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

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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 (bismuth-glucose-glycine-yeast 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 H_2S 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 H_2S. Replacing the *MET10* allele of high-H_2S-producing strains with *MET10-932* prevented H_2S formation by those strains. A single mutative change, corresponding to T662K, in *MET10-932* resulted in a loss of H_2S 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 H_2S formation conferred by this allele. In addition to T662K, arginine, tryptophan, and glutamic acid substitutions similarly reduced sulfide formation.

Formation of hydrogen sulfide (H_2S) 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 H_2S 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 H_2S 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 H_2S during fermentation is therefore highly desirable.

The formation of H_2S varies widely across strains (1, 20, 25, 26, 32, 42, 55). Production of H_2S 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 incorporate reduced sulfur into organic compounds and suggests that alterations in internal enzyme regulation or activity may affect H_2S production (8, 32, 37, 38, 43, 44, 45). Several theories as to the reason for release of bound sulfide as H_2S 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 H_2S (18, 19, 32, 44, 45). Under this scenario, reduced sulfur is released from the enzyme active site as H_2S. 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, H_2S 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.

H_2S is also thought to play a role in population signaling serving to coordinate the metabolic activities of the individual

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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_2S 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_2S 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 of *Saccharomyces*.

Growth conditions also impact the appearance and retention of H_2S. 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_2S 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_2S 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_2S-producing strains (20, 26, 32, 37, 42, 55). Although these screens have been successful in identifying strains that do not form H_2S, 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_2S (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_2S 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 *CYS4* gene, which encodes cystathionine beta-synthase, has been shown to reduce H_2S formation in brewing strains (46) but not in wine strains (21, 43).

Our strategy for generating commercial strains with reduced abilities to form H_2S 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_2S 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 $\alpha_2\beta_2$ 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 *MET1* 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_2S 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

TABLE 1. Strains used in this study

Strain[a]	Known genotype or description	Reference or source[b]
UCD932 (Ba2)	Native isolate	UCD culture collection
UCD934 (Ba25)	Native isolate	UCD culture collection
UCD938 (Ba86)	Native isolate	UCD culture collection
UCD939 (Ba99)	Native isolate	UCD culture collection
UCD940 (Ba111)	Native isolate	UCD culture collection
UCD942 (Ba126)	Native isolate	UCD culture collection
UCD950 (Ba196)	Native isolate	UCD culture collection
UCD956 (Ba224)	Native isolate	UCD culture collection
UCD957 (Ba229)	Native isolate	UCD culture collection
UCD522	Industrial isolate	UCD culture collection
YKR069W BY4742	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 met1*Δ::G418^r	Open Biosystems
YJR137C BY4742	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 met5*Δ::G418^r	Open Biosystems
YBR213W BY4742	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 met8*Δ::G418^r	Open Biosystems
YFR030W BY4742	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 met10*Δ::G418^r	Open Biosystems
ALY38	UCD932 *MET10-288*	This study
ALY39	UCD932 *MET10-932*	This study
ALY95	UCD932 *MET10-950*	This study
ALY72	BY4742 *MET10-950*	This study
ALY40	UCD950 *MET10-288*	This study
ALY41	UCD950 *MET10-932*	This study
ALY126	UCD950 *MET10-950*	This study
ALY127	UCD939 *MET10-939*	This study
ALY128	UCD939 *MET10-288*	This study
ALY130	UCD940 *MET10-288*	This study
ALY129	UCD940 *MET10-940*	This study
ALY131	UCD940 *MET10-932*	This study
ALY132-1A	UCD522 *met10*Δ::*KanMX4*	This study
ALY133-1B	UCD522 *MET10-288*	This study
ALY134-1C	UCD522 *MET10-288*	This study
ALY135-1D	UCD522 *met10*Δ::*KanMX4*	This study
ALY136-1A	UCD522 *MET10-522*	This study
ALY137-1B	UCD522 *met10*Δ::*hphNT1*	This study
ALY138-1C	UCD522 *met10*Δ::*hphNT1*	This study
ALY139-1D	UCD522 *MET10-522*	This study
ALY140-1A	UCD522 *MET10-932*	This study
ALY141-1B	UCD522 *MET10-932*	This study
ALY142-1C	UCD522 *met10*Δ::*KanMX4*	This study
ALY143-1D	UCD522 *met10*Δ::*KanMX4*	This study

[a] Ba designations are those from reference 28.
[b] UCD, University of California, Davis.

mg/ml) was used for maintenance of deletion strains carrying the G418^r (KanMX) or HphMX marker. Minimal YNB medium was made with 0.67% yeast nitrogen base without amino acids and supplemented with Casamino Acids as recommended (40). Selective methionine dropout media were 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 S288C), *MET10-522* (from UCD522), *MET10-932* (from UCD932), *MET10-939* (from UCD939) *MET10-940* (from UCD940), and *MET10-950* (from UCD950).

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 H_2S 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 1.3×10^5 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 CO_2 production. Replicate 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. H_2S was measured by using lead acetate columns purchased from Figasa International Inc. (Seoul, South Korea) that allow quantitative assessment of H_2S 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/PCR-MET10-R for *MET10*, HOM2-F/HOM2-R for *HOM2*, HOM6-F/HOM6-R for *HOM6*, SER33-F/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 Sequencing Facility at the University of California, Davis, by using an ABI 3730 capillary electrophoresis genetic analyzer and ABI BigDye Terminator version 3.1 cycle sequencing chemistry (Foster City, CA); primers used are listed in Tables S2 and S3 in the supplemental material. Sequence data were edited and analyzed with the BioEdit sequence alignment editor (version 5.0.9) (14a).

Genetic manipulations. Genetic manipulations, including crosses, sporulation, and tetrad analysis, were conducted using standard procedures (14).

Plasmids, DNA manipulations, allele swapping, and transformation methods. The plasmids pAL51 (*MET10-288*) and pAL52 (*MET10-932*) were used in this study. Primers PCR-MET10-F/PCR-MET10-R (Table S1 in the supplemental material), carrying the restriction sites BamHI and SacII, were designed to amplify *MET10* from yeast strain UCD932 and S288C chromosomal DNA (Invitrogen, Carlsbad, CA). Plasmid pYC130 (33) is a centromeric vector carrying the G418^r selectable marker and was digested with BamHI and SacII (New England Biolabs, Ipswich, MA) to allow the ligation of *MET10*. The resulting plasmids, pAL51 (*MET10-288*) and pAL52 (*MET10-932*), were used for transformation.

MET10 deletion mutants were created using a PCR-based technique (4). A KanMX-containing deletion cassette specifying G418 resistance (yeast deletion collection) with overhangs of noncoding regions on either side of *MET10* was PCR amplified using primers MET10-F-KO/MET10-R-KO, and the linear PCR fragment was transformed into yeast diploid strains UCD522, UCD932, UCD939, UCD940, and UCD950. By homologous recombination, one copy of the intact *MET10* was replaced with the knockout cassette, generating strains carrying both an intact copy of *MET10* and a KanMX marker. All of the strains, except UCD522 *met10*Δ::*KanMX*, were then sporulated, and those homologous for G418^r (KanMX) were used for further experiments. Gene deletions were confirmed by PCR using the upstream forward primer and an internal reverse primer to the KanMX disruption marker, JKKanRE (see Table S1 in the supplemental material).

To knock out the remaining intact copy of *MET10* in UCD522 *met10*Δ::*KanMX*, an HphMX cassette was amplified from BamHI-linearized pYC140 (15) by using primers MET10-hphMX-F/MET10-hphMX-R, and the linear PCR fragment was transformed into ALY29. A methionine-auxotrophic colony displaying both G418^r and Hph^r was selected and the HphMX deletion confirmed by PCR using the upstream forward primer and an internal reverse primer to the HphMX disruption marker HYGROB CHK_R (see Table S1 in the supplemental material).

Allele swaps of *MET10* were also performed using a PCR-based technique (4). Alleles of *MET10* were amplified from S288C, UCD932, UCD940, UCD950, and UCD522 using primers MET10-F-KO/MET10-R-KO. The linear PCR fragments amplified from S288C and UCD932 were then transformed into the methionine-auxotrophic strains. The other fragments were transformed into individual strains to create the corresponding control strains. Strains displaying the ability to grow in the absence of methionine were selected and sporulated to create strains homozygous for *MET10* for further experiments.

S. cerevisiae was transformed using the lithium acetate method (39). *Escherichia coli* was transformed as described previously (17). *E. coli* INVαF′ (Invitrogen, Carlsbad, CA) was used for plasmid preparations. Luria-Bertani medium (27) with ampicillin was used for selection for transformed *E. coli* cells.

PCR-mediated site-directed mutagenesis. Initially, the *MET10* alleles from UCD932 and UCD950 were cloned into the pUG6 vector (13) and each single nucleotide base difference was systematically converted to the base of the opposite allele by using QuikChange PCR-mediated site-directed mutagenesis (9). The primers and locations of the changes are listed in Table S4 in the supplemental material. Further amino acid substitutions at amino acid position 662 in *MET10-950* were also made using PCR-mediated site-directed mutagenesis. The primers, with their corresponding amino acid, are listed in Table S5 in the supplemental material.

Nucleotide sequence accession numbers. GenBank accession numbers (listed in parentheses) were determined for the UCD932 *MET10* (EF058164), UCD938 *MET10* (EF058165), UCD939 *MET10* (EF058166), UCD940 *MET10* (EF058167), UCD942 *MET10* (EF058168), UCD956 (EF058169), UCD522 *MET10* (EF058170), UCD957 *MET10* (EF058171), UCD934 *MET10* (EF058172), UCD950 *MET10* (EF058173), UCD932 *SER33* (EF058174), UCD939 *SER33* (EF058175), UCD940 *SER33* (EF058176), UCD956 *SER33* (EF058177), UCD950 *SER33* (EF058178), UCD932 *HOM6* (EF058179), UCD932 *MET1* (EF058180), UCD939 *MET1* (EF058181), UCD940 *MET1* (EF058182), UCD950 *MET1* (EF058183), UCD956 *MET1* (EF058184), UCD956 *MET5* (EF058185), UCD932 *MET5* (EF058186), UCD940 *MET5* (EF058187), UCD939 *MET5* (EF058188), UCD932 *MET8* (EF058189), UCD939 *MET8* (EF058190), UCD940 *MET8* (EF058191), UCD950 *MET8* (EF058192), and UCD956 *MET8* (EF058193) genes.

RESULTS

Identification of *MET10* as the gene responsible for low-level H_2S formation in UCD932. UCD932, a native strain isolated from an Italian vineyard (28) displayed an inability to produce both H_2S 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_2S, 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_2S 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 deletant 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_2S 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, *MET1*, *MET5*, *MET8*, or *MET10* (22), and loss of sulfite reductase activity has been shown to be inversely correlated with H_2S 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 *met1*Δ, *met8*Δ, *met5*Δ, or *met10*Δ 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 *met10*Δ deletant strain YFR030W BY4742 displayed white colonies on BiGGY agar, indicating that UCD932 likely contained a mutation in *MET10*.

To assess the dominance of this allele, UCD932 was transformed with a plasmid carrying *MET10-288*, the *MET10* allele from S288C (pAL51). For controls, this strain was also transformed with the pYC130 vector and with a construct carrying its native *MET10-932* 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/h, respectively. Transformation of UCD932 with *MET10-288* resulted in tan colonies, suggesting that the white colony phenotype of *MET10* is recessive. However, these diploids retained 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_2S, and suggests that het-

TABLE 2. Sequence analysis results for genes of the sulfate reduction pathway[a]

Yeast strain	Colony color on BiGGY agar	Level of H_2S production	Gene(s) displaying allelic differences
UCD932	White	None	*HOM6*, *MET5*, *MET10*
UCD934	Tan	Moderate	*MET10*
UCD938	Tan	Moderate	*MET10*
UCD939	Light tan	High	*MET5*, *MET8*, *MET10*
UCD940	Brown	High	*MET5*, *MET10*
UCD942	Light tan	Low	*MET10*
UCD950	Brown	High	*MET10*
UCD956	Light tan	Low	*MET1*, *MET5*, *MET8*, *MET10*
UCD957	Tan	High	*MET10*
UCD522	Brown	High	*MET10*

[a] H_2S formation was determined in MMM by using lead acetate detection tubes. If no discoloration of the detection tube occurred, the strain was categorized as a nonproducer. Low-level production ranged from detectable levels to <2 μg/liter of H_2S. Moderate production ranged from ≥ 2 to ≤ 15 μg/liter. High production was greater than 15 μg/liter.

TABLE 3. Amino acid differences identified in proteins of the sulfate reduction pathway

Gene	Amino acid position	Consensus residue	Amino acid difference (yeast strain[s])[a]
HOM6	54	N	S (UCD932)
SER33	60	K	N (S288C)
MET1	216	M	I (UCD956)
	296	A	V (S288C)
	502	G	E (S288C)
MET5	464	K	K or T (UCD956)
	1092	I	S (UCD932)
	1222	F	L (UCD940)
	1227	R	R or K (UCD939)
MET8	15	R	K (S288C)
	61	K	E (S288C)
	80	S	P (S288C)
	246	D	H (UCD939, UCD956)
MET10	135	T	N (UCD932) T or N (UCD940)
	172	None	T (UCD522, UCD932, UCD940, UCD938, UCD942, UCD956) A (S288C, UCD934, UCD957, UCD950) A or T (UCD939)
	314	P	P or S (UCD940)
	475	D	A (UCD938, UCD942)
	511	None	I (UCD934, UCD950, UCD957) T (S288C, UCD932, UCD938, UCD939, UCD940, UCD942, UCD956) I or T (UCD522)
	590	None	K (UCD934, UCD950, UCD957) E (S288C, UCD522, UCD932, UCD938, UCD940, UCD942, UCD956) Q (UCD939)
	662	T	K (UCD932)
	896	P	S (UCD956)

[a] Strains designated as having two possible amino acid changes at a specific site were heterozygous for the corresponding gene, carrying two different alleles.

erozygosity at this locus leads to a reduction in sulfide formation. However, these results would also be consistent with more than one tightly linked gene in combination with *MET10* being responsible for the low-H_2S phenotype of UCD932. Thus, further analysis was needed to confirm the role of *MET10-932* in H_2S production in UCD932.

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-H_2S-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 H_2S production in synthetic juice (Table 2) (42).

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, H_2S producers with tan to brown colonies on BiGGY agar, carried the same allele. UCD938 and UCD942, producers of low to moderate levels of H_2S yielding tan colonies on BiGGY agar, also carried identical alleles. UCD522 and UCD940, H_2S 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-H_2S producers with white colonies on BiGGY agar, and UCD939, an H_2S 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 H_2S production. The genetic diversity of *MET10* alleles and the apparent correlation with H_2S production supported the hypothesis that this subunit of sulfite reductase may be responsible for the H_2S phenotype in

TABLE 4. Properties of hydrogen sulfide production and fermentations in strains with different *MET10* alleles[a]

Strain	Allele	Maximum fermentation rate (g/h)	Total H_2S (μg)[b]
UCD932	*MET10-288*	0.37	<1
	MET10-932	0.34	<1
	MET10-950	0.41	<1
UCD950	*MET10-288*	0.42	32
	MET10-932	0.40	<1
	MET10-950	0.41	29
UCD940	*MET10-288*	0.40	54
	MET10-932	0.42	<1
	MET10-940	0.42	49

[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.

TABLE 5. Properties of hydrogen sulfide production and fermentations in spores of UCD522 with different *MET10* alleles[a]

Spore designation[b]	*MET10* allele	Maximum fermentation rate (g/h)	Total H_2S (μg)[c]
UCD522-1A (ALY132)	*met10*Δ::*KanMX4*	0.35	<1
UCD522-1B (ALY133)	*MET10-288*	0.35	16
UCD522-1C (ALY134)	*MET10-288*	0.42	33
UCD522-1D (ALY135)	*met10*Δ::*KanMX4*	0.24	<1
UCD522-2A (ALY136)	*MET10-522*	0.43	26
UCD522-2B (ALY137)	*met10*Δ::*hphNT1*	0.24	<1
UCD522-2C (ALY138)	*met10*Δ::*hphNT1*	0.34	<1
UCD522-2D (ALY139)	*MET10-522*	0.38	4
UCD522-3A (ALY140)	*MET10-932*	0.36	<1
UCD522-3B (ALY141)	*MET10-932*	0.37	<1
UCD522-3C (ALY142)	*met10*Δ::*KanMX4*	0.36	<1
UCD522-3D (ALY143)	*met10*Δ::*KanMX4*	0.22	<1

[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.

UD932. The effect of the specific *MET10* allele on H_2S production in different genetic backgrounds was therefore evaluated. The *MET10* alleles of H_2S-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_2S producers (Tables 4 and 5). The strains carrying an allele from S288C or their own allele maintained their H_2S-producing phenotype.

UCD522 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_2S production evaluated in tetrads displaying four viable spores (Table 5). The UCD522 strain carrying the double knockout of *MET10* (*met10*Δ::*KanMX*/*met10*Δ::*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_2S 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_2S 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 deletant allele (methionine auxotrophs) or the *MET10-932* allele were non-H_2S producing. The presence of either *MET10-288* or *MET10-522* resulted in production of H_2S. Thus, allele swap of the native *MET10* gene with *MET10-932* was sufficient to confer the phenotype of nonproduction of H_2S in UCD522 similar to the case in UCD940 and UCD950.

Identification of the residue responsible for elimination of H_2S 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 135 is a threonine in UCD950 and S288C but an asparagine in UCD932, amino acid 172 is an alanine in UCD950 and S288C but a threonine in UCD932, amino acid 511 is a threonine in UCD932 and S288C but an isoleucine in UCD950, amino acid 590 is a glutamate in UCD932 and S288C but a lysine in UCD950, and amino acid 662 is a threonine in both UCD950 and S288C but a lysine in UCD932. The sixth dissimilarity, an A/G difference at nucleotide 1278, did not result in a change of amino acid.

UCD950 is a high-level producer of H_2S regardless of growth conditions, whereas UCD932 is a non-H_2S 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_2S to the *MET10* polypeptide. To this end, each of the single-base-pair differences of the *MET10* alleles from UCD932 and

TABLE 6. Modifications of *MET10-932* and *MET10-950* alleles

Base pair change		Amino acid residue
MET10-932	*MET10-950*	
A404C	C404A	135
A514G	G514A	172
A1278G	G1278A	429
C1532T	T1532C	511
G1768A	A1768G	590
A1985C	C1985A	662

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 carrying either the wild-type *MET10-932* or *MET10-950* allele or one of the 12 modified alleles. These strains were examined for H_2S production in duplicate during small-scale fermentations (10 ml) in synthetic juice medium. H_2S 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 H_2S production (Table 7, data shown for this allele only). The unchanged UCD950 *MET10-950* allele and the UCD932 allele with the mutation to the UCD950 allele at position 1985 (UCD932 *MET10* 1985A-C) changing the lysine back to a threonine resulted in H_2S production, while the unchanged allele UCD932 *MET10-932* and the UCD950 allele with the change to UCD932 at position 1985 (UCD950 *MET10* 1985C-A) of threonine to lysine resulted in no detectable H_2S production (Table 7). The results indicate that the single base change at position 1985 is the key determinant of the difference in H_2S production of these alleles. These findings were then strengthened by examining the production of H_2S when the single mutant alleles were placed into two high-H_2S-producing commercial strains UCD522 and UCD940 (Table 7). Both of these strains produced H_2S with the UCD932 *MET10* 1985-A-C allele, but no H_2S was detected with the UCD950 *MET10* 1985C-A allele. Thus, switching the threonine at position 662 to lysine eliminated H_2S formation in the *MET10-950* allele when placed into UCD932, UCD950, UCD940, and UCD522. UCD932 did not switch to a H_2S 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.

TABLE 7. Impact of *MET10-932* and *MET10-950* alleles modified at position 1985 on H_2S production

MET10 allele	Nucleotide at position 1985	Strain background	H_2S production phenotype
UCD932	Adenine	UCD522	Negative
		UCD932	Negative
		UCD940	Negative
		UCD950	Negative
UCD950	Cytosine	UCD522	Positive
		UCD932	Negative
		UCD940	Positive
		UCD950	Positive
UCD932 1985A-C	Cytosine	UCD522	Positive
		UCD932	Negative
		UCD940	Positive
		UCD950	Positive
UCD950 1985C-A	Adenine	UCD522	Negative
		UCD932	Negative
		UCD940	Negative
		UCD950	Negative

TABLE 8. Properties of H_2S production with different amino acids at site 662[a]

Amino acid substitution	Maximum fermentation rate (g/h)	Total H_2S (μg)[b]
Lysine	0.15	<1
Threonine	0.15	36
Alanine	0.15	24
Arginine	0.15	<1
Aspartic acid	0.15	6
Glutamic acid	0.15	<1
Glutamine	0.13	12
Leucine	0.15	4
Phenylalanine	0.15	5
Serine	0.16	36
Tryptophan	0.15	<1
Tyrosine	0.15	8

[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.

To investigate the possible mechanism and extent to which the amino acid side chain at residue 662 affects H_2S 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_2S 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_2S production. These observations suggest that a bulky charged residue at position 662 impacts H_2S formation or release. Position 662 lies within the sulfite reductase catalytic domain of the α subunit.

DISCUSSION

Genetic analyses and allele swap experiments identified *MET10-932* as an allele capable of conferring the phenotype of non-H_2S 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_2S. This site lies within the electron transfer domain of sulfite

reductase. This mutation does not lead to methionine auxotrophy, as strains bearing this mutation are able to grow normally in the absence of this amino acid. Although the exact mechanism by which H_2S release is averted is not known, it is tempting to speculate that this mutative change alters the kinetics of dissociation of reduced sulfur, with the sulfur being retained in the active site until the amino compound acceptor is also bound to the enzyme complex, facilitating transfer to the next enzyme in the pathway and the organic molecule acceptor. Alternately, these differences in protein sequence may slightly decrease the specific activity of the whole enzyme complex to a degree that limits sulfite reduction to a level that does not exceed incorporation by the next enzyme in the pathway. The allele differences of subsequent enzymes in the pathway, the *CYS4* and *MET6* gene products, may then serve to boost formation of the S-containing amino acids. As a result, the strains remain fully prototrophic for sulfur-containing amino acids yet fail to release H_2S at a detectable level. The decrease in the rate of sulfite reduction could be accomplished by a slowing of the electron transfer to the sulfur moiety at the active site of the complex. Other models are also possible.

Previous research also identified mutative change of *MET10* as an underlying cause of reduction of sulfide formation (8, 45). However, in those studies, the mutations obtained impacted catalytic activity to a level leading to a growth requirement for S-containing amino acids. *MET10-932* is an allele that arose in nature, and thus, there would be selective pressure to maintain sufficient catalytic activity for S-containing amino acid biosynthesis. The parental strain, UCD932, was isolated from an Italian vineyard (28), and previous research suggested that it grows under nutrient-limiting conditions not supporting the growth of other wine strains of *Saccharomyces* (42). The production of H_2S 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 H_2S may have occurred in the wild.

H_2S production in UCD522 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-H_2S 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 H_2S production in UCD932 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 UCD932 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 H_2S release. Selective pressures to both increase and decrease H_2S 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 propose a simple inefficiency of incorporation as the explanation for appearance of high-level H_2S production.

In conclusion, the screen of native isolates led to the identification of a genetic determinant leading to a non-H_2S-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.

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