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Conversion of Wine Strains of *Saccharomyces cerevisiae* to Heterothallism

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A general method to convert homothallic strains of the yeast *Saccharomyces cerevisiae* to heterothallism is described which is applicable to genetically well-behaved diploids, as well as to strains that sporulate poorly or produce few viable and mating-competent spores. The heterothallic (*ho*) allele was introduced into three widely used wine strains through spore × cell hybridization. The resultant hybrids were sporulated, and heterothallic segregants were isolated for use in successive backcrosses. Heterothallic progeny of opposite mating type and monosomic for chromosome III produced by sixth-backcross hybrids or their progeny were mated together to reconstruct heterothallic derivatives of the wine strain parents. A helpful prerequisite to the introduction of *ho* was genetic purification of the parental strains based on repeated cycles of sporulation, ascus dissection, and clonal selection. A positive selection to isolate laboratory-wine strain hybrids requiring no prior genetic alteration of the industrial strains, coupled with a partial selection to reduce the number of spore progeny needed to be screened to isolate heterothallic segregants of the proper genotype made the procedure valuable for genetically intractable strains. Trial grape juice fermentations indicated that introduction of *ho* had no deleterious effect on fermentation behavior.

Wine yeasts are strains of *Saccharomyces cerevisiae* valued principally for their exceptional ethanol tolerance, resistance to sulfur dioxide, and ability to rapidly and efficiently ferment grape juice containing 20 to 25% sugar without producing off flavors or aromas. They share traits with related baking, brewing, and distilling strains that make them awkward subjects for genetic study and improvement (2, 39). These include homothallism, inefficient sporulation, poor spore viability, variable mating competence, undetermined chromosomal constitutions, and lack of selectable markers (37, 40).

Unlike laboratory strains, whose breeding has required efficient sporulation and mating ability, current industrial strains have existed and survived for the ability to produce bread, beer, or wine, not for a regular genetic disposition. Kielland-Brandt et al. (24) have suggested that the poor fertility of brewing strains may be the unintended consequence of continual selection for stability and constancy. Similarly, the presumed aneuploid nature of many industrial strains may simply be the result of the tolerance that *S. cerevisiae* has for an unbalanced chromosome complement (30, 35). For these reasons, production strains are rarely initially amenable to genetic analysis or manipulation.

Since the strains are homothallic, even success in inducing a diploid to sporulate will result in spore progeny that remains haploid only transiently. The major disadvantage of this condition is that only haploids are able to mate, while diploids and cells of higher ploidy—unless homozygous for *MAT*—cannot. Matings in which at least one parent is a homothallic spore can be accomplished only by laboriously pairing the cells by micromanipulation and, in the absence of selection, visually monitoring zygote formation (46). Analogous crosses between heterothallic strains are routinely done by simply mixing cells of opposite mating types and either picking a zygote by micromanipulation several hours later or selecting the hybrid on an appropriate medium. Essentially every zygote formed in such cell mixtures is a true hybrid. In contrast, zygotes formed in mass mixtures of homothallic spores and heterothallic haploid cells are not all hybrids. A significant fraction results from matings that occur between the spores and their own progeny. Identification of the desired hybrid without prior inclusion of appropriate markers is a tedious undertaking. Thus, the introduction of desirable alleles by hybridization is made even more time-consuming by the necessity of performing the requisite backcrosses to eliminate the undesirable alleles of the non-industrial parent by spore-to-cell matings. The study of the inheritance of any valued trait of industrial importance involving standard crosses will be similarly tedious.

Homothallism in *S. cerevisiae* was first described by Winge (45), who observed diploid cells in a culture grown from a single haploid spore. The cells were able to sporulate but were unable to mate. The genetic basis for homothallism has since been shown to be a single gene, of which the dominant allele confers homothallism and the recessive allele confers heterothallism (47). The mechanism by which homothallic haploid spores eventually give rise to diploid cells has already been described (19, 20). More recently, the *HO* gene product has been shown to be a site-specific endonuclease involved in catalyzing the recombination event which initiates a mating type switch (25). Homothallism among industrial strains of *S. cerevisiae* is the wild-type condition, although it is not obvious why this should be so. Assuming that diploidy is advantageous to haploidy, one would imagine that the former state could be readily reestablished through matings between the germinated meiotic products of opposite mating type without need for a switching mechanism. If spores were not formed within an ascus but were dispersed, reducing the likelihood that two of opposite mating types would be within close enough proximity to mate, then homothallism would provide a clear advantage. If spore viability were poor, which is very often the case among industrial strains, then the probability that a germinating spore will find itself in an ascus containing

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another viable spore of opposite mating type may be very low. In such a circumstance, a homothallic spore will be far more likely to re-establish the diploid state successfully than will a heterothallic one. It is also possible that the conditions under which homothallism evolved have since changed so that the former advantages are no longer obvious or even advantageous. One could explain the present predominance of homothallism in the absence of a current selective advantage if this condition is at least not disadvantageous.

To make wine yeasts more genetically accessible, we converted three commercially important strains from the homothallic to the heterothallic state after first obtaining pure-breeding derivative clones that sporulated more efficiently and produced fewer inviable spores. In this report, we describe simple, general methods to derive such clones from genetically heterogenous industrial strains and to select hybrids formed between wild-type homothallic spores and heterothallic haploids. The selection requires no prior alteration of the genotype of the industrial strain and greatly simplifies both the task of performing a series of backcrosses and that of obtaining desired segregants.

MATERIALS AND METHODS

Strains. The homothallic wine strains of *S. cerevisiae* UCD 505 (California Champagne) and UCD 595 (Pasteur Champagne) were obtained from Universal Foods Corporation, which obtained the progenitor strains from the Department of Viticulture and Enology, University of California, Davis. Strain UCD 522 (UCD Enology 522 or Montrachet) was from the wine yeast collection at the Department of Viticulture and Enology, University of California, Davis. Derivative strains 522X (36), AB2, and AB5 were obtained from the parental strains indicated below after repeated cycles of sporulation, ascus dissections, and single-spore isolations. Thus, 522X was derived from UCD 522 after two cycles, AB2 was derived from UCD 595 after five cycles, and AB5 was derived from UCD 505 after four cycles. Heterothallic haploids 2720-1d (**a** *ho ade2 cyh2*) and 2746-1b (**a** *ho ade2 cyh2 ura4* [E^R]) were obtained by tetrad dissection of appropriate diploids from our laboratory. Erythromycin resistance arose spontaneously and was originally obtained in strain 2720-1d. Strains S1795A (**a** *his4 ura1 trp5 ade6*) and S1799D (α *his4 trp5 ade6*) were obtained from the Yeast Genetic Stock Center, Department of Molecular and Cellular Biology, Division of Biophysics and Cell Physiology, University of California, Berkeley. All other strains are either hybrids formed between spores of the wine yeasts and the heterothallic haploids or meiotic segregants of these hybrids.

Media. YEPD is 1% Difco yeast extract–2% Difco Peptone–2% glucose. M is minimal medium (equivalent to 0.67% Difco yeast nitrogen base without amino acids) containing 2% glucose. SM is supplemented minimal medium containing the following additions (micrograms per milliliter): L-arginine, 20; L-histidine, 20; L-isoleucine, 30; L-leucine, 30; L-lysine, 30; L-methionine, 20; L-threonine, 200; L-tryptophan, 20; adenine sulfate, 20; uracil, 10. For diagnostic replica plates, one of the amino acids or bases was omitted from SM, or cycloheximide (Sigma Chemical Co.) was added to a final concentration of 3 μg/ml to either M or SM. When required, L-phenylalanine and L-tyrosine were added to M or SM at final concentrations of 50 and 30 μg/ml, respectively. SM plates containing SO_2 were prepared by addition of an appropriate volume of a freshly prepared 100× to 200× $K_2S_2O_5$ solution to prepoured plates (1 g of $K_2S_2O_5$ yields 0.5 g of SO_2). YEPA is 1% Difco yeast extract–2% Difco Peptone–2% potassium acetate. Sporulation medium is 2% potassium acetate. Petite medium is 1% Difco yeast extract–2% Difco Peptone–0.025% glucose–3% glycerol. M-P+ER medium is equivalent to 0.67% Difco yeast nitrogen base without amino acids containing 0.1% glucose, 3% glycerol, and 0.4% erythromycin (Sigma) added after autoclaving from a 10% stock solution in 70% ethanol. Media were solidified by addition of 1.7% agar (Moorhead).

Sporulation. Cells were streaked onto a YEPD plate and grown overnight at 30°C. On the following day, they were transferred to a YEPA plate, kept at room temperature overnight, and then transferred to solid sporulation medium, where they were incubated at room temperature for 3 to 20 days, until sporulation was adequate for tetrad dissections.

Genetic purification of homothallic wine yeasts. The sporulated wine yeasts were subjected to tetrad dissection. Spore clones that exhibited even and fast colony growth on the YEPD dissection slab were sporulated in turn, and their spore progeny was selected by the same criteria. Thus, each cycle of purification consisted of sporulation, ascus dissection, and clonal selection. The procedure was continued until segregation for growth rate was either eliminated or minimized and further improvement in spore viability was apparently unattainable.

Spore-to-cell matings. The method of Winge and Laustsen (46) was used for spore-to-cell matings. Asci were dissected as described by Johnston and Mortimer (22). Each spore of four to five dissected tetrads produced by the homothallic wine yeasts was paired with 1 to 10 cells of a heterothallic strain. Fewer cells—one to three—of an actively growing strain taken from an overnight streak on YEPD were used than when cells were taken directly from a refrigerated YEPD slant.

Detection of matings. (i) UV-stimulated mitotic crossover. Cells heterozygous for *CYH2* taken from colonies arising from the spore-cell mixtures were transferred in a standard grid pattern to a YEPD plate. A YEPD replica was irradiated with UV light a dose of 50 J/m^2, incubated for 24 h at 30°C, and replica plated to M+cycloheximide. Papillae that arose within 2 to 3 days on the cycloheximide plate were taken as evidence that mating had occurred. Cells from colonies on the unirradiated master grid corresponding to those that produced papillae on M+cycloheximide were restreaked on M. Single-colony isolates were sporulated, and 2:2 segregation for input markers was confirmed among dissected tetrads.

(ii) Direct selection. Colonies that arose from spore-cell mixtures in which the wild-type spores were erythromycin-sensitive prototrophs and the cells were erythromycin-resistant auxotrophs were streaked onto M-P+ER. Isolated colonies picked after 3 to 5 days of incubation at 30°C were sporulated, and their random spores were prepared as described below.

Generation of random spores. (i) Heterozygosity for a recessive drug marker. Asci of diploids heterozygous for *CYH2* were digested in 1 or 2% glusulase for 3 to 24 h at either room temperature or 30°C, washed twice with 5% Tween 80, suspended in phosphate buffer (0.07 M, pH 7.0), sonicated in 30- to 60-s intervals on ice for a total of 1 to 2 min, and diluted before being plated on SM plus cycloheximide.

(ii) Ether treatment. The ether treatment method of Dawes and Hardie was used (13). Sporulated cultures were transferred from solid sporulation medium to 1.5 ml of 0.2 M potassium acetate (pH 5.5) in 4-ml screw-cap tubes. After

addition of 1.5 ml of diethyl ether, the tubes were taped to a shaker platform and agitated at room temperature at 200 rpm for 20 min. Cultures were not pretreated with glusulase. The ether-treated cultures were centrifuged at 400 to 500 × *g*, washed twice with distilled water, and suspended in 2 ml of distilled water. Appropriate dilutions and platings were performed to yield less than 200 colonies on YEPD plates. All colonies from single plates were spotted onto master plates to constitute a random sample.

Detection of heterothallic segregants. Heterothallic auxotrophic segregants among the random spore or dissected tetrad progeny of hybrids were identified by complementation in matings with auxotrophic *MAT***a** and *MAT*α tester strains. Master plates containing spore-derived colonies were replica plated to separate lawns on YEPD consisting of *MAT***a** and *MAT*α tester strains carrying complementary nutritional markers. The mating plates were incubated overnight at 30°C before being replica plated to M. The heterothallic segregants always yielded confluent growth on M after 24 h when mated to one but not the other tester. In contrast, homothallic segregants showed no growth or papillae in separate matings to both testers. Heterothallic wild-type segregants were identified by microscopic observation of zygotes in mixtures of cells on YEPD with one but not the other tester. Observations were made 4 to 7 h after mixing cells taken from an overnight streak on YEPD incubated at 30°C.

Genetic analysis. Segregations for markers among the spore progeny of hybrids formed in crosses between laboratory and wine strains were detected and analyzed by standard genetic methods (26).

Fermentations. Sixteen-liter fermentations were performed in triplicate at 20°C in 20-liter glass carboys fitted with fermentation locks. The grape juice used was a 1987 Chenin blanc from Davis, Calif., harvested at 19.1°Brix (% soluble solids, which is approximately equal to % sugar), 0.87% titratable acidity, and pH 3.15. When the grapes were crushed, sulfur dioxide was added as $K_2S_2O_5$ to a final concentration of 50 mg/liter. The juice was settled overnight, racked off the lees, and kept at 0°C for 3 to 4 months before use. Starter yeast cultures were begun from overnight YEPD plates and prepared by inoculating juice samples of increasing volume and sulfur dioxide content for 3 to 4 days such that the final 5% inoculum consisted of 800 ml to which had been aseptically added 200 mg of yeast extract per liter, 200 μg of thiamine per liter; and 50 mg of sulfur dioxide per liter. The 16-liter juice lots were supplemented at the time of inoculation with a sterile mixture of powdered cellulose, yeast extract, yeast hulls, and thiamine at final concentrations of 100, 200, and 100 mg/liter and 200 μg/liter, respectively. The wines were racked off the yeast lees when successive Brix measurements indicated no further fermentative activity.

Small-scale laboratory fermentations, 100 ml or less, were performed in sidearm flasks or test tubes at room temperature without agitation.

RESULTS

Genetic purification. Obtaining pure-breeding derivative clones of the three wine strains with improved sporulation efficiency and greater spore viability was an essential preliminary step. By subjecting the strains to repeated cycles of sporulation, ascus dissection, and clonal selection, advantage was taken of their homothallism. Homozygosity at all loci except *MAT* was assured, as was the elimination of all recessive lethal mutations that may have accumulated undetected during propagation by innumerable previous mass transfers. With strain UCD 522, purified clone 522X, obtained in a previous study, sporulated as efficiently and produced viable spores at a level that was qualitatively indistinguishable from that of laboratory diploids after two cycles of purification (36). The most probable explanation for this result is that the strain is diploid (12). The two cycles of genetic purification simply permitted elimination of accumulated recessive lethal mutations. On the basis of recent genetic evidence, UCD 522 is disomic for 15 of the 17 yeast chromosomes and either disomic or trisomic for chromosome VII, whereas 522X is disomic for all 16 tested (Bakalinsky and Snow, Yeast, in press).

TABLE 1. Genetic purification of UCD 505 and UCD 595

Clone and strain	No. of dissected tetrads that yielded the following no. of colony-producing spores:					% Spore viability
	4	3	2	1	0	
Parental						
UCD 505	0	0	0	8	118	2.0[a]
UCD 595	0	0	0	6	156	0.9
First cycle						
UCD 505	7	6	3	3	15	40.4
UCD 595	0	1	2	9	7	21.0
Second cycle						
UCD 505	3	6	4	3	0	64.1
UCD 595	3	5	6	2	0	64.1
Third cycle						
UCD 505	7	2	7	2	0	69.4
UCD 595	7	1	8	1	0	70.6
Fourth cycle						
UCD 505	3	7	7	3	0	62.5
UCD 595	10	1	6	1	0	77.8
Fifth cycle						
UCD 505	ND[b]	ND	ND	ND	ND	ND
UCD 595	12	0	5	1	0	81.9

[a] This calculation is based on the finding that about 90% of the asci contained three spores and the remaining 10% contained four spores.
[b] ND, Not done.

With the two other wine yeasts, UCD 505 and UCD 595, the sporulation efficiency obtained in the genetically purified clones after four and five cycles, respectively, was qualitatively equivalent to or better than that of 522X (data not shown). However, spore viability, while improved, never attained the nearly 100% reached by 522X (Table 1). The fourth-cycle clone derived from UCD 505 has been designated AB5, and the fifth-cycle clone derived from UCD 595 is AB2. We have since determined that both parental strains are aneuploid (Bakalinsky and Snow, in press). Although one might presume that subjecting a homothallic aneuploid strain to repeated cycles of genetic purification would eventually produce progeny whose chromosome complement would be stabilized although not necessarily euploid, the persistent spore inviability that we observed suggested lingering heterogeneity. A possible explanation is presented in the Discussion.

Genealogy of the heterothallic derivatives. Heterothallic segregants from the initial outcross and all subsequent backcross hybrids were identified by complementation in matings with auxotrophic tester strains. In addition to being

heterothallic, selected segregants were also auxotrophic, at least *ade2*, and resistant to cycloheximide, *cyh2*. UCD 505 and UCD 595 derivatives were resistant to erythromycin (E^R). Inclusion of *cyh2* among UCD 522 backcross segregants allowed us to use the UV-stimulated formation of cyh^r papillae as a signal that matings had occurred. The same marker served a different purpose in the derivation of heterothallic segregants from UCD 505 and UCD 595. Since *cyh2* is recessive, it allowed us to generate random spores from the hybrids by standard methods.

(i) UCD 522. Spores of 522X were initially outcrossed to the heterothallic haploid 2720-1d. The resultant hybrids were sporulated, and the *ho ade2 cyh2* progeny of either mating type was isolated for use in subsequent backcrosses to 522X spores. The final reconstructed heterothallic derivative of UCD 522, 2799, was produced by crossing the progeny of the sixth-backcross hybrid (*MAT***a** *ho* × *MAT*α *ho ade2 cyh2*). The nearly 100% viability of spores produced by 522X allowed us to dissect tetrads from each backcross to isolate the heterothallic segregants used in successive crosses and in the final strain reconstruction. Since *cyh2* is a recessive mutation, we could not select directly for heterozygous hybrids on M+cycloheximide plates, since both the wild-type diploid and the heterozygote were equally sensitive (cyh^s). That the irradiation stimulated a mitotic crossover or gene conversion converting *CYH2*/*cyh2* heterozygotes to *cyh2*/*cyh2* homozygotes was confirmed by tetrad analysis. A single-cell isolate taken from a cyh^r papilla was sporulated and shown to segregate 2+:2− for *ADE2*:*ade2* and *HO*:*ho* but 0+:4− for *CYH2*:*cyh2*, the four spores being resistant. In contrast, a single-cell isolate taken from the corresponding colony on the unirradiated master plate segregated 2+:2− for *ADE2*:*ade2*, *HO*:*ho*, and *CYH2*:*cyh2*. The method efficiently detected hybrids and spared us the tedium of testing each of the spore-cell mixtures individually for clones segregating for input markers. Matings would not have occurred in at least half of the mixtures in which the spores would have been of the same mating type as the paired heterothallic cells. Since we isolated hybrids taken from the unirradiated master plate, we were able to avoid the potentially damaging consequences of UV irradiation. The dose used to stimulate induction of *cyh2*/*cyh2* (cyh^r) homozygotes from *CYH2*/*cyh2* (cyh^s) heterozygotes was never found to induce cyh^r clones from the diploidized cyh^s spores that failed to mate. The sole false-positive clone selected by this method failed to sporulate and was probably of the genotype *ho ADE2 cyh2*, having arisen by UV-stimulated reversion from the *ho ade2 cyh2* cells initially mixed with the spores.

Exclusive 2+:2− segregations among tetrads for each of the outcross and backcross hybrids indicated that all are disomic for chromosomes III (*MAT*), IV (*HO*), VII (*CYH2*), and XV (*ADE2*). Since only *ade2* segregants were scored for mating, complete tetrad data were not available for segregation at *MAT* or *HO*. Instead, pooled scores—**a**, α, or nonmating—for the two *ade2* segregants from each tetrad indicated that segregation at *HO* was 2*HO*:2*ho*, since half of the *ade2* segregants mated and half did not, and that segregation at *MAT* was 2**a**:2α, since half of the mating segregants were **a** and half were α. Final heterothallic versions of 522X were constructed by mating heterothallic sister spores produced by the sixth-backcross hybrid. Segregation for all markers, including *MAT*, among tetrads of 2799, the final construct that exhibited the highest spore viability, was 2+:2− in all cases (Table 2).

In preliminary 100-ml juice fermentations with UCD 522, 522X, and 2799, the latter two strains did not ferment juice—demonstrated by a failure to increase turbidity—to which 100 μg of SO_2 per ml had been added. In a subsequent effort to isolate SO_2-tolerant clones from the parents, the two sensitive strains were transferred separately from a fresh streak on YEPD to juice without SO_2. After 24 h, a sample was transferred to juice containing 25 μg/ml. When increases in turbidity and CO_2 evolution were obvious, successive transfers were made over a period of days to juice containing increasingly higher concentrations of SO_2 to a final value of 125 μg/ml. Single-cell isolates were taken from the final fermentations for use in the 16-liter trials. To determine whether there was a genetic basis for the increased resistance as opposed to purely physiologic adaptation, the resistant clones were sporulated and subjected to tetrad analysis. In both cases, segregation for SO_2 tolerance was 2+:2−. Results obtained by replica plating were identical to those obtained by visual determination of the presence or lack of vigorous fermentation in grape juice to which 100 μg of SO_2 per ml had been added. With homothallic strain 522X, the resistant segregants and the parental isolate grew on SM plates containing 200 μg of SO_2 per ml, whereas with heterothallic strain 2799, the resistant segregants and the parental isolate grew in 100 but not 200 μg of SO_2 per ml, the only two concentrations tested. These results suggest that a single, dominant mutation which confers resistance was selected. The work of Guerra et al. (16) and Thornton (43) suggests that two genes or multiple dominant genes, respectively, are involved in SO_2 tolerance. The clone of 522X used in the original outcross must have lost this or some other allele that confers resistance, since 522X has been used before in grape juice fermentations containing added SO_2 (34). We did not determine whether the mutations in 522X and 2799 are allelic.

(ii) UCD 505 and UCD 595. Spores of UCD 505 and UCD 595 were initially outcrossed to heterothallic haploid strain

TABLE 2. Tetrad analysis of the final heterothallic wine strain constructs

Strain[a] (genotype)[b] and ascal classes	No. of segregants					
	*Mat***a**: Matα	*ADE2*: *ade2*	*CYH2*: *cyh2*	*LEU2*: *leu2*	*URA4*: *ura4*	E^R: E^S
2799 (**a**/α *ho*/*ho* *ade2*/+ *cyh2*/+); 2:2	13	13	13	NS[c]	NS	NS
2896 (**a**/α *ho*/*ho* *leu2*/+ [E^R])						
2:2	17	0	0	17	4	0
3:1	0	0	0	0	4	0
4:0	0	17	17	0	9	16
0:4	0	0	0	0	0	1[d]
2921 (**a**/α *ho*/*ho* [E^R])						
2:2	6	0	2	NS	0	0
3:1	0	0	1	NS	0	1[d]
4:0	0	6	3	NS	6	5

[a] Strain 2799 was from UCD 522, 2896 was from UCD 595, and 2921 was from UCD 505.

[b] Neither parent of 2896 required uracil, but the segregation data suggest that one or both are disomic for chromosome XII, which carries *URA4*, and that at least one is heterozygous. Similarly, neither parent of 2921 was resistant to cycloheximide but the segregation data suggest that one or both are disomic for chromosome VII, which carries *CYH2*, and that at least one is heterozygous. Strains 2799, 2896, and 2921 had spore viabilities of 95.3, 94.7, and 65.8%, respectively.

[c] NS, Not scored.

[d] The E^S segregants were petite.

2746-1b. The resultant hybrids were sporulated, and *ho ade2 cyh2* [E^R] or *ho ade2 cyh2 ura4* [E^R] progeny of either mating type was isolated for use in subsequent backcrosses. As for UCD 522, construction of final heterothallic versions of UCD 505 and UCD 595 was initiated by mating heterothallic sister spores produced by the sixth-backcross hybrid. The spore viability of the first such reconstruction of UCD 505, 2910, was 62%—identical to that of the purified homothallic parent, AB5. Of 27 tetrads dissected, only 8 produced four viable spores. Since the mating phenotype of a few of the segregants was ambiguous, we performed a second reconstruction by mating heterothallic sister spores of one of the tetrads from 2910 that produced four viable spores. Tetrad data for this final construct, 2921, are shown in Table 2. With respect to its homothallic progenitor, a slight increase in spore viability and a marginal improvement in the number of tetrads that gave rise to four spore colonies was observed, i.e., 6 of 19. More importantly, an unambiguous mating phenotype was observed for all of the segregants. On the basis of the observed 2+:2−, 3+:1−, and 4+:0− segregations for cycloheximide resistance and the sensitivity of both of the parents of 2921 and of 2921 itself to cycloheximide, we conclude that at least one parent, if not both, is disomic for chromosome VII and heterozygous for *CYH2*.

Attempts to use the UV-induced cyh^r phenotype to signal matings between heterothallic haploid cells and spores of the purified UCD 505 clone, AB5, and the purified UCD 595 clone, AB2, were unsuccessful. Neither doubling the dose of irradiation to 100 J/m^2 nor pairing cells with twice the number of spores produced a different result. In consequence, a simple and more powerful direct selection based on the method used by Spencer and Spencer to select hybrids derived from "rare matings" between laboratory and industrial strains (38) was developed. The method effectively selected for hybrids in all of the crosses attempted. To isolate heterothallic, E^R, auxotrophic segregants from the selected hybrids for use in successive backcrosses, random spores were generated by plating sonicated, glusulase-treated spores onto SM+cycloheximide plates. Red colonies formed by *ade2* clones were subsequently patched onto a master grid to permit identification of the heterothallic segregants among them by complementation. Cytoplasmic inheritance of E^R was a distinct advantage, since all of the spore progeny of the hybrids was expected and indeed observed to be resistant.

Random spore and tetrad analysis of outcross and backcross hybrids suggests that in contrast to 522X, the purified homothallic versions of UCD 505 and UCD 595 (AB5 and AB2, respectively) are aneuploid (Table 3). Among random spores, the frequency of heterothallic segregants detected by a mating phenotype should have been 0.5 had the hybrids been disomic for both chromosomes III (*MAT*) and IV (*HO*). Although among progeny of some hybrids derived from UCD 505 the frequency approached 0.5, in most cases and among all of the progeny of hybrids derived from UCD 595, the frequency was significantly less. The frequency of *MAT***a** segregants appears to be approximately twice that of *MAT*α. Similar observations have been made concerning the more frequently found *MAT***a** allele among the meiotic progeny of other industrial strains and laboratory strain-industrial strain hybrids (1, 3, 4, 9, 23, 41). Among dissected tetrads produced by the last two backcross hybrids derived from UCD 505 and UCD 595, aberrant segregations for *MAT*, *CYH2*, *ADE2*, and *URA4* indicated that the marked chromosomes were present as trisomes (data not shown).

The first heterothallic reconstruction of UCD 595, 2840, produced tetrads containing nonmating progeny. Our initial assumption was that *HO* was still segregating and that at least one of the parents was only apparently heterothallic. Closer inspection of the segregation data (not shown) suggested that both parents were indeed heterothallic but that the *MAT***a** parent was disomic for chromosome III—*MAT***a**/*MAT***a**. Had the *HO* allele been segregating, it is doubtful that every tetrad would have produced two *MAT***a** segregants, as was observed. To test the possibility of disomy, both parents of 2840 were crossed to laboratory haploids and the random spore progeny of the resultant hybrids was scored for segregating markers and mating type. Segregation at *MAT* indicated that the *MAT*α parent was monosomic (data not shown). In contrast, segregation data obtained for *MAT* among progeny of the analogous cross (2902) involving the *MAT***a** parent confirmed its disomic constitution (Table 4).

Since we did not want expression of a mating phenotype obscured by disomy for chromosome III, we constructed a second derivative by crossing two heterothallic segregants from 2840. The two segregants were not sister spores, since no single tetrad yielded segregants of the proper genotype. As expected for a heterothallic **a**/α diploid, the second reconstruction, 2896, produced spores of high viability that segregated 2**a**:2α (Table 2). In addition to *MAT*, a second chromosome III marker, *leu2*, which was first discovered among the progeny of 2840 and identified by complementation, was also found to segregate 2+:2−, confirming the finding that both parents were monosomic for chromosome III. Since we did not deliberately introduce *leu2* into any of the crosses, it must have arisen spontaneously or have been

TABLE 3. Occurrence of mating segregants among random spores produced by UCD 505 and UCD 595 outcross and backcross hybrids selected on SM+cycloheximide plates[a]

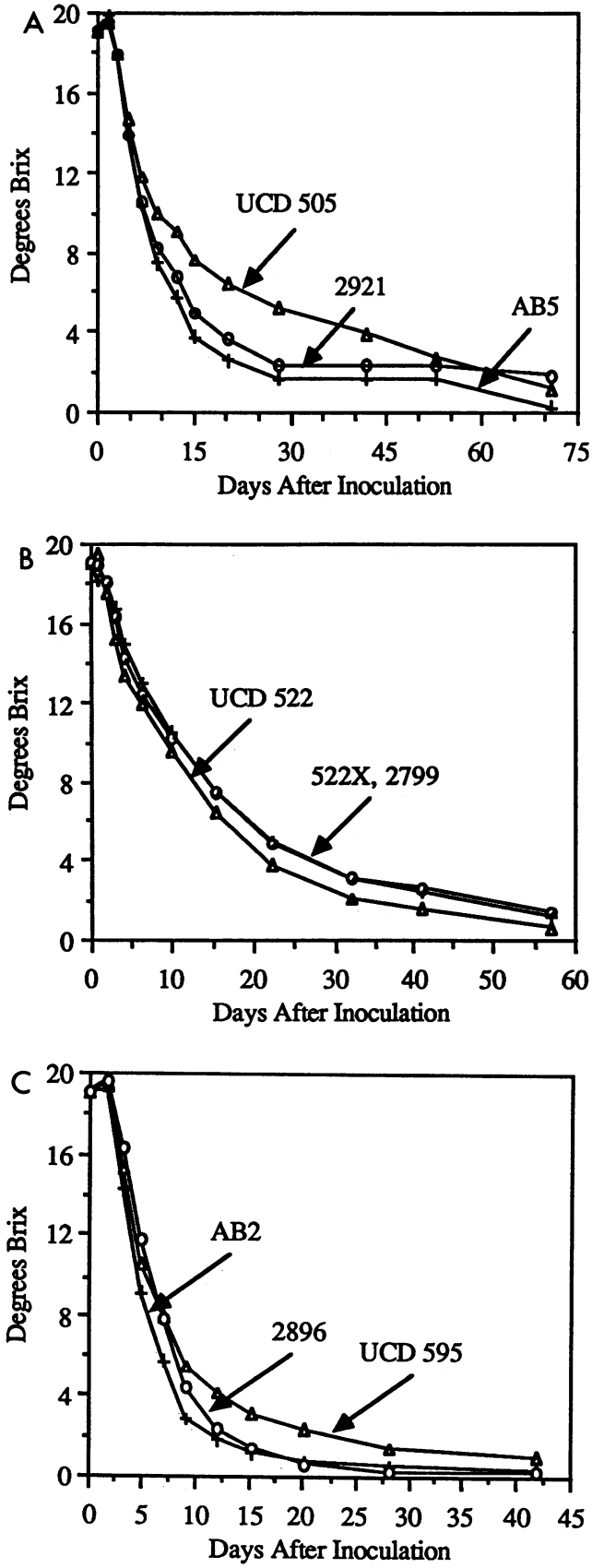
Hybrid	No. of segregants that were:					
	UCD 505 derived			UCD 595 derived		
	*MAT***a**	*MAT*α	Non-mating	*MAT***a**	*MAT*α	Non-mating
Outcross	7	3	16	4	6	54
Backcross 1	9	3	15	4	6	16
Backcross 2	4	5	25	7	3	18
Backcross 3	1	0	7	1	1	18
Backcross 4	1	0	19	8	0	68
Backcross 5	4	0	49	3	1	36

[a] Only *ade2 cyh2* segregants were scored for mating.

TABLE 4. Theoretical[a] and observed phenotypic frequencies for mating and nonmating segregants among random spores[b] of hybrid 2902[c] if it is trisomic for chromosome III[d]

Segregation	Phenotypic frequency			χ^{2e}
	a	α	Nonmating	
Chromosomal	0.50	0.17	0.33	0.50
Maximum equational	0.54	0.21	0.25	1.96
Observed	0.54	0.15	0.31	

[a] Frequencies are based on the analysis of Burnham (5).
[b] A total of 72 auxotrophic segregants were scored for mating type.
[c] Strain 2902 is 2831-14b × S1799D, a cross between the *MAT***a** parent of 2840—the first heterothallic reconstruction of UCD 595—and a laboratory haploid.
[d] *MAT***a** /*MAT***a**/*MAT*α.
[e] The χ^2 value at the 95% level of confidence for 2 degrees of freedom is 5.99.



present in heterozygous condition among the hybrid progeny containing multiple copies of chromosome III. The observed 2+:2−, 3+:1−, and 4+:0− segregations for *URA4*, a chromosome XII marker, taken together with the observation that neither parent required uracil, indicates that at least one, if not both, is disomic for chromosome XII and heterozygous for *URA4*.

Trial fermentations. Plots of degrees Brix versus time for single replicates of grape juice fermented by the parental and purified homothallic strains and the heterothallic derivatives are shown for UCD 505, UCD 522, and UCD 595 in Fig. 1. Because of an apparent nutritional deficiency in the grape juice that has since been implicated as a possible cause of stuck fermentations in other trials with other strains, none of the fermentations went to completion. In all of the trials, including those inoculated with the parental strains, residual, unfermented sugar remained in the wines. As a result, sensory evaluation of the partially fermented, oxidized wines was not undertaken. The oxidation was an unintended but inevitable consequence of having allowed the fermentations to proceed for 7 to 11 weeks in small lots in which surface area-to-volume ratios were comparatively high. The fermentations had been prolonged in the vain hope that they would eventually finish. Results of a sensory analysis of wines made from a different lot of grape juice will be reported elsewhere.

The comparative fermentations shown in Fig. 1A indicate that parental strain UCD 505 fermented significantly more slowly than either the purified homothallic derivative or the heterothallic derivative. The fermentation rates of the latter two strains did not appear to differ significantly from one another, although at the end of 70 days, the purified homothallic derivative had converted more sugar to ethanol than had its heterothallic counterpart. This suggests that genetic purification itself and not heterothallic conversion may have eliminated alleles from the homothallic parent that had a fermentation- or growth-retarding effect. The fermentation curves for UCD 522 and its derivatives (Fig. 1B) indicate that the purified homothallic and heterothallic versions fermented at identical rates, while the homothallic parental strain fermented at a slightly but insignificantly faster initial rate. Data for UCD 595 and its derivatives (Fig. 1C) showed the homothallic parent lagging slightly behind the two derivative strains after approximately 8 days. The initial rates of all three strains appeared to be very similar, although AB2 appeared to have begun fermenting sooner or slightly faster than either of the other two. After approximately 12 days, the rates of AB2 and 2896 appeared to be identical.

DISCUSSION

A method for converting homothallic wine strains to heterothallism with no apparent ill effect on fermentation behavior was developed. Aspects of the procedure are general and can be used to introduce any desirable recessive allele into production strains through hybridization. A useful

FIG. 1. Comparative grape juice fermentations. (A) Symbols: △, homothallic wine strain UCD 505; +, genetically purified homothallic derivative AB5; ○, heterothallic derivative 2921. (B) Symbols: △, homothallic wine strain UCD 522; +, genetically purified homothallic derivative 522X; ○, heterothallic derivative 2799. (C) Symbols: △, homothallic wine strain UCD 595; +, genetically purified homothallic derivative AB2; ○, heterothallic derivative 2896.

prerequisite to the initial outcross was genetic purification of the heterogeneous parental strains based on repeated cycles of sporulation, ascus dissection, and clonal selection. As a result, sporulation efficiency and spore viability were improved and recessive lethal mutations were eliminated. Since the final purified clones were essentially homozygous, we were able to evaluate the effect of introducing *ho* in a reasonably uniform genetic background. The marked improvement in spore viability made tetrad dissection productive even for strains that were initially hopeless. When further improvement in spore viability and further reduction in segregation for clonal growth rates were apparently unattainable, selected clones were sporulated and their spores were mated to heterothallic haploid cells. Use of a positive selection to isolate hybrids was found to greatly simplify the task of performing the initial crosses and the subsequent series of backcrosses. Backcrosses were undertaken to minimize the genetic contribution of the laboratory strains (used in the initial crosses) in the final heterothallic constructs.

If the wine strain spores were monosomic—which is apparently true for UCD 522 and 522X but not for UCD 505 or UCD 595 (Bakalinsky and Snow, in press)—then the genetic constitution of the first-generation hybrids and their spore progeny was 50% wine strain and 50% laboratory strain. Each subsequent backcross should theoretically have reduced the laboratory strain component by a factor of one-half so that after six backcrosses the resultant hybrids would retain less than 0.8% of the laboratory genome. First-generation hybrids formed in crosses between spores of UCD 505 and UCD 595 and the laboratory haploid had a 60% wine–40% laboratory genome based on findings concerning the chromosomal constitution of these two strains (Bakalinsky and Snow, in press). Thus, after six backcrosses, the genetic contribution of the laboratory strain in the final heterothallic versions of UCD 505 and UCD 595 was far less than in the corresponding derivatives of UCD 522 and 522X. Comparative grape juice fermentations involving the final heterothallic constructs and purified and parental homothallic strains indicated that *ho* had no deleterious effect on fermentation behavior.

Other workers have used mutagenesis to isolate heterothallic mutants from homothallic strains (21, 29). While this method has been used to obtain a heterothallic derivative from a wine yeast, it is not clear in this case whether the mutation was in the *HO* gene or in one of many other possible loci which would confer the same phenotype (14). Potentially undesirable mutations induced by the mutagen were not eliminated by a series of backcrosses. However, first-generation hybrids displayed kinetics of glucose consumption that were identical to those of the industrial parents.

Hybridization has been exploited by others as a means of introducing into production strains desirable traits controlled by one or few genes or to produce new combinations of genotypes involving crosses between segregants from different superior commercial strains. Thornton has converted a nonflocculent homothallic wine strain into a flocculent one by introducing the single dominant gene *FLO1* by mating wine strain spores with heterothallic flocculent haploid cells. Flocculent hybrids subjected to a series of backcrosses were shown to ferment grape juice as rapidly as the wine strain parent. No information was given regarding the spore viability of the latter strain, although 2+:2− segregation was observed for the input markers *FLO1* and *ade1* among dissected tetrads of the first-generation and backcross hybrids (44). Romano et al. (33) have constructed flocculent, non-H_2S-producing hybrid wine strains by spore-to-spore matings between homothallic wine strains possessing only one or the other of the two desirable traits. Both parents sporulated well, produced viable spores at high frequency, and were essentially homozygous. Instead of subjecting the parents to repeated cycles of purification, the chosen strains were selected from among many based on the relative uniformity of behavior of spore progeny with respect to valued enological traits. Hara et al. (17, 18) have had the advantage of possessing heterothallic parental strains in constructing killer wine yeasts. Crosses undertaken between haploid segregants of wine strains and those of a wild killer sake strain produced hybrid killers which, after a series of backcrosses, were shown to have retained the desirable wine-making traits of the wine strain parent. Hybridization between mating-competent spores of wine (43), brewing (11, 15), distilling (9, 10), and fuel alcohol-producing strains (42) has been used to produce hybrids with improved characteristics. Since a large and unknown number of genes is involved in controlling many of the traits considered important industrially, the hybrids so produced were evaluated empirically in small-scale fermentations.

Since two of the three parental strains into which we introduced the heterothallism allele initially sporulated poorly, produced few viable spores, and showed obvious segregation for alleles that have an effect on growth rate, we subjected the strains to a form of genetic purification involving repeated cycles of sporulation, ascus dissection, and clonal selection. This procedure helped select clones with an improved sporulation capacity and an improvement in spore viability. A qualitative and relative estimate of colonial growth of the dissected spore clones on a YEPD plate served as a basis for rejecting or choosing segregants for further purification. In retrospect, it may have been more prudent to test for ethanol tolerance and fermentation vigor in the presence of sulfur dioxide to assure the maintenance of these vital traits. As the number of purification cycles increased arithmetically, the number of prospective clones would have increased geometrically had we not deliberately limited our choice to the most promising. When it was determined that further cycles of purification resulted in no apparent improvement in either spore viability or elimination of the observed segregation for growth rate, selected clones were outcrossed to appropriately marked heterothallic laboratory haploids. While the backcrosses were performed to minimize the genetic contribution of the laboratory strain in the final heterothallic derivatives, several prospective segregants were identified for potential use in case those chosen later displayed undesirable genetic or fermentation behavior.

We believe that the aneuploid constitution of UCD 505 and UCD 595 may explain in whole or in part our inability to identify hybrids involving these two strains by use of the UV-induced cyh^r phenotype. UCD 505 is trisomic for chromosome III, which carries *MAT*, and either trisomic or tetrasomic for chromosome VII, which carries *CYH2*. UCD 595 is disomic for chromosome III and either disomic or tetrasomic for chromosome VII (Bakalinsky and Snow, in press). Thus, in addition to the spores of UCD 505 unable to mate with paired cells because they were of the same mating type were those unable to mate because they were of the genotype *MAT***a**/*MAT*α. Moreover, successful matings involving spores disomic for chromosome VII derived from either UCD 505 or UCD 595 would not have been detected by our method, since either multiple mitotic gene conversion events or a single such event, in addition to a mitotic

crossover, would have been required to produce *cyh2/cyh2/cyh2* homozygotes from the trisomic *CYH2/CYH2/cyh2* hybrid. It is also possible that the chromosome VII homologs of these strains do not share sufficient homology to chromosome VII of the laboratory strain to permit recombination to occur. Chromosomes III (24, 31), V (24, 28), X (8), XII, and XIII (32) of various lager strains of *S. cerevisiae* (referred to as *S. carlsbergensis*) either do not recombine at all or recombine very poorly with their laboratory counterparts. The positive selection for E^R, prototrophic hybrids in crosses between wild-type spores of UCD 505 and UCD 595 and E^R, auxotrophic cells on M-P+ER plates proved to be a far more powerful method. Although we paired the spores with cells by micromanipulation, this latter selection does not require it. Mass matings between haploid cells and ether-treated spore suspensions, in which all unsporulated cells (as well as a fraction of the spores) are killed, is far more convenient (Bakalinsky and Snow, in press).

All of the segregation data obtained for hybrids derived from UCD 522 are consistent with our later finding that the strain is diploid (Bakalinsky and Snow, unpublished data). Data obtained for hybrids derived from UCD 505 and UCD 595 indicating aneuploid chromosome complements are also consistent with these findings. While the aneuploidy resulted in a decrease in the frequency of heterothallic segregants of the proper genotype used in successive backcrosses (*ho ade2 cyh2* [E^R]), screening only the red clones that arose on SM+cycloheximide plates, coupled with the advantage that E^R is inherited cytoplasmically, made the search practical.

The persistent spore inviability that we observed in progeny derived from UCD 505 and UCD 595, even after repeated cycles of genetic purification, may be explained in the following manner. Based on the tolerance of *S. cerevisiae* for aneuploidy (30), the known instability of some multiple disomes (6, 7), and the large number of yeast chromosomes, (27), we postulate that multiple and repeated chromosome loss events occurring just after spore germination or after *HO*-induced diploidization may have generated both viable and inviable progeny with different aneuploid chromosome complements. Meiosis in an aneuploid strain in which some chromosomes are present in three copies gives rise to spore progeny that differ in chromosomal constitution. On average, half of the spores independently inherit a single copy and half inherit two copies of the chromosomes present in three copies in the aneuploid parent. Since the strains are all homothallic, subsequent *HO*-induced diploidization in progeny monosomic for chromosome III or disomic and homozygous for *MAT* gives rise to new aneuploid chromosome complements. If some spores are particularly slow growing because of the growth-retarding effects of their aneuploid constitution, spontaneous mitotic chromosome loss events may be selected to yield faster-growing mitotic segregants that will produce colonies consisting of a mixture of genotypes. When cells from such a colony are sporulated during repeated cycles of genetic purification, their spore progeny may undergo similar chromosome loss events, thus maintaining a form of genetic heterogeneity. Spores that do not initially undergo *HO*-induced diploidization because of heterozygosity at *MAT* may do so upon loss of one of the chromosome III homologs, resulting in a delayed source of further heterogeneity. Evidence has been obtained for frequent mitotic chromosome loss in triploid-derived spores (7, 8) and in the spore progeny of hybrids formed between laboratory haploids and spores of aneuploid wine strains UCD 505 and UCD 595 (Bakalinsky and Snow, in press). Some persistent spore inviability may be accounted for by the delayed expression of recessive lethal mutations carried on chromosomes present in multiple copies. A homothallic disomic spore heterozygous for a recessive lethal mutation would be expected to express the lethality either as a result of a gene conversion or a mitotic crossover leading to homozygosity or as a result of loss of the homolog carrying the wild-type allele. If the lethality were not expressed before *HO*-induced diploidization, the resulting tetrasome would carry the lethality in the duplex state, +/+/−/−. In a subsequent meiosis, the −/− disomic spores would be expected to be inviable.

With respect to the trial fermentations, differences between the derivative and parental strains appear to be relatively minor. When differences were noted, they indicated a greater similarity between the heterothallic derivatives and their genetically purified homothallic progenitors than between the homothallic parental strains and either the purified or heterothallic derivatives. This is not surprising, since the heterothallic clones were derived from repeated backcrosses to the purified homothallic versions of the parental strains and not to the parents themselves. The genetic purification was undertaken to rid the parental strains of alleles having growth-retarding or otherwise apparent deleterious effects on growth rate. If the purification was successful, one would almost certainly expect to observe some subsequent differences between the parental and derivative strains. At a minimum, *ho* appears not to have an obvious growth- or fermentation-retarding phenotype. This conclusion undoubtedly will be strengthened if similar findings are obtained with fermentations that do finish to produce wines that a trained sensory panel cannot distinguish from one another. The importance of subjecting the wines to sensory evaluation cannot be minimized, since we know so little about the genetic basis for many of the traits valued in wine strains. Consequently, one cannot predict in advance the unintended practical consequences of undertaking genetic alterations in the absence of trial fermentations.

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