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 urea 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 522^{EC-} 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 CO_2. Urea is first carboxylated into allophanate by urea carboxylase in

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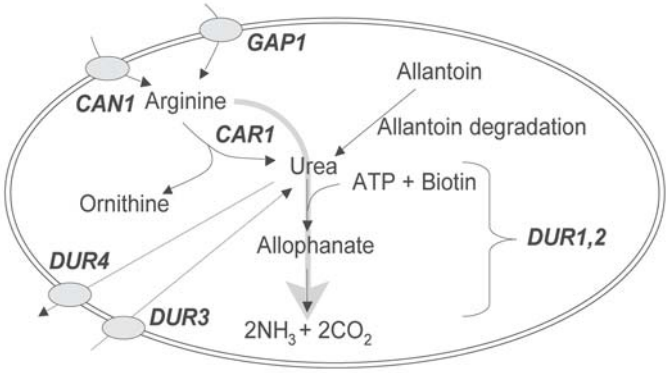

Figure 1 Arginine transport and catabolism during yeast alcoholic fermentation. Arginine is transported into *S. cerevisiae* by the general amino acid permease (Gap1p) or the arginine specific permease (Can1p). Arginase, encoded by *CAR1,* cleaves arginine into ornithine and urea. Urea can either be further catabolized to ammonia and CO_2 or excreted from the yeast. Urea degradation is an energy-dependent, two-step process catalyzed by urea amidolyase, the product of the *DUR1,2* gene. Two urea transporters exist in *S. cerevisiae*, a facilitated transporter presumably for excretion of urea from the cell down its concentration gradient, Dur4p, and an active transporter Dur3p, encoded by *DUR3,* for urea uptake when the cell requires additional nitrogen.

an adenosine 5'-triphosphate (ATP) and biotin-dependant reaction. Allophanate is subsequently degraded into CO_2 and NH_4^+ by allophanate hydrolase (Figure 1) (Whitney and Cooper 1972, 1973). Both activities are performed by a bi-functional enzyme, urea amidolyase, encoded by the *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 *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, El Berry et al. 1993, Rai et al. 1987). Although *CAR1* (arginase) and *DUR1,2* (urea amidolyase) are both subject to NCR (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 CO_2 due to a lack or lower level of *DUR1,2* expression in comparison to the expression of the *CAR1* gene. Therefore, constitutive expression of *DUR1,2* in a wine yeast should allow for the degradation of urea produced during grape must fermentations.

We expressed the *DUR1,2* gene constitutively under control of the *PGK1* promoter and terminator signals in a laboratory strain of *S. cerevisiae*. Under conditions that repress the expression of the endogenous gene, the recombinant urea amidolyase was functionally expressed in the laboratory strain of *S. cerevisiae* during growth on ammonia at 10 times the activity level of that found in the wild type strain. The *DUR1,2* expression cassette, flanked by ura3 sequences, was subsequently integrated into the *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 (522^{EC-}) is genetically stable and substantially equivalent to the industrial strain 522.

Materials and Methods

Strains and media. *Saccharomyces cerevisiae* strain TCY1 (*MAT*α*, ura3, lys2*) was used for isolation of genomic DNA. *Saccharomyces cerevisiae* strain GVY400 (*MAT*a*/MAT*α*, ura 3-52/ura3-52, leu2*Δ*1/leu2*Δ*1,TRP1/trp1*Δ*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 *ura3-PGK1*$_p$*-DUR1,2-PGK1*$_t$*-ura3* cassette into the *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/L, 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 SO_2 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 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.

Escherichia coli strain F$^-$Φ80d*lac*ZΔM15Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17(r_k^-, m_k^+) *pho*A *sup*E44 λ$^-$ *thi*-1 *gyr*A96 *rel*A1 was grown aerobically at 37°C in LB media. *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'-TTAAAAAA**ATG**ACAGTTAGTTCCGA TACA-3' (start codon indicated in bold) and 5'-TCGAA AAAGGTATT**TCA**TGCCAATGTTATGAC-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 *Eco*RI and *Bgl*II 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 *Sca*I-*Bgl*II phleomycin resistance cassette from pUT332 (Cayla, Toulouse, France) into the *Bam*HI and *Sma*I 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'-TTAGACTGCGTCTCCA TCTTTG-3' and 5'-TGCTGGCTTTACTGAAGA AGAG-3' for *DUR1,2*, and 5'-GGCCGGATCCATGTCCGGTAGAGGTA AAGG-3' and 5'-GGCCGAATTCTTAACCACCGAAACCG 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 streptavidin 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.

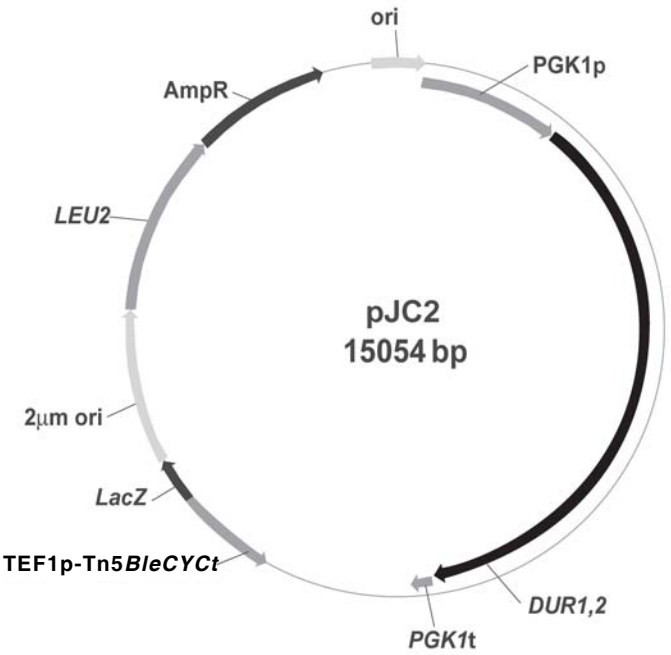

Figure 2 The shuttle plasmid pJC2 contains the *DUR1,2* gene under control of the *PGK1* promoter and terminator signals and the Tn5*Ble* gene and *bla* gene encoding resistance to phleomycin and ampicillin (in *E. coli*), respectively. pJC2 was used to transform the GVY400 laboratory yeast strain. GVY400:pJC2 thus contained three copies of the *DUR1,2* gene: the two native chromosomal genes, which are subject to NCR, and the plasmid borne *DUR1,2* constitutively expressed by the *PGK1* promoter and terminator signals in pJC2.

Urea amidolyase activity assays, on the same extracts as above, were carried out by measuring the rate of NH_4^+ 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 $MgSO_4$, 80 mM KCl, 4 mM ATP, 8 mM $KHCO_3$, 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'-AAGGAAAAAA**GCGG CCGC**AAAAGCTTTCTAACTGATCTAT-3' and 5'AAGGA AAAAA**GCGGCCGC**AAAAGCTTTAACGACGAGAAT T-3' (*Not*I sites indicated in bold) and ligated into pHVJH1 (Volschenk et al. 2004) yielding pJC3. *Srf*I digestion of pJC3 resulted in the excision of a linear 9191 bp *ura3-PGK1$_p$-DUR1,2-PGK1$_t$-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 pUT332Δ*URA3*, which contains the Tn5*ble* gene for selection of phleomycin resistance (Gatignol et al. 1990, Wenzel et al. 1992). Plasmid pUT332Δ*URA3* was constructed by digesting pUT332 with *Bgl*II, 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'-AGCTACAGCAATTAATACTTGATAAGAAGAGTATT 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 *Bgl*II-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'-TTAGACTGCGTCTCCATCTTTG-3') and DUR1,2F (5'-TGCTGGCTTTACTGAAGAAGAG-3').

Loss of phleomycin resistance in transformants. Genomic DNA from 522^{EC-}, 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 *Eco*RI, 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 Tn5*Ble* probe, generated by PCR using pUT332Δ*URA3* (used for co-transformation) as template and primers PhleoF (5'-AATGACCGACCAAG CGACG-3') and PhleoR (5'-ATCCTGGGTGGTGAGCAG-3'). A second Southern blot was performed using a *bla* probe, generated by PCR using pUT332Δ*URA3* as template and primers AmpRF (5'-TTGCGGCATTTTGCCTTCCT-3') and AmpRR (5'-GTTGCCGGGAAGCTAGAGTA-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 Dyedeoxy 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 522^{EC-}. The 522^{EC-} 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-mL sterile-filtered Chardonnay grape juice were inoculated to a final concentration of 1 x 10^6 cells/mL with either 522 or 522^{EC-}. 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 transcriptomes of the 522 and 522^{EC-} 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, fluidics, 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 H_2O 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: 5min). 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

Figure 3 The *ura3-PGK1*$_p$*-DUR1,2-PGK1*$_t$*-ura3* cassette integrated into the *URA3* locus of *S. cerevisiae* UC Davis 522. The linear cassette was co-transformed with pUT332Δ*URA3* that contains the Tn5*ble* gene that encodes for resistance to phleomycin. Colony PCR was performed on phleomycin-resistant colonies to confirm integration of the cassette at one of the *URA3* loci. During subsequent culturing on a nonselective medium, pUT332Δ*URA3* was lost.

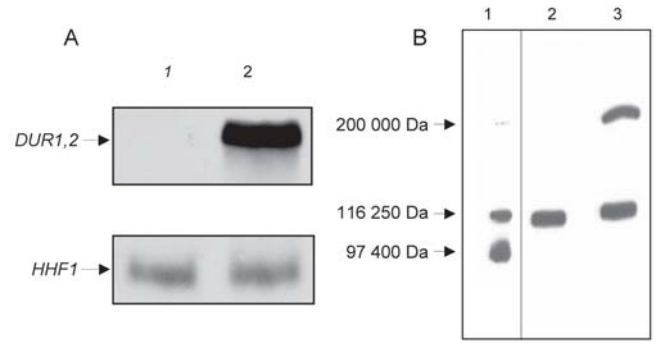

Figure 4 Up-regulation of *DUR1,2* transcription and translation in yeast engineered with the *DUR1,2* expression cassette. (**A**) *DUR1,2* expression in GVY400 cells transformed with pJC2 containing the *DUR1,2* gene under control of the *PGK1* promoter and terminator signals (lane 2) or pHVX2 containing the *PGK1* promoter and terminator signals but without the *DUR1,2* gene (lane 1) were compared by Northern analysis of total RNA using chemiluminescence. *HHFI* encoding histone 4 was used as the RNA loading standard. (**B**) Urea amidolyase expression in GVY400 cells transformed with pJC2 containing the *DUR1,2* gene (lane 3) or with pHVX2 lacking the *DUR1,2* gene (lane 2) were visualized by a Western blot of the biotinylated proteins present in total protein extracted from the transformants. Detection was by chemiluminescence onto X-Ray film using streptavidin coupled to horseradish peroxidase. High molecular weight biotinylated standards (5 µg per lane) were included as molecular weight markers (lane 1).

the expression data. 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$_p$-DUR1,2-PGK1$_t$-ura3* cassette (Figure 3) into the *URA3* locus of the industrial wine yeast 522.

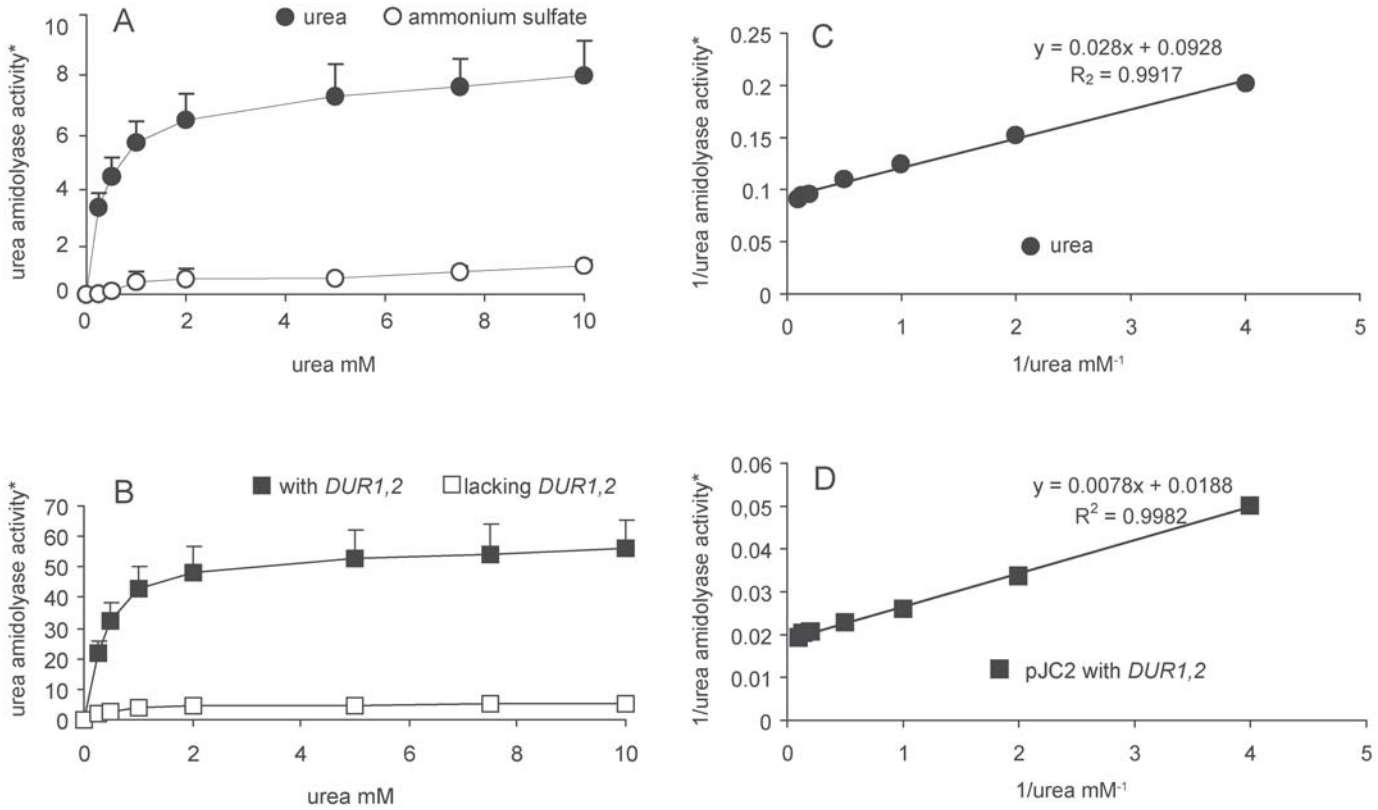

Figure 5 Urea amidolyase activity and its dependence on nitrogen source in the yeast growth media. (**A**) Enzyme activity of endogenous urea amidolyase in yeast strain GVY400 was compared between cells grown in media containing urea or ammonium sulfate as sole nitrogen source. (**B**) Recombinant urea amidolyase activity was compared to the endogenous activity in GVY400 cells transformed with pJC2 containing the *DUR1,2* gene or pHVX2 lacking the *DUR1,2* gene. All transformed cells were grown under NCR using ammonium sulfate as a nitrogen source. Double reciprocal plots were derived from data reported in (**A**) and (**B**) to determine the apparent K_m values for the endogenous (**C**) and recombinant (**D**) urea amidolyase activities, respectively. Enzyme assays were described in the text. Graphical points represent mean ± standard deviation (n = 6). *indicates nmol ammonia released/min/mg protein.

The entire integration cassette, including the homologous ura3-flanking regions and the *DUR1,2* expression cassette, was obtained from *S. cerevisiae* donor strains. Comparison of the recombinant 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 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 *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 $PGK1_p$-*DUR1,2*-$PGK1_t$ expression cassette since it was fully functional and urea was actively degraded in the 522^{EC-} strain (Figure 6).

***S. cerevisiae* 522^{EC-} 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 522 and 522^{EC-} strains are shown in Figure 6. The WT 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 522^{EC-} 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 522^{EC-} strain thus reduced the final amount of urea produced by 90.3% compared to the WT 522 yeast strain.

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

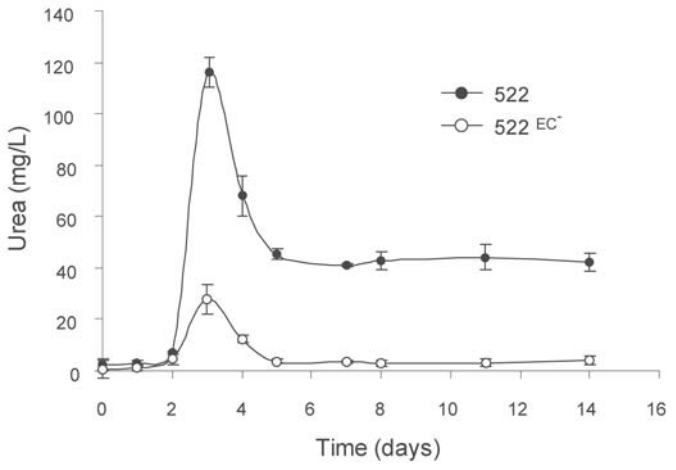

Figure 6 In vivo production and degradation of urea in Riesling grape juice by *S. cerevisiae* 522 and 522^{EC-} containing the integrated *ura3*-$PGK1_p$-*DUR1,2*-$PGK1_t$-*ura3* cassette. The industrial wine yeast 522 and the genetically enhanced 522^{EC-} derivative were grown in Riesling grape juice spiked with 2.5 g/L arginine. Data points represent means and standard deviations (n = 3).

Genetic and phenotypic characterization of the 522^{EC-} strain. The correct integration of the *DUR1,2* cassette into the *URA3* locus was confirmed with targeted Southern blots against both the *DUR1,2* and *URA3* loci of the 522^{EC-} yeast (Figure 7). Also, pUT332Δ*URA3*, used for cotransformation with the linear *ura3*-$PGK1_p$-*DUR1,2*-$PGK1_t$-*ura3*, was lost after continuous subculturing in nonselective media (Figure 8). Neither Tn5*ble* (phleomycin resistance) nor the *bla* gene (ampicillin resistance in *E. coli*) could be detected in 522^{EC-}. All 97 randomly chosen colonies tested positive for the *DUR1,2* cassette after 100 generations of nonselective growth of 522^{EC-}, indicating that integration of the *ura3*-$PGK1_p$-*DUR1,2*-$PGK1_t$-*ura3* cassette in the *URA3* locus of 522^{EC-} strain is sufficiently stable for industrial applications.

We studied the possible effect of this integration event on the transcriptome of the 522^{EC-} strain. Microarray data indicate that transcription of the *DUR1,2* gene was upregulated 17-fold. Furthermore, the integration event down-regulated only the *THI4, PET18,* and *RPL9B* genes more than 2-fold in the 522^{EC-} strain; the *URA3* gene was down-regulated 1.6-fold (Table 2). The introduction of the *ura3*-$PGK1_p$-*DUR1,2*-$PGK1_t$-*ura3* cassette in the *URA3* locus of 522^{EC-} strain did not affect growth and ethanol production when compared to the parental 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 1 Production of ethyl carbamate (µg/L) in Chardonnay wine produced with UC Davis 522 and 522^{EC-}.

	522	**522^{EC-}**
Replicate 1	89.50	9.89
Replicate 2	92.67	9.20
Replicate 3	81.38	9.74
Average (n = 3)	**87.85**	**9.61**
SD	5.82	0.36
CV (%)	6.6	3.80
% Reduction		**89.1**

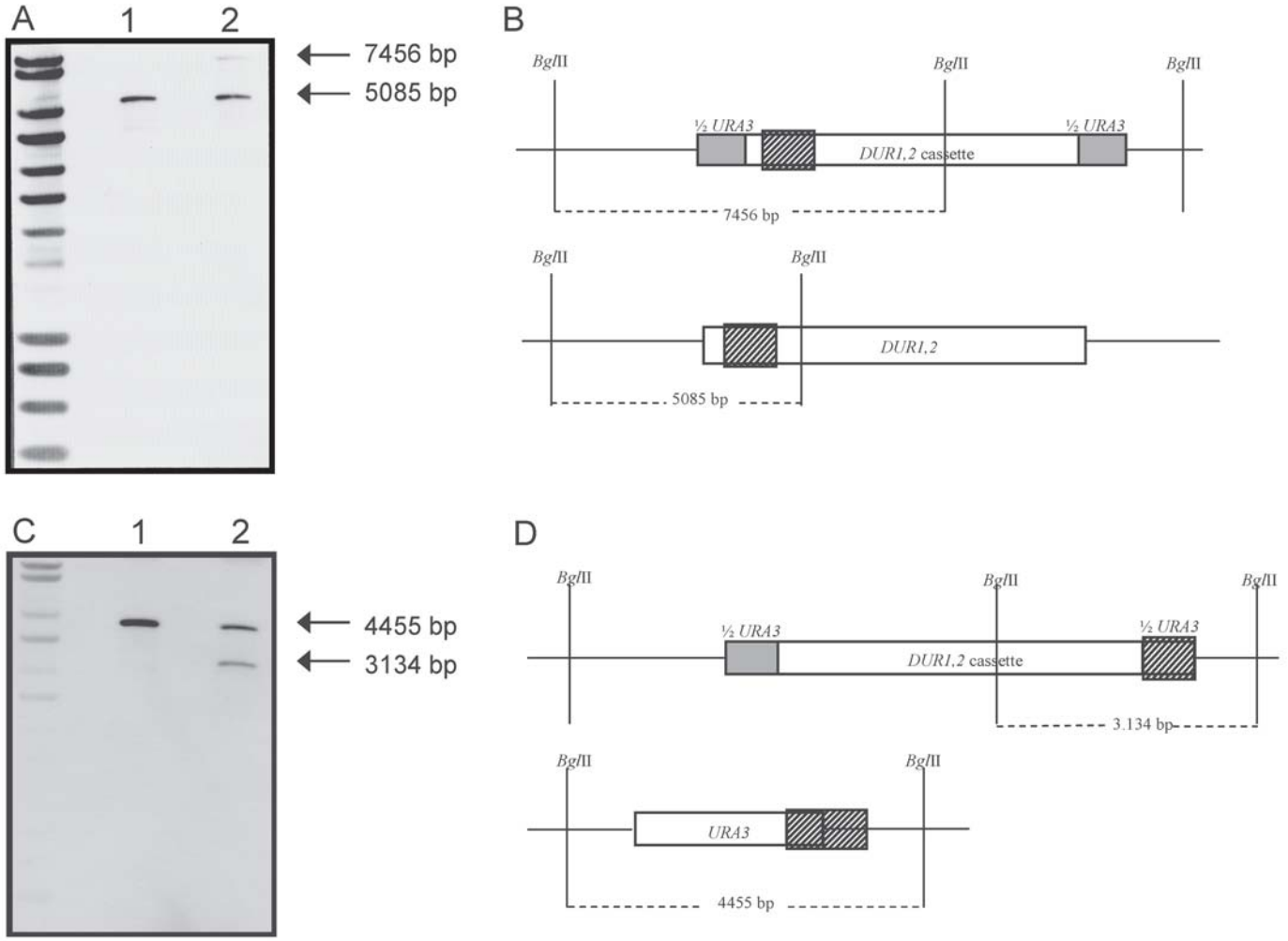

Figure 7 Southern blot confirming integration of the *DUR1,2* cassette into the *URA3* locus of 522: (**A**) *DUR1,2* probe on *Bgl*II: digested genomic DNA of (1) 522 and (2) 522^{EC-}. (**B**) Schematic representation of the integrated and wild type *DUR1,2* genes. Hatched boxes indicate the area of the *DUR1,2* gene used as probe. (**C**) *URA3* probe on *Bgl*II restriction enzyme digested genomic DNA of (1) 522 and (2) 522^{EC}. (**D**) Schematic representation of the integrated and wild type *URA3* loci. Hatched boxes indicate the area of the *URA3* gene used a probe.

Discussion

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 Heath 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/jecfa64_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.

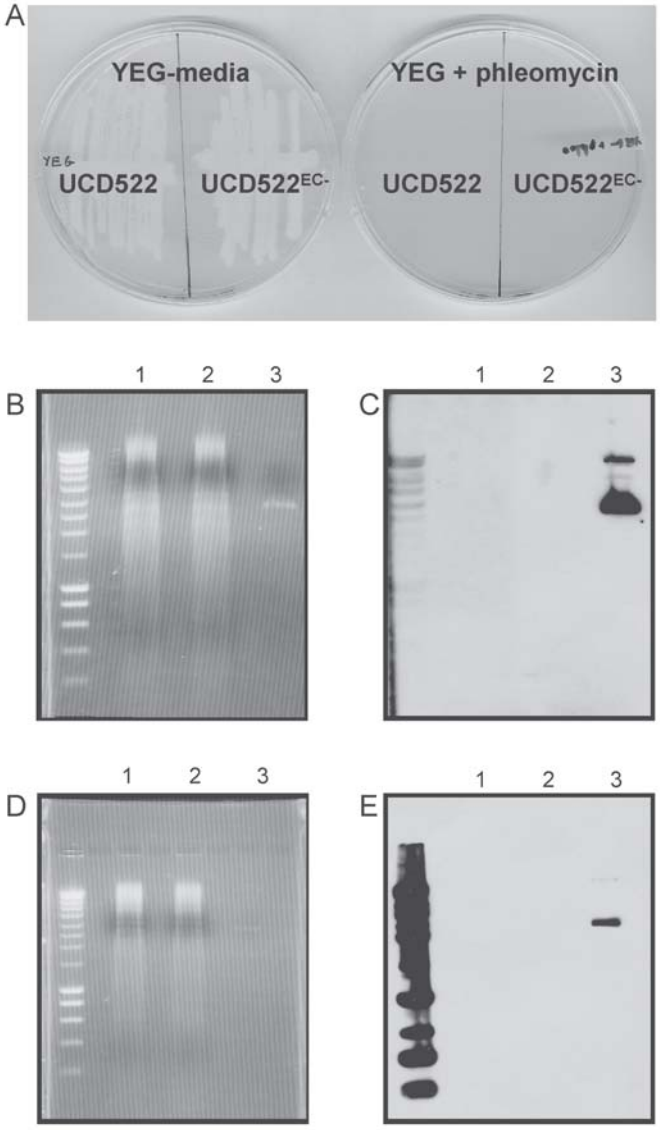

Figure 8 Phleomycin and ampicillin resistance genes are absent in the genome of 522^{EC-}. (**A**) Growth of 522 and 522^{EC-} yeast strains on YEG media with or without 100 mg/mL phleomycin. (**B**) Agarose gel showing fractionated genomic DNA of (1) 522 and (2) 522^{EC-} digested with *Eco*RI restriction enzyme and (3) pUT332Δ*URA3* containing the Tn5*ble* gene. (**C**) Southern blot probing for the presence of the Tn5*Ble* gene in the genomes of (1) 522 and (2) 522^{EC-} and (3) plasmid pUT332Δ*URA3*. (**D**) Agarose gel of fractionated genomic DNA of (1) 522 and (2) 522^{EC-} digested with *Eco*RI and (3) pUT332Δ*URA3* containing the *bla* gene. (**E**) Southern blot probing for the *bla* gene in the genomes of (1) 522 and (2) 522^{EC-} and (3) plasmid pUT332.

Table 2 Genes expressed at least 2-fold differently when comparing the transcriptomes of 522 and 522^{EC-}. One copy of the *URA3* gene was destroyed by integration of the *DUR1,2* cassette; expression of *URA3* was down-regulated 1.6-fold in 522^{EC-}.

Gene	ORF	Biological process	Average SLR[a]	Fold change[b]
DUR1,2	YBR208W	Urea metabolism	4.1	+17.2
THI4	YGR144W	Thiamine biosynthesis	-2.6	-6.0
PET18	YCR020C	Mitochondrial organization and biogenesis	-2.1	-4.2
RPL9B	YNL067W	Protein biosynthesis	-1.5	-2.9
URA3	YEL021W	Pyrimidine base biosynthesis	-0.7	-1.6

[a]Signal log ratio.
[b]Calculated from SLR.

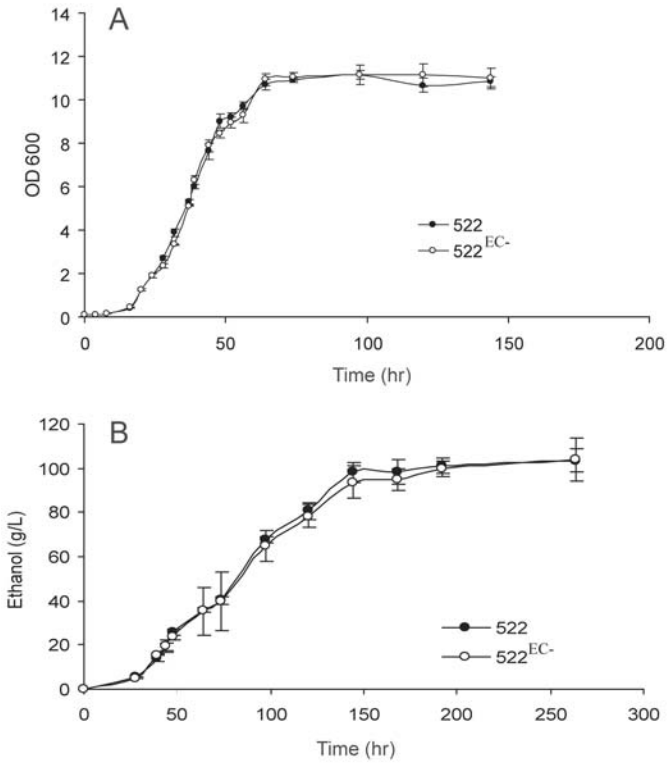

Figure 9 Growth and ethanol production by 522 and 522^{EC-} yeast strains (n = 3). (**A**) Growth of and (**B**) ethanol formation by 522 and 522^{EC-} in Chardonnay grape must fermented at 20°C were not affected by introduction of the linear *ura3-PGK1$_p$-DUR1,2-PGK1$_t$-ura3* cassette at one of the *URA3* loci in 522.

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 3 GC-MS data obtained for standard solutions of ethyl carbamate. All samples were analyzed at least in triplicate and GC-MS data were highly reproducible. The response of the GC-MS to standard solutions of EC was linear from 5 to 91 µg/L as indicated by the correlation coefficient.

Sample	Concn (µg/L)	Area 1	Area 2	Area 3	Mean area	SD	RSD[a] (%)	RF[b] (µg/L)	Corr. coeff.	Slope	Intercept
1	5.70	2524	2797	2792	2704	156	5.78	474.77	0.99932	231.770	1592.528
2	11.40	4058	4246	3842	4049	202	4.99	355.15			
3	22.80	7378	6604	7215	7066	408	5.77	309.90			
4	45.60	13201	11776	12811	12596	736	5.85	276.23			
5	91.20	23567	22338	21600	22502	994	4.42	246.73			

[a]Relative standard deviation.
[b]Response factor.

Table 4 Maximum potential ethyl carbamate detected by GC-MS in 20 wines (after heating at 70°C for 48 hr) from six countries.

Wine	Concn (µg/L)	Area 1	Area 2	Area 3	Mean peak area	SD	RSD[a] (%)
1	11.83	4274	4542	4185	4334	186	4.29
2	9.12	3649	3586	3887	3707	159	4.28
3	14.63	5043	4959	4948	4983	52	1.04
4	38.64	10262	10112	11269	10548	629	5.97
5	57.57	14342	15833	14632	14936	791	5.29
6	66.15	16610	16734	17430	16925	442	2.61
7	59.52	15890	15015	15260	15388	451	2.93
8	46.39	11208	13611	12213	12344	1207	9.78
9	55.41	13865	14820	14618	14434	503	3.49
10	24.36	7012	6808	7895	7238	578	7.98
11	44.76	11650	12576	11677	11968	527	4.40
12	41.32	11186	10952	11368	11169	209	1.87
13	27.50	7664	8366	7868	7966	361	4.53
14	38.22	10375	10471	10508	10451	69	0.66
15	57.68	14069	14839	15974	14961	958	6.41
16	33.82	9034	9428	9832	9431	399	4.23
17	51.99	14146	12880	13899	13642	671	4.92
18	18.61	5734	5935	6047	5905	159	2.69
19	34.99	9396	9295	10417	9703	621	6.40
20	36.57	9635	9756	10811	10067	647	6.43

[a]Relative standard deviation.

al. 1991). Furthermore, the enzyme is expensive and necessitates an incubation time of 8 to 12 weeks, which considerably increases delays in wine processing (Ough and Trioli 1988). Thus, while both of the above approaches seem feasible to limit EC formation in wine in theory, their practical applications have not been adopted widely by the wine industry.

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

Integration of the *DUR1,2* 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 522^{EC-} 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 *DUR1,2* gene in *S. cerevisiae*, we have reduced the production of EC in wine by 89.1%. The metabolically engineered 522^{EC-} 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 522^{EC-} 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 *DUR1,2* gene in yeasts to limit or prevent the formation of EC in alcoholic beverages and food. *Saccharomyces cerevisiae* 522^{EC-} has received Generally Regarded As Safe status from the U.S. FDA.

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