Metabolic Engineering of *Saccharomyces cerevisiae* to Minimize the Production of Ethyl Carbamate in Wine

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**Abstract:** *Saccharomyces cerevisiae* metabolizes arginine, one of the major amino acids in grape musts, to ornithine and urea during wine fermentations. Wine yeast strains of *S. cerevisiae* do not fully metabolize ura during grape must fermentation. Urea is secreted by yeast cells and it reacts spontaneously with ethanol in wine to form ethyl carbamate, a potential carcinogenic agent for humans. The lack of urea catabolism by yeast in wine may be ascribed to the transcriptional repression of the *DUR1,2* gene by good nitrogen sources present in the grape must. We expressed the *DUR1,2* gene under control of the *S. cerevisiae* PGK1 promoter and terminator signals and integrated this *DUR1,2* expression cassette, flanked by ura3 sequences, into the *URA3*-locus of the industrial wine yeast UC Davis 522. In vivo assays showed that the metabolically engineered industrial strain reduced ethyl carbamate in Chardonnay wine by 89.1%. Analyses of the genotype, phenotype, and transcriptome revealed that the engineered yeast 522EC is substantially equivalent to the parental 522 strain.

**Key words:** wine, ethyl carbamate, *DUR1,2*, carcinogen

Many potentially toxic substances can be present in food and beverages. Advances in detection methods have lead to the discovery of many compounds in wines, such as ethyl carbamate, that could be harmful to humans. This naturally occurring compound is considered potentially dangerous to humans, as it exhibits carcinogenic activity in a variety of laboratory animals; degradation products of this compound bind covalently to DNA and subsequently induce mutations (Bartsch et al. 1994, Hübner et al. 1997, Leithauser et al. 1990, Schlatter and Lutz 1990). Ethyl carbamate can be found in many fermented foods and beverages in the μg/L or μg/kg range (Canas et al. 1989, Ough 1976, Wittkowski 1997), and consumption of alcoholic beverages can substantially increase daily exposure to ethyl carbamate (Zimmerli and Schlatter 1991). As a consequence, regulatory authorities in several countries have imposed or suggested restrictions on the maximum concentration of this compound in wine and other alcoholic beverages.

Ethyl carbamate (EC) forms spontaneously during wine storage because of the reaction of ethanol with carbamyl compounds released during alcoholic and malolactic fermentation. Citrulline, released by lactic acid bacteria, or carbamyl phosphate can react with ethanol to form EC. However, urea released by yeast cells during alcoholic fermentation is the major precursor for EC in wine (Monteiro et al. 1989, Ough et al. 1988a). The most significant source of urea in wine is arginine (Monteiro and Bisson 1991), one of the major amino acids found in grape must and an important nitrogen source for yeast. Arginine is cleaved by arginase (encoded by the *CAR1* gene) into ornithine and urea and both of these compounds can be used as a nitrogen source by *Saccharomyces cerevisiae* (Figure 1). During wine fermentation, urea degradation does not always immediately follow arginine metabolism (Bisson 1996). Consequently, urea is progressively excreted by yeast cells and can be re-absorbed later to be utilized as a nitrogen source. Urea excretion and re-absorption both affect the amount of urea in the wine at the end of the fermentation (An and Ough 1993, Ough et al. 1988b, 1990, 1991). In musts containing higher amounts of assimilable nitrogen (especially arginine), urea can remain in the wine (An and Ough 1993, Ough et al. 1990).

*Saccharomyces cerevisiae* degrades urea in a two-step reaction yielding ammonia, which can be used to synthesize new complex nitrogenous molecules, and CO₂. Urea is first carboxylated into allophanate by urea carboxylase in...
an adenosine 5′-triphosphate (ATP) and biotin-dependant reaction. Allophanate is subsequently degraded into CO₂ and NH₄⁺ by allophanate hydrolase (Figure 1) (Whitney and Cooper 1972, 1973). Both activities are performed by a bi-functional enzyme, urea amidolysis, encoded by the \textit{DUR1,2} gene (Genbauffe and Cooper 1986). Arginine and urea are considered as secondary nitrogen sources and are used by the cell only when better ones, such as ammonia and glutamine, are no longer present in the growth medium (Cooper 1982). The ability of \textit{S. cerevisiae} to discriminate between nitrogen (N) sources is called nitrogen catabolite repression (NCR), which leads to the repression of genes encoding enzymes that degrade poor N-sources in the presence of a good N-source (Cooper 1982, Genbauffe and Cooper 1986). Distinctive regulatory mechanisms allow unique expression patterns of these genes (Dubois and Messenguy 1997, Kovari et al. 1993, Messenguy et al. 2000, Smart et al. 1996, Viljoen et al. 1992). Urea accumulation in wine could hence be the result of the incomplete degradation of arginine to ammonia and CO₂ due to a lack or lower level of \textit{DUR1,2} expression in comparison to the expression of the \textit{CAR1} gene. Therefore, constitutive expression of \textit{DUR1,2} in a wine yeast should allow for the degradation of urea produced during grape must fermentations.

We expressed the \textit{DUR1,2} gene constitutively under control of the \textit{PGK1} promoter and terminator signals in a laboratory strain of \textit{S. cerevisiae}. Under conditions that repress the expression of the endogenous gene, the recombinant urea amidolysis was functionally expressed in the laboratory strain of \textit{S. cerevisiae} during growth on ammonia at 10 times the activity level of that found in the wild type strain. The \textit{DUR1,2} expression cassette, flanked by \textit{ura3} sequences, was subsequently integrated into the \textit{URA3}-locus of the industrial wine yeast UC Davis 522 (commercially available to the wine industry as Montrachet and SC22). The metabolically engineered strain efficiently degraded urea and minimized the production of EC in Chardonnay wine by 89%. Genetic and phenotypic data as well as global gene expression patterns showed that the metabolically engineered yeast (522EC) is genetically stable and substantially equivalent to the industrial strain 522.

Materials and Methods

\textbf{Strains and media.} \textit{Saccharomyces cerevisiae} strain TCY1 (MAT\(a\), \textit{ura3}, lys2) was used for isolation of genomic DNA. \textit{Saccharomyces cerevisiae} strain GGY400 (MAT\(a\)/MAT\(a\), \textit{ura} 3-52/\textit{ura3}-52, \textit{leu}2\(\Delta\)/\textit{leu2}A1, \textit{TRP1}/\textit{trp}1\(\Delta\)63) was used for in vitro analyses of the expression cassette. The industrial wine yeast UC Davis 522 was used for the integration of the linear \textit{ura3-PGK1\_DUR1,2-\textit{PGK1,}-ura3} cassette into the \textit{URA3} locus. Yeasts were cultured in YPD medium and yeast transformants cultured aerobically at 30°C on either solid synthetic complete medium (0.17% yeast nitrogen base without ammonium sulfate and amino acids [Difco, Sparks, NV], 2% glucose, 0.5% ammonium sulfate, and all the required amino acids except for leucine) or in liquid minimum medium (0.17% nitrogen base without ammonium sulfate or amino acids, and 2% glucose; amino acids were added to compensate for auxotrophies as required; urea [0.1%], ammonium sulfate [0.1%], or glutamine [0.1%] were provided as sole nitrogen sources). Phleomycin (100 µg/mL) was added to agar plates for selection of industrial yeast transformants.

Riesling grape juice (22 Brix, pH 2.85, ammonia 79 mg/mL, FAN 112 mg/L) was obtained from Hawthorne Mountain Vineyards, Okanagan Valley, BC, Canada. The juice was treated with 0.02 mL pectinase (PEC5L; Scott Labs, Petaluma, CA) for 3 hr at 40°C. Gelatine was added to a final concentration of 0.2 g/L and incubated at 7°C for 12 hr to precipitate particulate matter. The juice was then filter-sterilized using a 0.22-µm filter (Millipore, Billerica, MA) and frozen at -20°C. No SO₂ was added to the juice. Prior to inoculation, 2.5 g/L of arginine was dissolved into the thawed juice and the pH was adjusted to 3.2 with KOH. The juice was then filter-sterilized (0.22-µm filter, Millipore).

Chardonnay grape juice (23.75 Brix, pH 3.41, ammonia 91.6 mg/L, FAN 309.6 mg/L) was obtained from Calona Vineyards, Okanagan Valley. The juice was filter-sterilized using a 0.22-µm filter (Millipore) and 1.5 mg/L of biotin was added to the Chardonnay grape juice inoculated with the modified yeast.

\textit{Escherichia coli} strain F\(\Phi\)80lacZAM15Δ(lacZY-argF) U169 recA1 endA1 hsdR17(r\(\gamma\), m\(\gamma\)) phoA supE44 \(\lambda\) thi-1 gyrA96 relA1 was grown aerobically at 37°C in LB media. \textit{Escherichia coli} transformants were grown in...
the same medium supplemented with 50 μg/mL ampicillin and used for plasmid selection. Standard procedures were used for the isolation of plasmids and genomic DNA and the transformation of E. coli (Ausubel et al. 1995) and S. cerevisiae (Gietz and Schiestl 1995).

Cloning of the DUR1,2 open reading frame and construction of expression cassette. DUR1,2 was amplified from S. cerevisiae TCY1 genomic DNA using heat-stable DNA polymerase Ex-Taq (Takara Biochemicals, Otsu, Japan). Primers 5’-TTAAAAATAATGACAGTTAGTTCGA TACA-3’ (start codon indicated in bold) and 5’-TCGAA AAAGGTATTTCATGCCAATTTAGC-3’ (antisense stop codon shown in bold) were designed to clone the resulting amplified product directly into plasmid pHVX2 between the PGK1 promoter and terminator signals (Volschenk et al. 1997) according to the DI-TRISEC method (Dietmaier and Fabry 1995). This resulted in the destruction of the EcoRI and BglII plasmid cloning sites. The sequence of the DUR1,2 gene in the resulting plasmid, pJC1, was verified by sequencing (Mobix, McMaster University, Canada), and the sequence of the deduced protein compared to that of two published urea amidolyase sequences (Feldmann et al. 1994, Genbauffe and Cooper 1991). Plasmid pJC2 (Figure 2) was constructed by cloning the Scal-BgII phleomycin resistance cassette from pUT332 (Cayla, Toulouse, France) into the BamHI and SmaI cloning sites of pJC1 (Coulon 2001).

Northern blotting. Total RNA was isolated from overnight cultures of S. cerevisiae GVY400 transformed with pJC2 (GVY400:pJC2) or pHVX2 (GVY400:pHVX2) grown in minimal medium containing ammonium sulfate as the nitrogen source, separated by electrophoresis on a 1% agarose-formaldehyde gel (Ausubel et al. 1995), transferred and fixed onto a Nylon membrane (Amersham Biosciences, Piscataway, NJ). PCR-generated double-stranded DNA probes, using primers 5’-TTCGACTCCGTTCACA TCTTTG-3’ and 5’-TGCTGGCTTTTACTGAAGA AGAG-3’ for DUR1,2, and 5’-GGCCGGATCCCATGGTCCGTAGAGGTA AAGG-3’ and 5’-GGCCGGATCCCTTAACCCGAGAACCG TATAAGG-3’ for HHF1. Prehybridization, hybridization of the probes, washing and detection was done using AlkPhos Direct Nucleic Acid Labelling and CDP-Star Detection System (Amersham).

Western blotting and biochemical analysis of the plasmid-based recombinant urea amidolyase protein. Wild type or S. cerevisiae GVY400:pJC2 or GVY400:pHVX2 were cultured to a density of A600 nm ~1.5 in minimal medium containing either ammonia or urea as the nitrogen source. Cells were washed twice in 1 volume of 0.9% NaCl, resuspended in 20 mM phosphate buffer pH 8 containing a complete protease inhibitor cocktail (Roche Molecular Biochemicals, Basel, Switzerland), and disrupted with 0.4-mm glass beads (BDH, Poole, United Kingdom). Extracts were centrifuged for 20 min at 4°C. Protein concentrations were estimated by the bichinchoninic acid method (Pierce Laboratories, Rockford, IL). Extracts were separated on an 8% SDS-PAGE gel and transferred to a polyvinylidene fluoride membrane (Bio-Rad, Hercules, CA). Chemiluminescent detection of naturally biotinylated proteins was performed using streptavadin conjugated to horseradish peroxidase (BM Chemiluminescence Blotting Kit Biotin/Streptavidin, Roche Molecular Biochemicals). A second SDS-PAGE gel was Coomassie blue-stained to confirm equal protein loading.

Urea amidolyase activity assays, on the same extracts as above, were carried out by measuring the rate of NH₄⁺ released from urea. Ammonia reacts with alkaline hypochlorite and phenol in the presence of the catalyst sodium nitroprusside to form indophenol. Urea hydrolysis reactions to release ammonia and carbon dioxide were started by addition of 20 μL crude extract to 480 μL of the reaction mixture (20 mM phosphate buffer pH 8, 5 mM MgSO₄, 80 mM KCl, 4 mM ATP, 8 mM KHCO₃, urea 0 to 10 mM, prewarmed at 30°C for 5 min). The hydrolysis reaction was allowed to continue at 30°C for 20 min then stopped by the addition of 1 mL phenol nitroprusside solution and 1 mL of alkaline hypochlorite solution (both Sigma Diagnostics, St. Louis, MO). Samples were left to incubate at 30°C for 30 min in order for phenol nitroprusside and alkaline hypochlorite to catalyze the formation of indophenol from the ammonia released. The concentration of ammonia released from urea hydrolysis is directly proportional to the absorbance of indophenol at 570 nm. The indophenol extinction coefficient was measured under our assay conditions and enzyme specific activities are given...
as nmol ammonia released/min/mg total protein. Results were corrected from background because of crude extract and substrate addition.

**Construction and integration of the linear** **ura3-PGK1_p-DUR1,2-PGK1_t-ura3 cassette into the URA3 locus of S. cerevisiae 522.** Construction of the *ura3-PGK1_p-DUR1,2-PGK1_t-ura3* cassette was done according to Volschenk et al. (2004). The *DUR1,2* expression cassette was amplified by the primers 5'-AAGGAAAAAACGGGC CGCAAAAGCTTTCTAATCACTGCTAT-3' and 5'-AAGGAAAAAACGGGCC CGCAAAAGCTTTCTAATCACTGCTAT-3' that produced a 959 bp *URA3* fragment flanked by 944 and 959 bp *URA3* sequences. Integration of the *ura3-PGK1_p-DUR1,2-PGK1_t-ura3* cassette into the *URA3* locus of the industrial wine yeast *S. cerevisiae* UC Davis 522, was obtained by co-transformation of the linear *ura3* flanked *PGK1_p-DUR1,2-PGK1_t* integration cassette and plasmid pUT332ΔURA3, which contains the Tns5ble gene for selection of phleomycin resistance (Gatignol et al. 1990, Wenzel et al. 1992). Plasmid pUT332ΔURA3 was constructed by digesting pUT332 with BglII, removal of the *URA3* sequences, and religation of the plasmid. Saccharomyces cerevisiae 522 transformants were plated out on YEG agar containing 100 μg/mL of phleomycin to minimize the number of colonies to be screened by PCR. Incubation was at 30°C for 72 hr. Colony PCR was performed on phleomycin-resistant colonies using the following primers 5'-AGCTACAGCAATTAACTTGATAAGAGTATT GAG-3' and 5'-CACAAATTACATTTGCCTGTGATAAACC TAATCAAG-3' that produced a 1059 bp fragment corresponding to the 5' end of the integrated fragment. Primers were specific for inside and outside of the cassette in order to detect integration.

Southern blots were performed using BglII-digested genomic DNA and the *URA3* probe to confirm integration of the *ura3-PGK1_p-DUR1,2-PGK1_t-ura3* linear cassette into the *URA3* locus. A 927 bp *URA3* probe and a 736 bp *DUR1,2* probe were used. The *DUR1,2* probe was generated by PCR using plasmid pDUR1,2 and primers DUR1,2probe5 (5'-TTGACTGCCTCTCCATCTTG-3') and DUR1,2F (5'-GGTCCGCTTTAAGTTAAGAG-3').

**Loss of phleomycin resistance in transformants.** Genomic DNA from 522ec- the 522 strain in which the *ura3-PGK1_p-DUR1,2-PGK1_t-ura3* linear cassette was integrated, and the host strain 522 was isolated, digested with EcoRI, and separated by electrophoresis in a 0.7% agarose gel. Following electrophoresis, the DNA was blotted onto positively charged Nylon membranes and fixed. A Southern blot was performed using a *Tns5*ble probe, generated by PCR using pUT332ΔURA3 (used for co-transformation) as template and primers PhleoF (5'-AATGACCGAACAAG CGAGC-3') and PhleoR (5'-ATACTGGTGTTGAGGACG-3'). A second Southern blot was performed using a *bla* probe, generated by PCR using pUT332ΔURA3 as template and primers AmpRF (5'-TTGCTGGCATTTTGGCCTTCTC-3') and AmpRR (5'-GTTGCGGGAAGCTAGTGA-3').

**DNA sequencing of the integrated expression cassette.** Sequencing was completed at the Nucleic Acid Protein Service Unit (NAPS Unit, University of British Columbia, Canada) using an PRISM 377 sequencer and AmpliTaq FS Dyedexoxy Terminator Cycle sequencing chemistry (Applied Biosystems, Foster City, CA). Using iPROOF DNA polymerase (Bio-Rad), two unique sets of templates spanning the entire *DUR1,2* cassette were obtained by PCR. The two complete sequences were aligned and analyzed for differences. If differences occurred, another round of PCR and sequencing of the region in doubt was run to determine the correct sequence. The sequence obtained from the genomic integrated locus was compared to previously published sequences. The published sequences for *URA3*, *PGK1_p*, and *PGK1_t* were obtained from the Saccharomyces Genome Database. The published sequences for *DUR1,2* were obtained from GenBank (M64926) and the Saccharomyces Genome Database.

**Genetic stability and phenotypic characterization of 522ec-.** The 522ec- strain was cultivated in YPD for 100 generations, after which the yeast cells were plated on YPD media and 97 random colonies were analyzed by colony PCR for the presence of the *DUR1,2* cassette. Kimax bottles (250 mL) capped with vapor locks containing 200-μL sterile-filtered Chardonnay grape juice were inoculated to a final concentration of 1 x 10^6 cells/mL with either 522 or 522ec-. Fermentations were conducted at 20°C. Fermentations (n = 3) were slowly stirred before samples were taken for growth and ethanol determination. Growth was determined by spectrophotometry (Abs 600 nm). Ethanol was measured by enzymatic analysis (K-ETOH; Megazyme International, Bray, Ireland).

**Analysis of the transcription levels of the 522 and 522ec- strains.** Cultures used for RNA extraction for microarray analyses were grown in 200 mL Chardonnay must to midlog phase (Abs 600 nm = 2.0) at 20°C. Yeast cells were rapidly harvested, washed and stored at -80°C until RNA extraction. Microarray analyses were done in triplicate, each with independently grown cells.

**RNA extraction and sample preparation.** Total RNA was extracted using the hot phenol method (Ausubel et al. 1995). Methods for poly(A)+ RNA purification, amplification and labeling, and cRNA fragmentation have been described previously (Erasmus et al. 2003).

**Hybridization, fluids, and scanning procedures.** Six oligonucleotide yeast genome arrays (YGS98; Affymetrix, Santa Clara, CA) were used as targets for hybridization. Preparation of hybridization solution, hybridization, and washing, staining, and scanning of yeast arrays were done as described by the manufacturer (Eukaryotic Arrays Gene Chip Expression Analysis and Technical Manual; Affymetrix). The following modifications to these procedures applied: hybridizations were performed at 45°C and arrays were scanned at 3 μm using a G2500A GeneArray Scanner (Agilent Technologies, Palo Alto, CA). The EukGE-WS2v4
fluidics protocol of Affymetrix MASv5.0 software was used to perform staining and washing procedures.

Data analyses. Data were analyzed using MASv5.0 and DMT (Affymetrix). All tunable parameters were set to default values (Affymetrix Statistical Algorithm Reference Guide). Only genes responding the same in all nine cross comparisons and with change p-values of <0.003 (genes with an increased call) or >0.997 (genes with a decreased call) were considered reproducible and statistically significant. The average of the Signal Log (base 2) Ratio (SLR) values were used to calculate the fold change. Genes were linked to their gene ontology (GO) annotations using the “orf_geneontology.tab” table (http://www.yeastgenome.org/gene_list.shtml).

Quantification of urea produced by yeast during fermentation of Riesling grape must. A single colony of S. cerevisiae 522 and a single colony of 522 EC were separately inoculated into 5 mL of YPD broth and grown overnight on a rotary wheel at 30°C. Cells were then subcultured into 50 mL of YPD and grown overnight at 30°C in a shaker bath (180 rpm). Cells were harvested by centrifugation (5000 x g, 4°C, 5 min) and washed once with sterile water. The cell pellets were resuspended in 5 mL of sterile water and optical densities (Abs 600 nm) were measured. Yeast cells were inoculated into sterile fermentation flasks containing 500 mL of Riesling grape juice to an optical density of 0.1 (Abs 600 nm) in triplicate. Fermentation flasks contained a magnetic stir bar and were closed with a disinfected (70% ethanol) vapor lock.

Prior to sampling, fermenting musts were stirred with a magnetic stirrer in order to obtain a homogenous sample. All samples were taken with minimal introduction of air. Using a 3-inch sterile needle (Air-Tite, Virginia Beach, VA) and a 3-mL syringe (Becton Dickinson, Franklin Lakes, NJ), the rubber stopper containing the vapor lock was pierced and ~500 μL of must was aseptically removed. The samples were immediately centrifuged and the supernatant was frozen for further analysis. Urea was measured by a Roche Urea/Ammonia assay kit. Urea determinations were done in triplicate.

Quantification of EC in wine by SPME and GC-MS. Chardonnay wine was heated at 70°C for 48 hr to stimulate EC production. A 10-mL wine sample was pipetted into a 20-mL sample vial. A small magnetic stirring bar and 3 g of NaCl were added and the vial was capped with PTFE/silicone septum. The vial was placed on a stirrer at 22°C and allowed to equilibrate, with stirring, for 15 min. A SPME fiber (65 μm carbowax/divinylbenzene) was conditioned at 250°C for 30 min before use. After sample equilibration, the fiber was inserted into the headspace. After 30 min, the fiber was removed from the sample vial and inserted into the injection port for 15 min. A blank run was performed before each sample run. Quantification was done using an external standard method.

An EC (Sigma-Aldrich, Milwaukee, WI) standard stock solution was prepared at 0.1 mg/mL in distilled H₂O containing 12% (v/v) ethanol and 1 mM tartaric acid at pH 3.1. Calibration standards were prepared with EC concentrations of 5, 10, 20, 40, 90 μg/L. The standard solutions were stored in the refrigerator at 4°C.

Ethyl carbamate in wine was quantified using an Agilent 6890N GC interfaced to a 5973N Mass Selective Detector. A 60 m x 0.25 mm i.d., 0.25 μm thickness DBWAX fused-silica open tubular column (J&W Scientific, Folsom, CA) was employed. The carrier gas was ultra-high-purity helium at a constant flow of 36 cm/s. The injector and transfer line temperature was set at 250°C. The oven temperature was initially set at 70°C for 2 min, then raised to 180°C at 8°C /min and held for 3 min. The temperature was then programmed to increase by 20°C/min to 220°C where it was held for 15 min. The total run time was 35.75 min. The injection mode was splitless for 5 min (purge flow: 5 mL/min, purge time: 5 min). The MS was operated in selected ion monitoring (SIM) mode with electron impact ionization; MS quad temperature 150°C and MS source temperature 230°C. The solvent delay was 8 min. Specific ions 44, 62, 74, 89 were monitored with a dwell time of 100 msec. Mass 62 was used for quantification against the mass spectrum of the authentic EC standard.

Results

Constitutive, functional expression of DUR1,2. Saccharomyces cerevisiae GVY400 (diploid) was transformed with pJC2 containing the S. cerevisiae DUR1,2 gene under control of the S. cerevisiae PGK1 promoter and terminator signals (Figure 3). The transformed yeast cells thus contained three copies of the DUR1,2 gene, two copies of the native chromosomal gene under control of the native DUR1,2 promoter, which is subject to nitrogen catabolite repression (NCR), and the plasmid-borne copy of DUR1,2 that is constitutively expressed under control of the PGK1 promoter and terminator signals in pJC2.

Northern blot analysis (Figure 4a) confirmed the NCR-sensitive expression of the chromosomal copies of DUR1,2 whereas the recombinant DUR1,2 transcript was abundant in GVY400:pJC2, independent of NCR. Western blot analysis (Figure 4b) showed a biotinylated protein similar in size to naturally biotinylated urea amidolyase (202 kD) detected only in cells expressing the recombinant transcript (GVY400:pJC2) but absent in control cells (GVY400:pHVX2), thereby confirming the presence of recombinant protein and absence of endogenous urea amidolyase in cells grown under NCR conditions.

Urea amidolyase activity measured in whole-cell extracts from cells grown under NCR conditions confirmed...
As expected, the endogenous activity of urea amidolyase decreased to background levels in cells grown under NCR conditions (Figure 5a,b) whereas the recombinant enzyme activity was 10-fold higher with a calculated Vmax of 53.2 nmol ammonia released/min/mg protein (Figure 5b). The affinity constant calculated for the recombinant enzyme ($K_m$ of 0.41 mM urea) agreed with the constant determined for the endogenous enzyme ($K_m$ of 0.30 mM urea) and with the previously reported constant ($K_m$ of 0.39 mM urea) for urea amidolyase (Whitney and Cooper 1973), indicating that the urea-degrading activity of the recombinant urea amidolyase is indistinguishable from the native activity (Figure 5c,d).

Construction of a commercial wine yeast strain that degrades urea efficiently. Data presented so far clearly indicate that the recombinant plasmid-based DUR1,2 gene under control of the PGK1 promoter and terminator signals (pJC2) is fully functional in S. cerevisiae cultured in a laboratory medium that prevents expression of the native DUR1,2 gene. However, plasmids are unstable in S. cerevisiae and we therefore integrated the linear ura3-PGK1-DUR1,2-PGK1-ura3 cassette (Figure 3) into the URA3 locus of the industrial wine yeast 522.
The entire integration cassette, including the homologous ura3-flanking regions and the \( \text{DUR1,2} \) expression cassette, was obtained from \( \text{S. cerevisiae} \) donor strains. Comparison of the recombinant \( \text{Dur1,2} \) protein sequence to previously reported sequences (Feldmann et al. 1994, Genbauff and Cooper 1991) revealed that three of the 1835 amino acids in the \( \text{Dur1,2} \) protein did not match one of the published sequences. These differences may well be due to strain genetic polymorphisms. These sequences were derived from different \( \text{S. cerevisiae} \) strains (S288C, RH218, and TCY1), thereby increasing the possibility of genetic polymorphisms. However, we cannot exclude that these anomalies are due to errors during the PCR amplification of DNA fragments. Alternatively, these differences can be attributed to errors in the published sequences. These changes, however, did not affect the function of the \( \text{PGK1-} \text{DUR1,2-PGK1} \) expression cassette since it was fully functional and urea was actively degraded in the \( \text{522EC} \) strain (Figure 6).

\( \text{S. cerevisiae} \) \( \text{522EC} \) decreases urea and EC production.

In vivo production and degradation of urea in Riesling grape must supplemented with 2.5 g/L of arginine by the wild type \( \text{522} \) and \( \text{522EC} \) strains are shown in Figure 6. The WT \( \text{522} \) strain produced 115.09 (±5.91) mg/L urea within 3 days, after which the urea was degraded to 42.21 (±3.33) mg/L; no further degradation of urea occurred. In contrast, the genetically enhanced \( \text{522EC} \) strain produced 27.91 (±5.82) mg/L after 3 days but continued to degrade urea until 4.08 (±1.79) mg/L remained in the wine. The metabolically engineered \( \text{522EC} \) strain thus reduced the final amount of urea produced by 90.3% compared to the WT \( \text{522} \) yeast strain.

Ethyl carbamate produced by the WT \( \text{522} \) and the \( \text{522EC} \) strains in Chardonnay grape must is shown in Table 1. The WT \( \text{522} \) strain produced 87.85 μg/L compared to 9.61 μg/L produced by \( \text{522EC} \) strain, a reduction of 89.1%.

Genetic and phenotypic characterization of the \( \text{522EC} \) strain.

The correct integration of the \( \text{DUR1,2} \) cassette into the \( \text{URA3} \) locus was confirmed with targeted Southern blots against both the \( \text{DUR1,2} \) and \( \text{URA3} \) loci of the \( \text{522EC} \) yeast (Figure 7). Also, \text{pUT332ΔURA3}, used for cotransformation with the linear \( \text{ura3-PGK1-} \text{DUR1,2-PGK1-} \text{ura3} \), was lost after continuous subculturing in nonselective media (Figure 8). Neither \text{Tn5bl}e (phleomycin resistance) nor the \text{bla} gene (ampicillin resistance in \( \text{E. coli} \)) could be detected in \( \text{522EC} \). All 97 randomly chosen colonies tested positive for the \( \text{DUR1,2} \) cassette after 100 generations of nonselective growth of \( \text{522EC} \), indicating that integration of the \( \text{ura3-PGK1-} \text{DUR1,2-PGK1-} \text{ura3} \) cassette in the \( \text{URA3} \) locus of \( \text{522EC} \) is sufficiently stable for industrial applications.

We studied the possible effect of this integration event on the transcriptome of the \( \text{522EC} \) strain. Microarray data indicate that transcription of the \( \text{DUR1,2} \) gene was up-regulated 17-fold. Furthermore, the integration event down-regulated the \( \text{THI4}, \text{PET18}, \) and \( \text{RPL9B} \) genes more than 2-fold in the \( \text{522EC} \) strain; the \( \text{URA3} \) gene was down-regulated 1.6-fold (Table 2). The introduction of the \( \text{ura3-PGK1-} \text{DUR1,2-PGK1-} \text{ura3} \) cassette in the \( \text{URA3} \) locus of \( \text{522EC} \) strain did not affect growth and ethanol production when compared to the parental \( \text{522} \) strain (Figure 9a,b).

Presence of EC in commercial wines.

To gain insight into the extent of the EC problem in commercial wines, we randomly analyzed 20 commercial wines from six major wine-producing countries. The five white wines included Riesling, Pinot Gris, and Chenin blanc, and the 15 red varietals included Pinot noir, Cabernet Sauvignon, Shiraz, Zinfandel, and Nebbiolo. GC-MS data obtained for standard EC solutions were highly reproducible (Table 3). Only six of the wines tested had potential EC levels less than 30 μg/L (Table 4), the legal limit allowed in Canada; the remaining 14 wines tested above this value. The voluntary EC limit adopted by the United States is 15 μg/L; only three of the white wines included in this study had potential EC levels below this limit but none of the red wines had EC content less than 15 μg/L.

<table>
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<tr>
<th>Table 1</th>
<th>Production of ethyl carbamate (μg/L) in Chardonnay wine produced with UC Davis 522 and 522EC.</th>
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<td>522</td>
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<tr>
<td>Replicate 1</td>
<td>89.50</td>
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<tr>
<td>Replicate 2</td>
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<td>Average (n = 3)</td>
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In 1985, the Liquor Control Board of Ontario, Canada discovered that local and imported wines and distilled beverages contained high levels of ethyl carbamate, a potent carcinogen in experimental animals and a suspected carcinogen in humans. This finding set off a chain of important events. In 1986, Canada adopted legislation limiting the maximum acceptable level of EC in wines to 30 μg/L. During 1988, the U.S. wine and distilled spirits industry set voluntary limits of EC in table wines for importation at 15 μg/L. In 1988 the U.S. National Institutes of Health National Toxicology Program (NTP) made a long-term EC study its highest priority. In 1997, the FDA, in collaboration with the Department of Viticulture and Enology at UC Davis, published an action manual to minimize EC in wines (http://vm.cfsan.fda.gov/~frf/ecaction.html). These measures included a ban on the use of urea as a source of nitrogen for grapevines or for yeast during fermentation.

Ethyl carbamate is genotoxic in vitro and in vivo (Zimmerli and Schlatter 1991). In 2004, the NTP released a report based on an extensive study and found that EC increased rates of cancer of the liver, lung, harderian gland, and hemangiosarcomas in both female and male mice. It also increased rates of cancer of the mammary gland and ovaries in female mice and the rates of skin cancer and cancer of the forestomach in male mice. In 2005, the World Health Organization and Food and Agriculture Organization Joint Expert Committee on Food Additives (JECFA) concluded that the intake of EC from food and alcoholic beverages is of concern and that mitigation measures to reduce EC in some alcoholic beverages should continue (ftp://ftp.fao.org/es/esn/jecfa/jecf64_summary.pdf).

In general, the wine industry has been responsive to recommendations, and EC levels in wines and brandy are considerably lower now than 20 years ago. However, this study showed that 14 out of 20 commercial wines from six of the major wine-producing countries contained EC levels that exceeded the legal limit of 30 μg/L in Canada; only three of the wines included in this study met the voluntary limit of 15 μg/L adopted by the United States.
This study clearly shows that NCR of the native DUR1,2 gene (Figure 4) is responsible for the inefficient hydrolysis of arginine-derived urea to ammonia and carbon dioxide during wine fermentations. Prevention of arginine degradation in S. cerevisiae was used as a strategy to prevent EC formation in sake (Kitamoto et al. 1991, Suizu et al. 1990). The researchers developed a yeast that was disrupted at the CAR1 locus and no longer capable of degrading arginine, thus preventing urea and EC from being formed. However, arginine is one of the major nitrogen sources in grape must, and the use of car1 yeast strains would likely lead to stuck fermentations or wine spoilage due to fermentation by opportunistic wild yeast strains.

Another approach to eliminate urea is by making use of a commercial preparation of urease derived from Lactobacillus fermentum; however, the efficacy of this treatment varies with the type of wine treated (Kodama et
Table 4 Maximum potential ethyl carbamate detected by GC-MS in 20 wines (after heating at 70°C for 48 hr) from six countries.

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<th>Area 1</th>
<th>Area 2</th>
<th>Area 3</th>
<th>Mean peak area</th>
<th>SD</th>
<th>RSD&lt;sup&gt;a&lt;/sup&gt; (%)</th>
<th>RF&lt;sup&gt;b&lt;/sup&gt; (μg/L)</th>
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<sup>a</sup>Relative standard deviation.

<sup>b</sup>Response factor.

The constitutive expression of the <i>S. cerevisiae DUR1,2</i> gene under control of the <i>S. cerevisiae PGK1</i> promoter and terminator signals into the <i>URA3</i> locus of the popular red wine yeast, UC Davis 522, up-regulated transcription of the <i>DUR1,2</i> gene 17-fold during the fermentation of Chardonnay grape must (Table 2). This was expected as the constitutive <i>PGK1</i> promoter is known to be a strong promoter when compared to the native inducible/repressible <i>DUR1,2</i> promoter.

Integration of the <i>DUR1,2</i> cassette had a minimal effect on global gene expression patterns in 522 EC-; no metabolic pathways were affected (Table 2) and growth and ethanol production in 522EC- were unaffected. The metabolically engineered 522 EC- strain is fully functional, degrades urea and produced Chardonnay wine with significantly less EC (9.61 μg/L) than wine produced using the parent strain (87.85 μg/L; see Table 1). A reduction of 89.1% in EC correlates well with the reduction of 90.3% in urea produced by the genetically enhanced 522 EC- strain (Figure 6).

Conclusions

The presence of EC in wine has been concern to the wine industry for more than two decades. By constitutively expressing the <i>DUR1,2</i> gene in <i>S. cerevisiae</i>, we have reduced the production of EC in wine by 89.1%. The metabolically engineered 522EC- urea-degrading yeast does not contain any antibiotic resistance marker genes or foreign DNA and is thus not transgenic. The yeast is genetically stable, and genotypic and phenotypic characterization and global gene expression patterns indicate that the 522EC- strain is substantially equivalent to the industrial 522 parental strain.

Metabolically engineered urea-degrading wine yeasts are therefore a feasible and affordable alternative to minimizing production of EC in alcoholic beverages. All of the currently used industrial wine, brandy, and sake yeast strains can be converted into urea-degrading strains, which will significantly lower the EC content of commercial wines, brandy, and sake.

Patent. An international PCT application (PCT/CA02/01719) has been submitted on the constitutive or regulated expression of the <i>DUR1,2</i> gene in yeasts to limit or prevent the formation of EC in alcoholic beverages and food. <i>Saccharomyces cerevisiae</i> 522EC- has received Generally Regarded As Safe status from the U.S. FDA.
Literature Cited


Leithauser, M.T., A. Liem, B.C. Stewart, E.C. Miller, and J.A. Miller. 1990. 1,N6-ethenodenedosine formation, mutagenicity and murine tumor induction as indicators of the generation of an electrophilic epoxide metabolite of the closely related carcinogens ethyl carbamate (urethane) and vinyl carbamate. Carcinogenesis 11:463-473.


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