Adaptation of *Candida albicans* to Reactive Sulfur Species

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ABSTRACT *Candida albicans* is an opportunistic fungal pathogen that is highly resistant to different oxidative stresses. How reactive sulfur species (RSS) such as sulfite regulate gene expression and the role of the transcription factor Zcf2 and the sulfite exporter Ssu1 in such responses are not known. Here, we show that *C. albicans* specifically adapts to sulfite stress and that Zcf2 is required for that response as well as induction of genes predicted to remove sulfite from cells and to increase the intracellular amount of a subset of nitrogen metabolites. Analysis of mutants in the sulfate assimilation pathway show that sulfite conversion to sulfide accounts for part of sulfite toxicity and that Zcf2-dependent expression of the *SSU1* sulfite exporter is induced by both sulfite and sulfide. Mutations in the *SSU1* promoter that selectively inhibit induction by the reactive nitrogen species (RNS) nitrite, a previously reported activator of *SSU1*, support a model for *C. albicans* in which Cta4-dependent RNS induction and Zcf2-dependent RSS induction are mediated by parallel pathways, different from *S. cerevisiae* in which the transcription factor Fzf1 mediates responses to both RNS and RSS. Lastly, we found that endogenous sulfite production leads to an increase in resistance to exogenously added sulfite. These results demonstrate that *C. albicans* has a unique response to sulfite that differs from the general oxidative stress response, and that adaptation to internal and external sulfite is largely mediated by one transcription factor and one effector gene.

KEYWORDS *Candida albicans*; sulfite; sulfide; ZCF2

REACTIVE sulfur species (RSS) play a role in cell signaling, redox homeostasis, and regulation of metabolism (Ali *et al.* 2006; Brannan 2010; Gruhlke and Slusarenko 2012). Similarly to reactive oxygen species (ROS) and reactive nitrogen species (RNS), redox active molecules containing oxygen and nitrogen, respectively, RSS are chemically reactive molecules containing sulfur atoms that are able to either oxidize or reduce biomolecules under physiological conditions (Giles *et al.* 2001). Reactive sulfur is formed as a byproduct of the reactions of biologically relevant thiol-containing molecules such as cysteine and glutathione, or as a result of sulfate (SO_4^{2-}) or sulfite (SO_3^{2-}) reduction (Brannan 2010). In bacteria, fungi, and plants, reactive sulfur-containing metabolites are

produced during reductive sulfate assimilation, where sulfate is reduced to sulfur-containing organic compounds (Thomas and Surdin-Kerjan 1997; Leustek *et al.* 2000). What controls the levels of toxic intermediates in this pathway is not known. One of these metabolites, sulfite, is of particular interest as a RSS due to its antimicrobial properties and wide use as a food preservative (Ogasawara *et al.* 2008; Rangan and Barceloux 2009).

In humans, sulfite is generated during oxidative catabolism of the sulfur-containing amino acid cysteine and is also released by neutrophils (Mitsuhashi *et al.* 2002; Stipanuk *et al.* 2006). The release of sulfite by neutrophils occurs in response to activation by bacterial lipopolysaccharides, suggesting that sulfite acts as an antimicrobial agent (Mitsuhashi *et al.* 1998). Sulfite is added to packaged food as an antimicrobial and anti-browning agent and in wine preparation as a selective inhibitor of bacteria (Taylor *et al.* 1986; Rangan and Barceloux 2009). The effective use of sulfite as a bacterial inhibitor in wine is due to the relative resistance of the wine yeast *Saccharomyces cerevisiae* to sulfite. Resistance of *S. cerevisiae* to either sulfite or RNS is mediated by transcriptional responses that require the

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Five Zinc Finger transcription factor FZF1 (Casalone *et al.* 1994; Avram *et al.* 1999; Park and Bakalinsky 2000; Sarver and DeRisi 2005; Engle and Fay 2012). Although the mechanism of sulfite resistance in *S. cerevisiae* has been investigated, responses to sulfite by other physiological relevant yeasts, such as the opportunistic fungal pathogen *C. albicans*, are less clearly understood (Nadai *et al.* 2016).

C. albicans inhabits the mouth, skin, and digestive tract of humans (Odds 1987). The ability to resist and adapt to stress, especially oxidative stress, allows *C. albicans* to inhabit these diverse niches and evade the host immune system (Jamieson *et al.* 1996; Enjalbert *et al.* 2007; Miramon *et al.* 2012). Pathways in *C. albicans* that protect against oxidative stress caused by ROS and nitrosative stress caused by RNS have been previously described. These ROS and RNS include chemicals such as hydrogen peroxide (H_2O_2) and nitric oxide (NO), respectively, which are used in humans as defense mechanisms against invading pathogens (Hromatka *et al.* 2005; dantas *et al.* 2015). Especially toxic are the products of the interactions between ROS and RNS, such as peroxy-nitrite, generated in macrophages by the reaction of nitric oxide with superoxide (McLean *et al.* 2010; Prolo *et al.* 2014). Moreover, NO reacts with cysteine thiols to form S-nitrosothiols (SNO), an effective inhibitor of Gram-positive bacteria and fungi (Finnen *et al.* 2007; Cariello *et al.* 2012). *C. albicans* is therefore exposed to a variety of toxic ROS, RNS, and RSS-related molecules and byproducts that it responds to in mechanistically distinct ways.

Nitrosative stress protection is mediated by the transcription factor Cta4 and the NO dioxygenase Yhb1 (Chiranand *et al.* 2008). NO also induces expression of *SSU1*, which encodes a sulfite transporter (Hromatka *et al.* 2005; Sarver and DeRisi 2005). However, *C. albicans* $\Delta ssu1$ mutants are not sensitive to NO, contrary to findings in *S. cerevisiae* (Sarver and DeRisi 2005; Chiranand *et al.* 2008). Pathways that specifically involve *C. albicans* adaptation to RSS such as sulfite remain to be elucidated. The transcriptional regulation in response to sulfite in *S. cerevisiae*, however, has been characterized and may provide an understanding of how *C. albicans* responds to sulfite.

Genome-wide transcriptional profiling in *S. cerevisiae* following exposure to sulfite showed upregulation of *SSU1* and the pyruvate decarboxylase gene *PDC1* (Park and Hwang 2008). Pdc1 catalyzes the reduction of pyruvate into acetaldehyde, which reacts with sulfite to form the nontoxic compound acetaldehyde hydroxysulfonate (Xu *et al.* 1994). Thus, *S. cerevisiae* is predicted to adapt to sulfite stress by increasing sulfite efflux from cells and detoxifying sulfite by its reactions with a chemical intermediate in ethanol fermentation. The genome-wide transcriptional responses to sulfite in *C. albicans* are unknown. *C. albicans* growth is inhibited by sulfite under acidic conditions and strains deleted for *SSU1* are hyper-sensitive to sulfite (Chiranand *et al.* 2008; Ogasawara *et al.* 2008).

Here we find that *C. albicans* adapts to sulfite and that this response requires the Zinc Cluster Transcription Factor (ZCF)

Zcf2 and the sulfite transporter Ssu1. Zcf2 has been previously implicated in cysteine-derived sulfite resistance and became our focus when we screened a collection of transcription factor deletion mutants from Homann *et al.* (2009) for increased sensitivity to sulfite (Hennicke *et al.* 2013). Transcript profiling revealed that sulfite induces a novel set of genes and that Zcf2 is required for induction of a subset of these genes. Remarkably, sulfide (S^{2-} , primarily present as HS^- and H_2S), a product of sulfite reduction, acts independently of sulfite to induce a Zcf2-dependent adaptation to sulfite. These results reveal a central role for Zcf2 in mediating cellular responses to different RSS.

Materials and Methods

C. albicans strains and growth conditions

C. albicans strains used in this project are listed in Supplemental Material, Table S1. Strain termed “Wild type” for all studies is $\Delta arg4$ and is derived from Homann *et al.* (2009). All deletion mutants were confirmed by PCR-based analysis. *C. albicans* strains were generally propagated at 30° in yeast extract–peptone–dextrose (YEPD) medium supplemented with uridine and adenine (1% yeast extract, 2% peptone, 2% dextrose, 40 mg/ml uridine, and 20 mg/ml adenine), or SD medium [Yeast Nitrogen Base: without amino acids or carbohydrate and with ammonium sulfate (YNB) from US Biological, 2% glucose, 80 mg/liter arginine].

For sulfite and sulfide growth assays, overnight cultures were diluted in YEPD pH 4.0 to an optical density (OD_{600}) of 0.03 and grown for 3 hr at 30° in a plate reader (Tecan) with continuous shaking. Cells were then challenged with various amounts of sulfite (0, 4, 8, or 12 mM) or sulfide (0, 1, or 2 mM) and incubated at 30° in the plate reader with continuous shaking overnight (16 hr). OD_{600} readings were taken every 30 min.

For adaptation assays, overnight cultures were diluted in YEPD pH 4.0 to an OD_{600} of 0.03 and grown for 3 hr at 30° in a plate reader with continuous shaking. Cultures were then pretreated with 0.25 mM Na_2SO_3 or 0.012 mM $Na_2S \cdot 9H_2O$ from a freshly prepared stock solution and grown at 30° for an additional 15 min. Following pretreatment, cultures were challenged with various concentrations of Na_2SO_3 (2, 4, and 8 mM) and grown overnight (16 hr) with continuous shaking at 30° in a plate reader. OD_{600} readings were taken every 30 min.

For microarray studies, overnight cultures were diluted to an OD_{600} of 0.03 in YEPD pH 4 medium and grown for 3 hr at 30°. Na_2SO_3 (0 or 0.25 mM) was added, and cultures were incubated at 30° for 15 min. The cultures were chilled on ice and pelleted by centrifugation. Finally, the pellets were flash frozen in liquid nitrogen and stored at –80°.

For luciferase assays, overnight cultures of the reporter strains in YEPD were diluted to an OD_{600} of 0.25 and grown to log phase for 4 hr in YEPD pH 4 before addition of sulfite, sulfide, cyanide, or nitrite, and in SD medium pH 4.8 for 4 hr before adding cysteine. For sulfate assimilation pathway flux

assays, reporter strains were diluted to an OD₆₀₀ of 0.25 and grown to log phase for 3 hr in YEPD pH 4 and subsequently washed in water and grown for 1 hr in defined SD medium without ammonium sulfate, containing or lacking 1% glucose, 10 mM ammonium chloride, or 0.5 mM methionine.

To test for methionine auxotrophy, overnight cultures were pelleted, washed once in water, resuspended in water, and incubated for 45 min at 30°. Cells were then diluted to OD₆₀₀ 0.03 in SD medium with or without 0.75 mg/ml methionine.

For testing the effects of sulfate assimilation pathway flux on sulfite resistance, strains were diluted to an OD₆₀₀ of 0.03 in YEPD pH 4, grown to log phase for 3 hr, and then resuspended and grown in SD medium (supplemented with 10 mM ammonium chloride and 1% glucose) with or without 0.75 mg/ml methionine for 1 hr. Cells were then washed and resuspended in YEPD pH 4 and challenged with 0 and 6 mM sulfite for 16 hr.

Analysis of sulfide formation

Sulfide production was evaluated using BiBAGY agar containing bismuth that will combine with sulfide to form a black (Bi₂S₃) pigment (Rikkerink *et al.* 1988). Each strain was plated onto BiBAGY agar, incubated at 30° for 48 hr, and assessed for color.

RNA isolation, microarray hybridization, and data processing

Total RNA was isolated from frozen pellets by hot phenol extraction (Schmitt *et al.* 1990; Chiranand *et al.* 2008) except that the RNA from the aqueous phase of the phenol extraction step was purified according to the Zymo Research Quick-RNA Miniprep protocol. Total RNA samples were eluted in RNase-free H₂O and analyzed using an Agilent 2100 Bioanalyzer.

RNA was reverse transcribed to Cy3 (green) and Cy5 (red) fluorescently labeled cDNA at a Baylor College of Medicine Genomic and RNA Profiling Core. The complementary DNA (cDNA) was hybridized to custom-designed oligonucleotide microarrays printed by Agilent Technologies (AMADID # 20166), as previously described (Nobile *et al.* 2012). cDNA from sulfite-treated wild-type cells was hybridized against differentially labeled cDNA from nontreated wild-type cells. Likewise, cDNA from sulfite-treated $\Delta zcf2$ cells was hybridized against differentially labeled cDNA from nontreated $\Delta zcf2$ cells. The log₂ ratios of red vs. green fluorescence were obtained from averages of two biological and two technical replicates in which the fluorescent dyes were swapped. Statistical significance was calculated through a standard *t*-test. The coefficient of determination (*R*²) was calculated by plotting replicates against each other: wild-type sulfite-treated green replicates 2 vs. 1, *R*² = 0.995; wild-type sulfite-treated red replicates 2 vs. 1, *R*² = 0.9957; wild-type nontreated green replicates 2 vs. 1, *R*² = 0.9904; wild-type nontreated red replicates 2 vs. 1, *R*² = 0.9921; $\Delta zcf2$ sulfite-treated green replicates 2 vs. 1, *R*² = 0.9965; $\Delta zcf2$ sulfite-treated red replicates 2 vs. 1, *R*² = 0.9955; $\Delta zcf2$ nontreated green replicates 2 vs. 1, *R*² = 0.9926; $\Delta zcf2$ nontreated red replicates 2 vs. 1, *R*² = 0.997.

Gene deletions

Homozygous deletion mutants of *MET16* were constructed in strains SN152 and *Δecm17* using methods previously described (Noble and Johnson 2005). The ORF of each *MET16* allele was replaced with cassettes containing *C. dubliniensis* *ARG4* and *C. albicans* *HygB*, whose 5'UTRs and ORFs were PCR amplified from plasmids pSN69 and pAU34-CaHygB, respectively (Basso *et al.* 2010).

Measuring sulfite

Sulfite concentrations were measured using MaxSignal Sulfite Assay Kit (Bioo Scientific). Sulfite concentrations were measured in water and YEPD pH 4 media supplemented with sulfide or sulfite. All samples were incubated at 30° for 30 min prior to being assayed for sulfite.

Construction of luciferase reporter strains

SSU1-gLUC and *ACT1-gLUC* plasmids were a modification of an *URA3*-marker-containing plasmid made by Anna Tillman. Nourseothricin resistance gene, *NAT*, from Clp10-*NAT* plasmid was inserted between *Bst*BI and *Sac*I restriction sites in place of the *URA3* marker. The luciferase reporter is a synthetic version of the *Gaussia princeps* luciferase gene fused to *C. albicans* *PGA59*, which encodes a cell-wall protein. Luciferase expressed from this fusion translocates to the cell surface, allowing for detection of luciferase luminescence from extracellular coelenterazine by intact cells (Enjalbert *et al.* 2009). The *MET3-gLUC* plasmid was created by inserting 1 kb of the *MET3* promoter region between *Xho*I and *Hind*III restriction sites in place of the *SSU1* promoter in the *SSU1-gLUC* plasmid. The plasmids were targeted to the *RPS1* locus by digesting *RPS1* at the *Stu*I site on the plasmid and then transforming the linearized plasmid into *C. albicans* strains by electroporation.

Luciferase assays

C. albicans cells were grown in liquid culture as reported. Sulfite (1.0 mM), sulfide (0.5 mM), or cyanide (0.25 mM) were added to the strains and grown for an additional 30 min, 0.5 mM nitrite was added to the strains and grown for 45 min, or 1.25 mM of cysteine was added to the strains and grown for an additional hour. The cells were subsequently collected by centrifugation, washed, and resuspended in luciferase assay buffer (LA buffer) as previously described (Enjalbert *et al.* 2009). The cells were deposited in solid white multiwell plates. Coelenterazine (Promega, Madison, WI) in LA buffer was added to a final concentration of 1.25 μM, and luciferase activity luminescence was recorded using a luminometer (Tecan). The RSS tested, sulfite or sulfide, caused only a small decrease in *ACT1p-gLUC* expression (Figure S1).

Construction of *SSU1-gLUC* mutants

Primers used in constructing mutant strains are listed in Table S2. Using site-directed mutagenesis by overlap extension as previously described (Ho *et al.* 1989), substitution

Figure 1 Sulfite-pretreated wild-type cells have an increase in sulfite resistance compared to non-pretreated cells. Cultures in log phase were pretreated for 15 min with 0.25 mM sulfite at 30° before being exposed to sulfite concentrations of 0, 2, 4, and 8 mM for 16 hr. Cell density readings were measured at a wavelength of 600 nm for (A) nontreated wild-type cells, (B) pretreated wild-type cells, (C) nontreated $\Delta zcf2$ cells, and (D) pretreated $\Delta zcf2$ cells. Results are averaged from three independent experiments ($n = 9$).

mutations were introduced in the *SSU1* promoter located in the plasmid containing the *SSU1p-gLUC* construct. First, two PCR reactions were carried out to amplify a region of the *SSU1* promoter and *gLUC* DNA on either side of the intended mutation, with the substituted nucleotides on a linker segment of the internal primers. Second, the two PCR products were fused in a third PCR reaction. The resulting fusion product was digested with enzyme *KpnI* and *BstXI* and ligated in the place of the native *SSU1* promoter in the plasmid containing the *SSU1p-gLUC* construct. The plasmids were confirmed by sequencing and transformed into *C. albicans* strains as previously described and targeted to the *RPS1* locus.

Motif discovery

One-kilobase promoter regions of *Zcf2*-dependent and independent genes were analyzed with the program The MEME Suite (Bailey and Elkan 1994) (<http://meme-suite.org/>).

Data availability

All strains and plasmids used in the study are available upon request. The raw array data files are available at Gene Expression Omnibus (GEO) (<http://www.ncbi.nlm.nih.gov/projects/geo/>) under accession no. GSE90819.

Results

Adaptation of *C. albicans* to sulfite requires the transcription factor *Zcf2*

Adaptation to stress occurs in a variety of cells: exposure to a subtoxic level of stress induces tolerance to normally toxic levels of stress (Guan *et al.* 2012). The human-resident fungus *C. albicans* adapts to different stresses (Brown *et al.* 2009), but whether and how it responds to RSS such as sulfite is poorly characterized. We confirmed the sensitivity of $\Delta zcf2$ and $\Delta ssu1$ to sulfite (Figure S2). To test for adaptation to sulfite, log phase *C. albicans* cultures were exposed for

15 min to a subtoxic level of sulfite (0.25 mM) – determined by growing *C. albicans* with various amounts of sulfite until the maximum concentration that gives little or no change in growth was found. Subsequently, cultures were challenged with higher, toxic levels of sulfite (2, 4, and 8 mM) and growth was measured for 16 hr (Figure 1). Pretreated cultures of the wild-type strain showed a greater resistance to sulfite than control cultures. Pretreated $\Delta zcf2$ cultures showed the same sensitivity to sulfite as control cultures. Reinsertion of the *ZCF2* gene in the $\Delta zcf2$ mutant complemented the sulfite adaptation phenotype (Figure S3). Therefore, *C. albicans* adapts to sulfite stress and *Zcf2* is necessary for this response. Similarly, pretreated $\Delta ssu1$ cultures also showed the same sensitivity to sulfite as control cultures (Figure S4).

Sulfite induces genes involved in alternative oxidation, sulfite detoxification, oligopeptide transport, and ribosome biogenesis in *C. albicans*

Zcf2 belongs to the fungal-specific zinc cluster transcription factor family, of which *S. cerevisiae* Gal4 is the best characterized (Traven *et al.* 2006). To determine which genes require *Zcf2* for sulfite induction, *C. albicans* strains were cultured for 15 min in the presence or absence of 0.25 mM sulfite and the amount of different mRNAs was analyzed by hybridization to *C. albicans* DNA microarrays. The transcript profiles of wild-type and $\Delta zcf2$ mutant strains grown in the presence and absence of 0.25 mM sulfite were examined. In the wild-type strain, ~1000 genes showed a statistically significant difference of at least twofold change in level of mRNA between the control and sulfite-treated cultures: 492 genes increased in expression and 511 genes decreased in expression. The 32 genes induced by at least eightfold in response to subtoxic sulfite include those predicted to be involved in alternative oxidation (e.g., *AOX2*), sulfite removal or detoxification (e.g., *SSU1*), nitrogen metabolism and oligopeptide transport (e.g., *AMO1*, *GAP2*, *CAN2*, *IFC3*, *ICF1*), and ribosome biogenesis

Table 1 Selected sulfite-induced genes involved in alternative respiration, sulfite removal, nitrogen catabolite repression, and ribosome biogenesis in wild-type *C. albicans*

Gene group and orf no. (name)	Proposed gene function	Fold induction
Alternative respiration orf19.4773 (AOX2)	Alternative oxidase	194
Sulfite removal orf19.7313 (SSU1)	Sulfite transporter	28
orf19.1867 (SSU12)	Sulfite transporter	13
Nitrogen metabolism orf19.5784 (AMO1)	Peroxisomal copper amine oxidase	26
orf19.6993 (GAP2)	Amino acid and polyamine permease	17
orf19.111 (CAN2)	Arginine transporter	16
orf19.3749 (IFC3)	Peptide transporter	14
orf19.3746 (OPT2)	Peptide transporter	14
orf19.313 (DAL4)	Allantoin permease	14
orf19.360 (FUR4)	Uracil permease	12
Ribosome biogenesis orf19.1862 (RTC3)	Ribosome metabolism	26

Selected genes significantly differentially expressed in sulfite-treated wild-type *C. albicans* cells (*t*-test: *n* = 8).

(orf19.1862) (Table 1). Twenty-five of 30 of the genes showing an eightfold or greater reduction in expression are predicted to have a role in ribosome or tRNA biogenesis, although no ribosomal protein genes showed altered expression (Table S3). Of the 492 most induced genes, 84 of them showed at least a twofold increase (19) or decrease (65) in the $\Delta zcf2$ strain relative to wild type.

The 65 genes that show Zcf2-dependent sulfite induction fit into categories similar to those observed to be induced in wild type in response to sulfite: sulfite reduction or removal, alternative oxidation, and nitrogen metabolism (Table 2).

Sulfite conversion to sulfide mediates the toxicity of sulfite in *C. albicans*

Induction of the alternative oxidase gene AOX2 suggests inhibition of cytochrome *c* (Xiao *et al.* 2010; Nicholls *et al.* 2013), in this case by sulfite. In the sulfate assimilation pathway (Figure 2), sulfate is metabolized to 3'-phospho-5'-adenylylsulfate (PAPS), which is subsequently reduced to sulfite via the Met16 PAPS reductase. Sulfite is then converted to sulfide by the Ecm17/Met10 sulfite reductase (Thomas and Surdin-Kerjan 1997; Li *et al.* 2013). Sulfide is toxic to *C. albicans*, and the $\Delta zcf2$ mutant is more sensitive to sulfide (Figure S5). An $\Delta ecm17$ mutant is highly resistant to sulfite (Figure 3), suggesting that sulfite conversion to sulfide mediates the toxicity of sulfite.

Although this $\Delta ecm17$ phenotype is consistent with the idea that sulfite conversion to sulfide mediates sulfite toxicity, there is another possible explanation. If there is flux of metabolites through the sulfate assimilation pathway, the block at the sulfite reductase step in the $\Delta ecm17$ strain is predicted to cause accumulation of sulfite that may lead to induction of sulfite adaptation. To test this possibility, strains were

constructed with homozygous deletions of *MET16* or of both *ECM17* and *MET16*. The natural sulfite accumulation in an $\Delta ecm17$ -deleted strain is predicted to be reduced when *MET16* is also deleted. As predicted, the $\Delta ecm17$ and $\Delta ecm17 \Delta met16$ strains fail to convert sulfite to sulfide, as assessed with a bismuth sulfide-based detection method (Figure S6). $\Delta met16$, $\Delta ecm17$, and $\Delta ecm17 \Delta met16$ also had the expected methionine autotrophy (Figure S6). The $\Delta met16$ strain displayed wild-type sulfite sensitivity while the $\Delta ecm17 \Delta met16$ strain showed a phenotype that was intermediate between the wild-type and the $\Delta ecm17$ strain (Figure 3). This result indicates that both sulfide and sulfite accumulation play a role in sulfite-induced toxicity. Even though natural sulfite accumulation was reduced in the $\Delta ecm17 \Delta met16$ strain, exogenous sulfite was still not as toxic as it would be in a sulfide-forming strain. Sulfite is also toxic to *S. cerevisiae* (Schimz 1980; Hinze and Holzer 1985); however, deletion of the *S. cerevisiae* *ECM17* ortholog *MET5* had no effect on the sulfite sensitivity of this yeast (Figure S7).

***C. albicans* senses sulfide**

To test whether *C. albicans* is adapting to sulfite stress by sensing the sulfide formed from sulfite added during pretreatment, *C. albicans* wild-type and $\Delta zcf2$ mutant strains were pretreated with a subtoxic amount of sulfide (0.012 mM) for 15 min before challenging with higher sulfite concentrations (Figure 4). The pretreated wild-type strain showed a greater resistance to sulfite than control cultures, while pretreated $\Delta zcf2$ mutant cultures showed no change in sulfite resistance. No change in sensitivity to sulfide was observed following sulfide or sulfite pretreatment. Zcf2 is therefore required for the increased sulfite resistance induced by sulfite or sulfide and suggests that both RSS induce sulfite resistance.

In the presence of oxygen, sulfide can oxidize to sulfite (O'Brien and Birkner 1977; Mishanina *et al.* 2015), thus raising the possibility that sulfide effects are mediated by sulfite. Measurement of sulfide conversion to sulfite using an enzyme-based assay showed that, after 30 min in water or YEPD medium at 30°, 2 mM sulfide yielded 4 or 6 μ M sulfite. Therefore, little sulfite is generated from sulfide under the experimental conditions used here. The dose dependence of the sulfite and sulfide pretreatment effect on sulfite resistance was assessed. The lowest concentration of sulfite or sulfide that gave a maximal increase in sulfite resistance was 31 or 6 μ M, respectively (Figure S8). Thus, even if the entire 6 μ M sulfide was converted to sulfite, that amount of sulfite is insufficient to induce sulfite resistance. These results argue that nonenzymatic conversion of sulfide to sulfite does not play a role in the ZCF2-dependent sulfite induction of sulfite resistance.

Zcf2-dependent expression of *SSU1* reporter is induced by several RSS

A luciferase-based reporter system was used to determine whether Zcf2 senses sulfite or sulfide since pretreatment with

Table 2 Several genes that show Zcf2-dependence in response to sulfite fit into three categories: sulfite removal or reduction, ammonium metabolism, and mitochondrial respiration

Gene group and orf no. (name)	Proposed gene function	Fold induction in wild type	Fold induction in $\Delta zcf2$	Ratio of fold induction in wild type vs. $\Delta zcf2$
Sulfite removal or reduction				
orf19.5811 (<i>MET1</i>)	Uroporphyrin-3 C-methyltransferase (siroheme)	15.6	1.4	11.1
orf19.7313 (<i>SSU1</i>)	Sulfite transporter	28.0	2.5	11.2
orf19.4076 (<i>MET10</i>)	Sulfite reductase	4.9	0.5	9.8
orf19.4099 (<i>ECM17</i>)	Sulfite reductase β -subunit	3.7	0.9	4.1
Ammonium metabolism				
orf19.6993 (<i>GAP2</i>)	Amino acid and polyamine permease	17.6	0.9	19.6
orf19.5784 (<i>AMO1</i>)	Peroxisomal copper amine oxidase	25.4	1.7	14.9
orf19.4063 (<i>GPT1</i>)	GABA/polyamine transporter	8.6	0.7	12.3
orf19.5672 (<i>MEP2</i>)	Ammonium permease	10.2	1.0	10.2
orf19.780 (<i>DUR1,2</i>)	Urea amidolyase	12.1	1.8	6.7
orf19.6570 (<i>NUP</i>)	Nucleoside permease	3.8	0.6	6.3
orf19.313 (<i>DAL4</i>)	Allantoin permease	14.0	2.7	5.2
orf19.781 (<i>DUR3</i>)	Spermidine transporter	4.5	1.0	4.5
orf19.2882 (<i>XUT1</i>)	Xanthine-uric acid transporter	2.2	0.5	4.4
orf19.111 (<i>CAN2</i>)	Arginine transporter	16.1	3.7	4.3
orf19.360 (<i>FUR4</i>)	Uracil permease	12.4	3.0	4.1
orf19.4716 (<i>GDH3</i>)	Glutamate dehydrogenase	3.0	1.0	3.0
orf19.97 (<i>CAN1</i>)	Amino acid permease	6.3	2.2	2.9
orf19.2474 (<i>PRC3</i>)	Carboxypeptidase	3.2	1.2	2.7
orf19.1275 (<i>GAT1</i>)	Transcription factor (regulator of nitrogen utilization)	10.1	4.3	2.3
orf19.6950	Arginine exporter from vacuole	3.2	1.5	2.1
Mitochondrial respiration				
orf19.1114	Cytochrome c oxidase subunit	2.0	0.6	3.3
orf19.4841 (<i>SHY1</i>)	Cytochrome c oxidase biosynthesis	3.5	1.3	2.7
orf19.1416 (<i>COX11</i>)	Cytochrome oxidase assembly	3.0	1.1	2.7
orf19.1667.1	Cytochrome oxidase assembly	2.9	1.2	2.4
orf19.409	Cytochrome c oxidase subunit	4.8	2.0	2.4
orf19.4773 (<i>AOX2</i>)	Alternative oxidase	198.5	84.1	2.4
orf19.1336.2	Mitochondrial respiratory chain complex assembly	2.3	1.0	2.3
orf19.4774 (<i>AOX1</i>)	Alternative oxidase	10.8	5.2	2.1

Twenty-eight of the 65 Zcf2-dependent genes that were downregulated more than twofold in the $\Delta zcf2$ mutant vs. wild type (t-test: $n = 8$).

either molecule resulted in sulfite adaptation. Wild type showed increased *SSU1-gLUC* induction in response to sub-toxic levels of sulfite (1 mM) or sulfide (0.75 mM) compared to control cultures, while the $\Delta zcf2$ strain showed no induction by either RSS (Figure 5A). Thus, Zcf2 is involved in sensing both these signals; however, it is possible that the sulfite induction of *SSU1-gLUC* expression was due to the conversion of sulfite to sulfide by sulfite reductase Ecm17. To test this possibility, the reporter gene induction was tested in a $\Delta ecm17 \Delta met16$ strain. *SSU1-gLUC* expression increased in the $\Delta ecm17 \Delta met16$ strain in response to exogenously added sulfite or sulfide, suggesting that both of these RSS can act as signals. Induction by sulfite is lower in $\Delta ecm17 \Delta met16$ than in wild type, supporting the idea that part of sulfite induction of *SSU1* is due to sulfite conversion to sulfide. Cyanide, another known inhibitor of cytochrome c oxidase, did not induce *SSU1* expression (Figure S9).

In addition to sulfite and sulfide, a third toxic sulfur-containing compound, cysteine, has been shown to induce

SSU1 expression in *C. albicans* in a Zcf2-dependent manner (Hennicke *et al.* 2013). To confirm whether *SSU1* activation by cysteine requires Zcf2, 1.25 mM cysteine was added to wild-type, $\Delta zcf2$, and $\Delta cdg1$ strains containing the *SSU1-gLUC* reporter in SD medium. Conversion of cysteine to sulfite is blocked in the $\Delta cdg1$ strain (Hennicke *et al.* 2013). *SSU1-gLUC* induction by cysteine was observed in all three strains at varying levels (Figure 5B); however, the $\Delta zcf2$ and $\Delta cdg1$ strains showed significantly reduced induction compared to wild type. These findings confirm previous work (Hennicke *et al.* 2013) showing that *SSU1* regulation by cysteine is partially dependent on Zcf2 as well as the conversion of cysteine to sulfite by Cdg1.

Sulfite induction of *SSU1* is independent of nitrite induction

The induction of *SSU1* in response to nitrite and sulfite by Cta4 and Zcf2, respectively, raises the possibility that Zcf2 regulates *SSU1* through different binding sites. Locating the

Figure 2 Sulfate assimilation pathway in *C. albicans*.

binding site will further determine whether nitrite, sulfite, and cysteine use common or parallel pathways to regulate *SSU1* expression. In *C. albicans*, the regulatory region of a gene is typically within a kilobase upstream of the start codon of the ORF. The effects of mutations in six upstream sequence motifs, or putative transcription factor binding sites, of the *SSU1* promoter region were tested. Three of the motifs (motifs 1–3) were found via motif-detecting software (Bailey and Elkan 1994) using data from the Zcf2-dependent sulfite regulon. The other three motifs were determined using existing information of binding motifs for other zinc cluster family members (Table S4).

Previously, a common binding element was found for the class of zinc cluster transcription family that includes the well-studied Gal4 transcription factor, as well as Zcf2 (MacPherson *et al.* 2006). The binding element consists of a pair of palindromic CGG nucleotide triplets separated by a variable number of nucleotides (Giniger *et al.* 1985; Liang *et al.* 1996). In the *C. albicans SSU1* promoter, two CGG triplets were found separated by 14 nt (motif 4). An additional variation of the CGG binding element was found containing CGG and GCG triplets (motif 5). Lastly, the putative nitric oxide-responsive element (NORE) motif of Cta4, which belongs to the same zinc cluster transcription regulatory family as Zcf2, was identified in the *SSU1* promoter region as CCTCGG (motif 6), differing from the NORE palindrome (CCGTCGG) by 1 nt (Chiranand *et al.* 2008).

Mutations were introduced in the *SSU1* promoter region of the *SSU1-gLUC* reporter gene expressed in *C. albicans* and the effect of the mutations was assessed in response to reactive chemicals known to activate *SSU1* such as sulfite, sulfide, cysteine, or nitrite. Mutating motifs 1–3 and 5 had no effect; however, mutating motifs 4 and 6 reduced *SSU1-gLUC* induction by nitrite and sulfite, respectively (Figure 6). Therefore,

Figure 3 *C. albicans* lacking *ECM17* are resistant to sulfite. Cell cultures in log phase were challenged for 16 hr with 0 and 12 mM sulfite (filled and open markers, respectively). Results are averages of three biological replicates ($n = 3$).

two putative zinc cluster regulatory sites have been identified that regulate *SSU1* in response to sulfite and nitrite. Mutating the motif 4 CGG and GCG triplets individually (motif 4A and motif 4B) still resulted in reduced *SSU1-gLUC* induction by nitrite (Figure 6B).

Internal production of sulfite mediates resistance to external sulfite

Sulfite and sulfide are metabolic intermediates in the sulfate assimilation pathway. In an efficient pathway, product rapidly becomes substrate for the next step and such channeling is observed in some pathways (Huang *et al.* 2001). But in other pathways, an overflow of diffusible, toxic pathway intermediates can occur. At least three mechanisms limit the damage of these toxins: compartmentation of the pathway (Kerfeld *et al.* 2010), enzymatic conversion to nontoxic side products (Xu *et al.* 1994), or transport of the toxic intermediate out of the cell. Removal of toxic metabolic intermediates by catalysis or transport is inducible by the toxin or part of an overall metabolic program for setting relative levels of pathway enzymes. This logic suggests that *SSU1* expression can be induced by overabundance of sulfite, or sulfide, produced by the sulfate assimilation pathway. To test this, we first grew *C. albicans* in rich medium and then switched to minimal medium lacking amino acids for an hour. Expression of *MET3*, encoding the first enzyme of the sulfate assimilation pathway, was induced by the minimal medium. This induction was inhibited by adding methionine to the medium (Figure S10), consistent with an expected induction of the sulfate assimilation pathway in minimal medium. Then, after growth in this minimal medium, *C. albicans* cultures were switched back to rich medium with or without added sulfite to test for sulfite resistance. Addition of methionine to the short-term minimal media step increased the growth sensitivity to sulfite in rich medium. Wild-type cells grown without methionine were less sensitive to sulfite than wild-type cells grown with methionine. The decrease in sensitivity to sulfite

Figure 4 Sulfide-pretreated wild-type cells have an increase in sulfite resistance compared to non-pretreated cells. Cultures in log phase were pretreated for 15 min with 0.012 mM sulfide at 30° before being exposed to sulfite concentrations of 0, 2, 4, and 8 mM for 16 hr. Cell-density readings were measured at a wavelength of 600 nm for (A) nontreated wild-type cells, (B) pretreated wild-type cells, (C) nontreated $\Delta zcf2$ cells, and (D) pretreated $\Delta zcf2$ cells. Results are averaged from three independent experiments ($n = 9$).

was not observed in a $\Delta met16$ mutant that cannot generate endogenous sulfite (Figure 7). Expression of the sulfite exporter gene *SSU1* showed a similar pattern in minimal medium: a growth-dependent, methionine-inhibited, induction of expression that was strongly reduced in $\Delta met16$ or $\Delta zcf2$ strains (Figure 8). Together these data suggest a model in which an excess of endogenous sulfite product by the Met16 PAPS reductase is reduced by a Zcf2-dependent sulfite (and sulfide)-induction of the sulfite exporter Ssu1, with an attendant decrease in sensitivity to extracellular sulfite. This result is consistent with a model where endogenous sulfite being produced via sulfate assimilation pathway flux promotes greater resistance to exogenously added sulfite.

Discussion

Sulfur-containing compounds are reduced or oxidized by presumably all organisms. Sulfur metabolism leads to the production of RSS, such as sulfide, which have a range of effects including microbial and environmental toxicity, to physiologically important signaling roles in mammals (Wang 2012). Here we show that the human pathogen *C. albicans* alters its sulfite sensitivity in response to different RSS, including those produced by its human host or released by neighboring bacteria. Zcf2 is necessary for *C. albicans* to respond to sulfite and sulfide suggesting that these RSS are ligands that are directly or indirectly sensed by Zcf2, a novel finding, as a specific receptor for sulfide is previously not known (Mishanina *et al.* 2015). Although *C. albicans* changes its sensitivity to sulfite in response to sulfite or sulfide, we were unable to detect adaptation of *C. albicans* to sulfide, that is, when *C. albicans* is pretreated with a subtoxic concentration of sulfide or sulfite, no increase in resistance to sulfide was observed. Increased sulfite resistance is correlated with an increase in expression of *SSU1*, which encodes the sulfite transport protein, Ssu1. Adjusting sulfide resistance by

changing the activity of a transporter protein is unlikely to be effective because the nonpolar H_2S molecule – abundant at neutral pH with its pKa of 6.9 – freely permeates the phospholipid bilayer.

Aside from sensing sulfide and sulfite, Zcf2 is required for cysteine inducible expression of *SSU1* (Hennicke *et al.* 2013). We extended and confirmed these results by analyzing sulfite adaptation and the molecules that induce *SSU1* expression. Using the *SSU1-gLUC* reporter, however, we show that *SSU1* regulation by cysteine is only partially Zcf2-dependent. Although *SSU1-gLUC* fold induction is reduced in the $\Delta zcf2$ strain compared to wild type, a significant cysteine-induced increase in *SSU1* expression was still observed, unlike when sulfite or sulfide was added to the $\Delta zcf2$ strain. Additionally, the reduction of *SSU1-gLUC* fold induction in the strain lacking *CDG1*, encoding the protein that converts cysteine to sulfite, suggests that cysteine activation of *SSU1* is due to cysteine conversion to sulfite or even sulfide. However, the increase in luminescence in the $\Delta cdg1$ strain in response to cysteine indicates that cysteine itself is also a signal. Therefore, it is possible that the reduced levels of *SSU1* induction in the $\Delta zcf2$ strain was because there was no Zcf2 present to sense the resulting sulfite formed from the added cysteine. To further examine gene induction in response to RSS, we examined the sulfite regulon of *C. albicans*.

Approximately 1000 genes are regulated by sulfite, roughly one-sixth of the protein coding genes reported in *C. albicans*. This large number of affected genes indicates the possibility of a transient inhibition of growth by the sulfite added, or that sulfur metabolism in *C. albicans* is involved in diverse cellular processes as shown previously (Garcia-Sanchez *et al.* 2004). Here we show that four functional sets of genes are regulated in response to sulfite: sulfite removal, ammonia metabolism, ribosome biogenesis, and alternative respiration. The genes involved in ribosome biogenesis were downregulated after exposure to sulfite. Under ROS stress, yeast downregulate

Figure 5 *SSU1-gLUC* induction, shown as Relative Luminescence Unit (RLU)/OD₆₀₀, by sulfite and sulfide is Zcf2-dependent. Cultures in log phase were grown in the presence or absence (white) of (A) 1.0 mM sulfite (gray) or 0.5 mM sulfide (black) for 30 min, or (B) 1.25 mM cysteine (black) for 1 hr, at 30°. *SSU1-gLUC* induction by sulfite and sulfide was observed in the wild-type strain, as well as in cells unable to endogenously produce sulfite ($\Delta met16$), sulfide ($\Delta ecm17$), or either sulfite and sulfide ($\Delta ecm17 \Delta met16$). No *SSU1-gLUC* induction was observed in the $\Delta zcf2$ mutant. Induction by cysteine was seen in all three strains tested. Results are averages of three biological replicates. *** and **** indicate that the detected differences were significant: $P < 0.001$ and $P < 0.0001$, respectively.

ribosome biogenesis to direct energy resources for other cellular processes (Loar *et al.* 2004). However, our microarray results demonstrated that Zcf2 does not play a role in down-regulating ribosome biogenesis genes. This is in contrast to other zinc cluster family members, such as Zcf29, which is involved in downregulating ribosome biogenesis genes (Issi *et al.* 2017). The sulfite removal genes and most of the

Figure 6 Mutating motifs 4 and 6 reduced *SSU1-gLUC* induction, shown as Relative Luminescence Unit (RLU)/OD₆₀₀, by nitrite and sulfite, respectively. Cultures in log phase were grown in the presence (black) or absence (white) of (A and B) 0.5 mM nitrite for 45 min, or (C) 1.0 mM sulfite for 30 min, at 30°. Results are averages of three biological replicates. * and ** indicate that the detected differences were significant: $P < 0.05$ and $P < 0.01$, respectively.

ammonia metabolism genes are Zcf2-dependent. Of the most upregulated genes, only $\Delta ssu1$ showed a sulfite-sensitive phenotype. When we examined the phenotype of four other strongly upregulated genes, *AOX2*, *AOX1*, *AMO1*, and Orf 19.3120, no sulfite sensitivity phenotype was observed.

The most induced gene in response to sulfite is *AOX2*. Aox2 provides a distinct oxidase pathway from cytochrome

Figure 7 Increased sulfate assimilation pathway flux induces *C. albicans* sulfite resistance. Wild-type and $\Delta met16$ mutant strains were challenged for 16 hr in YEPD pH 4 with 0 and 6 mM sulfite (filled and open markers, respectively) after being grown to log phase for 3 hr in YEPD pH 4, and then in SD medium (supplemented with ammonium chloride and glucose) with or without methionine for 1 hr. Results are averages of four experiments, each with three biological replicates for each condition.

c oxidase located in the mitochondrial electron transport chain. The upregulation of *AOX2* suggests that respiration, or more specifically cytochrome c oxidase, is being inhibited by sulfite. This observation could be explained by the fact that in *C. albicans*, sulfite is converted by sulfite reductase, *Ecm17*, to sulfide, a well-known cytochrome c oxidase inhibitor (Khan *et al.* 1990; Nicholls *et al.* 2013). The reason for the induction of other gene sets, however, such as nitrogen catabolism, is not as easily understood.

Genes that are likely to be upregulated under nitrogen-poor conditions were upregulated in response to sulfite despite the use of nitrogen-rich YEPD media. We speculate that the exogenously added sulfite reacts with free cysteine to form S-sulfocysteine, a metabolite that structurally resembles glutamate and is an antagonist of the glutamate receptor (Olney *et al.* 1975; Zhang *et al.* 2004). Glutamate is one of the initial products of ammonium assimilation in *C. albicans* and a major regulator of ammonia metabolism (Holmes *et al.* 1991). Therefore, an investigation of the induction of nitrogen catabolism genes in response to S-sulfocysteine will be of future interest. It is not certain whether the blocking of ammonium assimilation, due to the formation of S-sulfocysteine from sulfite and cysteine, contributes to sulfite-induced toxicity. However, the enzymatic conversion of sulfite to sulfide does contribute to sulfite-induced toxicity.

The *C. albicans* $\Delta ecm17$ mutant, deficient in forming sulfite from sulfide, is less sensitive to sulfite; supporting the hypothesis that sulfite-derived sulfide contributes to sulfite toxicity. However, this result could be due to increased *SSU1* levels in the $\Delta ecm17$ mutant from endogenous sulfite accumulation. Even so, the $\Delta ecm17 \Delta met16$ mutant, which has lower levels of *SSU1* than $\Delta ecm17$, had a sulfite sensitivity phenotype that was intermediate between the wild type and $\Delta ecm17$, suggesting that both sulfite and sulfide are directly toxic to *C. albicans*. Interestingly, deletion of *S. cerevisiae*

Figure 8 Increased sulfate assimilation pathway flux increases endogenous sulfite levels. *SSU1-gLUC* reporter luminescence, shown as Relative Luminescence Unit (RLU)/OD₆₀₀, was measured in *C. albicans* wild-type (white), $\Delta zcf2$ (gray), and $\Delta met16$ (black) strains that were grown for 1 hr at 30° in SD medium containing or lacking ammonium chloride, glucose, and methionine. Nutrients essential for growth, ammonia (NH₃) or glucose, are required for *SSU1-gLUC* induction, consistent with growth-dependent activity of the sulfur assimilation pathway (Figure S11). Results are averages of three biological replicates. ** indicates that the detected differences were significant: $P < 0.01$.

ECM17 homolog *MET5* did not result in a sulfite-resistant phenotype. This observation, along with differences in genes most upregulated in response to sulfite (*PDC1* in *S. cerevisiae* and *AOX2* in *C. albicans*), emphasize how distinctly these two yeasts respond to sulfite toxicity.

Metabolic pathways involving reduction and oxidation often have toxic intermediates and the regulation of their levels is not known. In *C. albicans*, there are no known enzymes that convert toxic sulfite to nontoxic sulfate, such as sulfite oxidase, an enzyme that occurs in humans, bacteria, and plants (Kappler and Enemark 2015). Therefore, *C. albicans* controls the levels of excess toxic sulfite produced in a different way. There are a variety of ways cells control the levels of excess toxic intermediates produced. One way is to control the concentration of enzymes in the pathway or through substrate channeling where intermediates in the pathway are passed directly from one enzyme to another without being released into solution (Huang *et al.* 2001; Bar-Even *et al.* 2012). Another way is by getting rid of the toxic substance, in this case, via *Ssu1*. When we increased flux through the sulfur assimilation pathway by growing *C. albicans* in media lacking methionine, resistance to exogenously added sulfite also increased. Increasing sulfate assimilation pathway flux and adding sulfite to *C. albicans* were both correlated with an increase in *SSU1* induction. From this we can conclude that similar mechanisms are used to avoid endogenous and exogenous sulfite toxicity.

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