Identification of MET10-932 and Characterization as an Allele Reducing Hydrogen Sulfide Formation in Wine Strains of Saccharomyces cerevisiae

Angela Linderholm, Kevin Dietzel,## Marissa Hirst, and Linda F. Bisson*

Department of Viticulture and Enology, University of California, Davis, Davis, California 95616

Received 12 July 2010/Accepted 25 September 2010

A vineyard isolate of the yeast Saccharomyces cerevisiae, UCD932, was identified as a strain producing little or no detectable hydrogen sulfide during wine fermentation. Genetic analysis revealed that this trait segregated as a single genetic determinant. The gene also conferred a white colony phenotype on BiGGY agar, which is thought to indicate low basal levels of sulfite reductase activity. However, this isolate does not display a requirement for S-containing amino acids, indicating that the sulfate reduction pathway is fully operational. Genetic crosses against known mutations conferring white colony color on BiGGY agar identified the gene leading to reduced H₂S formation as an allele of MET10 (MET10-932), which encodes a catalytic subunit of sulfite reductase. Sequence analysis of MET10-932 revealed several corresponding amino acid differences in relation to laboratory strain S288C. Allele differences for other genes of the sulfate reduction pathway were also detected in UCD932. The MET10 allele of UCD932 was found to be unique in comparison to the sequences of several other vineyard isolates with differing levels of production of H₂S. Replacing the MET10 allele of high-H₂S-producing strains with MET10-932 prevented H₂S formation by those strains. A single mutative change, corresponding to T662K, in MET10-932 resulted in a loss of H₂S production. The role of site 662 in sulfide reduction was further analyzed by changing the encoded amino acid at this position. A change back to threonine or to the conservative serine fully restored the H₂S formation conferred by this allele. In addition to T662K, arginine, tryptophan, and glutamic acid substitutions similarly reduced sulfide formation.

Formation of hydrogen sulfide (H₂S) by Saccharomyces cerevisiae (yeast) during fermentation has been well documented in wine, beer, and sake production (1, 10, 12, 19, 20, 25, 26, 32, 36, 53). This compound confers an odor reminiscent of rotten eggs and is considered a defect (35). Although H₂S is a volatile compound and can be removed by aeration, it has the potential and is considered a defect (35). Although H₂S is a volatile compound and can be removed by aeration, it has the potential to form ethyl mercaptan and other S-containing volatiles in wine (49), thus leading to other off-odors not easily removed from wine. Current practices for the removal of H₂S include precipitation by copper and inert gas stripping. Both of these methods are problematic. Copper in excess of legal allowable limits (0.5 mg/liter in the United States) must be removed. Volatile stripping may remove other important positive volatiles and therefore impact wine aroma in undesired ways. Prevention of the appearance of H₂S during fermentation is therefore highly desirable.

The formation of H₂S varies widely across strains (1, 20, 25, 26, 32, 42, 55). Production of H₂S ranges from 0 μg/liter to 300 μg/liter, below to well above the detection threshold of 50 to 80 μg/liter in wine (35). This dissimilarity in sulfide production levels has been attributed to differences in abilities to incor-porate reduced sulfur into organic compounds and suggests that alterations in internal enzyme regulation or activity may affect H₂S production (8, 32, 37, 38, 43, 44, 45). Several theories as to the reason for release of bound sulfide as H₂S exist. Sulfite reductase reduces sulfite to sulfide via a series of electron transfer steps. The reduced sulfide moiety can then be transferred and incorporated into O-acetyl-L-homoserine, producing homocysteine. An uncoupling of the substrate availability of O-acetyl-L-homoserine and sulfide is thought to lead to release of sulfide as H₂S (18, 19, 32, 44, 45). Under this scenario, reduced sulfur is released from the enzyme active site as H₂S. Strain variation in sulfide production would be caused by differences in dissociation kinetics or levels of activity of sulfite reductase. Alternately, reduction of sulfate may serve to balance the redox status of the cell, in which case, H₂S serves as the least toxic intermediate (23). In this case, biological demands other than amino acid biosynthesis control the activity of the sulfate reduction pathway.

In addition to meeting S-containing amino acid biosynthesis demands of the cell, sulfate reduction is needed for stress tolerance under nonproliferating conditions (2, 11, 51). Two secondary products of the formation of S-containing amino acids, glutathione and S-adenosylmethionine, are both required for stress tolerance and to buffer the redox status of the cytoplasm. The release of sulfide at this time could serve as a signal of severe environmental stress and the inability to make the required protective cofactors.

H₂S is also thought to play a role in population signaling serving to coordinate the metabolic activities of the individual
cells (23, 24, 30, 41). Hydrogen sulfide leads to an inhibition of oxidative metabolism coordinating fermentative activity. This synchronization of the yeast population may enable a more rapid domination of the yeast fermentation, as end products of sugar catabolism ethanol, carbon dioxide and heat, produced during fermentation are inhibitory to most organisms present on the surface of fruit. H$_2$S is a general inhibitor of respiration (23), and it may also serve to block oxidative metabolism of competing organisms, again fostering the dominance of Saccharomyces in the ecosystem. Given the multiple roles of S-containing metabolites and the importance of H$_2$S as a signaling molecule, the regulation of the sulfate reduction pathway is of necessity intricate (6, 7, 25, 29, 47). The complexity of regulation and variable demand for different end products of sulfate reduction provide ample opportunities for mutative alteration that would account for the observed variation in sulfide production across strains ofSaccharomyces.

Growth conditions also impact the appearance and retention of H$_2$S. Environmental and nutritional factors, such as levels of elemental sulfur (36), the presence of sulfur dioxide (44, 49) or organic compounds containing sulfur (1), nitrogen limitation (12, 19, 25), and vitamin deficiency (5, 50, 52, 54), have been associated with the production of volatile sulfur compounds. Not surprisingly, strains respond differently to these variations in growth conditions, with some producing little or no H$_2$S regardless of the condition and others producing high levels likewise regardless of the conditions (20).

Three approaches for the development of wine strains with a reduced tendency to produce H$_2$S have been taken: these are selection of natural low-production variants, reduction of sulfite reductase activity, and overexpression of enzymes downstream of sulfite reductase. Several groups have undertaken isolation and screening of naturally arising non-H$_2$S-producing strains (20, 26, 32, 37, 42, 55). Although these screens have been successful in identifying strains that do not form H$_2$S, there are problems with this approach. Genetic analysis, when performed, indicates that these traits are often quantitative, meaning multiple genes must cosegregate in crosses for the phenotype to be maintained (42). The involvement of quantitative trait loci restricts the ability to transfer this phenotype to other genetic backgrounds. Thus, these strains can be used as is, but this eliminates other desirable traits found in other genetic backgrounds. The second approach to obtaining low-sulfide-producing commercial strains involves generation of mutations, via either directed or spontaneous mutagenesis, that target sulfite reductase (32, 45). These approaches have also been successful in leading to the creation of mutants with both reduced enzyme activity and no release of sulfide. The main problem with this strategy, however, is that these changes often lead to a reduction or loss of sulfite reductase activity, generating strains that require supplementation of the medium with S-containing amino acids. Methionine and cysteine supplementation of grape juice is problematic, as under wine production conditions, these amino acids degrade to alternative S-containing volatiles that are equally objectionable as, if not more objectionable than, H$_2$S (35). Also, since wine fermentations are not conducted under sterile conditions, any genetically altered strain must be fully competitive with wild isolates that would not have an auxotrophy.

The final approach to generating commercial strains with a reduced ability to produce H$_2$S is the overexpression of enzymes downstream of sulfite reductase. Overexpression of enzymes that generate homocysteine could be expected to lead to enhanced fixation of reduced sulfur, thus resulting in more efficient transfer of reduced sulfur to organic molecules. The MET17 gene (also known as MET25) encodes O-acetyl homoserine-O-acetyl serine sulfhydrylase, forming homocysteine. Overexpression of this enzyme was found to decrease sulfide release in some but not all brewing and wine strains (34, 43). In the case of the wine strain, the strain showing a reduction in sulfide formation carried a defective allele of MET17 (21). Likewise the CYST4 gene, which encodes cystathionine beta-synthase, has been shown to reduce H$_2$S formation in brewing strains (46) but not in wine strains (21, 43).

Our strategy for generating commercial strains with reduced abilities to form H$_2$S was to likewise screen commercial and native isolates for the phenotype of inability to form sulfide under a variety of growth conditions (20, 42) and to then genetically characterize the isolates, seeking to identify one displaying simple genetic segregation properties for the trait. From screens of commercial and native yeast strain isolates (20, 42), UCD932 was identified as a strain that produces little or no detectable hydrogen sulfide under a variety of environmental conditions. This strain also forms white colonies on BiGGY agar (bismuth-glucose-glycine-yeast agar). Genetic crosses demonstrated that the traits for low-level H$_2$S production and white colony color were linked in UCD932 and that in many genetic backgrounds, the trait segregated as a single nuclear gene.

A screen of the deletion set of S. cerevisiae strains showed four possible mutations resulting in white colonies, and the relevant genes all encoded components of sulfite reductase (22) and were therefore candidate genes for the mutation in UCD932. The sulfite reductase enzyme complex is composed of catalytic and regulatory subunits. The catalytic subunits form an α$_2$β$_2$ core tetramer, with the α subunit encoded by the MET10 gene and the β subunit encoded by MET5. Activity also requires the two regulatory subunits encoded by MET17 and MET8 (48). The catalytic tetramer binds two flavin adenine dinucleotide (FAD) molecules, two flavin mononucleotide (FMN) molecules, and two siroheme molecules (48). Three molecules of NADPH are required to reduce one molecule of sulfite to sulfide for incorporation into amino acids. Earlier steps of sulfate reduction require ATP, making this a metabolically expensive pathway to operate.

As reported herein, genetic crosses revealed that the BiGGY agar white colony phenotype of UCD932 was due to an alteration of the MET10 gene. Subsequent analysis led to the identification of MET10-932 as a genetic alteration also leading to reduced H$_2$S formation in this strain. The specific genetic change of the Met10 protein responsible for this phenotype was identified. Allele swap experiments demonstrated that MET10-932 was sufficient in several genetic backgrounds to reduce sulfide formation to levels below detection.

MATERIALS AND METHODS

Yeast strains, culture conditions, and allele designations. The yeast strains used for this study are listed in Table 1. Yeast strains were maintained and grown on yeast extract-peptone-dextrose medium with 2% glucose (YPD) (40). The same medium (YPD) with Geneticin (G418; 0.2 mg/ml) or hygromycin (Hph; 0.3
mg/ml) was used for maintenance of deletion strains carrying the G418s (KanMX) or HphMX marker. Minimal YNB medium was made similar to YNB medium but without the methionine. MET10 alleles were given allele designations based upon the strain of origin, as follows: MET10-288 (from YKR069W), MET10-932 (from YFR030W), and MET10-939 (from YJR137C). MET10-940 (from UCD942), MET10-950 (from UCD950), and MET10-957 (from UCD957) were designated allele. For the screening experiments, lead acetate strips as previously described were used (20).

Fermentation conditions. The synthetic grape juice medium minimal must medium (MMM) (42) was used in fermentation experiments with either 208 mg or 123 mg of nitrogen equivalents (NE)/liter. A value of 208 mg NE/liter was used in all of the fermentation trials with the exception of the amino acid substitution analysis, in which the lower nitrogen level was used to better differentiate the moderate H2S producers. The nitrogen level was generated using 0.2 g of l-arginine/liter and 0.5 g or 0.1 g of ammonium phosphate/liter. Fermentations were initiated at a density of 1.3 x 10^5 cells/ml by inoculation with stationary-phase cells from a culture pregrown in MMM. Fermentations were conducted in either 500-ml or 250-ml Erlenmeyer flasks containing 300 ml or 150 ml of medium, respectively, depending upon the experiment. Control experiments indicated that levels of weight loss and sulfide formation were identical at these two volumes. Each flask was outfitted with a silicone stopper with a lead acetate tube attached. The flasks were incubated at 25°C with shaking at 120 rpm. Fermentations were monitored for 7 days by using weight loss as an estimate of CO2 production. Replica fermentations varied in weight by less than 10% in these studies. Uninoculated controls were run simultaneously to account for weight loss due to evaporation, which was less than 1% of the total weight lost over the time course of fermentation. For the screening experiment of the modified alleles, 10-ml medium volumes were used and samples were incubated on a roller drum to maintain suspension of the culture.

Hydrogen sulfide production. H2S was measured by using lead acetate columns purchased from Figasa International Inc. (Seoul, South Korea) that allow quantitative assessment of H2S formation over time. Fermentations were conducted in duplicate or triplicate, and weights of replicates did not differ by >10%. Hydrogen sulfide was monitored for 7 days in MMM (synthetic juice medium) (42) with strains transformed with the empty vector or carrying the designated allele. For the screening experiments, lead acetate strips as previously described were used (20).

Colony color screen of yeast strains. Yeast strains were screened on BigGY agar (bismuth-glucose-glycine-yeast agar) (31) supplemented with Casamino Acids as reported previously (22). Each strain was plated onto BigGY agar and incubated at 30°C for 48 h. The resulting colonies were assessed for color.

Sequence analysis. The sequences of MET10, HOM2, HOM6, SER33, MET1, MET5, and MET8 were determined in native and industrial strains of yeast. Chromosomal DNA was extracted from the cell pellets by using the smash and grab protocol (16), and amplification of the genes was carried out using high-fidelity Platinum Taq (Invitrogen, Carlsbad, CA) and primers PCR-MET10-F/HOM2-F/HOM6-F/HOM6-R for HOM6, SER33-R/SER33-R for SER33, MET1-F/MET1-R for MET1, MET5-F/MET5-R for MET5, and MET8-F/MET8-R for MET8 (see Table S1 in the supplemental material). Amplification conditions were as follows: 30 cycles of 94°C for 1 min, 94°C for 30 s, 50°C for 30 s, 68°C for 4 min, and a final extension at 68°C for 7 min. All sequencing was carried out at the College of Biological Sciences.
RESULTS

Identification of MEI0 as the gene responsible for low-level H,S formation in UCD932. UCD932, a native strain isolated from an Italian vineyard (28) displayed an inability to produce both H,S and white colonies on BiGGY agar. UCD932 was sporulated, and all four spores of three tetrads analyzed yielded white colonies and failed to produce H,S, indicating that this trait was homozygous in UCD932. In order to define the number of genes in UCD932 responsible for the white colony color and low-level H,S formation, a stable haploid derivative of UCD932 was created by knocking out the HO gene, preventing diploidization of spores. Spore derivatives were crossed against the dependent parent strains, BY4741 and BY4742, which yield tan colonies, and against UCD950, a strain that yields brown colonies on BiGGY agar. A total of 74 complete tetrads were examined (17 from the cross with BY4741, 18 from the cross with BY4742, and 39 from the cross with UCD950). The white colony phenotype showed normal Mendelian (2:2) segregation in all tetrads, indicating that a single gene was responsible for the white colony phenotype in UCD932. The white colony phenotype cosegregated with the loss of production of H,S in all 74 tetrads, suggesting that the same gene was responsible for both phenotypes.

White colony formation is a characteristic trait of mutations of one of the four genes encoding subunits of sulfite reductase, MEI1, MEI5, MEI8, or MEI10 (22), and loss of sulfite reductase activity has been shown to be inversely correlated with H,S formation (8, 32); thus, it was likely that UCD932 carried a mutation in one of the subunits of sulfite reductase. However, UCD932 was not auxotrophic for methionine or cysteine and growth was not stimulated by the addition of either of these amino acids (data not shown). Therefore, the strain did not contain a loss-of-function allele of one of these genes. Haploid derivatives of UCD932 were crossed with strains with either met1Δ, met8Δ, met5Δ, or met10Δ and assessed for the appearance of white colony color. If the deletant strain was able to complement the defect in UCD932, a pigmented colony would be expected. If there was no complementation, the colony color would remain white, indicating that the knockout allele was in the same gene as the original mutation resulting in white diploid colonies. Only the diploids derived from crosses of UCD932 with the met10Δ deletant strain YFR030W BY4742 displayed white colonies on BiGGY agar, indicating that UCD932 likely contained a mutation in MEI10.

To assess the dominance of this allele, UCD932 was transformed with a plasmid carrying MEI0-288, the MEI0 allele from S288C (pAL51). For controls, this strain was also transformed with the pYC130 vector and with a construct carrying its native MEI0-932 allele (pAL52). The maximum fermentation rates (determined as described in Table 4) for UCD932 with the pYC130 vector, pAL51, and pAL52 were 0.44, 0.45, and 0.41 g/l, respectively. Transformation of UCD932 with MEI0-288 resulted in tan colonies, suggesting that the white colony phenotype of MEI0 is recessive. However, these diploids contained the low-sulfide-production phenotype of UCD932 (no detectable discoloration of the lead acetate column), suggesting that this phenotype may be dominant in this cross. This finding was not surprising, as S288C is itself a low-level to moderate producer of H,S, and suggests that het-
H2S producers with tan colonies on BiGGY agar, genes of the pathway (Table 3). There were no differences in each carried unique alleles.

Thus, further analysis was needed to confirm the role of MET10 reduction pathway in UCD932 and to evaluate the allele at the locus, several additional genes from the sulfate reduction pathway, HOM2, HOM6, SER33, MET1, MET5, MET8, and MET10, were sequenced from UCD932 as well as from nine other native and industrial strains, UCD522, UCD934, UCD938, UCD939, UCD940, UCD942, UCD950, UCD956, and UCD957, that differ in color on BiGGY agar and in H2S production in synthetic juice (Table 2) (21).

Sequence analysis of genes in the sulfate reduction pathway. UCD932 was previously shown to carry mutations in CYS4 and MET6, both encoding enzymes of the sulfate reduction pathway downstream of sulfite reductase (21). However, introducing wild-type alleles of these genes into the UCD932 genetic background did not alter the low-H2S-production phenotype (21). In order to assess the genetic diversity of the sulfate reduction pathway in UCD932 and to evaluate the allele at the MET10 locus, several additional genes from the sulfate reduction pathway, HOM2, HOM6, SER33, MET1, MET5, MET8, and MET10, were sequenced from UCD932 as well as from nine other native and industrial strains, UCD522, UCD934, UCD938, UCD939, UCD940, UCD942, UCD950, UCD956, and UCD957, that differ in color on BiGGY agar and in H2S production in synthetic juice (Table 2) (21).

Sequence analysis of MET10 demonstrated that this gene shows higher allelic variation among yeast strains than other genes of the pathway (Table 3). Six different alleles were found in the 10 strains that were analyzed. No strain carried an allele identical to that of the laboratory reference strain S288C (MET10-288). UCD934, UCD957, and UCD950, H2S producers with tan to brown colonies on BiGGY agar, carried the same allele. UCD938 and UCD942, producers of low to moderate levels of H2S yielding tan colonies on BiGGY agar, also carried identical alleles. UCD522 and UCD940, H2S producers with brown colonies on BiGGY agar, were heterozygous at the MET10 locus, but both alleles in each strain were identical to those found in other strains. UCD932 and UCD956, non-H2S producers with white colonies on BiGGY agar, and UCD939, an H2S producer with tan colonies on BiGGY agar, each carried unique alleles.

Allelic differences were also detected in some of the other genes of the pathway (Table 3). There were no differences in the corresponding amino acids or DNA sequences of HOM2 (encoding aspartic β-semialdehyde dehydrogenase), one amino acid difference in the gene products of HOM6 (encoding homoserine dehydrogenase) in UCD932, and one amino acid difference in the gene products of SER33 (encoding 3-phosphoglycerate dehydrogenase) for S288C and all of the other wine strains and several amino acid differences between their MET1, MET5, and MET8 gene products (all components of the sulfite reductase enzyme) (Table 3). Thus, the sulfite reductase subunits display less conservation and greater genetic variation than other steps of the pathway. Naturally arising differences in alleles of the subunits of sulfite reductase may therefore explain the variation in sulfide formation observed among wine strains.

**Role of MET10 alleles in H2S production.** The genetic diversity of MET10 alleles and the apparent correlation with H2S production supported the hypothesis that this subunit of sulfite reductase may be responsible for the H2S phenotype in...
UD932. The effect of the specific MET10 allele on H$_2$S production in different genetic backgrounds was therefore evaluated. The MET10 alleles of H$_2$S-producing yeast strains were replaced with the allele MET10-932. The native MET10 genes in UCD950, UCD940, UCD522, and UCD932 were deleted using either the KanMX or HphMX cassette as a selectable marker. Drug-resistant diploids were then sporulated to obtain strains homozygous for the disruption of the native MET10 gene. Strains carrying the knockout alleles were then transformed with the native (as a control) or nonnative alleles selecting for methionine prototrophy. All of the strains carrying MET10-932 fermented at the same rate as the parental and control strains but became non-H$_2$S producers (Tables 4 and 5). The strains carrying an allele from S288C or their own allele maintained their H$_2$S-producing phenotype.

UDC522 is a commercial wine strain that has been characterized as aneuploid and has difficulty sporulating (3). Therefore, to evaluate the impact of MET10-932 in this genetic background, both native MET10 alleles needed to be individually disrupted in the diploid and the diploid transformed directly with a functional MET10 allele. Diploids prototrophic for methionine could then be sporulated and H$_2$S production evaluated in tetrads displaying four viable spores (Table 5). The UCD522 strain carrying the double knockout of MET10 (met10Δ::KanMX/met10Δ::HphNT) was transformed with MET10-522, MET10-288, or MET10-932. The resulting heterozygous diploids were then sporulated and tetrads displaying four viable spores taken for analysis of both fermentation rate and H$_2$S production. Data for one representative tetrad for each transformation are presented in Table 5. The strains fermented to completion and behaved as expected in terms of H$_2$S production. The MET10-932 and MET10-288 insertions displaced the hygromycin resistance cassette, and the MET10-522 insertion displaced the Geneticin resistance cassette in these tetrads. All spores from each tetrad were able to complete fermentation, although the maximal fermentation rates differed somewhat. More replicates would be needed to determine if these observed differences are statistically significant in the null alleles. There were no differences in the fermentation rates for the methionine prototrophs carrying different MET10 alleles. Spores carrying a deletion allele (methionine auxotrophs) or the MET10-932 allele were non-H$_2$S producing. The presence of either MET10-288 or MET10-522 resulted in production of H$_2$S. Thus, allele swap of the native MET10 gene with MET10-932 was sufficient to confer the phenotype of nonproduction of H$_2$S in UCD522 similar to the case in UCD940 and UCD950.

**Identification of the residue responsible for elimination of H$_2$S production in MET10-932.** The MET10 gene of UCD932 was sufficient to confer the phenotype of loss of sulfide formation to high-sulfide-producing native isolates and the commercial strains. The MET10-932 allele contained eight base pair substitutions leading to changes in amino acid sequences in relation to S288C. The alleles of MET10 from UCD932 and UCD950 differ by six nucleotides; five of those differences result in changes in the primary protein sequence: amino acid 172 is an alanine in UCD950 and S288C but a threonine in UCD932, amino acid 511 is a threonine in both UCD950 and S288C but a threonine in UCD932, amino acid 662 is a threonine in both UCD950 and S288C but a threonine in UCD932, and amino acid 1278 is a histidine in both UCD950 and S288C but a glutamate in UCD932. The sixth dissimilarity, an A/G difference at nucleotide 1278, did not result in a change of amino acid.

UDC950 is a high-level producer of H$_2$S regardless of growth conditions, whereas UCD932 is a non-H$_2$S producer likewise regardless of growth conditions (42); thus, comparing the differences of these two alleles should identify the precise substitutions conferring the property of nonrelease of H$_2$S to the MET10 polypeptide. To this end, each of the single-base-pair differences of the MET10 alleles from UCD932 and

<table>
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<th>Strain</th>
<th>Allele</th>
<th>Maximum fermentation rate (g/h)</th>
<th>Total H$_2$S (µg)</th>
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<td></td>
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</table>

$^a$ Values represent the averages of independent determinations of two replicates. All fermentations reached dryness (defined by <0.5% sugar remaining). The maximum fermentation rate was calculated from the fermentation rate data by using time points corresponding to the steepest decline in weight.

$^b$ $<1$ means no detectable discoloration of the lead acetate column was observed.

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<table>
<thead>
<tr>
<th>Spore designation</th>
<th>MET10 allele</th>
<th>Maximum fermentation rate (g/h)</th>
<th>Total H$_2$S (µg)</th>
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<tr>
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$^a$ Values represent the averages of independent determinations of two replicates. All fermentations reached dryness (defined by <0.5% sugar remaining). The maximum fermentation rate was calculated from the fermentation rate data by using time points corresponding to the steepest decline in weight.

$^b$ A, B, C, and D designate different spores of the same tetrad; there are three tetrads, each derived from an independent transformation event to methionine prototrophy. The basal medium contained methionine, and no further supplement was added.

$^c$ <1 means no detectable discoloration of the lead acetate column was observed.
UCD950 was systematically converted to the base of the opposite allele by using site-directed mutagenesis, including as a control the neutral change of base pair 1278 (Table 6). The resulting alleles were identical to the parent allele with the exception of the single swapped-base change. The modified alleles were then inserted into each strain, UCD932 and UCD950. This resulted in 14 versions of each of the strains or one of the 12 modified alleles. These strains were examined for H$_2$S production in duplicate during small-scale fermentations (10 ml) in synthetic juice medium. H$_2$S was detected by the use of lead acetate columns after 4 days of fermentation (20). Only the modification at position 662 led to a change in the use of lead acetate columns after 4 days of fermentation.

The maximum fermentation rate was calculated from the fermentation rate data/H$_{11021}$cates. All fermentations reached dryness (defined by <0.5% sugar remaining). The maximum fermentation rate was calculated from the fermentation rate data and by using time points corresponding to the steepest decline in weight.

The change from a hydroxyl-bearing amino acid side chain (threonine) to a bulky side chain carrying a charge apparently impacts the ability to release reduced sulfide as H$_2$S. This site lies within the electron transfer domain of sulfite reductase.

The single mutant alleles were placed into two high-H$_2$S-producing commercial strains UCD522 and UCD940 (Table 7). Both of these strains produced H$_2$S with the UCD932 MET10 1985-A-C allele, but no H$_2$S was detected with the UCD950 MET10 1985C-A allele. Thus, switching the threonine at position 662 to lysine eliminated H$_2$S formation in the MET10-950 allele when placed into UCD932, UCD950, UCD940, and UCD952. UCD952 did not switch to a H$_2$S producer, regardless of the allele it carried (Table 7), suggesting that other factors may also be important in limiting sulfide formation in this genetic background.

To investigate the possible mechanism and extent to which the amino acid side chain at residue 662 affects H$_2$S production, PCR-mediated site-directed mutagenesis was used to replace the threonine encoded by the UCD950 MET10 allele with other amino acids. All of the fermentation profiles were comparable to that of the wild type (Table 8). Replacement of threonine with glutamic acid, tryptophan, or arginine resulted in the elimination of H$_2$S production similar to the case with lysine. Replacement with serine or alanine retained the high level of production of sulfide similar to the case with threonine. Other amino acid substitutions resulted in moderate H$_2$S production. These observations suggest that a bulky charged residue at position 662 impacts H$_2$S formation or release. Position 662 lies within the sulfite reductase catalytic domain of the $\alpha$ subunit.

### DISCUSSION

Genetic analyses and allele swap experiments identified MET10-932 as an allele capable of conferring the phenotype of non-H$_2$S production in a variety of genetic backgrounds. Amino acid substitution data indicate that a single-residue change at amino acid position 662 of Met10p is responsible for this trait. The change from a hydroxyl-bearing amino acid side chain (threonine) to a bulky side chain carrying a charge apparently impacts the ability to release reduced sulfide as H$_2$S.

The maximum fermentation rate was calculated from the fermentation rate data and by using time points corresponding to the steepest decline in weight. $<1$ means no detectable discoloration of the lead acetate column was observed.

<table>
<thead>
<tr>
<th>Amino acid substitution</th>
<th>Maximum fermentation rate (g/h)</th>
<th>Total H$_2$S (µg) $^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysine</td>
<td>0.15</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Threonine</td>
<td>0.15</td>
<td>1.36</td>
</tr>
<tr>
<td>Alanine</td>
<td>0.15</td>
<td>24</td>
</tr>
<tr>
<td>Arginine</td>
<td>0.15</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>0.15</td>
<td>6</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>0.15</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Glutamine</td>
<td>0.13</td>
<td>12</td>
</tr>
<tr>
<td>Leucine</td>
<td>0.15</td>
<td>4</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>0.15</td>
<td>5</td>
</tr>
<tr>
<td>Serine</td>
<td>0.16</td>
<td>36</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>0.15</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.15</td>
<td>8</td>
</tr>
</tbody>
</table>

$^a$ Values represent the averages of independent determinations of two replicates. All fermentations reached dryness (defined by <0.5% sugar remaining).
replacement of their native in the wild. Of the models proposed in the literature to explain the growth of other wine strains of that it grows under nutrient-limiting conditions not supporting the growth of other wine strains of Saccharomyces (42). The production of H₂S may confer a growth disadvantage under nutrient-restrictive conditions. Further, if sulfur is itself a limiting nutrient in the environment, selective pressure for more efficient incorporation and less loss as H₂S may have occurred in the wild.

H₂S production in UCDS22 and in several of the native isolates behaves as a quantitative trait (42). The fact that the replacement of their native MET10 alleles with MET10-932 is sufficient to convert these strains to non-H₂S producers suggests that the specific change to MET10 by substitution of the threonine at position 662 for lysine behaves in a dominant manner over the other quantitative trait loci leading to reduced sulfide formation. An alteration of sulfide association/dissociation kinetics would explain these observations. The finding that replacement of the MET10-932 allele with other alleles of MET10 restored colony color on BiGGY agar but not H₂S production suggests that this strain also carries other mutative changes that reduce sulfide release. It is interesting that 10 genes of the sulfate reduction pathway have been sequenced (CYS4, HOM2, HOM6, MET1, MET5, MET6, MET8, MET10, MET17, and SER33) and UCDS92 carries mutations in five of them (CYS4, HOM6, MET5, MET6, and MET10). These alleles may also play a role in modifying both sulfite reductase activity and H₂S release. Selective pressures to both increase and decrease H₂S formation appear to exist in the wild. Of the models proposed in the literature to explain the release of sulfide, we favor those in which altering release levels would confer a selective advantage over those that pose a simple inefficiency of incorporation as the explanation for appearance of high-level H₂S production.

In conclusion, the screen of native isolates led to the identification of a genetic determinant leading to a non-H₂S-producing phenotype. The MET10-932 allele eliminated production of detectable sulfide in a variety of genetic backgrounds. The white colony phenotype of this allele provides a convenient marker for transfer of this allele to other strains via classical genetic breeding as well as via recombinant DNA technologies. This discovery has broad commercial potential for the creation of strains lacking sulfide production for fermentation. Since a single allele transfer is all that is needed, these strains would otherwise retain their innate genetic diversity.

ACKNOWLEDGMENTS

We generously thank the Carlsberg Research Center for providing plasmid pYC130. Vidhya Ramakrishnan and Yeun Hong are thanked for useful discussions. Y. Hong is also thanked for helping with some of the screening experiments.

This research was supported by grants from the American Vineyard Foundation and the California Competitive Grant Program for Research in Viticulture and Enology and the Maynard A. Amerine Enology Endowment. A. Linderholm was supported by the Paul Monk Scholarship and scholarships from the American Society of Enology and Viticulture and Confrérie de la Chaine des Rotisseurs.

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