryotolerant (Rainieri et al., 1999; Salvadó et al., 2011). The oenological properties of the species and its hybrids have also been studied (Masneuf et al., 1998; Tosi et al., 2009; Masneuf-Pomarède et al., 2010). A microsatellite-

Abbreviations: PCR, polymerase chain reaction; YPD, yeast extract peptone dextrose; SGM, synthetic grape medium; ITS, internal transcribed spacer; EDTA, ethylenediaminetetraacetic acid.

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based system for genetic identification of individuals within *S. uvarum* has been reported (Masneuf-Pomarède et al., 2007), as well as studies on the chromosomal and genetic constitution of natural and industrial hybrids involving *S. uvarum* as one parent (Nguyen et al., 2000; Le Jeune et al., 2003; Rainieri et al., 2006; Dunn and Sherlock, 2008).

Here we present results of a genetic characterization of individual strains of *S. uvarum* isolated from grape juice samples from New Zealand vineyards and wineries. Species were identified using ITS sequences analysis and by their genetic compatibility with the sequenced strain of *S. uvarum*, CBS7001, and one strain was completely sequenced. In addition, we characterized the sulfite tolerance and cold fermentation capability of strains of *S. uvarum* and assessed the utility of microsatellite genotyping to identify strains.

2. Methods

2.1. Strains

Haploid marked strains derived from CBS7001 were kindly provided by Sanford Silverman of the Botstein lab (Princeton University), as follows: ACY338 (MATα *ho*::HygB *ura3*::Nat) and ACY339 (MATa *ho*::HygB *ura3*::Nat). New Zealand strains were isolated by direct plating from commercial juice or wine, after dilution as appropriate, on YPD plates (Burgers and Klein, 1986) containing chloramphenicol (100 μg/mL). In total over 300 yeast colonies were identified as *S. uvarum* by PCR amplification and restriction digestion of the ITS region (Zhang et al., 2010a). The *S. uvarum* colonies were all obtained from samples from the Marlborough region, and were derived from five different commercial juices (sampled prior to their inoculation with commercial wine yeast) or from samples taken during or at the end of five different fermentations (one wild ferment, four inoculated). For all but one of the grape juices sampled, *S. uvarum* was a minority component of the yeast flora (see Zhang et al., 2010a), but it comprised the majority of several ferment samples. A subset of 65 of these isolates was genetically characterized by microsatellite analysis and phenotyped for sulfite tolerance and growth in the cold. In addition, individual isolates were analyzed by sequencing specific loci or by crossing to CBS7001, a strain of *S. uvarum* that has been completely sequenced. The 65 isolates were as follows: eight colonies were obtained from the end of a wild ferment from Coopers Creek (labeled A1-A10), 23 colonies from a starting grape juice (prior to inoculation of a commercial yeast strain) from Delegat's winery (prefix 7), 25 colonies from a Saint Clair starting juice (prefix 8), as well as four and five colonies each from two juices from Pernod-Ricard NZ Ltd (prefixes 1 and 2).

Sulfite tolerance of strains was measured by spotting aliquots from cultures that had been grown overnight in YPD medium onto fresh YPD plates (pH 3.5 with succinate) containing 7.5–25 mM sodium sulfite; 15 mM sulfite gave clear segregation of tolerance/sensitivity for the A9xAC338 progeny.

2.2. Polymerase chain reaction

DNA extraction method was modified from Ling et al. (1995). Polymerase chain reaction (PCR) mixtures (25 μL) were set up with the following components: 2.5 μL 10× PCR buffer (including Mg^{2+}), 1 μL each of 10 μM primers, 0.5 μL of 10 mM of each nucleotide, 0.2 μL of 5 U/μL *Taq* DNA polymerase, 2 μL of template DNA and distilled water up to 25 μL. Programs used for PCR were: 5 min of 95 °C; followed by 35 cycles of 30 s of 95 °C, 30 s of 50–63 °C annealing and 60 s of 72 °C; followed by 7 min of 72 °C. The sequences of the primers used and the annealing temperatures are listed in Table 1. Yields of PCR products were assayed by agarose gel electrophoresis. Fragments were separated on 1% or 3% agarose gels made in 1× Tris-borate–EDTA buffer. Gels were stained with ethidium bromide, visualized, and photographed under ultraviolet light (302 nm). Fragment sizes were estimated by comparison against a DNA standard (1 Kb plus ladder; Invitrogen).

Table 1
Sequence of the primers and the annealing temperature used in the experiments.

Fragments amplified	Primer sequence	Annealing temperature
ITS	ITS1: TCC GTA GGT GAA CCT GCG G ITS4: TCC TCC GCT TAT TGA TAT GC	55 °C
PAD1	PAD1-F: AGT CCA ACG CAT TGA GCA G PAD1-R: TCT GTC GGG GCT AAA AGA AA	54 °C
IRC7	IRC7-F : GGC AAT CAA GAT CGG AAA GA IRC7-R :GTC AAG CTC AAG CCT GGT TC	55 °C
SSU1	L1: AAA GCG ACG TCC GCT AAG TA R1: CCC CAA GCG GTT AGT AAA CA	60 °C
FZF1	L1: TAC GGG TTG ACC ACT CCA AT R1: CAC CGC GTT CAT ATC ATC AG	60 °C
MNT2	L1: GCT TAA CCG TGA AGG TTT AT R1: TGC TCA TAA TCA TAT TCC CA	51 °C

2.3. Microsatellite analysis

The program SERV was used to select the best repeats in the genome sequence of CBS7001 for amplification (Legendre et al., 2007). Primer sequences and concentrations used in the microsatellite multiplex are given in Table 2. Amplifications were performed using the Qiagen multiplex PCR kit (#206143) with standard reaction conditions (54 °C annealing). The PCR products were assayed for their yield on agarose gels and analyzed on an Applied Biosystems DNA Sequencing 3130XL machine using Genescan software.

2.4. Purification of PCR products and sequencing

PCR products for sequencing were purified using High Pure PCR Product Purification Kit (Roche) according to the manufacturer's instructions and analyzed on an Applied Biosystems DNA Sequencing 3130XL machine. All sequences with accession numbers were sequenced completely on both strands in this way. Whole genome sequencing was performed using an Ion Torrent Personal Genome Machine.

Table 2
Sequence of the microsatellite primers used in the experiments.

Name	Repeat	Primer sequences	Comment
NB10H	TG	GTGCTCTCGATGTATATATTTATGTAG	Chromosome 10
NB10R		TGATTG-hex CAACATTCTTGCGTCACATA	
NB9F	AT	GTGCTCAAACAAGAAACTGTGGTCGT-Fam	Chromosome 15
NB9R		TGCTTTAATTTCAAGAAACA	
NB5H	GCT	GTGCTCGGTGACCGTTTCCACTATT-hex	Chromosome 2
NB5R		CACTAAGAATGATGGGGAAG	
NB6F	AAT	GTGCTCGTGGACACAAACGATAACAA-fam	Chromosome 7
NB6R		GGATAAACATCGCTCCATAG	
NB1H	ATG	GTGCTCCATGGACTTGTATGAAGCAA-hex	Chromosome 10
NB1R		GTTCGTTACCTTCAGTGCTC	
NB2F	CAA	GTGCTCCAACTTCGAAAGGATTGACT-fam	Chromosome 5
NB2R		TTGCCAATCAATATGTTCAC	
NB7H	TAT	GTGCTCGGTATTATTCCGGGGTAAAG-hex	Chromosome 16
NB7R		ATCCTTTAAGTCGCTCAGTG	
NB3F	AAC	GTGCTCAGTCAGAAATTAACCGCAAC-fam	Chromosome 15
NB3R		GGTTGGTTATGCTCATCTGT	
NB4H	TGT	GTGCTCGACATTGTAAAAGCACAGCA-hex	Chromosome 10
NB4R		ACGGGGCTTCTCTAGATATT	
NB8F	TGT	GTGCTCTGCATGAAAGATTGTAAAGG-fam	Chromosome 16
NB8R		TCCACAACGATATCAAGACA	