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Exploration of Sulfur Assimilation of *Aspergillus fumigatus* Reveals Biosynthesis of Sulfur-Containing Amino Acids as a Virulence Determinant

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Fungal infections are of major relevance due to the increased numbers of immunocompromised patients, frequently delayed diagnosis, and limited therapeutics. To date, the growth and nutritional requirements of fungi during infection, which are relevant for invasion of the host, are poorly understood. This is particularly true for invasive pulmonary aspergillosis, as so far, sources of (macro)elements that are exploited during infection have been identified to only a limited extent. Here, we have investigated sulfur (S) utilization by the human-pathogenic mold *Aspergillus fumigatus* during invasive growth. Our data reveal that inorganic S compounds or taurine is unlikely to serve as an S source during invasive pulmonary aspergillosis since a sulfate transporter mutant strain and a sulfite reductase mutant strain are fully virulent. In contrast, the S-containing amino acid cysteine is limiting for fungal growth, as proven by the reduced virulence of a cysteine auxotroph. Moreover, phenotypic characterization of this strain further revealed the robustness of the subordinate glutathione redox system. Interestingly, we demonstrate that methionine synthase is essential for *A. fumigatus* virulence, defining the biosynthetic route of this proteinogenic amino acid as a potential antifungal target. In conclusion, we provide novel insights into the nutritional requirements of *A. fumigatus* during pathogenesis, a prerequisite to understanding and fighting infection.

Nutrient supply is an essential prerequisite for the onset and manifestation of infection by any pathogen (1). The acquisition of nutrients by the invading microorganism is a requirement for its proliferation at the intimate host-pathogen interface. Despite their nonspecific character, efficient and versatile metabolic pathways have to be considered virulence determinants (2). The infected host organism, in turn, might restrict the access of pathogens to certain trace elements and metabolites in order to subvert their growth and invasion, a strategy that was recently called "nutritional immunity" (3). Elucidating the mechanisms that are in place on either side is a prerequisite for deducing general principles of pathogenesis for any infectious disease (4) and identifying possible therapeutic targets.

Aspergillus fumigatus is a ubiquitous filamentous fungus commonly found in organic debris, where it plays an essential role in macroelement (carbon and nitrogen, etc.) recycling (5, 6). However, this mold is also a prominent opportunistic pathogen that can cause invasive infections in immunocompromised individuals. Invasive pulmonary aspergillosis (IPA), the most severe infection caused by *A. fumigatus* (7), has a very high mortality rate, which can reach up to 90% depending on the immune status of a susceptible patient (8–10). This pronounced lethality can be attributed in part to the relative inefficiency of current chemotherapies (11).

As an opportunist, *A. fumigatus* appears not to express specific virulence factors (12). It is widely accepted that its virulence is a multifactorial trait that can be understood only in the context of the host-pathogen interaction (4). As a consequence, many research efforts are now directed toward the host-pathogen interplay to develop novel immunotherapies against infection. However, in recent years, it has also become clear that nutrient uptake and metabolic versatility are important, although nonspecific, virulence determinants of pathogenicity (2). Consequently, to fully understand *A. fumigatus* virulence, it is necessary to further decipher the metabolic status of the fungus during intrapulmonary growth. Knowledge of the fungal metabolome might lead to the discovery of valid and promising antifungal targets (13).

For *A. fumigatus*, it known that several biosynthetic pathways of amino acids and vitamins as well as the acquisition of several elements from invaded tissues are crucial determinants for its virulence. For instance, biosynthesis of lysine (14, 15), aromatic amino acids (16), and folate (17); siderophore-dependent acquisition of iron (18); and transporter-mediated uptake of zinc (19) or calcium (20) are essential for the fungus to grow within the lungs and, therefore, determine its pathogenic effects. On the other hand, any sources of nitrogen in pulmonary tissue have not yet been clearly identified, despite many studies addressing N me-

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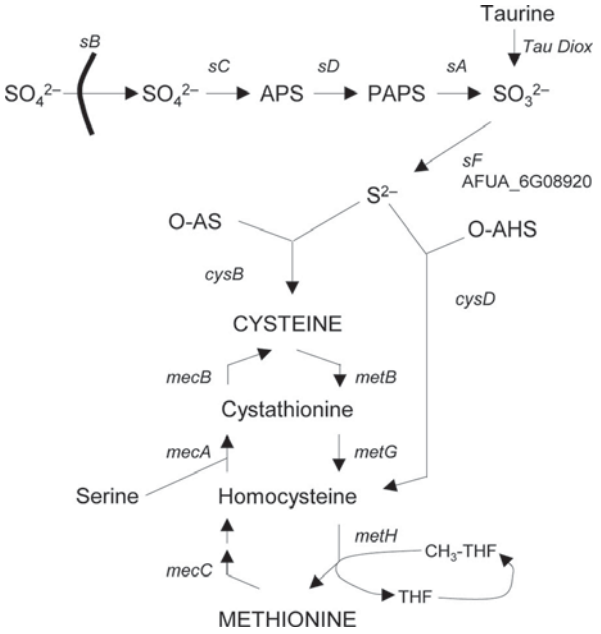

FIG 1 Schematic representation of sulfur metabolism in filamentous fungi. The sulfate assimilation and transsulfuration pathways are depicted. *sB*, sulfate transporter; *sC*, ATP sulfurylase; *sD*, adenosine 5′-phosphosulfate (APS) kinase; *sA*, 3′-phosphoadenosine-5′-phosphosulfate (PAPS) reductase; *sF*, β-subunit of the sulfite reductase; AFUA_6G08920, α-subunit of the sulfite reductase;*cysB*, cysteine synthase; *metB*, cystathionine-γ-synthase; *metG*, cystathionine-β-lyase; *metH*, methionine synthase; *mecC*, *S*-adenosylmethionine synthetase; *mecA*, cystathionine-β-synthase; *mecB*, cystathionine-γ-lyase; *cysD*, homocysteine synthase; O-AS, *O*-acetylserine; O-HS, *O*-acetylhomoserine.

tabolism (21–26), and there are indications that *A. fumigatus* endures nitrogen starvation during intrapulmonary growth (27). This is probably due to the complexity of nitrogen metabolism and the high degree of redundancy of proteolytic and transport activities encoded in the *A. fumigatus* genome. In addition, it can be speculated that the variety of nitrogen-containing compounds offered by the lung tissue facilitates and/or diversifies the acquisition of this macroelement by the fungus. Sulfur is another essential macroelement that, conversely to nitrogen, is present in a limited number of molecules within host tissue and whose metabolism is likely not as complex.

The element sulfur is an integral component of several fundamental organic molecules, such as the proteinogenic amino acids methionine and cysteine, the antioxidant glutathione (GSH), or coenzyme A (CoA). For incorporation into these compounds, there is a variety of assimilatory pathways in living cells, with significant differences between fungi and humans making this metabolic route relevant for the development of antifungal substances (28). In essence, there are two kinds of sources that deliver sulfur for fungal metabolism: inorganic sulfur compounds and organic S-containing molecules. While the former can be incorporated only after reductive assimilation, the latter ones may be utilized directly. Mammals, however, are incapable of incorporating reduced sulfur derived from inorganic sources and, moreover, require methionine as an essential ingredient in their diet to serve as a source of sulfur (29). Methionine can generally be converted by the so-called transsulfuration pathway to cysteine (Fig. 1) (30, 31), which serves as a precursor for a variety of fundamental structural components, among them iron-sulfur clusters or disulfide bridges in proteins. While the conversion between the S-containing amino acids is reversible in fungi, human cells are incapable of generating methionine from cysteine (32); thus, humans cannot exploit cysteine or glutathione as an organic sulfur source.

We recently demonstrated that the transcription factor MetR, which is a central regulator of sulfur assimilation, affects *A. fumigatus* virulence in a positive manner (33). However, due to the broad transcriptional remodeling for which MetR accounts, it was not possible to definitively conclude that impaired sulfur assimilation is the only reason for the decreased virulence of the MetR mutant. Here, we investigated if *A. fumigatus* is able to assimilate some of the most abundant sulfur sources that might be encountered within the lung tissue. It is known that sulfated glycosaminoglycans (GAGs) are highly abundant in the lungs (34), and although the *A. fumigatus* genome does not seem to encode heparanases, the level of sulfation of GAGs in lung fibroblasts has been related to the level of extracellular free sulfate (SO_4^{2-}) (35). This suggests that there might be a pool of free sulfate in the extracellular matrix (35), pointing to this inorganic compound as a putative S source. Moreover, tissue free S-containing amino acids (methionine, cysteine, and taurine) have been detected in human lungs (36), and taurine has been shown to be highly concentrated in the bronchoalveolar lavage fluid of both healthy and asthmatic patients (37). Therefore, we scrutinized the fungal transsulfuration pathway by generating and characterizing various *A. fumigatus* mutants impaired in the utilization of different S sources. We were able to exclude inorganic sulfur compounds and taurine as sources of sulfur exploited by *A. fumigatus in vivo*. In addition, we found that the bioavailability of cysteine in the murine lung is limited, arguing against its role as the predominant sulfur source. Moreover, our data identify methionine synthase enzymatic activity as being essential for the growth of *A. fumigatus* within lung tissue.

MATERIALS AND METHODS

Ethics statement. Mice were bred and kept in accordance with the principles outlined by the *European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes* (38). All infection experiments were performed in compliance with a protocol approved by the Government of Lower Franconia (files numbers 55.2-2531.01-90/09 and 55.2-2531.01-86-13).

Strains, media, and culture conditions. *Escherichia coli* strain DH5α (39) was used for cloning procedures. Plasmid-carrying *E. coli* strains were routinely grown at 37°C in LB liquid medium (1% peptone, 0.5% yeast extract, 0.5% NaCl) under selective conditions (100 $\mu g \cdot ml^{-1}$ ampicillin or 50 $\mu g \cdot ml^{-1}$ kanamycin); for growth on plates, 1.5% agar was added to solidify the medium. All plasmid constructs used in the course of this study were generated by using the Seamless Cloning technology (Invitrogen), as previously described (33).

Wild-type *Aspergillus fumigatus* strain ATCC 46645 served as the reference recipient (40). *A. fumigatus* strains were generally cultured in nitrate-based minimal medium (MM) (41) (1% glucose, 1× AspA [1:50 dilution of 50× AspA {3.5 M $NaNO_3$, 350 mM KCl, 550 mM KH_2PO_4, pH 5.5}]) with 0.25 mM $MgSO_4$, 1× trace elements solution, and 2% agar (Serva, for solid media) at 37°C. For selection in the presence of resistance markers, 50 $\mu g \cdot ml^{-1}$ of hygromycin B or 100 $\mu g \cdot ml^{-1}$ of pyrithiamine (InvivoGen) was applied. For sulfur-free medium, $MgCl_2$ replaced $MgSO_4$, and a modified mixture of trace elements lacking any sulfate salt was used. When indicated, media were supplemented with L-cysteine, albumin fraction V, bovine serum albumin (BSA) (Biochemica), L-methionine, L-homocysteine, taurine, reduced glutathione, folic acid (folate),

casein hydrolysate (Casamino Acids), potassium 4-nitrophenyl sulfate, sodium sulfide (Sigma), L-serine, anhydrous sodium sulfate, anhydrous sodium sulfite, or sodium thiosulfate pentahydrate (Roth). For all growth assays using solid medium, the culture medium was inoculated with 10 μl of a freshly prepared *A. fumigatus* spore suspension (10^5 conidia · ml^{-1} in water supplemented with 0.9% NaCl and 0.02% Tween 80) and incubated at 37°C for 3 days.

A. fumigatus liquid medium shifts were performed according to methods described previously by Narendja et al. (42), with adjusted medium to modify the sources of sulfur: 200 ml minimal medium lacking sulfur and supplemented with 5 mM methionine was inoculated with 10^8 freshly harvested, 5-day-old *A. fumigatus* ATCC 46645, *mecA*Δ, *cysB*Δ, or *mecA*Δ; *cysB*Δ conidia and propagated at 37°C at 150 rpm for 16 to 22 h. Mycelia from such precultures were then harvested, washed extensively with water, and split into equal aliquots on a sterile surface. These aliquots were then added to 100 ml of minimal medium base without a sulfur source or supplemented with 2 mM SO_4^{2-}, 2 mM S^{2-}, 5 mM cysteine, or 0.25 mM cysteine and incubated at 37°C at 150 rpm for 1 to 8 h.

Extraction and manipulation of nucleic acids. Standard protocols for recombinant DNA technology were used (43). Phusion high-fidelity DNA polymerase (Fermentas) was generally used in PCRs, and essential cloning steps were verified by sequencing. Fungal genomic DNA was prepared according to protocols described previously by Kolar et al. (44), and Southern analyses were carried out as described previously (45, 46).

Glutathione measurement. GSH and GSSG levels were measured as described previously by Carberry and colleagues (47), with the exception that 200 mg of mycelia was used for glutathione extraction and whole-cell lysates. Samples were diluted 1/10 for determination of GSSG levels and 1/50 for determination of GSH levels.

Sensitivity against reactive oxygen species. Sensitivity against diamide, menadione, and H_2O_2 was measured as described previously (48, 49). Briefly, 10^6 *A. fumigatus* conidia were mixed with 25 ml minimal medium agar previously cooled to 50°C and poured into petri dishes. In the center of the agar plate, a hole with a diameter of 10 mm was created, which was filled with a solution of 3% H_2O_2 (30%, vol/vol; Fluka, Germany), 100 mM diamide (*N*,*N*,*N*′,*N*′-tetramethylazodicarboxamide; Sigma, Germany), or 2 mM menadione (2-methyl-1,4-naphthoquinone; Sigma, Germany). After incubation at 37°C for 20 h, the diameter of the inhibition zone was determined. The experiments were repeated twice in triplicate. Total values for each strain were analyzed by using statistical Student's *t* test. Differences were considered significant when *P* values were <0.05. Other stressors were added directly to the agar plates to the indicated final concentrations; the fungus was inoculated as explained above and incubated for 3 days at 37°C. Fludioxonil and 2,5-dihydroxybenzoic acid (2,5-DHBA) were purchased from Sigma-Aldrich. Fe^{3+} was added as chloride salt. Gliotoxin was purchased from Cayman Chemical Europe and dissolved in DMSO prior to addition to the medium.

Extracellular proteolytic activity. For determining the amounts of extracellular proteases secreted by relevant mutant isolates, a semiquantitative assay on solid culture medium was employed: strains were point inoculated as 500 conidia onto single minimal medium plates containing cysteine as the permissive sulfur source together with 0.4% casein as the sole nitrogen source and incubated at 37°C. Radial growth of the fungal mycelium was led by a protein precipitation zone due to acidification of the culture medium, which was cleared depending on the amount of extracellular proteolysis. The relative degree of proteolytic activity is given by the ratio of the growth zone diameter to the clearing zone diameter measured after defined periods of time, with wild-type isolate ATCC 46645 serving as the matching standard.

Conidiocidal assays. Bone marrow (BM) cells from a BALB/c donor mouse were flushed from femur and tibia bones with phosphate-buffered saline (PBS; PAN, Aidenbach, Germany). Neutrophils were isolated from this cell suspension by using the EasyStep negative-selection mouse neutrophil enrichment kit (Stemcell Technologies Inc.) according to the manufacturer's instructions. Purified neutrophils were set in culture in RPMI medium supplemented with 10% inactivated fetal bovine serum (FBS), and conidia were added on top at a multiplicity of infection (MOI) ratio of 1:10. After 6 h, medium was collected and centrifuged at 4,000 rpm for 5 min; the pellet was resuspended in distilled water and finally resuspended in 1 ml 0.9% NaCl plus 0.01% Tween 20. Different dilutions were plated onto MM in triplicates, and CFU were counted.

Infection experiments. Outbred female BALB/c mice (Charles Rivers Breeders) were used for infection experiments. Immunosuppression was carried out by subcutaneous injection of 112 mg · kg^{-1} of body weight hydrocortisone acetate and intraperitoneal injection of 150 mg · kg^{-1} cyclophosphamide according to a sequential protocol described previously (50), with the modification that 2 doses of cortisone on days −3 and −1 were applied. Bacterial infections were prevented by adding 2 g · $liter^{-1}$ neomycin to the drinking water. Inocula were prepared by harvesting conidia from 5-day-old slants of solid medium followed by filtration through Miracloth tissue and washing with saline. Mice were anesthetized by intraperitoneal injection of a ketamine (1%)-xylazine (0.2%) solution, and pulmonary tissue was infected by intranasal instillation of 2×10^5 conidiospores suspended in 50 μl of saline. Disease progression was monitored daily by recording the weight and behavior of the animals. Mice showing respiratory distress, hunched posture, poor mobility, or a loss of >20% of their body weight were euthanized.

Statistical analyses. Measurements are expressed as the means ± standard deviations (SD), except for CFU, for which, due to the high inherent deviation, standard errors of the means (SEM) are shown. Analyses were performed by using GraphPad Prism 6 software. To perform statistical comparisons between individual biological measurements, the unpaired Student *t* test was applied. To compare survival curves of infected mice, the log rank (Mantel-Cox) test was applied.

RESULTS

The *sB* gene of *Aspergillus fumigatus*, which encodes its sole sulfate transporter, is dispensable for virulence. Sulfate (SO_4^{2-}), one preferred sulfur source for filamentous fungi (31), is widespread in nature and also relatively abundant. Furthermore, several sulfate-containing glycosaminoglycans can be found in pulmonary tissue. Among them, heparan sulfate, a major component of the extracellular matrix, has to be considered a potential S source for *A. fumigatus* during invasion. In the past, a specific sulfate transporter was identified in the closely related species *Aspergillus nidulans* (51). Recent investigations characterized this protein as being a member of the SulP family of sulfate transporters (52, 53), whose encoding *sB* gene (AN2730) was shown to be regulated by MetR (52–54). We previously identified its *A. fumigatus* orthologue (AFUA_1G05020) in a BLAST query (55–57) and confirmed that the expression of the encoding gene is regulated by MetR (33). To explore the hypothesis that sulfate is exploited as an S source during intrapulmonary growth, we deleted the complete *sB* coding sequence, making use of a self-excising genetic marker cassette (22, 33, 58) (see Fig. S1 in the supplemental material). Phenotypic inspection of the deletant strain AfS177 (Fig. 2A) revealed that the mutant is indeed unable to grow in the presence of SO_4^{2-} or nitrophenylsulfate as the sole S source. This demonstrates that, in contrast to *A. nidulans* (52), the *sB*-encoded transporter appears to be functionally unique in *A. fumigatus*. Moreover, the *sB*Δ strain grew poorly on sulfite (SO_3^{2-}) and thiosulfate, suggesting that the SB transporter is also involved in the uptake of partially reduced inorganic S sources. In addition, the deletant became resistant to chromate, a toxic sulfate analogue that is commonly used to isolate and validate mutants impaired in the uptake of sulfate (51, 52, 59). As expected, AfS177 grew normally on organic S sources such as methionine or cysteine.

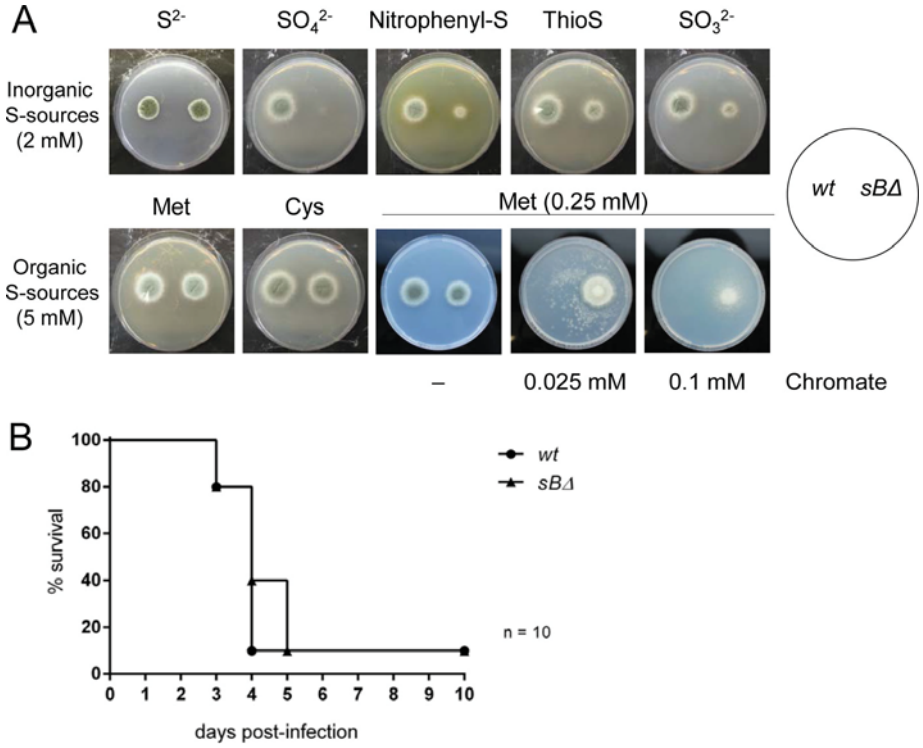

FIG 2 The sulfate transporter-encoding gene *sB* is not required for virulence. (A) Phenotypic characterization shows that the *sB*Δ mutant strain did not grow on sulfate (SO_4^{2-}), grew very poorly on nitrophenylsulfate, and was resistant to chromate, demonstrating that this gene encodes the sole and specific sulfate transporter of *A. fumigatus*. In addition, it grew worse than the wild type (wt) on sulfite (SO_3^{2-}) and thiosulfate, suggesting that it participates in the uptake of other inorganic sulfur sources. On the contrary, it grew well on organic sources and sulfide (S^{2-}). (B) The *sB*Δ mutant is fully virulent in a leukopenic murine model of IPA, which excludes sulfate as the sole exploited sulfur source in murine lungs.

To investigate any role of SB in *A. fumigatus* virulence in an established infection model of IPA, susceptible mice were inoculated by pulmonary instillation of conidia of the wild-type or the AfS177 strain, and survival was monitored over time (Fig. 2B). As the *sB*Δ mutant was fully virulent and taking into account that SB is the sole sulfate transporter in this *Aspergillus* species, this indicates that sulfate does not serve as an essential S source for *A. fumigatus* within the murine lung.

Sulfite reductase activity of *A. fumigatus* mediates assimilation of inorganic S sources or taurine but is not a virulence determinant. Taurine is a cysteine-derived sulfonic acid that has been shown to be highly concentrated in the bronchoalveolar lavage fluid of both healthy and asthmatic patients (37). This compound can be used as a sulfur source by many bacterial and fungal species, including *A. fumigatus* (33). The enzyme responsible for the degradation of taurine, a taurine/α-ketoglutarate dioxygenase activity, has been described for *Escherichia coli* (60) and *Saccharomyces cerevisiae* (61). The *A. fumigatus* genome encodes up to seven putative taurine/α-ketoglutarate dioxygenases (data not shown). Therefore, to directly test whether taurine can be utilized as a sulfur source during *in vivo* growth, all seven genes need to be inactivated. To circumvent the necessity of constructing a septuple mutant, we decided to analyze the potential of taurine as a sulfur source in a more attainable manner: The action of a taurine/α-ketoglutarate dioxygenase releases sulfite, which must be further reduced to sulfide (S^{2-}) to become incorporated into organic S compounds (Fig. 1). Therefore, elimination of the sulfite reductase activity not only would block the assimilation of inorganic sulfur sources (excluding S^{2-}) but also might interfere with the utilization of taurine as an S source. Consequently, we deleted the entire coding sequence of the β-subunit of the sulfite reductase-encoding *sF* gene (AFUA_2G15590), again making use of the self-excising marker for gene replacement (see Fig. S2 in the supplemental material). As expected, the resulting mutant strain, AfS179, was not able to grow in the presence of any oxidized inorganic S source, such as sulfate or sulfite, but resumed growth on sulfide-containing culture medium (Fig. 3A). Moreover, the mutant could not assimilate taurine, confirming the requirement for the encoded enzyme for the catabolism of this pseudo-amino acid, but grew in the presence of several other organic S sources tested. Interestingly, the *sF*Δ strain also grew poorly on methionine-containing medium, despite the fact that it may be converted to other S compounds by the transsulfuration pathway. Surprisingly, the *sF*Δ mutant could not grow in the presence of bovine serum albumin (BSA) as the sole source of sulfur. As this protein contains 35 cysteine and 5 methionine residues (62), a mutant that is able to assimilate these amino acids should be able to utilize BSA as a source of sulfur. However, further supplementation of BSA medium with cysteine restored the growth of the AfS179 strain (see Fig. S3 in the supplemental material), which makes any potential toxic effects of BSA on this strain unlikely. We previously observed that the presence or absence of a nitrogen source affects the assimilation of some S sources (33). Therefore, we decided to test whether the utilization of BSA as a complex S source might be achieved under conditions of nitrogen deprivation (see Fig. S3 in the supplemental material). We observed that the inability of the

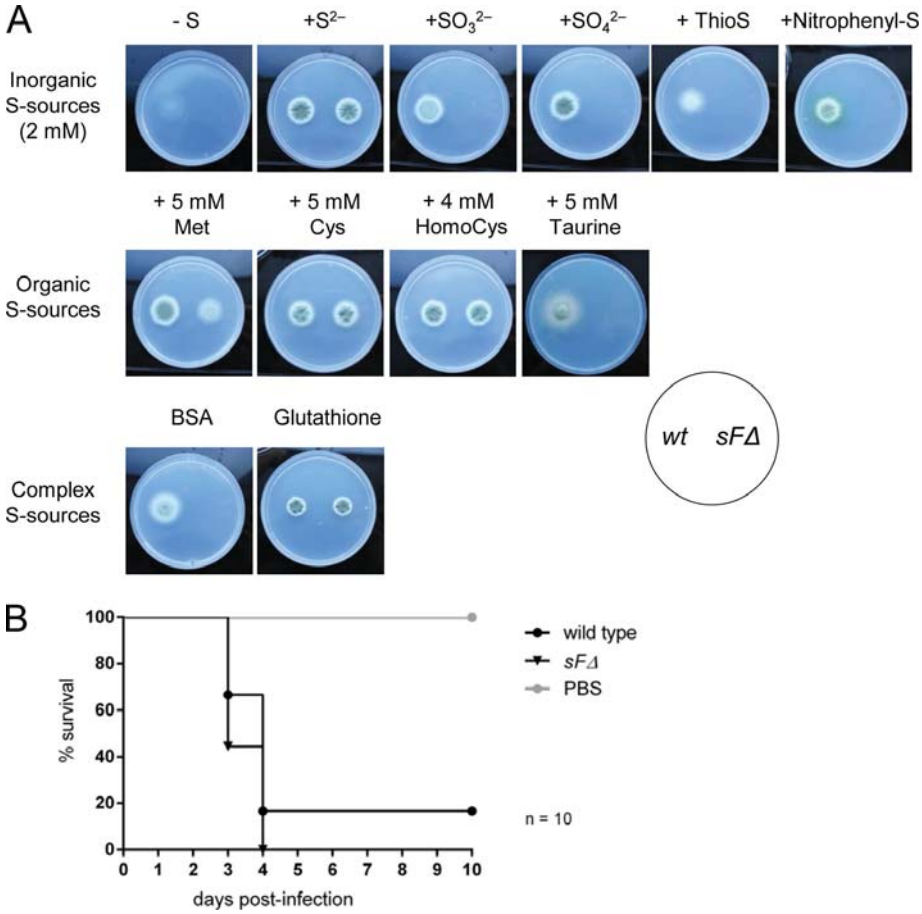

FIG 3 The sulfite reductase-encoding gene *sF* is required for utilization of taurine but dispensable for virulence. (A) Phenotypic analysis proves that the *sF*Δ mutant is unable to grow on any of the inorganic sulfur sources except sulfide. As expected, it grew on organic sources except taurine, whose catabolism releases sulfite. Surprisingly, it was unable to grow on BSA. (B) The *sF*Δ mutant is fully virulent in a leukopenic murine model of IPA. Therefore, inorganic sulfur compounds (except sulfide) and taurine can be excluded as essential S sources in pulmonary tissue.

*sF*Δ deletant to utilize BSA as an S source was independent of the availability or source of nitrogen.

Furthermore, we tested the virulence of the *sF*Δ mutant in the leukopenic murine model of IPA. Mice were infected with conidia of the wild-type and AfS179 mutant strains, and the onset of pulmonary aspergillosis was recorded. Despite its inability to utilize several S compounds *in vitro*, the *sF*Δ strain was fully virulent *in vivo* (Fig. 3B). From these findings, we conclude that assimilation of taurine or oxidized inorganic S compounds is not required for virulence and, therefore, that these compounds are not indispensable sulfur sources for the fungus within lung tissue.

Low cysteine availability in murine lungs limits virulence of an auxotrophic *A. fumigatus* strain. As a proteinogenic amino acid, cysteine may be present in lungs at elevated levels. Although the importance of extracellular proteolysis for *A. fumigatus* virulence is not clarified (22, 23, 25, 26), it is known that several proteases are secreted during intrapulmonary growth (reviewed in reference 13). Thus, cysteine is a reasonable candidate to be exploited by *A. fumigatus* as an S source during invasive growth. Transporters of amino acids entail a great degree of redundancy, making it difficult to selectively block the uptake of a distinct one. Therefore, we decided to generate a cysteine auxotroph with the aim of investigating whether *A. fumigatus* acquires cysteine during intrapulmonary growth. For this surrogate strategy, both the main and alternative pathways of cysteine synthesis need to be shut down in *Aspergillus* (63) (Fig. 1). We again made use of the self-excising genetic marker to consecutively delete the annotated coding sequences of the presumed *mecA* and *cysB* genes, encoding cystathionine-β-synthase and cysteine synthase, respectively, corresponding to the gene identifiers AFUA_2G07620 and AFUA_4G03930 (see Fig. S4A in the supplemental material). The resulting double mutant showed the same growth capacities as those of the *mecA*Δ single mutant strain AfS182 (see Fig. S4B in the supplemental material), calling into question the correct annotation of the CysB-encoding gene locus. An *in silico* analysis of the *A. fumigatus* genome searching for orthologues of the previously described *A. nidulans* CysB protein (AN8057) (64) revealed that, indeed, the most similar protein is encoded by the AFUA_5G02180 locus (see Fig. S5A in the supplemental material). Based on this result, we targeted this putative CysB-encoding gene in the *mecA*Δ background (see Fig. S5B in the supplemental material) to discover that the corresponding double mutant strain, AfS185, displays cysteine auxotrophy (Fig. 4A). For confirmation and to demonstrate that the observed phenotype relies on the absence of both targeted genes, we reintroduced the *mecA* gene to construct a *cysB*Δ single mutant, resulting in strain AfS186.

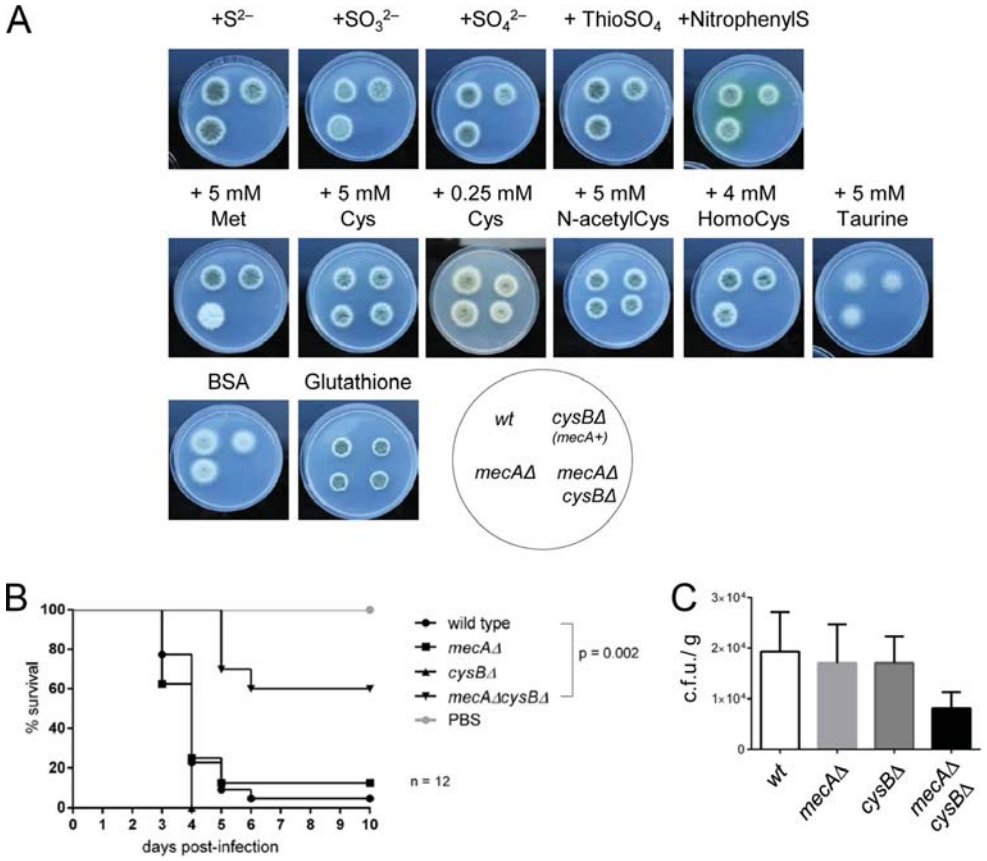

FIG 4 A *mecA*Δ*cysB*Δ double mutant constitutes a cysteine auxotroph and displays reduced virulence in leukopenic mice. (A) The *mecA*Δ *cysB*Δ double mutant grows only on cysteine-containing compounds, proving that it is a tight auxotroph. Both the main and alternative pathways need to be blocked in order to obtain auxotrophy, as proven by the wild-type phenotype of the single mutants. Interestingly, the *mecA*Δ strain grew worse on methionine but not on homocysteine, suggesting that the release of sulfur from methionine is not efficient. (B) Survival analyses in leukopenic mice show a significant reduction in the virulence of the *mecA*Δ *cysB*Δ double mutant. This reduction could be attributed to auxotrophy since the single *cysB*Δ and *mecA*Δ mutants were fully virulent. (C) Calculation of fungal burden in lungs of infected mice as CFU corroborates the decreased growth capacity. Data for CFU are displayed as medians and SEM.

Phenotypic inspection of this set of recombinant *A. fumigatus* isolates confirmed that, indeed, both the *mecA* and *cysB* genes must be absent to result in cysteine auxotrophy (Fig. 4A) and that the actual CysB cysteine synthase in *A. fumigatus* is encoded not by the AFUA_4G03930 locus but by the gene assigned to AFUA_5G02180. The Cys$^-$ auxotrophic strain was able to grow in the presence of low concentrations (0.25 mM) of cysteine, proving that trace amounts of this amino acid are sufficient to support fungal growth. However, the auxotroph was not able to utilize BSA (Fig. 4A) as a sole sulfur source. It has been described that an *A. fumigatus* lysine auxotroph cannot germinate on nonhydrolyzed proteins but can utilize them as a carbon source after germination (15). To test whether the same situation is true for the cysteine auxotroph, we pursued two approaches: first, we supplemented BSA-containing medium with a very low concentration of cysteine (0.001 mM) that is sufficient to support germination but not sustained growth, and second, we inoculated germinated conidia onto BSA as the sole S source (see Fig. S6A in the supplemental material). In both cases, the mutant was able to grow, demonstrating that cysteine is required to trigger the germination of AfS185 conidia. Nevertheless, under both conditions, the growth of the auxotroph was considerably reduced compared to that of the wild-type or the single mutant strain, suggesting that BSA, despite containing a higher-than-average percentage of cysteines, does not completely rescue the auxotrophy for this amino acid.

To assess whether cysteine is sufficiently available in lung tissues to be assimilated by *A. fumigatus* and to serve as an S source, virulence studies in leukopenic mice were conducted. These mice were inoculated with conidia of the wild-type isolate, each single-deletion mutant, or the Cys$^-$ auxotrophic double deletant to monitor disease progression and survival for a period of 10 days (Fig. 4B). While the *mecA*Δ or *cysB*Δ single mutant was as pathogenic as the wild-type isolate, the *mecA*Δ;*cysB*Δ double mutant displayed a significant reduction in virulence. The increased survival of mice infected with the double mutant correlated with a decrease of the fungal burden in pulmonary tissue (Fig. 4C). These data indicate that the amount of cysteine readily available, as free amino acid or bound in glutathione, in lung tissue is insufficient to trigger germination and/or to rescue the auxotrophy of the mutant, suggesting that cysteine is not an abundant source of sulfur during invasive pulmonary aspergillosis in this murine infection model.

To assess any correlation of the observed phenotypes with extracellular proteolysis executed by the mutant strains, semiqualitative assays on solid, casein-supplemented culture medium were carried out to reveal that none of the strains displayed any altered capacities compared to their wild-type progenitor (data not

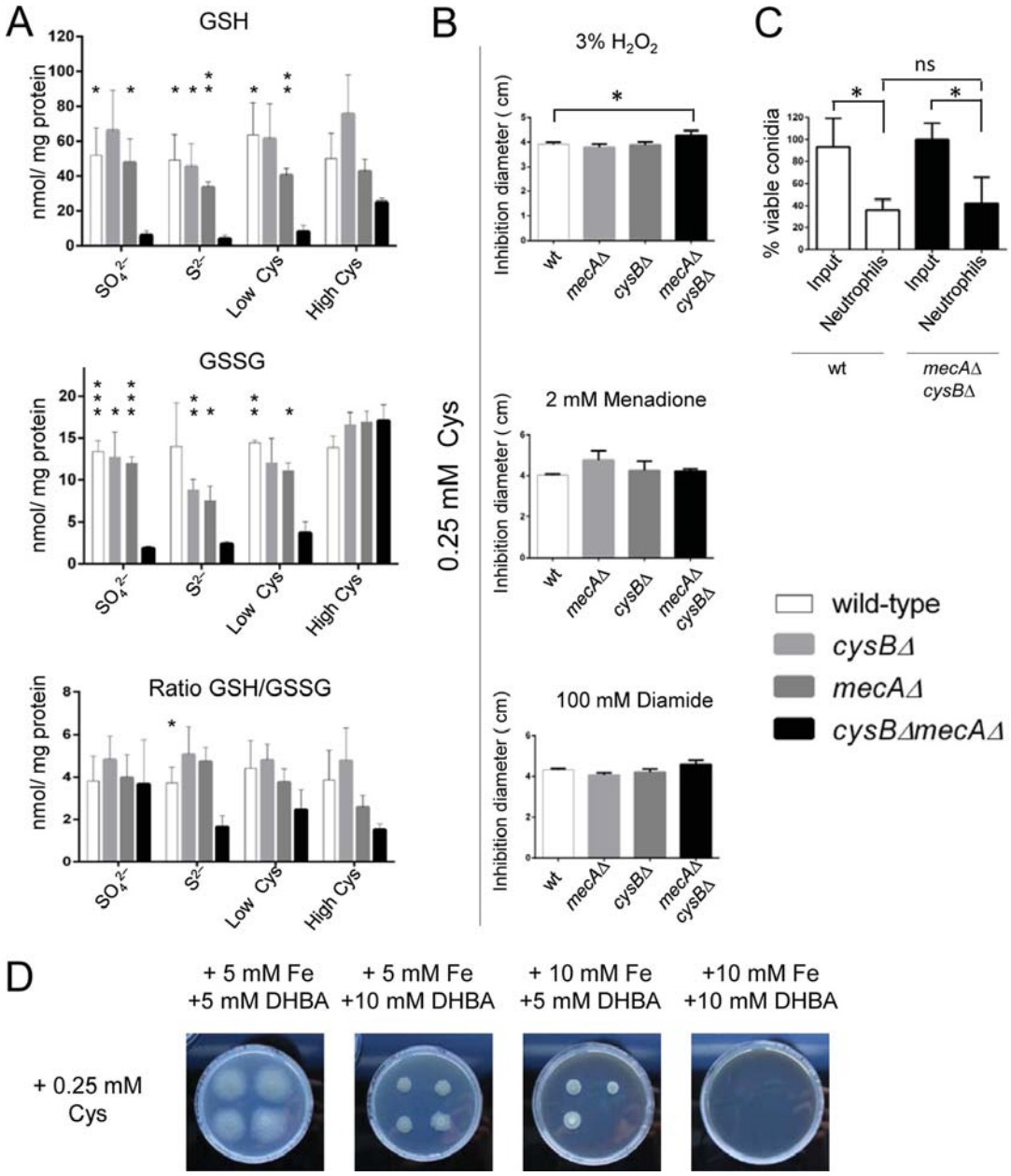

FIG 5 The cysteine auxotroph contains less glutathione but does not show increased sensitivity to oxidative agents or neutrophil killing. (A) The Cys^- auxotrophic strain has significantly reduced levels of GSH and GSSG in the presence of sulfate, sulfide, and a low concentration (0.25 mM) but not a high concentration (5 mM) of cysteine, yet the GSH/GSSG ratio is slightly but not significantly reduced. (B) The double mutant shows slight susceptibility to H_2O_2 in the presence of a low concentration (0.25 mM) of cysteine but not to menadione or diamide. (C) Neutrophils isolated from murine bone marrow kill conidia of the wild type and the cysteine auxotroph at similar rates (significant differences are marked with asterisks [*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significant]). (D) Out of many oxidative and glutathione stressors tested, the double mutant shows higher sensitivity than the wild-type only to the combination of high iron excess and DHBA (see legend for Fig. 4A for strain identification).

shown; see also Fig. S6 in the supplemental material). Accordingly, the limited virulence potential of the Cys^- auxotroph appears not to be linked primarily to a reduced proteolytic capacity.

Cysteine supplementation does not interfere with oxidative stress resistance of *A. fumigatus*. Cysteine is a constituent of the tripeptide glutathione, which is the most important regulator of cellular redox status and is involved in oxidative stress resistance (65). Therefore, it might be speculated that the reduced virulence of the cysteine auxotroph is due to its higher susceptibility to oxidative stressors released by the host. To investigate this possibility, we measured the amounts of reduced glutathione (GSH) and oxidized glutathione (GSSG) to deduce the GSH/GSSG ratio in the wild-type and mutant strains grown under different conditions of sulfur supplementation in order to monitor whether supplementation would affect the intracellular glutathione pool (Fig. 5A). Levels of both GSH and GSSG were significantly decreased in the *mecA*Δ;*cysB*Δ double mutant strain AfS185 grown in the presence of sulfate, sulfide, and a low (0.25 mM) cysteine supply but

not at high concentrations (5 mM) of this amino acid. Interestingly, the deduced GSH/GSSG ratio did not vary significantly, reflecting the parallel decrease in the levels of both reduced and oxidized glutathione as well as the robustness of glutathione homeostasis in *A. fumigatus*. In line with this observation, we tested whether there was any sensitivity of the cysteine auxotroph to oxidative stress by growth inhibition assays (Fig. 5B; see also Fig. S6 in the supplemental material). The single mutants showed the same resistance capacity as the wild-type isolate under all tested conditions. The auxotroph strain was slightly more sensitive to H_2O_2 under conditions of low-cysteine supplementation (Fig. 5B). Surprisingly, no increased sensitivity to diamide, a thiol-oxidizing drug that is able to indirectly induce oxidative stress by oxidizing GSH (66), was observed. Fludioxonil is an antifungal drug whose effect proved to be enhanced by the disturbance of glutathione homeostasis (67). Therefore, we tested whether the cysteine auxotroph is more sensitive to this compound under low-cysteine conditions (see Fig. S6B in the supplemental material) but did not observe an altered sensitivity. We further tested the action of 2,5-dihydroxybenzoic acid (2,5-DHBA), which disrupts cellular GSH/GSSG homeostasis (67), alone or in combination with fludioxonil, but again, we did not observe a more susceptible phenotype (see Fig. S6C in the supplemental material). Iron excess can cause oxidative stress via Haber-Weiss/Fenton chemistry; furthermore, regulation of iron homeostasis involves posttranslational glutathione-mediated regulation. When we tested the sensitivity of the cysteine auxotroph to excess iron alone (see Fig. S6D in the supplemental material) or in combination with 2,5-DHBA (see Fig. S6E in the supplemental material), we observed that the mutant was not more sensitive to excess iron alone; however, the combination of a high iron excess and 2,5-DHBA completely inhibited the growth of the auxotroph. Thus, glutathione homeostasis is sufficiently maintained in the mutant to cope with single stressors, but the combination of excess iron and further disruption of the glutathione balance appears to negatively affect the growth of this strain. Finally, to assess whether the reduced levels of glutathione have an impact on the resistance of the conidia of the cysteine auxotroph against attack by immune effector cells, we performed a conidiocidal assay using neutrophilic granulocytes isolated from murine bone marrow (Fig. 5C). Neutrophils killed conidia of the wild-type and double mutant strains to the same extent, demonstrating that the cysteine auxotroph is not more sensitive to phagocytic elimination by these effector cells.

Gliotoxin is a sulfur-containing mycotoxin produced by *A. fumigatus* (68). It has been reported that elevated levels of GSH may potentiate the autotoxic effects of gliotoxin (69); thus, we hypothesized that the AfS185 strain, which contains reduced levels of GSH, might be more resistant to this toxin. However, when we tested the sensitivity of the cysteine auxotroph to exogenous gliotoxin (see Fig. S6F in the supplemental material), the mutant strain did not show increased resistance, suggesting that decreased levels of reduced glutathione do not affect the intrinsic mechanisms of resistance of *A. fumigatus* to its major mycotoxin.

In conclusion, the oxidative resistance capacities of the cysteine auxotroph remain virtually as strong as those of the wild type, even under conditions of cysteine limitation. Therefore, even if the action of the immune system in neutropenic mice is just marginal, this result reinforces the conclusion that the reduced virulence of the mutant is due to the insufficient amount of readily available cysteine in the murine lung but not to altered oxidative stress susceptibility.

Methionine biosynthesis of *A. fumigatus* represents a target for antifungal interference. Methionine, as a proteinogenic amino acid, is also present in proteins found at the site of infection and, therefore, might serve as a sulfur source for *A. fumigatus*. As is the case for cysteine, transport of methionine entails a great degree of redundancy, and therefore, we decided to construct a Met^- auxotrophic strain. To do so, the methionine synthase-encoding gene *metH* (AFUA_4G07360) (Fig. 1) was targeted for deletion. However, after several unsuccessful attempts, it was suspected that this gene might be essential in *A. fumigatus*. To test this hypothesis, a heterokaryon rescue analysis (70, 71) with *akuA*Δ recipient strain AfS35 (72, 73) was performed to reveal that homologous replacement of the *metH* locus could not be achieved (Fig. 6A) when transformed protoplasts were regenerated on *Aspergillus* minimal medium supplemented with methionine. To further confirm that the *metH* gene is indispensable for *A. fumigatus*, we constructed a conditional expression strain, AfS180, in which the expression of the coding sequence is driven by a doxycycline-dependent Tet-ON module (see Fig. S7 in the supplemental material) (74, 75), a system that has successfully been used in *A. fumigatus* to prove gene essentiality (76). In the presence of doxycycline, the *metH* gene is properly expressed to support growth of AfS180 (Fig. 6B), independent of the presence of methionine (see Fig. S8 in the supplemental material). In the absence of doxycycline, however, AfS180 did not grow, even though methionine had been added to nitrogen-depleted culture medium to force its uptake. This result contrasts with data for *A. nidulans*, in which the *metH* gene can apparently be manipulated to result in methionine auxotrophy (77). The absence of MetH enzymatic activity might trigger two deleterious effects for fungal cell metabolism, which could explain why a deletant cannot be isolated. First, its elimination may result in a toxic accumulation of homocysteine (78, 79), and second, its absence might provoke a shortage of folate, since it cannot be recycled from methyl-tetrahydrofolate (80) (Fig. 1). To minimize the impact of these negative effects, we grew the conditional mutant in the presence of serine (to facilitate the conversion of homocysteine to cystathionine) (Fig. 1) and/or folate (see Fig. S8 in the supplemental material). However, in the absence of doxycycline, AfS180 did not grow independently of the supplemented compounds. Finally, we tried to compensate for the absence of MetH with other amino acids or complex substrates; surprisingly, Casamino Acids partially recovered the growth of AfS180, and further addition of methionine restored the growth capacity almost completely (Fig. 6C).

Given this growth phenotype under such specialized conditions, we were interested in whether *metH* is required under *in vivo* conditions during infection. We inoculated mice with conidia of the conditional promoter replacement strain AfS180, which resulted in wild-type-like virulence in the cohort that had been fed doxycycline in the drinking water (Fig. 6D). In contrast, a highly significant reduction in virulence was noted for mice that had not been treated with doxycycline: only 2 out of 10 mice succumbed to infection, and a postmortem diagnostic PCR analysis revealed that the genome in these fungal isolates had undergone a recombination event that eliminated the Tet-ON module (not shown), a leakiness of the system which was described previously (81). Furthermore, the obtained avirulence correlated with an almost complete elimination of the fungus in pulmonary tissue (Fig. 6E). In

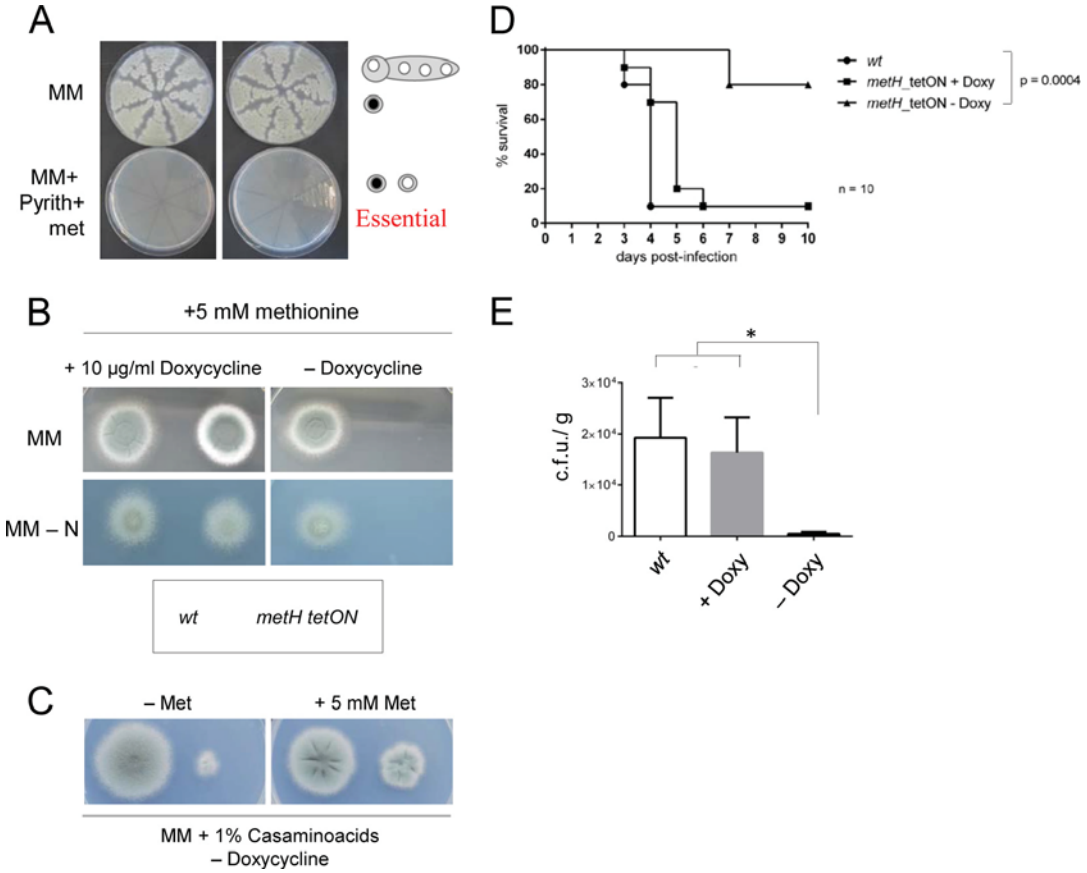

FIG 6 The methionine synthase-encoding gene (*metH*) is essential for *A. fumigatus in vivo*. (A) Heterokaryon rescue technique using the *akuA* background strain. Primary transformants were able to grow on nonselective *Aspergillus* minimal medium (where wild-type nuclei are able to grow) but not on selective medium containing the selectable marker pyrithiamine (where the mutant nuclei should grow), even if there was methionine in the medium. (B) A mutant expressing the *metH* gene under the control of the Tet-ON inducible system is able to grow in the presence of doxycycline but not on minimal medium without the inducing antibiotic, although methionine is present and even if it is the sole nitrogen source. (C) In the absence of doxycycline, supplementation with a Casamino Acids mixture partially recovers growth of the conditional mutant, which is almost complete with further supplementation with methionine. (D) The conditional strain is virulent when infected leukopenic mice are fed doxycycline in drinking water, but it is avirulent when mice are not fed the antibiotic, proving that *metH* is essential under *in vivo* infection conditions. (E) Calculation of fungal burden in lungs of infected mice as CFU corroborates the decreased growth capacity. Data for CFU are displayed as medians and SEM.

conclusion, the *metH* gene is essential for *A. fumigatus* under conditions of infection, proving that the amount of readily available amino acids in lung tissue is rather scarce and making its gene product a promising target for the development of antifungal substances in the context of aspergillosis.

DISCUSSION

Microbial metabolism is crucial for pathogenesis, and several biosynthetic pathways have been established in the past to represent valid targets for chemotherapeutic intervention. In this respect, routes of sulfur assimilation have been studied to some degree for pathogenic microbes (82) but insufficiently for fungal pathogens. We previously demonstrated that proper regulation of sulfur assimilation is essential for *A. fumigatus* virulence (33). In the present study, we aimed to scrutinize sulfur utilization during intrapulmonary growth, which would significantly advance our understanding of *A. fumigatus* virulence. We demonstrate that neither inorganic S sources nor taurine is required to cause infection, excluding them as exploited S sources during *in vivo* growth. We furthermore show that a cysteine auxotrophic strain cannot invade the murine lung, proving that this amino acid is limiting in pulmonary tissue. This mutant strain also revealed that the subordinate glutathione oxidative homeostasis system is robust, since a limiting cysteine supply is not reflected by increased sensitivity toward oxidative agents or neutrophil killing. Finally, we demonstrate that the methionine synthase-encoding gene is essential for *A. fumigatus in vivo*, pointing to the methionine biosynthetic pathway as an attractive target for antifungal therapy.

Transporters represent promising druggable candidates for antimicrobial therapy, based on their cellular localization. In fungi, however, high degrees of redundancy for uptake activities are evident, which reflect the osmotrophic feeding style common to these organisms. The fact that the *sB* gene encodes the sole sulfate transporter in *A. fumigatus*, as proven by the inability of the respective deletant to grow on sulfate as an S source and its resistance to the toxic analogue chromate, is exceptional in relation to other fungal organisms: *Saccharomyces cerevisiae* (83), *Neurospora crassa* (84), and *Penicillium chrysogenum* (85) appear to contain more than one sulfate transporter, and for the closely related species *A. nidulans*, an alternative sulfate transporter (GenBank accession number ABA28286.1) encoded by the *astA* gene has been described (52). A BLAST query failed to identify an AstA ortho-

logue in *A. fumigatus* (not shown), and we therefore conclude that the capacity of *A. fumigatus* to take up sulfate completely depends on the SB transporter. However, an *sB*Δ deletant is fully virulent, demonstrating that acquisition of sulfate is dispensable in the murine lung, eliminating the respective transport activity as a suitable target for the development of antifungals.

We have previously shown that *A. fumigatus* can grow on taurine as a sole S source, and by generating the respective *sF*Δ mutant, we demonstrated that this capacity indeed requires the sulfite reductase activity. This mutant is completely virulent, which excludes inorganic S compounds (except H_2S) and taurine as being required S sources *in vivo*. This result correlates with previously reported information on the pathogenic fungus *Paracoccidioides brasiliensis*: this fungus also penetrates the human body through the respiratory tract to infect lung alveoli, and it is known that the yeast form, the parasitic morphotype, cannot assimilate inorganic S sources (86–88) and, thus, must feed on organic S compounds. Interestingly, the *sF*Δ strain was furthermore unable to grow on BSA as a source of sulfur and/or nitrogen. We previously showed that the assimilation of certain sulfur sources varies depending upon the availability of nitrogen (33); also, sulfite reductase activity is known to be affected by the nitrogen source (89). We therefore tested the growth capacity of the mutant on different nitrogen sources, which did not utilize BSA under any of the tested conditions. Finally, we tested the ability of germinated spores to grow on BSA, to rule out the possibility that the mutant could have a defect in germination rather than growth in this proteinaceous substance, but these germlings were unable to assimilate BSA (not shown). However, the mutant was able to grow on single S-containing amino acids and on the tripeptide glutathione, which suggests that the *sF*Δ strain is somehow impaired in the degradation of proteins rather than in the utilization of amino acids. To our knowledge, this is the first report of a connection between sulfite reductase activity and protein degradation/assimilation.

We demonstrate that the main and alternative pathways of cysteine synthesis are active in *A. fumigatus*, since construction of a double mutant blocking both routes is required to obtain a cysteine auxotroph. Interestingly, when the alternative pathway was blocked, this *mecA*Δ mutant grew on homocysteine but worse than the wild type on methionine. This suggests that the release of sulfur from methionine is less effective, a hypothesis which could not be further investigated since the enzymes that participate in the catabolism of those compounds have not been identified. One possibility could be that part of the sulfur released from methionine catabolism is lost in the form of volatile sulfur compounds that are liberated into the air. Interestingly, the cysteine auxotroph, which absolutely requires free cysteine to germinate and to be able to release this amino acid from proteinaceous substrates, displayed a significant reduction in virulence. This strongly suggests that the amount of readily available cysteine in the lungs, which includes tissue free amino acid and glutathione, is limited and not sufficient to trigger germination and/or to rescue auxotrophy.

Taking into account that the cysteine auxotroph shows decreased GSH and GSSG levels, it was surprising that it is not significantly sensitive to diamide. It is important to remark that the GSH/GSSG ratio is maintained, indicating that the cells do not encounter oxidative stress *per se* and suggesting that the glutathione reductase may be sufficiently effective to counteract the action of diamide. On the contrary, the mutant was slightly more sensitive to H_2O_2, which can be due to the fact that diamide is an oxidant that gives rise only to disulfides (90), whereas peroxides generate disulfides as well as sulfenates (Cys-SOH), sulfinates (Cys-SO_2H), and sulfonates (Cys-SO_3H) (91). Despite the smaller amounts of glutathione, the auxotroph was basically as resistant as the wild type to all oxidative stressors tested, which led us to propose that the GSH/GSSG ratio is decisive to maintain a proper redox status rather than the absolute levels of each component.

Methionine synthase has been described to be essential for *Candida albicans* (79) and *Cryptococcus neoformans* (92) virulence, indicating that this enzymatic activity is relevant for yeast pathogenicity. Here, we have demonstrated that the methionine synthase activity is also required for the filamentous fungus *A. fumigatus* to invade a susceptible host, suggesting that this enzyme may be especially important for all pathogenic fungal species. We observed that a conditional mutant is able to grow under noninducing conditions in the presence of Casamino Acids and excess methionine (but not methionine only), which suggests that the absence of methionine synthesis causes an imbalance in the amino acid pool and/or sensing that can be compensated for by the addition of free amino acids. Actually, a role of methionine in amino acid sensing through protein phosphatase 2A (PP2A) methylation has been described (93). Interestingly, our observations not only demonstrate that the methionine synthase activity is essential for *A. fumigatus* to cause infection but also prove that the amount of readily accessible amino acids in lung tissue is limited.

Methionine synthases catalyze the transfer of a methyl group from N^5-methyl-5,6,7,8-tetrahydrofolate (CH_3-THF) to L-homocysteine (Hcy), the terminal step in methionine biosynthesis. Two apparently unrelated families of proteins that lack similarity in their amino acid sequences (94, 95) catalyze this reaction: cobalamin-dependent methionine synthase (EC 2.1.1.13) and cobalamin-independent methionine synthase (EC 2.1.1.14). Mammals utilize the cobalamin-dependent methionine synthase, while plants and fungi draw on the cobalamin-independent enzyme. Therefore, having demonstrated that the methionine synthase is essential for *A. fumigatus* pathogenicity, we propose that it constitutes a specific and promising fungal target for chemotherapy, as has been suggested previously (79, 96), which would actually have a broad antifungal spectrum.

In essence, this study not only emphasizes the importance of primary metabolism for fungal pathogenesis but also underscores the need to unravel the nutritional needs of the pathogen during infection and invasion in order to circumscribe the respective virulome in an increasingly defined fashion. The achieved insights make a case for probing the host niche as a nutritional source and furthermore highlight the necessity for testing virulence in appropriate model systems of infection, given that *in vitro* data allow predictions to only a limited extent, as exemplified by the presumed essentiality of the *A. fumigatus metH* gene.

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