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Identification of Genes Affecting Hydrogen Sulfide Formation in *Saccharomyces cerevisiae*▽

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A screen of the *Saccharomyces cerevisiae* deletion strain set was performed to identify genes affecting hydrogen sulfide (H_2S) production. Mutants were screened using two assays: colony color on BiGGY agar, which detects the basal level of sulfite reductase activity, and production of H_2S in a synthetic juice medium using lead acetate detection of free sulfide in the headspace. A total of 88 mutants produced darker colony colors than the parental strain, and 4 produced colonies significantly lighter in color. There was no correlation between the appearance of a dark colony color on BiGGY agar and H_2S production in synthetic juice media. Sixteen null mutations were identified as leading to the production of increased levels of H_2S in synthetic juice using the headspace analysis assay. All 16 mutants also produced H_2S in actual juices. Five of these genes encode proteins involved in sulfur containing amino acid or precursor biosynthesis and are directly associated with the sulfate assimilation pathway. The remaining genes encode proteins involved in a variety of cellular activities, including cell membrane integrity, cell energy regulation and balance, or other metabolic functions. The levels of hydrogen sulfide production of each of the 16 strains varied in response to nutritional conditions. In most cases, creation of multiple deletions of the 16 mutations in the same strain did not lead to a further increase in H_2S production, instead often resulting in decreased levels.

The appearance of hydrogen sulfide in wine as a consequence of yeast metabolism is considered to be a sensory defect. From a commercial standpoint, the availability of strains unable to produce this defect would be highly desirable. There are various mechanisms through which hydrogen sulfide may be produced by *Saccharomyces cerevisiae*. H_2S may be generated through the degradation of sulfur-containing amino acids, the reduction of elemental sulfur, or the reduction of sulfite or sulfate (2, 21, 31, 34). When other preferred nitrogen sources are depleted, *Saccharomyces* can degrade sulfur-containing amino acids to utilize the nitrogen, resulting in the release of H_2S or other volatile sulfur compounds as by-products. However, the concentration of sulfur-containing amino acids in grape juice is generally not high enough to be responsible for the observed levels of H_2S. Residual elemental sulfur remaining on the grapes after sulfur dusting to control fungal blooms contributes to H_2S production and therefore can be easily prevented by ceasing sulfur-containing fungicide application in close proximity to harvest time (32, 44). In this case the yeast is only indirectly responsible for the formation of H_2S, which occurs spontaneously as a consequence of the reductive conditions established in the anaerobic fermentation at low pH. H_2S is most commonly produced as a result of the activity of the sulfate reduction pathway, in which *S. cerevisiae* reduces sulfate or sulfite for the synthesis of the sulfur-containing amino acids methionine and cysteine and their derivatives. Inefficiency of incorporation of the reduced sulfur into the precursors of these amino acids has been proposed to result in leakage of sulfide from the pathway and the formation of H_2S (10, 17, 31, 40). Mutation of this pathway accompanied by methionine supplementation of the grape juice is not a viable strategy for the elimination of reduced sulfide formation since both cysteine and methionine are reactive chemically under these reductive fermentation conditions, leading to a host of other equally undesirable sulfur-containing spoilage compounds.

During wine production, the level of H_2S in the finished wine is the most important parameter determining the acceptability of the product. In addition to the level of H_2S produced by the strain, other factors, such as loss resulting from volatilization and entrainment due to carbon dioxide release, temperature of fermentation, and timing of formation versus cessation of fermentative activity, all impact the residual levels of reduced sulfide in the wine. Thus, environmental factors, in addition to production levels, affect the appearance and retention of H_2S in wine. In order to specifically address the role of strain genetic background in H_2S formation independent of the environmental factors impacting retention in wine, a systematic analysis of the yeast deletion set was undertaken to define the genes that when mutated influence sulfide formation under controlled growth conditions. The goal of this research is to define the genetic factors leading to sulfide formation so that genetic strategies can then be used to identify or create commercial strains that will not produce hydrogen sulfide under winemaking conditions.

Control of the sulfate reduction pathway is multifaceted and responsive to numerous regulatory inputs (6, 8, 14, 26, 29, 30). As a consequence, mutational change of a wide array of genes may impact sulfide formation and release. Sulfite reductase is responsible for reducing sulfite to sulfide and is regulated by

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general amino acid control, as well as methionine (26). Other research suggests that cysteine or its derivatives, rather than methionine, is the main end product regulating pathway activity (14, 29). It is likely that the regulation of the pathway varies by media and growth conditions, with pathway activity controlled by the factor for which there is the greater cellular demand (40).

Previous research has identified *MET17* (also known as *MET25* and *MET15*) and *CYS4*, both located downstream of sulfite reductase, as controlling genes in the pathway affecting sulfide leakage (13, 28, 42, 43). The *MET17* gene encodes the enzyme *O*-acetyl homoserine-*O*-acetyl serine sulfhydrylase and is responsible for incorporating the sulfide, along with *O*-acetylhomoserine, into homocysteine. The gene *CYS4* encodes cystathionine beta-synthase, which converts homocysteine into cystathionine. Overexpression of *MET17* in wine strains reduced H_2S formation in one strain but had no effect in another (39). The strain in which overexpression had an effect was later shown to carry a disrupted allele of *MET17* (24). Likewise, overexpression of *CYS4* in four native isolate strains did not lead to any significant reduction in H_2S production (24), although overexpression of *CYS4* did reduce H_2S in a brewing strain (43). These findings suggest that simple increases in enzyme activity of reactions that consume reduced sulfide are insufficient to reduce sulfide formation and therefore not a viable strategy for the construction of genetically modified strains with a reduced capacity to produce sulfide.

Numerous nutritional factors have been demonstrated to affect the quantity of hydrogen sulfide produced by *Saccharomyces* during fermentation. Elevated H_2S production may occur as a response to deficient nitrogen concentration in must (12, 16, 17, 46). Further, *Saccharomyces* has been demonstrated to produce elevated levels of H_2S as a response to high levels of nitrogen as well as low levels, depending on the strain (40). The time at which nitrogen is depleted is significant, and the highest levels of H_2S have been shown to be produced when the nitrogen supply was exhausted during the rapid growth phase (17). Deficiencies in vitamins and micronutrients essential for the synthesis of sulfur containing amino acids may also contribute to H_2S production. Certain strains of *Saccharomyces* were found to produce increased H_2S as a consequence of deficiencies of pantothenate and vitamin B_6 (pyridoxine) (48).

Various environmental and fermentation conditions also influence the amount of hydrogen sulfide that is produced. Fermentation temperature (21, 31), juice turbidity (21), the level of soluble solids (46), and titratable acidity (46) have been shown to significantly affect H_2S levels. The presence of various constituents such as metal ions have also been suggested to result in increased H_2S in wine, although this has not been conclusively demonstrated (10). High-level additions of the antimicrobial compound sulfite (SO_2) have also been reported to result in increased formation of H_2S (21). The sulfate reduction pathway is also induced as part of the general yeast stress response (11) under conditions in which the expression of genes involved in amino acid biosynthesis in general is being repressed. This likely reflects the important protective role of the cysteine-containing tripeptide glutathione that is required to maintain the redox balance of the cytoplasm.

Extensive research has provided evidence that the yeast strain, and therefore the genetic background, is an important variable in H_2S production and that strains respond differently to physiological and environmental factors in the production of reduced sulfide (1, 12, 13, 16, 31, 41, 44, 46). As a consequence of this strain variability, it has not been possible to devise fermentation management strategies guaranteed to reduce or eliminate the appearance of H_2S. Spiropoulos et al. (40) grew 29 *Saccharomyces* commercial and natural isolate strains under three different nutritional conditions and demonstrated not only that H_2S production among strains differed but also that the response of the strains to the medium conditions varied dramatically. The diversity in physiological patterns of sulfide production in combination with the natural variability of the composition of grape juice makes it difficult to predict sulfide production behavior of individual yeast strains. A study of *S. cerevisiae* in continuous culture suggested that H_2S may play a role in population signaling (38). Therefore, there may be selective advantages to the production of higher levels of volatile sulfide under certain growth conditions, which may serve as the driving force for the selection of variation in H_2S production ability among wild populations.

The goal of the present study was to systematically define the genetic elements both increasing and decreasing the level of sulfide formation. A commercially available set of yeast deletion mutants was screened in order to comprehensively identify all genetic lesions affecting production of H_2S. The possible additive effects of these mutations on H_2S formation were also evaluated.

MATERIALS AND METHODS

Yeast strains and culture conditions. The yeast knockout deletion strain set used was the haploid set BY4742 (*MAT*α) and was obtained from Open Biosystems (Huntsville, AL). The deletion strains were generated by a PCR-based gene deletion strategy (49; *Saccharomyces* Genome Deletion Project [http://www-sequence.stanford.edu/group/yeast_deletion_project/deletions3.html]). Strains designated by the manufacturer as quality control failures were not utilized, nor were strains that displayed markers inconsistent with their reported genotypes. The wild-type strain, BY4742, carries the genotype *MAT*α *his3D1 leu2D0 lys2D0 ura3D0* (ATCC 201389). Yeast strains were maintained and grown on yeast extract-peptone-dextrose medium with 2% glucose (YPD) (36). The same medium (YPD) with Geneticin (G418; 0.2 mg/ml) was used for the maintenance of deletion strains carrying the $G418^r$ marker. Both BiGGY agar and the synthetic grape juice contained methionine and all other amino acid and nutrient requirements of the yeast so that all mutants could be cultivated in these media. The levels of sulfur-containing amino acids did not lead to sulfide formation in the control (parental) strain.

DNA and genetic manipulations. Genetic manipulations, including crosses, sporulation, and tetrad analysis, were carried out by using standard procedures (35, 36). Gene deletions were confirmed by PCR using the upstream forward primer and an internal reverse primer to the KanMX disruption marker-JKKanRE. Amplification conditions were as follows: 30 cycles of 94°C for 2 min, 92°C for 45 s, 56°C for 30 s, and 72°C for 1 min, followed by a final extension at 72°C for 7 min. Primer sequences are listed in Table 1.

Screen of deletion set and native strains. The deletion set (Open Biosystems, Huntsville, AL) was screened on BiGGY agar, a bismuth glucose glycine yeast agar (27). The strains were also screened in the synthetic grape juice medium "minimal must media" (MMM) (13, 40), initially with 123 mg of nitrogen eq/liter by using a headspace assay. The nitrogen level was generated by using 0.2 g of L-arginine/liter and 0.5 g of ammonium phosphate/liter. For the BiGGY agar screen, cells were initially grown in microtiter wells on YPD medium and then replica plated to BiGGY agar and evaluated for color 4 days later. For screening in MMM, cells were initially grown on YPD plates (36). The same medium (YPD) with G418 at 0.2 mg/ml was used for maintenance of the deletion strains carrying the $G418^r$ marker. Individual colonies were used to inoculate 50-ml test tubes containing 5 ml of MMM incubated at 25°C on shaker tables at 120 rpm, and detection was done with lead acetate strips as described below.

TABLE 1. Primers

Primer	Sequence (5′→3′)
ATP11-F	CTCATCGAGCACCCTTTG
CGR1-F	CTATTCATTAACCTTATTTTATT
CYS4TOFWD	GCCAAAGTAAAAGGCAACAC
FCY22-F	CATCACGGCTCATTCATTG
GOS1-F	CCAAATTTTCTAGGCGTTG
HHT2-F	CAGAACGTCCTGCCATACAAA
HOM2-F	CACTTAAGTACACATACAAA
HOM6-F	CCTGGTGGTAAAGTTGGG
IKI3-F	CTGGCCAATTACTACTACAT
MET17-BEGF	CCTTTTTCTTGCTCTCTTGTC
PSY4-F	GCCTATCACAAAGTGCTCT
RXT2-F	CTATACCCAAGAGGACCGA
SER33-F	GGAATCTCCCAGGTTTAAT
SIT4-F	GAAACTTATCGCTGGGAA
TPO2-F	CAACTTCCTTCTCCTACTATA
YPL035C-F	CCTTTCCCGTATTTCTTCCA
JKKanRE	GGGCGACAGTCACATCAT

Analysis of hydrogen sulfide formation. Hydrogen sulfide production was evaluated by using the lead acetate method (12, 40). Volatile sulfide released as hydrogen sulfide interacts with the lead in the lead acetate matrix to form a dark precipitate. This method detects volatile H_2S in the headspace of the fermentation. Since not all H_2S is trapped, this represents a convenient qualitative method to distinguish strains producing H_2S from those that do not and allows strains to be characterized as high, moderate, or low producers or nonproducers of H_2S. Hydrogen sulfide formation was initially detected by using paper strips (2 by 10 cm, 3 mm; Whatman filter paper) that had been previously treated with 50 μl of 5% lead acetate solution and allowed to dry at room temperature. The lead acetate strips were folded in half and inserted into 50-ml culture tubes with the culture tube cap securing either end of the strip, enclosing the mid-portion of the lead acetate strip in the gaseous environment over the liquid medium. Tubes were inoculated directly from colonies of each of the deletants. Hydrogen sulfide formation was qualitatively measured based on the degree of blackening of the lead acetate strip. The entire yeast deletion set was screened by using this rapid detection procedure. This method was also used for the screening of relative levels of sulfide formation as a consequence of growth and medium conditions among the mutants identified as sulfide producers.

All strains showing a darkening of the lead acetate strip were confirmed by using a more sensitive and quantitative method. Fermentations were conducted in 500-ml Erlenmeyer flasks containing 300 ml of MMM, with a lead acetate column secured to the top of the flask in a rubber stopper. For this purpose, 123 mg of nitrogen/liter of MMM was used to more accurately emulate low-nitrogen grape juice conditions. Fermentations were initiated at a density of 1.33×10^5 cells/ml by inoculation with stationary-phase cells from a 24-h culture pregrown in 5 ml of MMM of the same composition. The fermentations were performed in triplicate, incubated at 25°C and 120 rpm, and monitored over 7 days based on weight loss and darkening on the lead acetate column. To quantify H_2S production, commercially available packed lead acetate columns were used in which each millimeter of blackening on the column denoted 4 μg of H_2S/liter. Lead acetate columns were purchased from Figasa International, Inc. (Seoul, Korea). There was excellent agreement between the qualitative screen and the quantitative column assay. In the quantitative lead acetate tube assays, triplicate independent experiments (independent batches of media and inocula, conducted sequentially but not simultaneously) were performed, and the results were averaged. The data were analyzed by using one-way analysis of variance and determining the standard error of the mean. Statistical data are reported for those cases in which the variation was greater than 10% or three standard deviations.

A modification of the packed lead acetate column method was used to screen the relative levels of H_2S produced for the strains carrying more than one mutation impacting sulfide formation. The double-deletion mutants were formed by genetic crossing. First, half of the deletion strains were crossed with the BY4741 (*MAT***a**) parental strain, and spores were selected that were *MAT***a** and carried the appropriate deletion. The spores were then genetically crossed against the other deletants in the opposite mating type, and the progeny were screened by PCR for the presence of both deletions. Strains carrying both deletants were then evaluated for H_2S production.

A Whatman filter paper strip (1.5 by 8.0 cm, 3 mm) was rolled and placed in a 1-ml bulb-less plastic pipette and treated with 250 μl of a 3% lead acetate solution. The paper was allowed to dry at room temperature, and the plastic lead column was then attached to the 50-ml culture tube with a silicone stopper. This arrangement forced more of the sulfide in the headspace into contact with the lead acetate paper and is therefore more sensitive than the assay used to screen the deletion set. Hydrogen sulfide formation was measured based on the millimeters of darkening on the paper. It is important to note that this assay is semiquantitative, but the sensitivity was comparable to the commercial lead acetate tubes. Strains that demonstrated significant H_2S production down to 1 mm with the commercial tubes showed consistent darkening of the rolled columns. Thus, this method provided a rapid and convenient assay of the relative levels of sulfide formation since the single parent controls were consistently run against the double mutants. The height of the darkening and the extent of the darkness varied across strains but within a given strain was consistent. This effect is likely due to the rate of the evolution of CO_2 during fermentation, which serves as a carrier gas for the H_2S.

TABLE 2. Modified MMM composition

MMM composition (g of N eq/liter)	Arginine (g/liter)	Ammonium phosphate (g/liter)	Casamino Acids (g/liter)	YNB (g/liter)
433	0.8	1	2	1.7
123	0.2	0.1	2	1.7
123 plus 1/5 YNB	0.2	0.1	2	0.34
65	0.107	0.015	1	1.7
65 plus 1/2 YNB	0.107	0.015	1	0.85

Fermentation conditions. To identify yeast strains and nutritional conditions impacting hydrogen sulfide formation, yeast cultures were grown in 5 ml of MMM in 50-ml culture tubes at 25°C on shaker tables at 120 rpm as described above. The synthetic grape juice medium, MMM, was used and modified from the original recipe (40) to produce seven different nitrogen and micronutrient compositions. Arginine, ammonium phosphate, and Casamino Acid additions were manipulated to adjust the nitrogen concentration, and YNB (i.e., yeast nitrogen base without amino acids and ammonium sulfate) additions were adjusted to control for nutrient and vitamin concentration. The MMM modifications are illustrated in Table 2. Hydrogen sulfide formation was evaluated after 4 days based on the degree of blackening of the lead acetate strip. Strains that did not grow in 4 days were tested again using a longer time course.

RESULTS

Screening of the deletion set and native isolates on BiGGY agar. In order to assess the H_2S production of the deletion strains and native isolates, they were initially all plated on BiGGY agar, and the color of the colonies was evaluated. BiGGY agar uses bismuth as an indicator for the production of sulfide; the more sulfide produced, the darker the colonies due to the precipitation of bismuth sulfide. The production of sulfide in this medium is thought to correlate with the basal level of activity of sulfite reductase (18, 27). The parental strain of the deletion set, BY4742, displayed a light tan to tan colony color on BiGGY agar, making it possible to distinguish increased (darker colony color) and decreased (lighter colony color) H_2S production. The colony colors observed for the set of strains were white, light tan, tan (colony color of the deletion set parental strain), light brown, brown, and black. Analysis of the deletion set identified 4 mutations resulting in white colonies, 258 were light tan, 4,478 were tan, 59 were light brown, 28 were brown, and 1 was black in color. The four mutations leading to white colonies encoded catalytic or regulatory genes of sulfite reductase (*MET1*, *MET5*, *MET8*, and *MET10*). The mutations leading to increased sulfide formation showed a bias toward genes involved in purine biosynthesis, methionine metabolism, tryptophan biosynthesis, vacuolar

TABLE 3. Darker-staining deletion strains

Function[a]	Gene(s)[b]
Purine biosynthesis	*ADE1*, *ADE2*, *ADE4*, *ADE5*, *7*, *ADE6*, *ADE8*
Stress response	*RTS1*
Tryptophan biosynthesis	*TRP3*, *TRP4*, *TRP5*
Amino acid metabolism	*GCV1*, *GCV2*
Protein metabolism	*DOA1*, *GCN1*, *UBP14*
ATP synthesis	*ATP11*, *CUP5*
Sporulation	*EMI5*, *RMD11*
Transcription	*DAL81*, *RPA34*, *SNF12*, *SNF5*, *SSN8*
Secretion	*GOS1*, *SSO1*
Gluconeogenesis	*CAT8*
Meiosis	*CBC2*, *UME6*
Chaperone regulator	*HLJ1*
Chromatin structure	*HHT2*, *SPT7*
mRNA splicing	*LEA1*, *MUD1*
Zinc metabolism	*IZH3*
Elongator complex subunit	*IKI3*
Nitrogen transport	*NPR2*
Glutathione biosynthesis	*GSH1*
Mitochondrial function	*YIA6*
Vacuolar acidification	*TFP1*, *VMA4*, *VMA5*, *VMA10*, *VMA13*, *VMA21*, *VMA22*, *VPH2*
Methionine metabolism	*CYS4*, *HOM2*, *HOM6*, *MUP1*, *MET17*, *MET31*, *SAM1*, *SAM2*
Protein binding	*ATG19*
Phospholipid metabolism	*DPL1*
Thiamine metabolism	*THI2*
Oxidative stress response	*MXR1*
Polyamine transport	*TPO2*
Histone acetylation	*SGF73*
Metal transporter degradation	*TRE1*
Protein folding	*ALF1*
ABC transporter	*MDL2*
Fatty acid elongase	*FEN1*
Fatty acid metabolism	*SUR4*
Actin filament organization	*SIW14*
Serine biosynthesis	*SER33*
Nuclear fusion	*VIK1*
Purine-cytosine transport	*FCY22*
RNA polymerase II transcription factor	*CUP9*
NADH kinase	*POS5*
Enzyme regulator	*RAI1*
Unknown	*FMP49*, *TED1*, *YHI9*, YBR239C, YCL007C, YDR008C, YDR048C, YDR095C, YKL118W, YLR358C, YMR244W, YOL138C, YOL075C, YOR331C, YPR115W

[a] Function as determined from *Saccharomyces* Genome Database (http://www.yeastgenome.org/).

[b] Underlined genes of unknown function are listed as "dubious" in the database. Of the dubious genes, only YOR331C overlaps with another gene (*VMA4*) that impacts H_2S production.

acidification, and transcription (Table 3). Several are also involved in the stress response. Thus, this assay appeared to identify a spectrum of genes impacting colony color that may directly or indirectly influence the basal level or activity of sulfite reductase in the cell.

Screening of deletion strains for H_2S formation under fermentation conditions. Previous studies (40) indicated that colony color on BiGGY agar was not predictive of H_2S production in actual and synthetic juice media. This is likely because of the differences in the media conditions and their impacts upon sulfate reduction. Therefore, it was also important to screen the deletion set directly for H_2S production using synthetic grape juice media with detection of volatilized H_2S in the headspace. Initial experiments were conducted to determine the appropriate medium conditions to use to screen the deletion set. The 88 deletion strains resulting in a colony color darker than that of the parental strain on BiGGY agar were taken as a subset and analyzed for H_2S production by using lead acetate strips under multiple nutritional conditions to identify the medium composition resulting in hydrogen sulfide production among the greatest number of strains. The 88 strains were grown in the five synthetic medium conditions listed in Table 3. The MMM with 65 mg of nitrogen/liter (1/2 Casamino Acids) plus 1/2 YNB was selected for further screening of the entire deletion collection because under this condition the largest subset of deletion strains used in the preliminary screen produced H_2S. Multiple quality control failures, representing strains containing an incorrect genotype, were identified, and these strains were not included in further analyses. Of the 4,828 strains evaluated, 16 positive strains (Table 4) were confirmed in triplicate. PCR analysis was used to confirm the presence of the expected deletion in each strain. Eleven of these strains produced dark-colored colonies, and five strains produced tan-colored colonies on BiGGY agar.

The relative amount of hydrogen sulfide produced by the 16 positive yeast strains was quantitatively measured by using the lead acetate columns (Table 5). Each strain was analyzed at a

TABLE 4. Genes affecting hydrogen sulfide production in *Saccharomyces*

Colony type and gene name	Function[a]
Dark-colored colonies	
MET17	*O*-Acetyl homoserine-*O*-acetyl serine sulfhydrylase
CYS4	Cystathionine beta-synthase
GOS1	v-Snare protein involved in Golgi transport
FCY22	Putative purine-cytosine permease
TPO2	Polyamine transport protein
HOM2	1-Aspartic beta semi-aldehyde dehydrogenase
HOM6	Homoserine dehydrogenase
ATP11	Molecular chaperone of mitochondrial ATP synthase
SER33	3-Phosphogycerate dehydrogenase
HHT2	DNA binding and chromatin assembly
IKI3	Killer toxin sensitivity, maintenance of membrane structural integrity
Tan-colored colonies	
RXT2	Unknown function
YPL035C	Unknown function
SIT4	Protein serine/threonine phosphatase activity, cell wall organization
PSY4	Unknown function
CGR1	Involved in nucleolar integrity and processing of pre-reran

[a] Function obtained from *Saccharomyces* Genome Database (http://www.yeastgenome.org/).

TABLE 5. Properties of hydrogen sulfide production fermentations[a]

Strain genotype	Fermentation rate (g/h)[b]	Total wt loss (g)[c]	Mean total H_2S (μg/liter) ± SEM
BY4742	0.182	19.62	0 ± 0.00
*met17*Δ	0.180	19.96	245 ± 120.28
*cys4*Δ	0.024	6.70	493 ± 108.10
*gos1*Δ	0.126	17.53	311 ± 16.65
*fcy22*Δ	0.197	17.23	307 ± 12.86
*tpo2*Δ	0.139	17.17	292 ± 13.86
*cgr1*Δ	0.151	18.73	232 ± 96.08
*hom2*Δ	0.003	6.70	198 ± 137.58
*hom6*Δ	0.024	8.35	113 ± 30.02
*atp11*Δ	0.118	14.94	20 ± 131.88
*ser33*Δ	0.149	15.85	73 ± 82.20
*hht2*Δ	0.166	18.71	44 ± 58.92
*iki3*Δ	0.100	15.60	7 ± 2.31
*rxt2*Δ	0.158	16.79	8 ± 0.00
*ypl035C*Δ	0.163	18.54	4 ± 0.00
*sit4*Δ	0.004	13.09	7 ± 2.31
*psy4*Δ	0.056	17.43	4 ± 0.00

[a] Values represent the average of independent determinations of three replicates. All strains are in the BY4742 background.
[b] The maximum fermentation rate was calculated from the fermentation rate data by using time points corresponding to the steepest decline in weight.
[c] Weight loss is expressed as total grams per 300 ml of medium, corrected for evaporative loss.

TABLE 6. Effects of MMM media compositions on hydrogen sulfide formation[a]

Strain genotype	Effect of:				
	N at 433 mg/liter	N at 123 mg/liter	N at 65 mg/liter	N at 65 mg/liter plus 1/2 YNB	N at 123 mg/liter plus 1/5 YNB
*met17*Δ	+	+	+	+	+
*cys4*Δ	+	+	+	+	+
*gos1*Δ	+	+	+	+	+
*fcy22*Δ	+	+	+	+	+
*tpo2*Δ	+	+	+	+	+
*hom2*Δ	+	+	+	+	+
*hom6*Δ	+	+	+	+	+
*atp11*Δ	+	+	0	0	+
*ser33*Δ	+	+	0	0	+
*hht2*Δ	+	0	0	+	tr
*rxt2*Δ	+	0	0	+	tr
*iki3*Δ	0	0	0	+	0
*ypl035C*Δ	0	0	0	tr	0
*sit4*Δ	tr	0	0	+	0
*psy4*Δ	0	tr	0	+	+
*cgr1*Δ	+	0	0	+	+

[a] H_2S production was evaluated with lead acetate strips: +, a detectable amount of H_2S was produced; 0, H_2S was not detected; tr, very light color change indicating trace amounts of sulfide formation. The data represent the findings of at least two independent replicates.

minimum in triplicate using independent experiments, and the values were averaged. Because of growth requirements, the BY4742 Δ*cys4* deletion strain was grown in 208 mg of nitrogen/liter with 1 g per liter of yeast extract. There was a large variation in the amounts of sulfide produced, ranging from 4 to 493 μg/liter. There were seven high H_2S producers (>200 μg/liter), four medium producers (>20 μg/liter), and five low producers (<10 μg/liter). One-way analysis of variance was used to determine the standard error of the means. The variation in fermentation rate and total weight lost was not significant across the strains and less than 10% of the average values reported in the table. There was significant variation observed for the two highest sulfide-producing strains, and accurate quantitation of these levels of sulfide appears to be outside of the dynamic range of the commercial columns. Several strains showed relatively little variation in sulfide production levels across the replicates, i.e., the *gos1*Δ, *fcy22*Δ, *top2*Δ, *iki3*Δ, *rxt2*Δ, *yp1035c*Δ, *sit4*Δ, and *psy4*Δ strains. Five strains—i.e., the *atp11*Δ, *hht2*Δ, *hom2*Δ, *hom6*Δ, and *ser33*Δ strains—were found to display hypervariable H_2S production, with three of these showing variations greater than the value of the mean. This hypervariability was reproducible and therefore likely due to uncontrolled factors such as sensitivity to the position of the flask in the incubator or the level of aeration of the medium during preparation. Although the levels produced varied significantly, all deletants consistently produced H_2S throughout all experiments. This hypervariability is an important clue to the type of physiological effect resulting in sulfide formation.

Five of the positive H_2S-producing strains were defective in genes encoding for enzymes involved in sulfur-containing amino acid or precursor biosynthesis and were associated with the sulfate assimilation pathway. Six strains were defective in genes involved in transport, secretion, or cell wall or membrane integrity or other functions. One strain contained a mutation affecting ATP synthesis. The remaining strains had deletions in genes of unknown function. The available information regarding gene function was obtained from the *Saccharomyces* Genome Database (E. L. Hong et al. [http://www.yeastgenome.org/]). The deletion of some genes that impacted hydrogen sulfide production also impacted fermentation rate and completeness compared to the parental strain BY4742 (Table 5). However, there is no significant correlation between fermentation rate and the amount of hydrogen sulfide produced.

Impact of growth conditions on H_2S production. All 16 positive strains were further analyzed for hydrogen sulfide production in various media to determine the relationship between genotype and pattern of H_2S formation. Fermentations were run, in duplicate, in 433 mg of nitrogen/liter, 123 mg of nitrogen/liter, 123 mg of nitrogen/liter plus 1/5 YNB, 65 mg of nitrogen /liter (1/2 Casamino Acids), or 65 mg of nitrogen/liter (1/2 Casamino Acids) plus 1/2 YNB to determine whether nitrogen and micronutrient concentrations or combinations would impact the production of H_2S (Table 6).

The deletion strains were demonstrated to follow six basic patterns in their response to various medium compositions under these conditions. Seven deletion strains, i.e., the *cys4*Δ, *met17*Δ, *gos1*Δ, *fcy22*Δ, *tpo2*Δ, *hom2*Δ, and *hom6*Δ strains, demonstrated consistent hydrogen sulfide production under all conditions tested. The two *atp11*Δ and *ser33*Δ strains displayed reduced levels of H_2S production when the nitrogen concentration was decreased below 123 mg/liter. The *iki3*Δ and YPL035CΔ strains displayed increased levels of H_2S production when the nitrogen and micronutrient concentrations were both low. The *psy4*Δ deletion resulted in increased levels of H_2S production when the micronutrient concentration was decreased. The three *hht2*Δ, *rxt2*Δ, and *cgr1*Δ strains produced H_2S when the nitrogen level was high and under other nitrogen conditions when the micronutrient level was reduced. The

*sit4*Δ deletion resulted in hydrogen sulfide production under conditions in which nitrogen and vitamin levels were both high and when the nitrogen concentration was at the lowest level and the vitamin level was reduced. Thus, the patterns of H_2S production in these different mutants resembles that observed for commercial and native isolates, suggesting that wine isolates carry a variety of lesions impacting sulfide production.

Additive effect of gene deletions on H_2S production. Previous genetic analysis of H_2S production in native wine isolates suggested that this phenotype was under the control of multiple genes (40). Therefore, the effect of multiple mutations of the genes identified as leading to sulfide production was investigated by generating strains carrying more than one mutation. Each H_2S-producing deletion strain was mated with each of the other H_2S-producing deletion strains, and the resulting haploid strain carrying both deletions was selected for fermentations in 123 mg of nitrogen/liter to assess H_2S production. The plastic tube lead acetate method was used to compare H_2S production levels among strains.

Overall, the majority of the double-deletion strains did not have an additive effect on H_2S production (Table 7). The double-deletion strains produced the same amount of H_2S as the parental strains, or the double-deletion strain did not produce any H_2S. Some of the strains that did not produce any H_2S were sickly and did not grow well, so their failure to produce H_2S could have been due to the growth defect. There were three strains (the *hht2*Δ *fcy22*Δ, *cgr1*Δ *iki3*Δ and *cgr1*Δ *sit4*Δ strains) that produced significant increases in H_2S compared to either single null parental strain.

TABLE 7. H_2S production of double deletion strains in the BY4742 background

Strain genotype	H_2S (mm)[a]
*atp11*Δ	0
*cgr1*Δ	0
*fcy22*Δ	23
*gos1*Δ	22.5
*hom2*Δ	16
*hom6*Δ	19.5
*hht2*Δ	8
*iki3*Δ	0
*psy4*Δ	0
*rxt2*Δ	0
*ser33*Δ	23.5
*sit4*Δ	0
*tpo2*Δ	26.5
*ypl035C*Δ	0
*gos1*Δ *fcy22*Δ	0
*gos1*Δ *hom2*Δ	18
*gos1*Δ *hom6*Δ	16
*gos1*Δ *hht2*Δ	0
*gos1*Δ *iki3*Δ	0
*gos1*Δ *ser33*Δ	0
*gos1*Δ *tpo2*Δ	30.5
*hom2*Δ *fcy22*Δ	20
*hom2*Δ *atp11*Δ	1.5
*hom2*Δ *hht2*Δ	13.5
*hom2*Δ *iki3*Δ	24
*hom2*Δ *ser33*Δ	9
*hom2*Δ *tpo2*Δ	16.5
*hom6*Δ *fcy22*Δ	20
*hom6*Δ *atp11*Δ	11
*hom6*Δ *hht2*Δ	9
*hom6*Δ *ser33*Δ	8
*hom6*Δ *tpo2*Δ	9
*hht2*Δ *fcy22*Δ	37
*hht2*Δ *iki3*Δ	0
*hht2*Δ *tpo2*Δ	0
*iki3*Δ *fcy22*Δ	0
*iki3*Δ *atp11*Δ	0
*iki3*Δ *tpo2*Δ	25
*ser33*Δ *fcy22*Δ	0
*ser33*Δ *atp11*Δ	0
*ser33*Δ *tpo2*Δ	0
*tpo2*Δ *fcy22*Δ	0
*tpo2*Δ *atp11*Δ	0
*rxt2*Δ *iki3*Δ	tr[b]
*rxt2*Δ *hom2*Δ	1
*rxt2*Δ *hom6*Δ	5.5
*rxt2*Δ *ser33*Δ	0
*rxt2*Δ *tpo2*Δ	0
*rxt2*Δ *hht2*Δ	0
*rxt2*Δ *atp11*Δ	tr
*rxt2*Δ *psy4*Δ	0
*rxt2*Δ *fcy22*Δ	0
*rxt2*Δ *gos1*Δ	0
*rxt2*Δ *sit1*Δ	0
*cgr1*Δ *tpo2*Δ	13
*cgr1*Δ *ser33*Δ	5
*cgr1*Δ *hom2*Δ	3.5
*cgr1*Δ *iki3*Δ	11
*sit4*Δ *ser33*Δ	0
*sit4*Δ *cgr1*Δ	10
*sit4*Δ *atp11*Δ	0
*sit4*Δ *hht2*Δ	0
*sit4*Δ *tpo2*Δ	0
*sit4*Δ *fcy22*Δ	0
*sit4*Δ *hom6*Δ	2.5
*sit4*Δ *iki3*Δ	0
*sit4*Δ *hom2*Δ	6
*sit4*Δ *gos1*Δ	0
*ypl035C*Δ *atp11*Δ	0
*ypl035C*Δ *fcy22*Δ	0
*ypl035C*Δ *tpo2*Δ	0
*ypl035C*Δ *hht2*Δ	0
*ypl035C*Δ *hom2*Δ	7
*ypl035C*Δ *gos1*Δ	0
*ypl035C*Δ *iki3*Δ	0
*ypl035C*Δ *hom6*Δ	3.5
*ypl035C*Δ *ser33*Δ	0
*ypl035C*Δ *psy4*Δ	0
*ypl035C*Δ *sit4*Δ	0
*psy4*Δ *ser33*Δ	0
*psy4*Δ *fcy22*Δ	0
*psy4*Δ *gos1*Δ	0
*psy4*Δ *hom2*Δ	0
*psy4*Δ *hom6*Δ	0
*psy4*Δ *hht2*Δ	0
*psy4*Δ *tpo2*Δ	0

[a] mm refers to millimeters of darkening of the lead acetate in the column inserted into the headspace of the culture tube.
[b] tr, trace amount.

DISCUSSION

Screening of the native strains on BiGGY agar and under fermentation conditions identified several mutations that impact the production of H_2S. Colony color on BiGGY agar is thought to reflect the basal level of activity of sulfite reductase and can be considered to indicate the relative gap between the formation and sequestration of reduced sulfide in S-containing components of the cell. The majority of mutations did not appreciably impact colony color on BiGGY agar, with only 4 displaying a significantly lighter and 88 displaying a significantly darker colony hue. That the four white colonies identified genes directly impacting sulfite reductase activity is not surprising and is consistent with the view that this medium detects the relative basal level of activity of this enzyme complex. There was no correlation between darker colony color and H_2S production during grape juice fermentation. The lack of correlation likely reflects the differences in the growth conditions and in the detection methods between media. Both assays may indeed be indicators of relative sulfite reductase levels, but those levels are not correlated between the two growth conditions. However, the headspace detection of volatile sulfide via lead acetate strips is more closely correlated with production under commercial conditions (40).

Genes leading to the production of high levels of H_2S. Deletion of eight genes resulted in high levels of H_2S sulfide being produced (>100 μg/liter). Seven of these deletants produced high levels of sulfide in all of the media evaluated. Four of these genes (*CYS4*, *HOM2*, *HOM6*, and *MET17*) encode proteins of the sulfate reduction pathway (Fig. 1). *MET17* is im-

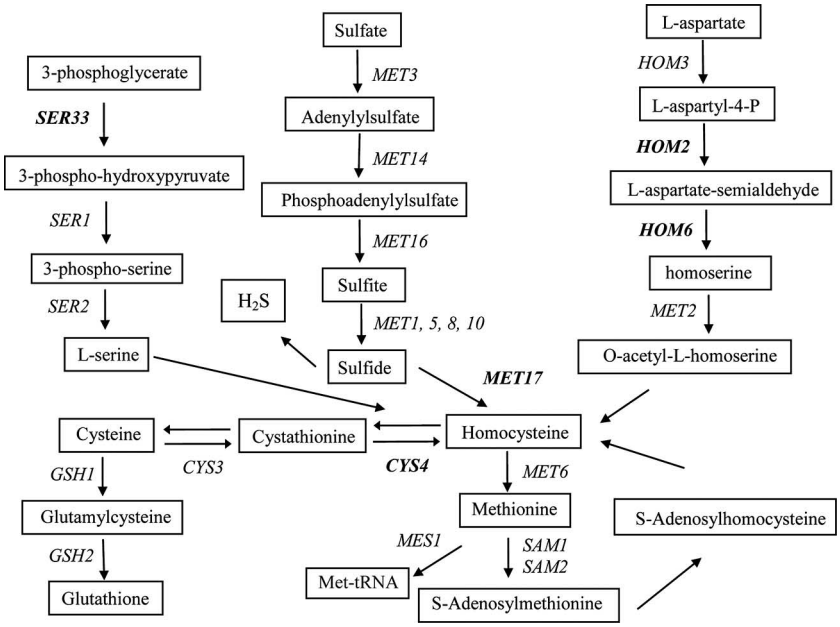

FIG. 1. Sulfate reduction pathway. Genes in boldface represent deletions that change the parental strain into an H_2S producer.

mediately downstream of sulfite reductase and is responsible for the incorporation of reduced sulfide into the acceptor molecule *O*-acetyl-L-homoserine. Loss of this activity would be predicted to lead to an accumulation of reduced sulfide, thus explaining the production of high levels of H_2S. *HOM2* and *HOM6* are required for the synthesis of *O*-acetyl-L-homoserine, and the inability to produce this compound would likewise be expected to result in the production of sulfide similar to the loss of *MET17*. However, it is interesting that the two other genes involved in *O*-acetyl-L-homoserine production, *MET2* and *HOM3*, did not affect sulfide formation under the conditions tested, nor did they lead to darker colonies on BiGGY agar. This finding suggests that it is not the mere absence of *O*-acetyl-L-homoserine that is responsible for the H_2S production phenotype. It is possible that L-aspartyl-4-P is an inducer of the sulfate reduction pathway or otherwise regulates sulfate reduction such that the loss of *HOM3* results in a decrease in activity of the pathway that compensates for the loss of incorporation of reduced sulfide. Similarly, homoserine may be a negative regulator of the pathway such that when homoserine levels accumulate sulfate reduction is reduced. The lack of homoserine in the *HOM2* and *HOM6* mutants may cause an increase in the activity of the sulfate reduction pathway rather than the lack of production of *O*-acetyl-L-homoserine leading to a decrease in the sequestration of reduced sulfide. We favor this latter hypothesis since it would offer an explanation of the hypervariability of the response seen in the production of sulfide. Conditions leading to subtle changes in the concentration of a regulatory metabolite for a pathway regulated by multiple metabolites could lead to a more variable phenotype sensitive to subtle environmental changes than would an absolute lack of substrate. Finally, the loss of the *CYS4* gene immediately downstream of *MET17* also resulted in high levels of production of H_2S. Since cysteine and methionine are both thought to be regulators of sulfate reduction, the imbalance between the two may drive sulfate reduction to increase pool levels of cysteine. Interestingly, the loss of *MET6* did not lead to an increase in sulfide formation. This suggests that under the anaerobic fermentation conditions evaluated, cysteine levels may play a more important regulatory role in sulfate reduction than does methionine. Loss of the genes encoding catalytic and regulatory subunits of sulfite reductase resulted in the elimination of sulfide formation, as would be expected. However, genes upstream of this activity had no apparent effect on sulfide formation, suggesting that the basal levels of sulfite reductase are not impacted by the loss of precursors.

The other three genes leading to a constitutively high level of sulfide are *FCY22*, *GOS1*, and *TPO2*. The gene *TPO2* encodes a polyamine transport protein in the cell membrane that displays specificity for spermine uptake (45). Polyamines are essential for cell growth, and spermine is involved in cellular metabolism. However, recent evidence has suggested that TPO genes encode a group of proton-motor-force-dependent multidrug transporters located in the plasma membrane (33). *TPO2* has also been demonstrated to be strongly induced by aldehyde, which suggests the protein is involved in transporting excess acetaldehyde out of the cell (4). Not only is acetaldehyde toxic to the cell, but it induces the sulfate reduction pathway (4). Because sulfur-containing compounds such as methionine, *S*-adenosylmethionine, and cysteine, as well as sulfite, an intermediate in sulfate reduction, bind to or otherwise interact with to prevent damage caused by acetaldehyde, insufficient amounts of one or more of these compounds may lead to induction of the sulfate reduction pathway, thus allowing more H_2S production. The role of cellular acetaldehyde levels in H_2S production merits further study as many commercial and wild strains show the interesting pattern of producing H_2S under nutrient-rich conditions or under limitation for micronutrients, both conditions that would be predicted to

lead to high cellular acetaldehyde levels. Research has shown that acetaldehyde rather than ethanol is the major factor limiting yeast fermentative ability (5, 19, 20).

The gene *GOS1* encodes for a type II membrane SNARE protein involved in transport and secretion and has been suggested to be involved in multiple transport steps, specifically endoplasmic reticulum-Golgi and intra-Golgi transport and to lead to defects in substrate transport (25; http://www.yeastgenome.org/). The gene *FCY22* encodes for a purine-cytosine permease that mediates the active transport of purine samples and cytosine (47). The role of these genes in hydrogen sulfide production is not apparent.

The final gene leading to high levels of H_2S in juice is *CGR1*. In contrast to the other seven high producers, sulfide production was not constitutive in this deletant. This strain only produced high levels of H_2S when medium levels of nitrogen were high relative to micronutrient content. As nitrogen was reduced keeping micronutrient levels high, the production of sulfide was likewise reduced. This is intriguing since many native isolates display this same pattern of behavior. *CGR1* encodes for a protein involved in nucleolar integrity and processing of pre-RNA (http://www.yeastgenome.org/). We found that sulfide formation by the *cgr1*Δ strain is dependent upon the volume and level of aeration of the medium, suggesting that the function of this gene in sulfide formation is related to the availability of oxygen, with more poorly aerated cultures yielding higher levels of sulfide, thus explaining the hypervariability in the sulfide production of this strain.

Genes resulting in moderate and variable production of H_2S. Deletion of three genes (*SER33*, *ATP11*, and *HHT2*) resulted in the production of, on average, moderate levels of H_2S, but the range of sulfide production of these strains was variable. The *SER33* gene encodes for the protein phosphoglycerate dehydrogenase, which catalyzes the first reaction of serine biosynthesis from the glycolytic metabolite 3-phosphoglycerate. *SER33* has been identified, based on mRNA data, to be the main isozyme of the phosphoglycerate pathway during growth on glucose (3); the other isozyme is encoded by *SER3*. In our study, sulfide levels were increased in the *ser33*Δ strain but not in the *ser3*Δ strain. Serine is utilized in the reaction catalyzed by *CYS4* and is incorporated, along with homocysteine, into cystathionine. This step may be a significant regulator of hydrogen sulfide production. Furthermore, phosphoglycerate dehydrogenase activity requires NAD^+ as a cofactor, and blockage of this reaction has been demonstrated to significantly affect redox metabolism (3). Increased sulfide was only detected when the first step of the serine biosynthesis pathway was blocked, suggesting that either 3-phosphoglycerate or 3-phospho-hydroxypyruvate is a regulator of the sulfate reduction pathway responsible for the appearance of sulfide, rather than a deficiency of serine. The effect of *SER33* on sulfide formation was dependent upon the nitrogen level of the medium. At low nitrogen levels, sulfide formation was greatly reduced. *SER33* expression has also been shown to be regulated by the available nitrogen source (3), which may explain why *ser33*Δ mutants did not produce H_2S at the lowest nitrogen concentration but did produce at the higher nitrogen concentrations where the serine biosynthesis pathway is more fully induced.

The *ATP11* gene encodes for a molecular chaperone of mitochondrial ATP synthase and is required for the assembly of alpha and beta subunits into the F1 sector of the F1F0 ATP synthase (1). The role of this specific gene in the production of hydrogen sulfide is likewise not clear.

HHT2 encodes a histone protein responsible for DNA binding and chromatin assembly. The role of this gene in sulfide formation is unclear since it, like *ATP11*, is predicted to affect multiple cellular processes. Hht2p has been reported to be associated with elongator, an RNA polymerase II-associated histone acetylase that facilitates transcription, and several other factors in a complex (23). Two other proteins identified in our study that affect sulfide production, Sit4p and Iki3p, are also known to form a complex with Hht2p (15, 23, 37). *SIT4* is involved in transcriptional regulation (9), and *IKI3* encodes for a subunit of the elongator complex (7, 22, 23). The fact that deletion of all three of these genes leads to increased H_2S suggests that the reported interaction of their gene products is of biological significance and this complex exerts some form of regulation on the reduction of sulfide, either directly or indirectly. Further research is needed to examine these interactions and the various other functions of *HHT2* to determine how mutation of these genes can impact sulfide formation. *SIT4* and *IKI3* only lead to H_2S formation in nutrient-depleted media. The *HHT2* deletant also led to increased sulfide under these conditions, but it did so under nutrient-rich conditions as well. *SIT4* encodes a serine-threonine phosphatase that functions in the G_1/S transition of the mitotic cycle, which is regulated by nutrient-induced signaling in yeast (50). This might explain why the *sit4*Δ strain produces H_2S only when the micronutrients and nitrogen were both reduced. However, the *SIT4* gene has been shown to affect multiple cellular functions: cell wall integrity and activity, actin cytoskeleton organization, and ribosomal gene transcription (9, 15, 37). The protein phosphatase encoded by *SIT4* is required for cell cycle progression and also functions as a cytoplasmic and nuclear protein that modulates functions mediated by Pkc1p, including cell wall and actin cytoskeleton organization (9, 15, 37). It is not clear which of its roles is important in minimizing H_2S formation.

Three other genes were also identified that impact H_2S formation under juice conditions. The role of deletions of these three genes—*YPL035C*, *RXT2*, and *PSY4*—in H_2S formation is not clear since all three encode proteins of unknown function (http://www.yeastgenome.org/). The effects of these genes on sulfide production may suggest a cellular role in sulfate reduction, or a secondary role impacting the regulation, activity, or precursor availability of sulfate reduction.

Additive effect of deletions on hydrogen sulfide formation. Overall, the majority of the combination of deletions affecting H_2S formation did not have an additive effect on hydrogen sulfide formation. There were three sets of double-deletion (*fcy22*Δ*hht2*Δ, *cgr1*Δ*iki3*Δ, and *cgr1*Δ*sit4*Δ) strains producing more H_2S than either parent. *FCY22* encodes for a purine-cytosine permease (47), and *HHT2* encodes for a protein involved in DNA binding and chromatin assembly. It is unclear how the simultaneous loss of *FCY22* and *HHT2* leads to elevated sulfide formation since the physiological roles of these two proteins have not been fully elucidated. Their effects on sulfide formation appear to be synergistic.

CGR1, as stated above, encodes for a protein involved with nucleolar integrity, and *IKI3* and *SIT4* have been shown to

physically interact in the nucleus. Again, the nature of that interaction with respect to sulfide formation has not been elucidated. That both of these genes lead to an increase in sulfide when present in a *cgr1*Δ further suggests that the complex that they are components of is the critical factor in increasing sulfide formation rather than other activities of these proteins.

Conclusion. Screening of the deletion set of *S. cerevisiae* identified several genes impacting H_2S formation. Five of these genes (*MET17*, *CYS4*, *HOM2*, *HOM6*, and *SER33*) encode proteins directly involved in the biosynthesis of the sulfur-containing amino acids. The fact that other genes involved in sulfate reduction did not demonstrate an impact on sulfide formation suggests that these genes or their substrates or products may play key regulatory roles in the reduction of sulfate. Other genes identified appear to have a more indirect role. Two key cellular activities are suggested in the present study as impacting sulfide production during anaerobic fermentation: accumulation of acetaldehyde and the elongator histone complex. The accumulation of acetaldehyde may be responsible for the increased expression of the sulfate reduction pathway and the increased levels of sulfide due to increased cellular demands for glutathione. This hypothesis can be tested by evaluating in detail the impact of the deletants identified on acetaldehyde accumulation and the oxidative stress response. The role of the elongator histone complex is unclear and merits further study. The other genes identified in the present study are of unknown or poorly characterized function. The mechanism by which loss of these genes affects the formation of H_2S will require a better understanding of their physiological roles in the cell.

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