

Factors Supporting Cysteine Tolerance and Sulfite Production in *Candida albicans*

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The amino acid cysteine has long been known to be toxic at elevated levels for bacteria, fungi, and humans. However, mechanisms of cysteine tolerance in microbes remain largely obscure. Here we show that the human pathogenic yeast *Candida albicans* excretes sulfite when confronted with increasing cysteine concentrations. Mutant construction and phenotypic analysis revealed that sulfite formation from cysteine in *C. albicans* relies on cysteine dioxygenase Cdg1, an enzyme with similar functions in humans. Environmental cysteine induced not only the expression of the *CDG1* gene in *C. albicans*, but also the expression of *SSU1*, encoding a putative sulfite efflux pump. Accordingly, the deletion of *SSU1* resulted in enhanced sensitivity of the fungal cells to both cysteine and sulfite. To study the regulation of sulfite/cysteine tolerance in more detail, we screened a *C. albicans* library of transcription factor mutants in the presence of sulfite. This approach and subsequent independent mutant analysis identified the zinc cluster transcription factor Zcf2 to govern sulfite/cysteine tolerance, as well as cysteine-inducible *SSU1* and *CDG1* gene expression. *cdg1* Δ and *ssu1* Δ mutants displayed reduced hypha formation in the presence of cysteine, indicating a possible role of the newly proposed mechanisms of cysteine tolerance and sulfite secretion in the pathogenicity of *C. albicans*. Moreover, *cdg1* Δ mutants induced delayed mortality in a mouse model of disseminated infection. Since sulfite is toxic and a potent reducing agent, its production by *C. albicans* suggests diverse roles during host adaptation and pathogenicity.

Although sulfite is potentially toxic, in yeasts the molecule is also a normal intermediate during reductive sulfate assimilation. Due to its antimicrobial and antioxidative properties, sulfite is also well known for its common use as a preservative in food industries, in particular during the production of wine (1). The molecular basis of sulfite tolerance has therefore been intensively studied in fermenting yeasts and led to the identification of the sulfite efflux pump Ssu1 in *Saccharomyces cerevisiae* (2, 3). Overexpression of *SSU1* in *S. cerevisiae* resulted in enhanced sulfite resistance, and the gene was shown to be positively controlled by the transcription factor Fzf1 (4–6). Beside a role in sulfite detoxification, other biological functions have been addressed for Ssu1 in *S. cerevisiae*, and with a pathobiological interest, in the human pathogenic yeast *Candida albicans*. Of particular note, transcriptional profiling in both *S. cerevisiae* and *C. albicans* during nitrosative stress identified a small set of upregulated genes, including *SSU1* (7, 8). This nitric oxide-induced response was found in *S. cerevisiae* to depend on Fzf1, which, however, is absent from *C. albicans*. Instead, Cta4 was identified in *C. albicans* as a major nitrosative stress regulator. Nitric oxide-induced expression of *SSU1* in *C. albicans* was to some extent dependent on this transcription factor but not sulfite resistance (9).

A sophisticated function of active sulfite secretion was hypothesized in dermatophytes. The particular ability of these specialized filamentous fungi to grow within keratinized host structures was correlated with sulfite secretion as a means to reduce the keratin stabilizing disulfide bonds (10–12). A putative role of Ssu1 in this process of sulfitolysis was supported by the observation that *SSU1* is constitutively expressed at a high level in these microorganisms

(13). In humans, sulfite is produced as an intermediate during oxidative cysteine catabolism, a process which is regulated by the key enzyme cysteine dioxygenase Cdo1. A homologous enzyme has recently been detected also in dermatophytes (14). Apart from potential functions in active sulfite formation, such a pathway may at the same time also represent an alternative means of cysteine detoxification, like in humans (15). Elevated concentrations of cysteine are known to be growth inhibiting not only for humans or dermatophytes, but also for a broad variety of other microorganisms (15–24). We have recently reported a functional role of Ssu1 and Cdo1 for keratin degradation and cysteine tolerance by targeted mutagenesis in the dermatophyte *Arthroderma benhaimiae* (25).

C. albicans, a common commensal of the human gastrointestinal tract, has also long been known for the growth sensitivity to increasing cysteine levels (23, 26). Cysteine and other thiols are present in the mammalian gut at considerable concentration, where they support eminent intestinal functions, including tissue

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redox homeostasis, mucus fluidity and nutrient absorption (27). Therefore, mechanisms which favor cysteine tolerance in *C. albicans* are likely to be important during host adaptation processes.

In order to uncover mechanisms supporting cysteine and sulfite tolerance in *C. albicans*, the present work was started by investigating a potential role of the putative sulfite efflux pump *Ssu1* in this process. It is known from prior work that *C. albicans ssu1Δ* mutants are sensitive to toxic sulfite, a phenotype shared with *S. cerevisiae* (9). Since in humans sulfite can be produced from cysteine, we found it reasonable to discover that *C. albicans ssu1Δ* mutants were also sensitive to cysteine and that cysteine induced the expression of the *SSU1* gene. In humans, the conversion of cysteine to sulfite depends on cysteine dioxygenase *Cdo1*. Therefore, we were attracted by a homologue of this key enzyme in *C. albicans*, *Cdg1*. Sulfite production from cysteine in *C. albicans* was abolished in a *C. albicans cdg1Δ* knockout mutant. A transcriptional regulator governing *CDG1* and *SSU1* expression as well as sulfite tolerance was identified.

MATERIALS AND METHODS

Strains and growth conditions. *C. albicans* strains used in the present study are listed in Table S1 in the supplemental material. All *C. albicans* strains were routinely propagated on YPD agar plates (10 g yeast extract, 20 g of peptone, 20 g glucose, and 15 g of agar per liter) at 30°C and stored as frozen stocks in liquid YPD medium with 15% glycerol at -80°C. A library of *C. albicans* transcription factor mutant strains (28) was obtained via The Fungal Genetics Stock Center (University of Missouri, Kansas City, MO) (29).

Plasmid constructions. A construct for the deletion of the *SSU1* gene was generated as follows: an *ApaI*-*XhoI* fragment with *SSU1* upstream sequences was cloned after amplification by PCR with the primers *SSU1-1* and *SSU1-2* (all of the primers are listed in Table S2 in the supplemental material). Genomic DNA from *C. albicans* strain SC5314 was used as a template. A *SacII*-*SacI* fragment containing *SSU1* downstream sequences was obtained with the primers *SSU1-3* and *SSU1-4*. The *SSU1* upstream and downstream fragments were successively substituted for *WOR1* upstream and downstream fragments in plasmid *pWOR1M2* via the introduced restriction sites, respectively (30), to result in *pSSU1M2*, in which the *SAT1* flipper is flanked by *SSU1* sequences. In the same way, constructs were generated for the deletion of *orf19.1867* (alias *SSU12* or *MAE1*) with the primers *SSU1L1-1/SSU1L1-2* and *SSU1L1-3/SSU1L1-4*, for the deletion of *CDG1* with the primers *CDG1-3/CDG1-4* and *CDG1-5/CDG1-6*, for the deletion of *ZCF2* with the primers *ZCF2-5/ZCF2-2* and *ZCF2-3/ZCF2-4*, and for the deletion of *CTA4* with the primers *CTA4-1/CTA4-2* and *CTA4-3/CTA4-4* to yield plasmids *pSSU1L1M2*, *pCDG1M2*, *pZCF2M2*, and *pCTA4M2*, respectively. For reinsertion of the *SSU1* gene at its original locus in the homozygous *ssu1Δ* mutants *SSU1M4A/B*, the *SSU1* gene of strain SC5314 was amplified by PCR with primers *SSU1-1* and *SSU1-6*. Primer *SSU1-6* introduced a *BamHI* site directly after the stop codon. The *ApaI*-*BamHI* fragment was cloned in the *ApaI*-*BglII*-digested plasmid *pSAP2KS1* (31) to result in *pSSU1KS1*. The *SacII*-*SacI* downstream *SSU1* fragment from *pSSU1M2* was cloned in *pSSU1KS1* to replace the *SacII*-*SacI* downstream *SAP2* fragment, resulting in plasmid *pSSU1KS2*. In the same manner, reinsertion of the *CDG1* gene at its wild-type locus in mutants *CDG1M4A/B* was performed, using primer *CDG1-3* and primer *CDG1-7*, which introduced a *BglII* site directly after the stop codon. The *ApaI*-*BglII* fragment was cloned in the *ApaI*-*BglII*-digested plasmid *pSAP2KS1*, resulting in *pCDG1KS1*. The *SacII*-*SacI* downstream *CDG1* fragment from *pCDG1M2* was cloned in *pCDG1KS1*, resulting in plasmid *pCDG1KS2*. A *P_{SSU1}-GFP* reporter construct was generated as follows: an *ApaI*-*SalI* fragment containing *SSU1* upstream sequences was amplified from genomic DNA of strain SC5314 with the primers *SSU1-1* and *SSU1-7*, and a *PstI*-*SacI* downstream *SSU1* fragment was amplified with the primers *SSU1-8* and *SSU1-4*. The upstream and

downstream *SSU1* fragments were successively cloned via the introduced restriction sites in the plasmid *pOPTIG22* (32), resulting in the plasmid *pSSU1G2*, in which the promoter *P_{SSU1}* is fused to the *GFP* gene.

***C. albicans* transformant construction.** *C. albicans* was transformed by an electroporation protocol (33) with the following gel-purified, linear DNA fragments: the *ApaI*-*SacI* fragments from *pSSU1M2*, *pSSU1L1M2*, *pCDG1M2*, *pZCF2M2*, and *pCTA4M2* were used to delete the genes *SSU1*, *SSU12*, *CDG1*, *ZCF2*, and *CTA4* in strain SC5314 (see Fig. S1 in the supplemental material), and strains *SSU1M4A/B* were used for the construction of *ssu1Δ ssu12Δ* and *ssu1Δ cdg1Δ* double-knockout mutants. The *ApaI*-*SacI* fragments from *pSSU1KS2* and *pCDG1KS2* were used to reinsert the *SSU1* and *CDG1* genes in the *ssu1Δ* and *cdg1Δ* deletion mutants, respectively, and the *ApaI*-*SacI* fragment from plasmid *pSSU1G2* was used to integrate the *P_{SSU1}-GFP* fusion into one of the two *SSU1* alleles in strain SC5314, *CTA4M4A/B*, and *ZCF2M4A/B*, respectively (see Fig. S1 and S2 in the supplemental material). Selection of transformants on nourseothricin (Werner Bioagents, Jena, Germany) and sensitive derivatives thereof was carried out by the *SAT1*-flipping method as described previously (34). The correct genomic integration of the constructs was confirmed by Southern hybridization.

Southern and Northern hybridization. Genomic DNA from *C. albicans* strains was isolated as described before (35). DNA (15 μg) was digested with appropriate restriction enzymes, separated in 1% (wt/vol) agarose gels, ethidium bromide stained, transferred by vacuum blotting onto nylon membranes, and fixed by UV cross-linking. Southern hybridization was performed with enhanced chemiluminescence-labeled probes, using the Amersham ECL direct nucleic acid labeling and detection system (GE Healthcare, Braunschweig, Germany) and according to the instructions of the manufacturer. Total RNA from *C. albicans* was isolated by the hot acidic phenol method (36), purified with the RNeasy Minikit (Qiagen), and DNase-treated on-column by use of the RNase-free DNase set (Qiagen) to remove contaminations with genomic DNA. A total of 20 μg of RNA per sample was separated on formaldehyde-containing agarose gels and blotted onto nylon membranes. For the detection of *SSU1* transcripts, an *SSU1*-specific probe was prepared by PCR with the primers *SSU1-10* and *SSU1-11*, using SC5314 DNA as a template, and labeled with digoxigenin-11-dUTP (Roche). In accordance with the instructions of the manufacturer, Northern hybridization was carried out in DIG-Easy Hyb buffer, followed by binding of antidigoxigenin-alkaline phosphate Fab fragments and the use of CDP-Star Ready-To-Use solution (Roche). Signals were detected on Amersham ECL-Hyperfilms (GE Healthcare).

Quantitative real-time reverse transcription-PCR (qRT-PCR). Total RNA (100 ng) was used to carry out qRT-PCR within a one-step approach using the Brilliant III SYBR green Ultra-Fast QRT PCR master mix kit (Agilent Technologies, La Jolla, CA) (for the primers used, see Table S2 in the supplemental material). RT-PCR was performed on a Stratagene Mx3005P and the threshold cycle was determined by the instrument's MxPro software version 4.10 (Agilent Technologies). Expression was calculated by the $\Delta\Delta C_T$ method as described previously (37). Expressions were normalized to the expression of the *ACT1* gene; for all samples, three biological replicates were analyzed.

Growth and sensitivity assays. Sulfite resistance of *C. albicans* strains was monitored on YPD-TA agar plates containing sulfite, prepared as described previously (13). Briefly, appropriate amounts of a freshly prepared Na_2SO_3 stock solution were spread by plating onto 25 ml of YPD agar containing 75 mM L-tartaric acid (pH 3.5) for a final sodium sulfite concentration as indicated in Results. Before use, the sulfite was allowed to dry and diffuse into the agar overnight at room temperature. To test for the ability of the *C. albicans* strains to grow in the presence of L-cysteine or L-methionine, overnight precultures grown at 30°C in YPD were diluted 1:100 in 10 ml of fresh SD medium (20 g glucose and 6.7 g of YNB with ammonium sulfate [MP Biomedicals] per liter) containing increasing concentrations of the amino acids as indicated in Results, followed by incubation at 30°C. The influence of cysteine on germ tube formation was

assayed in 1 ml of Dulbecco modified Eagle medium (DMEM; 1 g of glucose/liter, without L-glutamine) cell culture medium (PAA Laboratories) at 37°C.

Detection of sulfite. A commercially available sulfite detection kit was used for sulfite identification in *C. albicans* culture supernatants (R-Biopharm, Darmstadt, Germany). In brief, precultures of strains SC5314, CDG1M4A, and CDG1KS2A were grown in 50 ml of liquid YPD medium. After 24 h, the cultures were centrifuged, washed once with 25 ml of SD medium and resuspended in 50 ml of fresh SD medium. The cultures were supplemented with 10 mM cysteine and grown for 24 h at 30°C. Afterward, supernatants were harvested and used for sulfite detection according to the instructions of the manufacturer. Each culture was prepared in triplicate.

In vivo experiments. Female BALB/c mice (Charles River Laboratories, 18 to 20 g) were housed in groups of five in individually ventilated cages and cared for in strict accordance with the principles outlined in the *European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes* (<http://conventions.coe.int/Treaty/en/Treaties/Html/123.htm>). Experiments were approved by the governmental ethics committee (reg. no. 03-007/07). Mice were infected via the lateral tail vein with 10^4 *C. albicans* yeast cells per g of body weight in phosphate-buffered saline. Animals were monitored at least twice daily and euthanized if moribund (defined by lethargy, inability to ambulate, and/or hypothermia). Gross pathological alterations were recorded during necropsy.

Fluorescence microscopy and flow cytometry. Fluorescence microscopy was performed with a Zeiss LSM 510 Meta laser scanning microscope. Imaging scans were acquired with an argon laser with a wavelength of 488 nm and corresponding filter settings for green fluorescent protein (GFP) and parallel/overlay transmission images. The cells were inspected with a $\times 63$ immersion oil objective. Flow cytometry of *C. albicans* wild-type and GFP reporter strains was performed using a BD FACSCanto II flow cytometer and the FACSDiva (BD Biosciences, Heidelberg, Germany) software to determine the median fluorescence values. The analyzed *C. albicans* samples were derived from SD medium overnight cultures which were diluted 1:100 in fresh SD medium supplemented with the indicated compounds and incubated at the conditions as specified in Results.

Statistical analysis. Unless otherwise indicated, data were expressed as the means \pm the standard deviations (SD). Differences were analyzed by the two-tailed unpaired Student *t* test, a *P* value of <0.05 was considered statistically significant. Mouse survival was analyzed by using Kaplan-Meier curves and the log-rank test.

RESULTS

***C. albicans ssu1Δ* mutants are growth sensitive to cysteine.** In order to test the possibility whether Ssu1 contributes to cysteine tolerance of *C. albicans*, specific *ssu1Δ* deletion mutants and reconstituted strains were constructed in the *C. albicans* wild-type strain SC5314 by the SAT1-flipping methodology (see Fig. S1 in the supplemental material) (34). Wild-type and transformants were assayed for their ability to grow in the presence of sulfite, because sulfite resistance has previously been reported to depend on *SSU1* in both *S. cerevisiae* and *C. albicans* (6, 9). Confirming prior findings, our *C. albicans ssu1Δ* mutants were strongly inhibited in growth on YPD-TA agar containing elevated sulfite concentrations (Fig. 1A). In the next step, *ssu1Δ* mutants were tested for growth in the presence of increasing cysteine levels. In this experiment, growth was also monitored in the presence of the sulfur containing amino acid methionine. Compared to wild-type and reconstituted strains, a growth-inhibitory effect was identified for *ssu1Δ* mutants in the presence of cysteine (Fig. 1B) but not for methionine (Fig. 1C). In order to find out whether other genes homologous to *SSU1* are present in the *C. albicans* genome, a

BLAST search with *SSU1* DNA sequences was carried out (<http://www.candidagenome.org/>). This approach identified the uncharacterized gene orf19.1867 (alias *SSU12* or *MAE1*) with considerable sequence similarity to *SSU1* (52.1% on the protein level). This gene was also deleted in the *C. albicans* wild type, as well as in the *ssu1Δ* mutant (Materials and Methods). In the presence of sulfite or cysteine, *C. albicans ssu12Δ* deletion mutants behaved like the wild type, and *ssu1Δ ssu12Δ* double knockout strains behaved like *ssu1Δ* single mutants (data not shown). These observations excluded a contribution of *SSU12* to the tolerance of cysteine and/or sulfite under the tested conditions.

Cysteine activates *SSU1* gene expression. The finding that Ssu1 supports cysteine tolerance in *C. albicans* instigated the question whether cysteine itself acts as an inducer of *SSU1* gene expression. *C. albicans* reporter strains were constructed which express the *GFP* gene under the control of the promoter P_{SSU1} in one of the wild-type *SSU1* alleles (see Fig. S2 in the supplemental material). Overnight cultures of the reporter strains *SSU1G1A/B* were diluted and grown in fresh SD medium in the absence and presence of cysteine. After 6 h, the samples were inspected by flow cytometry for quantification of the GFP intensity as a measurement of gene expression (Fig. 2A). Herein, *SSU1* was found to be strongly activated only during growth of the cells in the presence of cysteine, but not in the presence of methionine. Enhanced P_{SSU1} -dependent *GFP* expression in the presence of cysteine was also demonstrated by fluorescence microscopy (Fig. 2B). As a positive control, *GFP* expression from the constitutively active *C. albicans ADH1* promoter was monitored (38). Northern analysis further proved *SSU1* gene induction in response to cysteine (Fig. 2C).

***C. albicans* produces sulfite in the presence of cysteine, relying on Cdg1.** In mammals, toxic concentrations of cysteine are eliminated by the oxidative cysteine catabolism, via the formation of sulfite (15). Given our knowledge that cysteine induced the expression of the sulfite efflux pump Ssu1, we reasoned that excess of cysteine may be converted to sulfite by *C. albicans* as well. Using a commercially available detection kit, sulfite was found in the supernatant of *C. albicans* wild-type cells in the presence of cysteine but not in the absence of cysteine or in the uninoculated control media (Fig. 3A). The *C. albicans* genome harbors a putative homologue of cysteine dioxygenase, *C. albicans Cdg1* (uncharacterized orf19.7314), and a putative cysteine degradation pathway resulting in sulfite formation has been predicted (<http://www.candidagenome.org/> [39]). Notably, the *CDG1* and *SSU1* genes are located next to each other in the genome, in divergent orientation, the two start codons separated by an intergenic region of 2378 bp. To clarify whether sulfite production by *C. albicans* in the presence of cysteine depends on Cdg1, the *CDG1* gene was deleted in the wild-type strain SC5314 (see Fig. S1 in the supplemental material). Wild-type, *cdg1Δ* mutant, and complemented strains were compared for the production of sulfite in the culture supernatant during growth in the presence of cysteine. Here, sulfite was specifically absent in the culture supernatant of the *cdg1Δ* mutant (Fig. 3A). This finding demonstrated a central role of cysteine dioxygenase Cdg1 for sulfite formation under the tested conditions. In contrast to the *ssu1Δ* mutant, *cdg1Δ* mutant cells displayed only a slightly reduced growth in the presence of elevated cysteine levels after 8 h and showed even enhanced growth after 24 h compared to the wild-type and reconstituted strains (Fig. 3B). This observation suggests that cysteine toxicity under these conditions largely results from the formation of sulfite, which is no

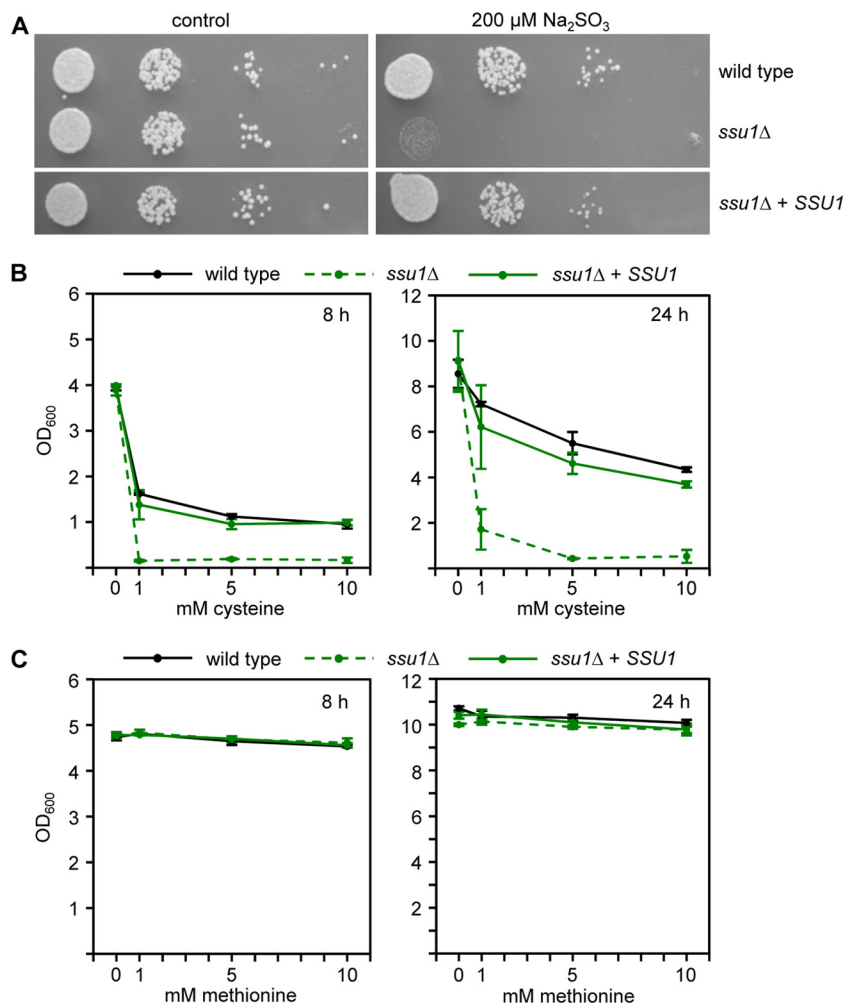


FIG 1 *C. albicans* *ssu1Δ* mutants are growth sensitive to sulfite and cysteine. (A) Overnight cultures of strains SC5314 (wild type), SSU1M4A/B (*ssu1Δ*) and SSU1KS2A/B (*ssu1Δ + SSU1*) were serially 10-fold diluted, and aliquots were inoculated on YPD-TA agar plates containing 200 μM sodium sulfite. As a control, YPD-TA agar plates without sodium sulfite were used. The agar plates were incubated for 28 h at 30°C. (B and C) *C. albicans* overnight cultures of the same strains were diluted 1:100 in fresh SD medium containing increasing concentrations of cysteine (B) or methionine (C). The optical densities of the cultures were measured after growth for 8 h and 24 h at 30°C. The addition of cysteine to the medium significantly reduced the growth of the *ssu1Δ* mutant compared to wild-type and reconstituted strains. The two independently constructed mutants and complemented strains behaved identically, and the results for only one of them are shown.

longer produced by *cdg1Δ* mutant cells. However, the possibility remained that cysteine itself could be a substrate of Ssu1, which would allow cysteine detoxification in *cdg1Δ* mutants independent from sulfite formation via cysteine dioxygenase. To exclude this alternative, we deleted the *CDG1* gene in the *C. albicans* *ssu1Δ* mutant background. This approach should rescue *ssu1Δ* mutant cells from increased cysteine sensitivity under these conditions, in case that cysteine toxicity is mediated via sulfite formation. Proving this assumption, the *ssu1Δ cdg1Δ* double-knockout strain displayed reduced sensitivity to cysteine, like the *cdg1Δ* single mutant (Fig. 3B). In order to monitor whether *CDG1* gene expression in *C. albicans* is also upregulated in the presence of cysteine, transcription of *CDG1* in the wild-type SC5314 was measured by qRT-PCR during growth in the absence or presence of cysteine (Fig. 3C). A strong upregulation of *CDG1* (and *SSU1*) expression in response to cysteine was detected, further supporting a role of *C. albicans* cysteine dioxygenase in the elimination of cysteine via sulfite.

Zcf2 governs sulfite resistance, as well as cysteine-inducible *CDG1* and *SSU1* expression. In order to identify a potential transcriptional regulator of sulfite tolerance in *C. albicans*, we screened a library of transcription factor mutants in the presence of sulfite for enhanced sulfite sensitivity. This library contained a collection of 165 genetically matched *C. albicans* strains, each one with the deletion of a specific transcription regulator (28). By this approach, we identified only one factor, Zcf2, the lack of which resulted in a strongly increased sensitivity to sulfite (Fig. 4A). In order to further verify the role of Zcf2 in sulfite tolerance, independent *zcf2Δ* deletion mutants were constructed in the *C. albicans* wild-type SC5314 (see Fig. S1 in the supplemental material). Phenotypic analysis of the resulting strains ZCF2M4A/B confirmed the increased sensitivity of the *zcf2Δ* mutant to sulfite (Fig. 4A). Inspection of the *zcf2Δ* mutant during growth in SD medium plus cysteine revealed that the absence of Zcf2 also resulted in increased cysteine sensitivity (Fig. 4B). Next, by using the *zcf2Δ* mutant, we

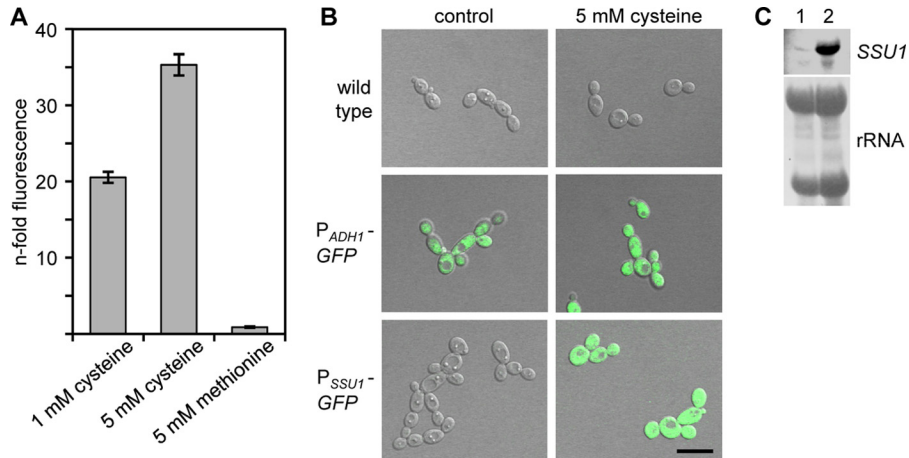


FIG 2 *SSU1* gene expression is upregulated in *C. albicans* in the presence of cysteine. (A) The fluorescence of *C. albicans* cells of reporter strains *SSU1G1A/B* was measured by flow cytometry after growth for 6 h in SD medium containing the indicated substances. The bars represent the n-fold fluorescence intensities measured in the presence versus absence of the tested compound for each strain. The two independently constructed reporter strains behaved identically, and only one of them is shown. (B) Detection of fluorescence on the level of single cells in the presence of cysteine. After 6 h of growth in SD medium in the absence or presence of 5 mM cysteine, respectively, overlay fluorescence microscopy pictures were taken from cells of the wild type, the positive control *SCADH1G4A* (*P_{ADH1}-GFP*), and the reporter strains *SSU1G1A/B* (*P_{SSU1}-GFP*). The two independently constructed *P_{SSU1}-GFP* reporter strains behaved identically, and the results for only one of them are shown (scale bar, 10 μ m). (C) Detection of *SSU1* mRNA by Northern hybridization analysis in response to cysteine. After 6 h of growth in SD medium in the absence (lane 1) or presence of 5 mM cysteine (lane 2), *SSU1* mRNA was detected in cells of the wild-type strain SC5314 (upper panel). rRNA is shown as a loading control (lower panel).

tested whether *Zcf2* is necessary for cysteine inducible *SSU1* and/or *CDG1* expression. To study *SSU1* expression, the *zcf2* Δ mutant was used as a host strain for integration of the *P_{SSU1}-GFP* reporter construct at the *SSU1* locus (strains *ZCF2SSU1G1A/B*). Flow cytometry measurements discovered that *SSU1* upregulation in the presence of cysteine is dependent on a functional *Zcf2* (Fig. 4C). Strongly decreased *SSU1* upregulation in *zcf2* Δ mutant cells under these conditions was confirmed by qRT-PCR (Fig. 4D). Using qRT-PCR, we also

monitored *CDG1* expression, showing that upregulation of the *CDG1* gene upon cysteine in the *zcf2* Δ mutant was not as strong as in the wild type (Fig. 4D). qRT-PCR analysis in wild-type cells further revealed that expression of *ZCF2* itself was also positively controlled in the presence of cysteine (Fig. 4D). In conclusion, these analyses suggest that *Zcf2* supports both sulfite and cysteine tolerance; second, *Zcf2* governs the cysteine inducible expression of *SSU1*, and at least to some extent, *CDG1*.

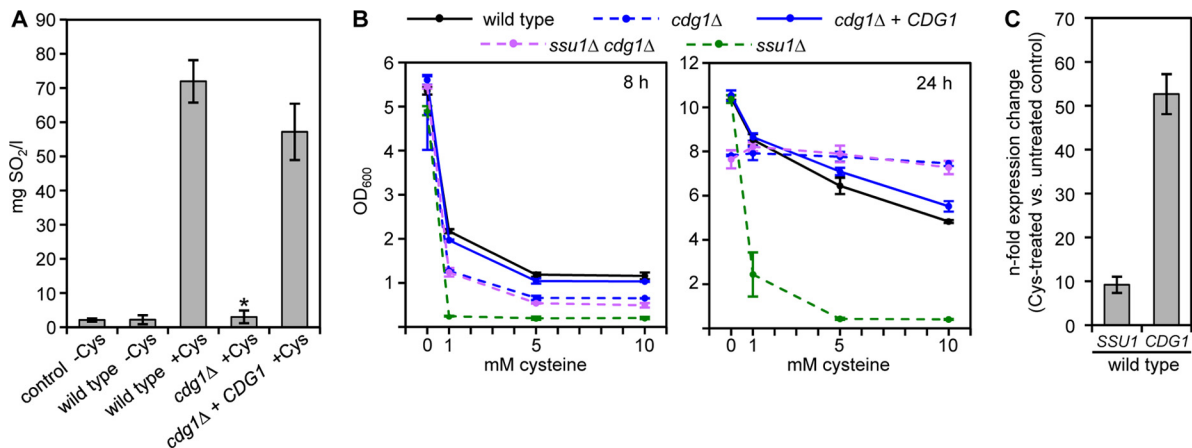


FIG 3 Growth of *C. albicans* in the presence of cysteine results in sulfite formation in culture supernatants. (A) Sulfite production was inspected in the supernatants of the wild type, mutants *CDG1M4A/B* (*cdg1* Δ), and complemented strains *CDG1KS2A/B* (*cdg1* Δ + *CDG1*) after 24 h of growth in SD medium containing 10 mM cysteine. Supernatants of the wild type grown for 24 h in SD medium without cysteine and uninoculated SD medium with 10 mM cysteine were used as controls. The results are means \pm the SD from three independent experiments. Sulfite as SO_2 was not detectable in supernatants of the *cdg1* Δ mutant compared to wild type and complemented strain. The asterisk (*) indicates that the detected difference was significant ($P < 0.05$). The independently constructed A/B mutants behaved identically, and the results for only one of them are shown. (B) Overnight precultures of wild type, *SSU1M4A* (*ssu1* Δ), *CDG1M4A/B* (*cdg1* Δ), complemented strains *CDG1KS2A/B* (*cdg1* Δ + *CDG1*), and *ssu1* Δ *cdg1* Δ double mutants were diluted 1:100 in fresh SD medium containing various concentrations of cysteine. The optical densities of the cultures were measured after growth for 8 h and 24 h at 30°C. The results are means \pm the SD from three independent experiments. (C) qRT-PCR measurements show an upregulation of *CDG1* and *SSU1* gene expression levels in the wild type in the presence or absence of 5 mM cysteine after 6 h of growth in SD medium.

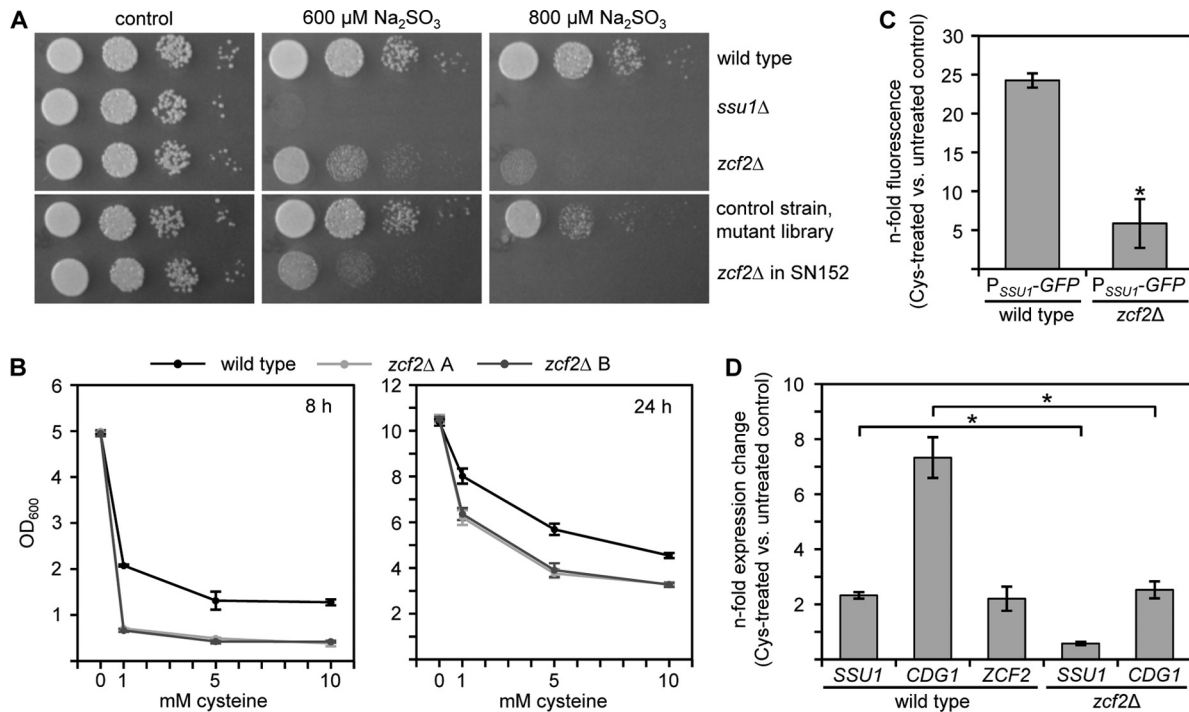


FIG 4 Zcf2 supports sulfite and cysteine tolerance and governs cysteine-inducible *SSU1/CDG1* expression. (A) Overnight cultures of wild type, *SSU1M4A* (*ssu1* Δ), and *ZCF2M4A/B* (*zcf2* Δ) were serially diluted and inoculated on YPD-TA agar plates containing sodium sulfite as indicated, or without sulfite for control. The two independently constructed *zcf2* Δ mutants behaved identically, and the results for only one of them are shown. The *zcf2* Δ mutants (*zcf2* Δ in SN152) and the corresponding control strain from the originally screened transcription factor mutant library were also included (lower panel). Photographs were taken after 28 h of growth at 30°C. (B) *C. albicans* overnight cultures of wild-type and *ZCF2M4A/B* (*zcf2* Δ A/B) strains were diluted 1:100 in fresh SD medium containing cysteine as indicated. The optical densities of the cultures were measured after growth for 8 h and 24 h at 30°C. The results are means \pm the SD from three independent experiments. The addition of cysteine to the medium significantly reduced the growth of the *zcf2* Δ mutant compared to the wild type. (C) The fluorescence of *C. albicans* cells of reporter strains *SSU1G1A/B* ($P_{SSU1-GFP}$ /wild type) and *ZCF2SSU1G1A/B* ($P_{SSU1-GFP}$ /*zcf2* Δ) was measured by flow cytometry after growth for 6 h in SD medium containing 1 mM cysteine. The bars represent the n-fold fluorescence intensities measured in the presence versus absence of cysteine for each strain. The results are means \pm the SD from three independent experiments. An asterisk (*) indicates that the detected differences were significant ($P < 0.05$). The two independently constructed A/B reporter strains behaved identically, and the results for only one of them are shown. (D) qRT-PCR measurements show an upregulation of *CDG1*, *SSU1*, and *ZCF2* gene expression levels in the wild type after growth for 6 h in SD medium in the presence or absence of 1 mM cysteine, whereas expression levels of *SSU1* and *CDG1* are decreased in the *zcf2* Δ mutant. The results are means \pm the SD from three independent experiments. An asterisk (*) indicates that the detected differences were significant ($P < 0.05$).

Cysteine-inducible expression of *SSU1* is not relying on the nitrosative stress regulator *Cta4*. Formerly, the nitric oxide-induced expression of *SSU1* in *C. albicans* was revealed to depend to some extent on the nitrosative stress regulator *Cta4* (9). Therefore, we also investigated whether *Cta4* contributes to cysteine inducible *SSU1* expression and/or *Ssu1* mediated cysteine/sulfite tolerance. Deletion of *CTA4* in the wild-type SC5314 (see Fig. S1 in the supplemental material) resulted in a slightly increased sensitivity of *C. albicans* to sulfite in our assay (see Fig. S3A in the supplemental material). This finding is in contrast to prior observations, where no phenotype of a *cta4* Δ mutant on sulfite was recorded (9). The phenotype we detected, however, was not comparable to the effect of *ZCF2* deletion. The sensitivity of *cta4* Δ mutant cells to cysteine was not increased under the tested conditions (Fig. S3B). Also, *SSU1* gene expression in the presence of cysteine was found to be induced in the *cta4* Δ mutant cells at a level comparable to wild-type cells, demonstrated by $P_{SSU1-GFP}$ reporter strains in the background of the *cta4* Δ mutants (see Fig. S3C in the supplemental material). qRT-PCR measurements confirmed this finding and also revealed that in the *cta4* Δ mutant *CDG1* is still strongly induced in the presence of cysteine, albeit to a decreased extent (see Fig. S3D in the supplemental material). In summary, our results

suggest that *Cta4*, unlike *Zcf2*, does not play a major role for cysteine and/or sulfite tolerance under the tested conditions.

***cdg1* Δ mutants display reduced hyphae formation in the presence of cysteine and are attenuated in virulence.** We also tested whether *C. albicans* hyphae development is compromised by the lack of *Cdg1* (or *Ssu1*) in the presence of cysteine. Cells of the wild-type, *cdg1* Δ and *ssu1* Δ mutant, and complemented strains were diluted in cell culture medium at 37°C for 6 h in the presence or absence of 5 mM cysteine. The presence of cysteine resulted in a strong repression of hyphae formation specifically in cultures of both mutants, *cdg1* Δ and *ssu1* Δ (Fig. 5A). Here, only the production of very short germ tubes was monitored. This observation stimulated the question whether *Cdg1* and *Ssu1* contribute to the pathogenicity of *C. albicans*. To test this possibility, the standard model of disseminated candidiasis in mice was applied. Immunocompetent mice were infected intravenously with the *C. albicans* wild type, *ssu1* Δ and *cdg1* Δ knockout mutants, and complemented strains, respectively. Mice infected with the *C. albicans* *cdg1* Δ mutant survived significantly longer than mice infected with either the corresponding complemented strain or wild-type *C. albicans* (Fig. 5B). We performed histological analysis of the kidneys of moribund mice. Histologically, the mutant strain

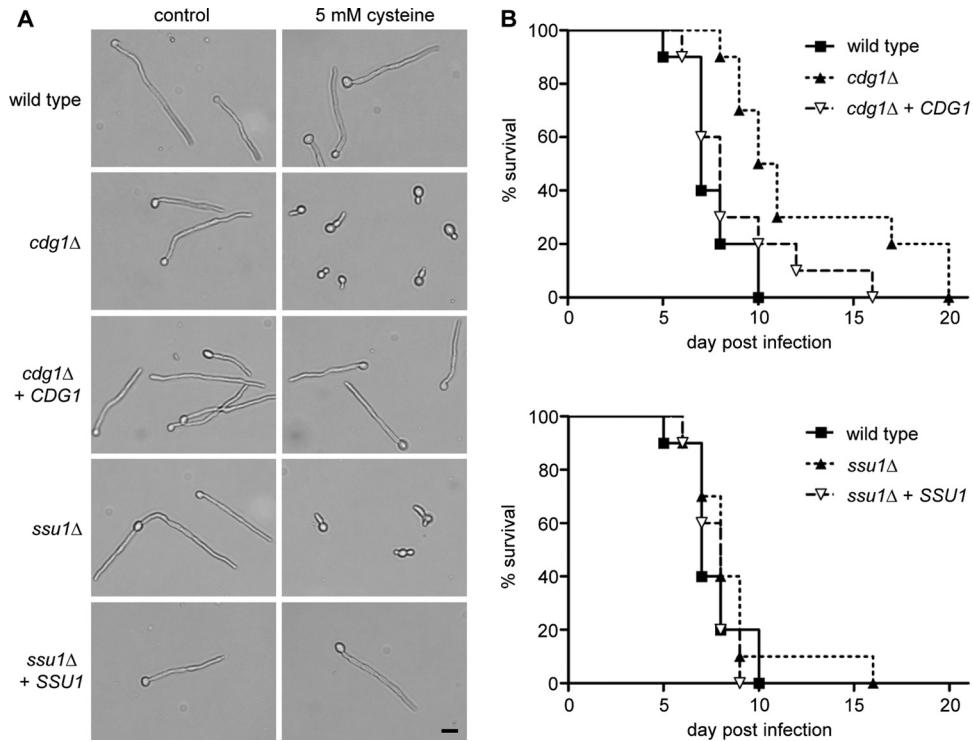


FIG 5 Cdg1 contributes to cysteine tolerance during hyphal development and to the pathogenicity of *C. albicans*. (A) Decreased hypha formation of *cdg1Δ* and *ssu1Δ* mutants in the presence of cysteine. *C. albicans* cells of SC5314 (wild type) and mutant strains SSU1M4A/B (*ssu1Δ*) and CDG1M4A/B (*cdg1Δ*), as well as complemented strains SSU1KS2A/B (*ssu1Δ + SSU1*) and CDG1KS2A/B (*cdg1Δ + CDG1*), were grown overnight at 30°C in SD medium and diluted 1:100 in DMEM in the absence or presence of 5 mM cysteine, followed by incubation at 37°C with gentle shaking. Microscopic photographs were taken after 6 h (scale bar, 10 μm). The two independently constructed mutants A/B and complemented strains A/B behaved identically, and the results for only one of them are shown. (B) Survival of mice infected with strain SC5314 (wild type), *cdg1Δ* mutant CDG1M4A, and complemented strain CDG1KS2A (*cdg1Δ + CDG1*), as well as *ssu1Δ* mutant SSU1M4A and complemented strain SSU1KS2A (*ssu1Δ + SSU1*) (*n* = 10 per group). Mortality induced by the *cdg1Δ* mutant is significantly delayed (log-rank test, *P* < 0.05 compared to the wild-type and complemented strains, respectively).

showed filamentation indistinguishable from wild-type cells. We therefore conclude that the mutant did not have a gross filamentation defect *in vivo*, at least in the inspected tissue samples (not shown). In contrast to knockout of *CDG1*, deletion of *SSU1* did not affect *C. albicans* virulence in this model.

DISCUSSION

From our findings, we conclude that elevated levels of cysteine are converted by *C. albicans* to sulfite. Central to this process is cysteine dioxygenase Cdg1. Sulfite in turn can be detoxified by *C. albicans* via support of the putative sulfite efflux pump Ssu1 (Fig. 6). The pathway of sulfite production from cysteine may support the tolerance of *C. albicans* to high cysteine levels under certain conditions, but at the same time, sulfite secretion and resistance *per se* may display specific roles during host adaptation and/or pathogenicity.

Putative function of cysteine and sulfite tolerance in the context of *C. albicans* commensalism and/or infection. The knowledge that sulfite is also a normal metabolite generated during reductive sulfate assimilation in bacteria, fungi and plants suggests mechanisms avoiding endogenous sulfite toxicity. On the other hand, active sulfite secretion may serve direct functions. A specific biological role of sulfite secretion by fungal microorganisms has been proposed for dermatophytes, i.e., acting as a reducing agent in the process of keratin degradation (12, 13). Testing this hypothesis for *C. albicans*, we were not able to compare the growth ability

of wild-type and mutant strains in keratin media in which dermatophyte growth is assumed to rely on sulfitolysis. We found that *C. albicans* was unable to grow under such conditions (data not shown). Considering that sulfite is a potent reducing agent and antimicrobial preservative, its secretion and/or resistance by microbes may in general interfere with the cellular survival, especially in habitats of complex communities. This situation may also

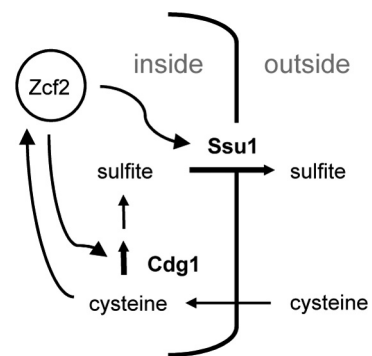


FIG 6 Proposed, simplified model for sulfite production by *C. albicans* from cysteine. Excess of environmental cysteine leads to induced expression of both cysteine dioxygenase Cdg1 and the sulfite efflux pump Ssu1, controlled by transcription factor Zcf2. Cdg1 manages a key step in the conversion of cysteine to sulfite, the export of which is supported by Ssu1.

occur in the human intestinal tract, where *C. albicans* resides as a common commensal together with a multitude of competing microorganisms. In addition, other molecules might be related to sulfite and cysteine metabolism, e.g., hydrogen sulfide and sulfur dioxide, both of which can act as crucial gasotransmitters in humans (40). Addressing the question of whether such gases or sulfite produced by *C. albicans* are involved in commensalism will be of future interest. In principle, sulfite can modify any protein in which function or stability relies on discrete disulfide bridges. Such putative targets also include certain antimicrobial peptides, key effectors in the protection of mucosal surfaces against infectious microbes. As an example, recent evidence suggested that the reduction of disulfide bonds of β -defensin 1 altered the antimicrobial activity of the molecule (41).

In mammals, cysteine and methionine are normally consumed as part of dietary proteins, being transported across the intestinal epithelium. Circulating free amino acids are removed from plasma by diverse tissues, by the liver, for example, for the synthesis of glutathione (42). In addition, experiments in rats detected also cysteine secretion from intestinal mucosa to the gut lumen. This process was assumed to explain the observation that cysteine accounted for 15 to 20% of the mucosal thiol pool in rat jejunum, putatively playing an important role in intestinal function (43); the intestinal mucosa of rats was found to contain millimolar thiol concentrations (44). It can be speculated that microbial pathogens such as *C. albicans*, which can induce endogenous, systemic infections via penetration of gastrointestinal mucosae, benefit from specialized mechanisms of cysteine tolerance.

Role of Ssu1 and Cdg1 in the process of cysteine tolerance in *C. albicans*. Intracellular cysteine levels in mammals are strictly maintained, in order to allow cysteine-dependent metabolism at concentrations below the threshold of cysteine toxicity, e.g., in rats in a range between 20 and 100 $\mu\text{mol/kg}$ in the liver and between 80 and 200 $\mu\text{mol/liter}$ in plasma, respectively (15). Cysteine dioxygenase has been described in mammals as a key enzyme in the process of catabolic elimination of toxic cysteine concentrations via sulfite. Homologues of cysteine dioxygenases have recently been identified in bacteria (45), in dermatophytes (14), and in the present study, in *C. albicans*. Interestingly, however, such sequences have not been detected in the model yeast *S. cerevisiae*. In mammals, sulfite is further oxidized by sulfite oxidase to nontoxic sulfate, which in turn is excreted by the urine. Sulfite oxidases are widespread also in filamentous fungi; however, homologous sequences or genes associated with the implied pathway (gene ontology GO: 0008482) have not been identified in the *C. albicans* genome (<http://www.candidagenome.org/>). The latter observation further supports a role of the putative sulfite efflux pump Ssu1 for the process of cysteine conversion and sulfite elimination. This assumption was supported by our findings that the *ssu1* Δ mutants were sensitive to both cysteine and sulfite. Further, we showed that cysteine sensitivity of *ssu1* Δ mutants during yeast growth was decreased by the deletion of *CDG1*, i.e., by inhibition of sulfite formation. During our virulence studies in a model of disseminated infection of mice, we observed significantly delayed mortality in mice infected with the *C. albicans* *cdg1* Δ , but not the *ssu1* Δ mutant. Nevertheless, it remains elusive whether the decreased virulence potential of the *cdg1* Δ mutant is a result of altered cysteine sensitivity or the failure of sulfite generation *per se* via this pathway. Interestingly, *in vitro*

inspection of the *cdg1* Δ mutant discovered strongly decreased hyphae formation in the presence of cysteine, whereas yeast growth was only slightly affected. Whether such phenotypic differences indicate a specific impact on the morphogenetic switch or merely reflect enhanced cysteine sensitivity of hyphae remains to be investigated. For the future, it may also be of particular interest to test additional infection models with the *cdg1* Δ mutant. Ideally, such experiments should elucidate the commensal state of *C. albicans* in the human intestinal tract and the penetration of gut mucosae.

***SSU1* and *CDG1* gene regulation.** Whereas *SSU1* was found to be induced in *C. albicans* in the presence of cysteine, a global gene expression analysis in the presence of cysteine did not detect increased *SSU1* expression levels in *S. cerevisiae* (46). In *S. cerevisiae*, the inducible response of *SSU1* gene expression to nitric oxide stress is known to be governed by the transcriptional regulator Fzf1, which, however, has not been identified in the *C. albicans* genome (8). Instead, the transcription factor Cta4 was recently shown to control the activation of the nitric oxide-inducible gene *YHB1* and also, to some extent, the gene *SSU1* in *C. albicans* (9). Our findings on *SSU1* expression in the *cta4* Δ mutant background suggest that *SSU1* gene activation in response to cysteine was not relying on Cta4. Nevertheless, in contrast to others (9), we cannot fully exclude a role of Cta4 in sulfite tolerance. We found a small but detectable growth inhibition of the *cta4* Δ mutant upon increasing sulfite concentrations; however, this was not comparable to the effect displayed by the *zcf2* Δ mutant. In addition, sensitivity to cysteine was not increased in *C. albicans* *cta4* Δ mutants. Therefore, it seems likely that the activation of *SSU1* upon cysteine as well as sulfite resistance are mainly under the control of a pathway different from the major nitrosative stress response in *C. albicans*. We have identified a candidate transcriptional regulator for this process, Zcf2, the lack of which resulted in an increased sensitivity to sulfite and cysteine. The discovered function of Zcf2 may be quite specific, since *C. albicans* *zcf2* Δ mutants did not exhibit any other growth defects either in our experiments or in any assays used for exhaustive phenotyping of this transcription factor mutant library (28). The role of Zcf2 in the control of *SSU1* expression and sulfite tolerance is further supported by our observation that *SSU1* expression upon cysteine was strongly decreased in the *zcf2* Δ mutant. In light of previous (9) and present findings on the putative regulation of *SSU1* in *C. albicans*, the gene appears to be a target of both Cta4 and Zcf2, depending on the stimulus. Nevertheless, it should be tested in the future if Zcf2 is involved in the nitric oxide stress response, and genome-wide analyses may identify additional target genes of this regulator.

Conclusion. Given the antimicrobial activity of cysteine and cysteine derivatives, these compounds have even been suggested as potential antimicrobial drugs. Nevertheless, the underlying molecular basis for such growth-inhibiting effects remains largely unknown. Different possible mechanisms of cysteine toxicity have been suggested in the past in diverse microbial species, including a putative interference of cysteine with the biosynthesis of branched-chain amino acids, a possible interaction with membrane bound respiratory enzymes, and the promotion of oxidative DNA damage (20, 22, 47, 48). Our finding that *C. albicans* can convert cysteine to sulfite adds to the knowledge on cysteine toxicity in this organism. At the same time, the fungus can detoxify

sulfite by the transporter Ssu1. Therefore, it appears to be of particular interest to find out whether active sulfite secretion by such mechanisms is used by *C. albicans* in the human host and what the consequences of sulfite secretion are during commensalism and/or during symptomatic infection.

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