Evaluation of Lysine Biosynthesis as an Antifungal Drug Target: Biochemical Characterization of *Aspergillus fumigatus* Homocitrate Synthase and Virulence Studies†‡

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*Aspergillus fumigatus* is the main cause of severe invasive aspergillosis. To combat this life-threatening infection, only limited numbers of antifungals are available. The fungal α-aminoadipate pathway, which is essential for lysine biosynthesis, has been suggested as a potential antifungal drug target. Here we reanalyzed the role of this pathway for establishment of invasive aspergillosis in murine models. We selected the first pathway-specific enzyme, homocitrate synthase (HcsA), for biochemical characterization and for study of its role in virulence. *A. fumigatus* HcsA was specific for the substrates acetyl-coenzyme A (acytalc-CoA) and α-ketoglutarate, and its activity was independent of any metal ions. In contrast to the case for other homocitrate synthases, enzymatic activity was hardly affected by lysine and gene expression increased under conditions of lysine supplementation. An *hcsA* deletion mutant was lysine auxotrophic and unable to germinate on unhydrolyzed proteins given as a sole nutrient source. However, the addition of partially purified *A. fumigatus* proteases restored growth, confirming the importance of free lysine to complement auxotrophy. In contrast to lysine-auxotrophic mutants from other fungal species, the mutant grew on blood and serum, indicating the existence of high-affinity lysine uptake systems. In agreement, although the virulence of the mutant was strongly attenuated in murine models of bronchopulmonary aspergillosis, virulence was partially restored by lysine supplementation via the drinking water. Additionally, in contrast to the case for attenuated pulmonary infections, the mutant retained full virulence when injected intravenously. Therefore, we concluded that inhibition of fungal lysine biosynthesis, at least for disseminating invasive aspergillosis, does not appear to provide a suitable target for new antifungals.

The opportunistic human pathogen *Aspergillus fumigatus* causes severe invasive infections in immunocompromised patients. Advances in transplantation medicine, the use of immunosuppressive drugs in cancer therapy (44), and immunosuppressing diseases such as AIDS (42) lead to increasing numbers of patients at risk to acquire invasive aspergillosis. Unfortunately, diagnostic and therapeutic tools are extremely limited. The antifungals currently used mainly target the fungal cell wall and membrane. Although these provide suitable targets, antifungal drug resistance has been observed, and the extensive use of antifungals may be hampered by severe side effects (15, 16, 29, 36, 40). Additionally, the unambiguous diagnosis of invasive fungal infections is sometimes difficult, but it is necessary to start therapy as soon as possible, using the best-suited drugs (52). Therefore, new broad-spectrum antifungals which display high effectiveness and reduced toxicity are required. New antifungals may be derived from the inhibition of fungal metabolism during pathogenesis. The nutrition of a pathogen during infection is essential for growth and maintenance within the host. Therefore, targeting of pathways required for nutrient acquisition and metabolism could lead to new antifungals. However, only limited information on carbon and nitrogen sources which serve as nutrients for the pathogen during the infection process is available (11).

To elucidate the nutritional conditions during fungal infections and to identify possible drug targets, we focus on the impact of fungal pathways that do not exist in humans, such as the methylcitrate cycle (24), the glyoxylate bypass (53), and lysine biosynthesis. Interestingly, it has been shown that the glyoxylate bypass is essential for full virulence of *Candida albicans* (5, 34, 45) but is dispensable for virulence of *Cryptococcus neoformans* (51) and *A. fumigatus* (41, 53). In contrast, the methylcitrate cycle, which is essential for the removal of toxic propionyl-coenzyme A (propionyl-CoA), has been shown to be important for full virulence of *A. fumigatus* (24), but unfortunately, no genes coding for enzymes of this cycle can be detected in the genome of *C. albicans*. Therefore, targeting the methylcitrate cycle would provide a tool against *A. fumigatus* but not *C. albicans*. Lysine is an essential amino acid for humans and must be obtained from the diet. In contrast, fungi synthesize lysine via the α-aminoadipate pathway (66), and most, if not all, fungi contain the key enzymes of this pathway. Therefore, inhibition of fungal lysine biosynthesis has been assumed to be a possible antifungal drug target (43, 64).

The α-aminoadipate pathway (for a schematic of this path-
way, see Fig. S1 in the supplemental material) is characterized by the initial condensation of acetyl-CoA and α-ketoglutarate, catalyzed by the homocitrate synthase, leading to the first key intermediate, homocitrate (63). Homocitrate is converted into homoconitrate by de- and rehydration reactions. Although the homoconitase encoded by lysF seems to play an important role in these reactions, only the rehydration of homoconitase to homocitrate has been confirmed experimentally (61), and further investigations are required to complete the knowledge on the conversion of homocitrate to homoconitrate. The last fungus-specific reaction in lysine biosynthesis is the oxidative decarboxylation of homoconitrate into α-ketoconitate, catalyzed by the homoconitase dehydrogenase. Homoconitase dehydrogenase was recently purified and characterized from Saccharomyces cerevisiae (31–33) but has not been obtained from any other fungal source, which keeps the data on the characteristics of this enzyme quite limited. α-Ketoadipate subsequently undergoes an aminotransferase reaction yielding α-aminoconitate, which gives the pathway its name and is a key metabolite in fungal penicillin biosynthesis (3). The subsequent steps in lysine biosynthesis are completely reversible and are also present in humans, for lysine degradation. Therefore, these “shared enzymes” are not assumed to provide suitable targets for new antifungals.

In former studies (30), a low-dose murine infection model of bronchopulmonary aspergillosis was described, in which a homoconitase mutant of A. fumigatus was analyzed for its virulence. The strongly reduced virulence of this mutant implied that the lysine content within infected tissues might be insufficient to promote growth of lysine auxotrophic mutants, implying that fungal lysine biosynthesis might serve as an attractive antifungal drug target. However, studies were limited to bronchopulmonary aspergillosis in neutropenic mice, and neither complementation of lysine auxotrophy by feeding lysine to mice nor intravenous infections, resembling disseminated aspergillosis, were investigated.

To obtain deeper insights into the impact of lysine biosynthesis in fungal virulence and its suitability for potential new antifungals, we investigated the biochemical properties of homocitrate synthase from A. fumigatus and compared its expression pattern with those described for the penicillin producer Penicillium chrysogenum and the yeast Saccharomyces cerevisiae. For biochemical characterization, the enzyme was produced as a recombinant protein in Escherichia coli and purified to homogeneity. 5′-Rapid amplification of cDNA ends (5′-RACE) allowed the identification of the transcriptional start point, and quantitative reverse transcription-PCR (qRT-PCR) enabled the investigation of gene expression under various growth conditions. To confirm the impact of homocitrate synthase on lysine prototrophy and to reinvestigate the impact of lysine biosynthesis on fungal virulence, a deletion mutant was created and phenotypically characterized under different in vitro and in vivo conditions. Our results show that the biochemical parameters differ slightly from those for other fungi and that gene expression seems not to be subjected to feedback inhibition by lysine. Even more, although a strong attenuation of virulence was observed under some infection conditions, we concluded that inhibition of lysine biosynthesis might not provide a suitable target to combat A. fumigatus infections.

**Materials and Methods**

**Media and growth conditions.** A. fumigatus strains were cultivated at 37°C on Aspergillus minimal medium (AMM) (http://www.fgsc.net/methods/asnidmed.html) with 50 mM glucose as the carbon source. Alternatively, 1% bovine serum albumin (BSA; Fluka, Sigma-Aldrich, Taufkirchen, Germany), 0.2% insoluble casein (Fluka, Sigma-Aldrich), 1% peptone (Applichem GmbH, Darmstadt, Germany), or 30% pork serum (Gibco-Invitrogen GmbH, Karlsruhe, Germany) served as a nutrient source. If not indicated otherwise, all media also contained 10 mM nitrate as a nitrogen source. When required, hygromycin B (Roche Diagnostics GmbH, Mannheim, Germany) was added at a concentration of 180 μg/ml and lysine was added to a final concentration of 5 mM. Blood agar was prepared by combining equal volumes of a 0.9% NaCl solution containing 3% agar equilibrated to 50°C with prewarmed sheep blood (Fiebig-Nährstoff-technik Animalblood Products, Idstein-Niederauern, Germany). Plates used for fungal transformations contained AMM-glucose medium with 0.6 M KCl and 240 μg/ml hygromycin B. Conidium suspensions were obtained from malt extract agar slant cultures as described previously (53). The number of conidia was determined by use of a hemocytometer. For testing the growth of different A. fumigatus strains on explanted lung tissue, an NMRI mouse (1 year of age) was sacrificed and the lung was removed. The lung was cut into five pieces and briefly washed in phosphate-buffered saline (PBS). The tissue was placed on a PBS-containing agar plate and inoculated with 1,000 conidia of the respective A. fumigatus strain. Plates were incubated at 37°C. E. coli strains were generally grown on LB medium. For recombinant protein production, BL21(DE3) Rosetta 2 cells (Novagen, Darmstadt, Germany) were used and were incubated in Overnight Express Instant TB medium (Novagen). When required, media were supplemented with ampicillin (final concentration of 0.1 mg/ml) and/or chloramphenicol (final concentration of 34 μg/ml).

**Protease enrichment and protein hydrolysis on agar plates.** A. fumigatus proteases were enriched from the culture broth of the ΔhcsAΔhcsFΔARKwild-type strain (14) grown for 50 h on BSA-containing medium. The mycelium was removed by filtration, and proteins were precipitated from 200 ml medium by ammonium sulfate precipitation, ranging from 0 to 70% saturation. The pellet was collected by centrifugation at 12,000 × g for 15 min and dissolved in a minimal volume of 50 mM HEPES, pH 7.5. The sample was dialyzed overnight against 5 liters of 50 mM HEPES, pH 7.5, leading to a final volume of 12 ml. The dialyzed solution was filtered sterilized (0.2-μm filter), and protease activity was confirmed by an azocasein assay (21). Hydrolytic activity of the protease solution was additionally confirmed by spreading 600 μl of the solution on agar plates containing insoluble casein. Protein hydrolysis was indicated by the disappearance of casein granules. Casein-containing plates without protease treatment served as negative controls. Plates were point inoculated with conidium suspensions of the different A. fumigatus strains and incubated at 37°C.

**Overproduction and purification of recombinant homocitrate synthase.** All oligonucleotides used in this study are listed in Table S1 in the supplemental material. For overproduction of homocitrate synthase, total RNA was isolated from AMM-glucose-grown mycelium of strain CBS 144.89, using a RiboPure yeast RNA purification kit (Applied Biosystems, Darmstadt, Germany). hcs4 cDNA was obtained by first-strand transcription with Bioscript reverse transcriptase (Bioline GmbH, Luckenwalde, Germany) and the sequence-specific oligonucleotides BamHsc4_up and NotHsc4_down. Amplification of the cDNA template was performed with Immolase polymerase (Bioline GmbH). The PCR product was cloned into the pCR4 TOPO vector (Invitrogen GmbH, Karlsruhe, Germany), excised by BamHI/NotI double digestion, and subcloned into a modified BamHsc4NotI-restricted PET34A H6 vector containing an N-terminal His tag (23), resulting in the plasmid pET43.1H6-Hsc4. The plasmid was transferred to E. coli BL21(DE3) Rosetta 2 cells (Novagen) by heat shock transformation. For overexpression of HscA cells were grown for 18 h at 30°C in Overnight Express Instant TB medium (Novagen). Cells were collected by centrifugation, resuspended in 20 mM HEPES, pH 7.5, with 30 mM KCl, and disrupted by sonication (3 times for 2 min each, with 50% pulse and 70% intensity) (Sonoplus; Bandelin Electronic GmbH & Co. KG, Germany). The cleared supernatant was loaded onto a Ni chelation column (GE Healthcare, Munich, Germany), using a MonoHscA column (GE Healthcare, Munich, Germany). The sample was dialyzed against 5 liters of 50 mM HEPES, pH 7.5, 100 mM guanidine hydrochloride, 100 mM α-cyclodextrin, 600 mM ammonium sulfate, 30 mM KCl, and 20% glycerol (1).
Determination of HcsA activity. HcsA activity was determined in a standard assay as previously described (1), with some modifications. In brief, the assay mixture contained 200 mM HEPES, pH 7.5, 0.2 mM dichloromethylphosphonate (DCPMP), 0.2 mM acetyl-CoA, 20 mM α-ketoglutarate, and enzyme-containing fractions in a final volume of 1 ml. The enzyme assay was preincubated at room temperature for 2 min before starting the enzymatic reaction by the addition of α-ketoglutarate. Activity was determined by measuring the absorbance at 600 nm. Using different concentrations of oxidized DCPMP at pH 7.5, the extinction coefficient at 600 nm was determined to be 8.2 mM⁻¹ cm⁻¹. One unit was defined as the reduction of 1 μmol of DCPMP per min. Protein concentrations were determined using a Bio-Rad protein assay concentrate (Bio-Rad Laboratories GmbH, Munich, Germany) as described in the manufacturer's protocol, with bovine serum albumin as the standard.

Biochemical characterization of recombinant homocitrate synthase, Kₚ values for the substrates acetyl-CoA and α-ketoglutarate were determined by keeping the concentration of one of the substrates constant while that of the other was varied. The effects of MgCl₂, CuSO₄, CaCl₂, Na₂SO₄, ZnSO₄, K₂SO₄, NaCl, KCl, EDTA, dipryridyl, phenanthroline, lysine, methylcitrate, and citrate on HcsA activity of the recombinant enzyme were tested in the standard assay mixture in the presence of the indicated substances added at various concentrations. Preincubating the enzyme at different temperatures, with measurement of the residuaal activity after different time points, was used to determine the temperature stability of homocitrate synthase. The temperature dependency of HcsA activity was tested in the standard assay for the range of 22°C to 62°C. The pH dependency of HcsA activity was measured by replacing the HEPES buffer used in the standard assay with a buffer combination containing 0.1 M boric acid, 0.1 M acetic acid, and 0.1 M phosboric acid. The pH of the buffer was adjusted in the range of 2 to 12. The pH optimum was determined at 6.5 mol of DCPIP per min. Protein concentrations were determined using a Bio-Rad protein assay concentrate (Bio-Rad, USA) as described in the manufacturer's protocol, with bovine serum albumin as the standard.

Generation of homocitrate synthase deletion mutants and complemented strains. For deletion of the homocitrate synthase coding region, a 1.278-bp upstream fragment was amplified from genomic DNA of the A. fumigatus H9004 wild type (6) using the oligonucleotides HindHcsDel_up and NotHcsDel_top. A 942-bp downstream fragment was amplified with oligonucleotides HindHcsDel_do and NotHcsDel_bot after gel purification, aliquots of both fragments were mixed, denatured, annealed, and elongated by Taq polymerase (BioTag Red; Bioline GmbH). After four cycles of fusion and elongation, the flanking oligonucleotides HindHcsDel_up and HindHcsDel_do were added and the fused fragments were amplified by 30 additional PCR cycles. The PCR product was subcloned into the pUC18 vector (MBI Fermentas, St. Leon-Rot, Germany) via HindIII restriction. The resulting vector was transformed into A. fumigatus strain CBS 144.89 grown on glucose minimal medium in the presence of 5 mM lysine or nitrate. Additionally, a glucose-nitrate-grown culture was shifted for 2 h to a medium containing glucose and lysine and vice versa. For each condition tested, five independent biological replicates were investigated, each containing two or three technical replicates. cDNA was synthesized by using Invitrogen's instructions for first-strand cDNA synthesis with anchored oligo(dT)₁₂ primers and SuperScript III reverse transcriptase (both from Invitrogen) as described previously (24). Quantitative real-time PCR was performed on a StepOne real-time PCR system, using a GeneAmp Fast PCR kit (both from Applied Biosystems) as described in the manufacturer's protocol, and data were evaluated with the StepOne real-time PCR software package. EvaGreen dye (VWR International GmbH) was used to visualize gene amplification. Amplification rates of the actin and β-tubulin housekeeping genes were used for data normalization. Actin and β-tubulin were amplified with oligonucleotides RT_Act1_f and RT_Act1_r and oligonucleotides RT_HcsA_f and RT_HcsA_r. Six independent cDNA clones were selected for sequence determination.

Quantitative real-time PCR. To analyze hcsA gene expression, RNA was isolated from strain CBS 144.89 grown on glucose minimal medium in the presence or absence of 5 mM lysine or nitrate. Additionally, a glucose-nitrate-grown culture was shifted for 2 h to a medium containing glucose and lysine and vice versa. For each condition tested, five independent biological replicates were investigated, each containing two or three technical replicates. cDNA was synthesized by using Invitrogen's instructions for first-strand cDNA synthesis with anchored oligo(dT)₁₂ primers and SuperScript III reverse transcriptase (both from Invitrogen) as described previously (24). Quantitative real-time PCR was performed on a StepOne real-time PCR system, using a GeneAmp Fast PCR kit (both from Applied Biosystems) as described in the manufacturer's protocol, and data were evaluated with the StepOne real-time PCR software package. EvaGreen dye (VWR International GmbH) was used to visualize gene amplification. Amplification rates of the actin and β-tubulin housekeeping genes were used for data normalization. Actin and β-tubulin were amplified with oligonucleotides RT_Act1_f and RT_Act1_r and oligonucleotides RT_HcsA_f and RT_HcsA_r. Six independent cDNA clones were sequenced for sequence identification.

Statistical analysis of hcsA expression levels. Within each of the biological replicates, the mean values of the technical replicates were used to calculate the relative hcsA gene expression level by dividing the data by the corresponding Ct value for actin or β-tubulin. Subsequently, normalization of data within each biological replicate was performed by calculating the mean value for all gene expression data from the corresponding experimental batch. All expression values from each experimental batch were divided by their respective mean value. This normalization was required to remove the experimental bias and to make the data from the different biological samples comparable. After this normalization, the mean values for the technical replicates from each experimental set were calculated, resulting in one value for each growth condition for each of the five biological replicates. The five mean values for each growth condition were used for final comparison of the expression levels. Since the five values for each growth condition were found to follow a Gaussian distribution, we used the paired t test for determination of statistical significance (48).
intranasal challenge were carried out as described previously (25). Briefly, mice were immunosuppressed by intraperitoneal injection of cortisone acetate (25 mg/mouse; Sigma-Aldrich) on days −3 and 0. On day 0, the mice were anesthetized with ketamine (100 mg/kg of body weight; Inresa Arzneimittel GmbH, Freiburg, Germany) and xylazine (2 mg/kg; Bayer Vital GmbH, Leverkusen, Germany) and infected intranasally with conidium suspensions containing either 1 × 10^8 or 1 × 10^9 conidia in 20 μL PBS. Survival was monitored for 14 days. For time-response studies, mice were infected as described above, and two mice per group were sacrificed at the indicated time points. For intravenous infection, 1 × 10^8 conidia in 200 μL PBS were injected into the lateral tail vein. Survival was monitored for 8 days. Immunosuppressed mice inoculated with PBS served as sham-infected controls in all experiments. For study of the effect of increased dietary lysine on the virulence of the homocitrate synthase deletion mutant, drinking water was supplemented with 100 mM lysine and 2.5 mM glucose. Control groups received drinking water supplemented with 2.5 mM glucose only.

Mice were examined clinically at least twice daily and weighed individually every day. Animals were considered moribund and were sacrificed humanely if they showed rapid weight loss (>20% of body weight at the time of infection), severe dyspnea, decreased body temperature, or other signs of serious illness. Necropsy was performed immediately after euthanasia, and lungs and internal organs were removed and fixed in neutral buffered formalin for histological analysis. Paraffin-embedded tissue sections were stained according to standard procedures with hematoxylin-eosin (HE) or periodic acid–Schiff stain (PAS). For better visualization of fungal elements in lungs, methenamine silver staining was used (56). Stained tissue sections were analyzed by bright-field microscopy, using a Zeiss Axiosmager.M1 microscope (Carl Zeiss MicroImaging GmbH) equipped with a Spot Flex shifting-pixel color mosaic camera (Diagnostic Instruments, Inc.).

Nucleotide sequence accession number. Details concerning all exon and intron sequences of hcsA can be found under GenBank accession number FN401071.

RESULTS

Identification of the hcsA homocitrate synthase gene from A. fumigatus. To identify the hcsA gene from A. fumigatus, the translated sequence of the Penicillium chrysogenum lys1 gene (GenBank accession no. O94225), annotated as the mitochondrial precursor of the homocitrate synthase (2, 26), was used as a template in BLAST analyses against fungal genomes. A protein with 94% sequence identity and with the locus tag a template in BLAST analyses against fungal genomes. A pro-

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Purification and stabilization of recombinant homocitrate synthase. To confirm that the identified open reading frame indeed coded for a functional homocitrate synthase and to ease the biochemical characterization of the enzyme, we produced recombinant homocitrate synthase (HcsA) with an N-terminal His tag in E. coli. This strategy was necessary because the enzyme activity in A. fumigatus cell extracts was close to the detection limit of our spectrophotometric assays. After over-

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FIG. 1. Purification and molecular mass determination for recombinant homocitrate synthase from A. fumigatus. (A) SDS-PAGE analysis of recombinant homocitrate synthase purification. The purified protein shows a molecular subunit mass of 51 kDa. Lane M, molecular mass marker; lane 1, cell extract (25 μg); lane 2, flowthrough (25 μg); lane 3, wash fraction (10 μg); lane 4, elution fraction (2.5 μg). (B) Blue native gel analysis for molecular mass determination for the native protein. Lane M, native molecular mass marker; lane 1, purified recombinant homocitrate synthase. The bands at 66 kDa, 148 kDa, 226 kDa, and 280 kDa indicate monomeric (*), dimeric (**), trimeric (***), and tetrameric (****) subunit compositions. The dimeric and tetrameric structures are most abundant. Note that the molecular mass of the native subunit appears to be larger than that of the denatured subunit.
inhibitory effect was observed in the presence of Cu²⁺. At 60°C, the half-life of the enzyme was 2 min. At 37°C, about 30% of the activity was lost after 270 min. When diluted 5-fold from the stabilization buffer, the enzyme was stable for at least 8 h within a temperature range of 4°C to 7.8 to 8.2 and was most active at temperatures of 50°C to 52°C. 

Homocitrate synthase activity was determined by the condensation of acetyl-CoA with $\alpha$-ketoglutarate. This was tested by two approaches. In one approach, MgCl₂ was added to an assay mixture containing EDTA-inactivated enzyme. In the second approach, we first desalted the EDTA-treated enzyme and incubated it in a buffer containing MgCl₂ or ZnCl₂ prior to the determination of enzymatic activity. None of these procedures restored the activity of the enzyme. Some inhibition of enzymatic activity was also observed in the presence of lysine, but a concentration of 5 mM was required to inhibit activity by 50%, which might not constitute an intracellularly conceivable concentration. The homocitrate isomer methylcitrate had only a minor effect, whereas a stronger inhibitory effect was observed when citrate was added to the assay mixture. Taken together, these data show that homocitrate synthase activity is sensitive toward several divalent cations, but it remained unclear which composition was most abundant and functional under in vivo conditions (1).

Biochemical characterization of recombinant homocitrate synthase. Homocitrate synthase activity was determined by detecting the reduction of DCPIP by CoA released during the condensation of acetyl-CoA with $\alpha$-ketoglutarate. Replacement of DCPIP with 5,5'-dithiobis-2-nitrobenzoate (DTNB) strongly inhibited enzymatic activity.

As stated above, the specific activity of the purified enzyme was 0.8 U mg⁻¹, which leads to a calculated turnover number of 0.68 s⁻¹. The $K_m$ values for the substrates acetyl-CoA and $\alpha$-ketoglutarate were 17 µM and 261 µM, respectively (Table 1). The enzyme exhibited its maximum activity at pH values of 7.8 to 8.2 and was most active at temperatures of 50°C to 52°C. When diluted 5-fold from the stabilization buffer, the enzyme was stable for at least 8 h within a temperature range of 4°C to 37°C. At 42°C, about 30% of the activity was lost after 270 min. At 60°C, the half-life of the enzyme was 2 min.

Homocitrate synthase activity was additionally tested for the requirement of cofactors and for inhibitors, including different metabolites, metal ions, and chelators (Table 2). A strong inhibitory effect was observed in the presence of Cu²⁺, Zn²⁺, EDTA, and phenanthroline, implying that enzymatic activity might depend on metal ions. However, the addition of Mg²⁺ or Ca²⁺ ions neither inhibited nor stimulated enzymatic activity, and Mg²⁺ ions did not restore EDTA-mediated inhibition. This was tested by two approaches. In one approach, MgCl₂ was added to an assay mixture containing EDTA-inactivated enzyme. In the second approach, we first desalted the EDTA-treated enzyme and incubated it in a buffer containing MgCl₂ or ZnCl₂ prior to the determination of enzymatic activity. None of these procedures restored the activity of the enzyme.

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**Deletion and complementation of the hcsA coding region.** To confirm that hcsA encodes an essential enzyme of the $\alpha$-aminoadipate pathway, we aimed for the generation of an hcsA deletion mutant. The A. fumigatus ΔakuBΔhcsA strain contains a deletion in the $\alpha$-keto acid dehydrogenase gene, responsible for the nonhomologous formation of acetyl-CoA and $\alpha$-ketoglutarate. Replace...
end-joining repair mechanism. Therefore, an akuB deletion strain yields an increased frequency of homologous integration in transformation approaches (14). The ΔakuB strain was tested for the ability to cause invasive aspergillosis in a murine model system and was indistinguishable from its parental strain (14). Therefore, the ΔakuB strain was used as the parental strain in our experimental approaches and is subsequently denoted the wild-type strain. In agreement, most transformants were auxotrophic for lysine. Five auxotrophic strains were selected for Southern analysis (Fig. 2A and B), confirming the deletion of the hcsA gene. (C) Southern blot analysis of genomic DNAs of wild-type and hcsA deletion mutant strains and of three independent complemented mutant strains. For detection, a probe directed against the hcsA gene was used. All complemented mutants maintained the hygromycin resistance cassette, as checked by resistance analysis and independent Southern analysis.

Lyssine prototrophy was restored by complementation of the ΔhcsA strain with the hcsA wild-type gene. Transformants which retained hygromycin B resistance but showed lysine prototrophy were selected. This ensured that resulting strains were derived from the transformation of the mutant and not from wild-type contaminations. Southern blot analysis revealed a single-copy integration of the hcsA gene, whereby the size of hybridizing bands was sometimes larger than that of the wild-type ΔakuB strain due to the retention of the hygromycin B resistance cassette (Fig. 2C). The complemented mutant strain chcsA hph 5.16 A1 (in further descriptions denoted the complemented mutant) was selected for comparative analyses.

Importance of de novo lysine biosynthesis for germination of conidia on different carbon sources. When grown on glucose minimal medium, HcsA was essential to support growth in the absence of externally added lysine (Fig. 3A). Interestingly, when lysine was used in the absence of nitrate as the sole nitrogen source, growth of all three strains was much less pronounced than that in the presence of additional nitrate. This indicates that lysine may either represent a bad source of nitrogen or exert toxic effects, as described for S. cerevisiae (62, 67). To address the question of whether proteins, blood, or serum was able to support growth of such a mutant, we inoculated the ΔhcsA strain, the complemented mutant, and the wild type on solid media containing either porcine serum (30% [vol/vol]), sheep blood (50% [vol/vol]), bovine serum albumin (1% [wt/vol]), or milk casein (0.2% [wt/vol]). On serum and blood, the mutant strain showed no altered growth phenotype (Fig. 3A). This indicates that blood contains sufficient available lysine to support germination and growth of a lysine-auxotrophic mutant. Interestingly, the mutant was not able to grow on media containing unhydrolyzed proteins such as bovine serum albumin and casein, although these proteins consist of approximately 10% and 5% lysine, respectively (Fig. 3A), which might be sufficient to restore growth. In agreement, the mutant grew perfectly on acid-hydrolyzed casein (Casamino Acids) (not shown). Therefore, we speculated that resting conidia of an amino acid-auxotrophic mutant might be unable to hydrolyze proteins in sufficient amounts to complement their auxotrophy. This hypothesis was tested in further experiments.

Impact of proteases on lysine supply and growth on explanted lung tissues. We recently presumed that proteins might provide one of the major nutrients for A. fumigatus during lung infection (24). Here we showed that conidia of lysine-auxotrophic mutants were unable to germinate on unhydrolyzed proteins. However, it remained questionable whether the hcsA mutant, once germinated, would be able to utilize proteins as a carbon source. This was tested by pregrowing all strains on either glucose-lysine or peptone medium. The mycelium was harvested, washed, and starved of lysine for 2 h before being transferred to protein-containing media. As shown in Fig. 3B, the mutant regained the ability to grow on protein-containing media. This clearly indicates that mycelium of the ΔhcsA mutant is able to hydrolyze proteins in a sufficient manner to relieve lysine auxotrophy, whereas conidia are not. To assign this growth to active protein hydrolysis, A. fumigatus proteases were partially purified from the growth medium of a wild-type strain. Protease-containing fractions were either plated directly on albumin-containing medium or subjected to heat inactivation prior to plating. Inoculation of the medium with resting conidia showed that the mutant grew on albumin only when active, but not heat-inactivated, proteases were added (Fig. 3C), confirming the requirement of protease activity for germination and growth of the lysine-auxotrophic A. fumigatus strain.

We additionally checked whether the hcsA mutant was able to grow on lung tissues explanted from an immunocompetent mouse and tested the growth in a manner similar to the procedure described for an A. fumigatus hcsA mutant (50).
this purpose, washed pieces of lung tissue were placed on PBS-agar plates, inoculated with the wild type, the ΔhcsA strain, and the complemented mutant, and incubated at 37°C. After 24 h of incubation, none of the strains showed visible growth on the surface. However, as shown in Fig. 3D, all strains covered the surface with a mycelial mat within 48 h, without displaying any obvious differences between the three strains. Therefore, autolysis of lung tissue (which is highly progressed after 48 h) provides sufficient lysine to support germination and growth of the hcsA mutant.

**hcsA expression analysis and localization studies.** To analyze the lysine-dependent expression of hcsA, we performed real-time PCR studies. Additionally, we monitored the fluorescence of a strain carrying a fusion of egfp with hcsA under the control of its natural promoter. For real-time PCR analysis, we compared hcsA expression after growth of wild-type mycelium in the presence or absence of lysine and after shifting of mycelium from medium without lysine to medium with lysine and vice versa, as indicated in Fig. 4. For normalization of data, we selected the actin and β-tubulin genes, which were constitutively expressed under all five conditions. Mycelia grown for 15 h in either the presence or absence of lysine showed similar expression levels. Interestingly, transcript levels were significantly higher when lysine served as the sole nitrogen source. Additionally, a culture shifted for 2 h from lysine-containing medium to a medium without lysine showed no increase in the hcsA transcript level. In contrast, hcsA transcription strongly increased when a culture was shifted from a medium without lysine to lysine-containing medium. Therefore, we concluded that hcsA transcription is not inhibited by lysine rather than induced. This contrasts with results obtained for *S. cerevisiae*, in which the addition of lysine strongly represses the expres-
sion of genes from the α-aminoadipate pathway (7, 20). The constitutive production of homocitrate synthase in A. fumigatus was additionally confirmed by fluorescence microscopy of the strain carrying a fusion of the hcsA gene with the gene coding for EGFP. Strong fluorescence was visible regardless of the presence or absence of lysine in the medium (data not shown). A more detailed analysis of the subcellular localization of the fusion protein revealed fluorescence exclusively from the cytoplasm. As shown in Fig. 5A to E, neither mitochondrial nor nuclear GFP fluorescence was detectable. These results indicate that (i) hcsA is constitutively expressed, with an increase in transcription when cells are grown in the presence of lysine; (ii) HcsA seems to be produced in the presence and absence of lysine; and (iii) the enzyme seems to be located only within the cytoplasm, confirming the absence of a mitochondrial import sequence.

Virulence in a murine infection model of bronchopulmonary aspergillosis and lung histopathology. Previously, an A. fumigatus lysF mutant, which is devoid of the homoaconitase of the α-aminoadipate pathway, was studied in a murine infection model for bronchopulmonary aspergillosis, using mice treated with the cytostatic drug cyclophosphamide. The mutant displayed a strongly attenuated virulence (30), and it was concluded that lysine biosynthesis is essential for fungal virulence. However, no histological analyses were performed to study the fate of conidia, and a model for disseminated aspergillosis was not tested.

To confirm the general importance of lysine biosynthesis in pathogenesis, we performed several additional virulence studies with our ΔhcsA strain. First, instead of using leukopenic mice, we tested virulence in mice immunosuppressed with cortisone acetate. This treatment allows the recruitment of neutrophils and monocytes, which, although phagocytosis is partially impaired, attack fungal cells and prevent rapid dissemination. Mice were infected with 1 × 10⁵ conidia by inhalation, and survival was monitored over a period of 14 days, followed by histological analyses of the lungs. As shown in the survival curves in Fig. 6A, both the wild type and the complemented mutant caused high mortality rates, which were not statistically significantly different by Kaplan-Meyer estimation and log rank tests (9, 24). In contrast, the ΔhcsA mutant was strongly attenuated, with only one mouse succumbing to infection (on day 8 postinfection) (Fig. 6A). Despite the different immunosuppression regimen used here, these results are in agreement with observations on the lysF mutant (30). Histopathology revealed diffuse hemorrhages and massive bronchial and peribronchial immune cell infiltrations (mainly neutrophils) surrounding invasive mycelium in the lungs of

FIG. 4. A. fumigatus homocitrate synthase expression analysis by qRT-PCR. The graph shows the normalized mean values for five independent biological replicates of hcsA expression standardized against transcript levels of actin and β-tubulin. The growth conditions are indicated below each column. Nitrate, 15 h in AMM-glucose with nitrate as the sole nitrogen source; nitrate/lysine, 15 h in AMM-glucose with nitrate and lysine as nitrogen sources; lysine, 15 h in AMM-glucose with lysine as the sole nitrogen source; nitrate/lysine → 2 h shift to nitrate, transfer of mycelium from a medium with nitrate and lysine to a medium with nitrate; nitrate → 2 h shift to nitrate/lysine, transfer of mycelium from a medium with nitrate to a medium with nitrate and lysine. hcsA was constitutively expressed in mycelium grown in the presence or absence of lysine. Shifting the mycelium from a medium without lysine to a medium with lysine significantly induced hcsA expression. P values from paired t test analyses are shown. For details on data normalization and statistical analysis, refer to Materials and Methods.
all mice infected with either the wild type or the complemented mutant (Fig. 7A). In contrast, mice infected with the ΔhcsA mutant showed no or very mild pneumonia without visible mycelium. The only exception in this group was the single mouse that succumbed to infection on day 8. Histopathology of this mouse resembled the findings for mice infected with the wild type and the complemented mutant (Fig. 7A), which tempted us to study the virulence of the ΔhcsA mutant in more detail.

Time-response analysis of inflammation and fungal invasion. To study the fate of ΔhcsA conidia in comparison to those of the wild-type and complemented strains, we performed a time-response study of infection. In this experiment, mice were sacrificed 8 h, 24 h, and 48 h after infection to visualize the time-dependent fungal biomass formation and the accompanying immune response. The infectious dose was increased to 1 × 10⁶ conidia, which allowed detection of fungal cells even at the onset of infection. Additionally, one group of mice infected with the ΔhcsA strain received supplementary lysine via the drinking water to investigate the effect of an elevated lysine pool on virulence of this mutant. Mice infected with the wild type and the complemented mutant, as well as mice with lysine supplementation and infection with the ΔhcsA mutant, developed clinical symptoms indicative of aspergillosis (lethargy and marked dyspnea) 36 to 48 h after infection. At necropsy, the lungs of mice from these groups showed extensive red hepatization and reduced retraction from 24 h postinfection onwards. Additionally, at 48 h postinfection, some of these animals showed signs of lung hemorrhage. In contrast, mice infected with the ΔhcsA mutant without lysine supplementation showed no clinical symptoms and very limited red hepatization 48 h after infection.

Interestingly, already at 8 h postinfection, germinating conidia could be observed in the lungs of mice infected with the wild type, the complemented mutant, and the ΔhcsA mutant with lysine supplementation (Fig. 7B). In these groups,
invasive mycelium and neutrophil infiltration could be observed as early as 24 h postinfection, which is in agreement with observations on a bioluminescent *A. fumigatus* strain (12). The extent of the lesions increased over time. Compared to these findings, the ΔhcsA mutant without lysine supplementation showed severely delayed germination. Swelling but not germination of conidia could be observed at 8 h postinfection, and mycelium was not detectable until 48 h after infection, implying that the free lysine content within the lung tissue upon normal feeding of mice is insufficient to fully complement the lysine auxotrophy of the ΔhcsA strain. Consistent with the delayed development of hyphae, the first immune cell infiltrates were observed 48 h after infection, whereby the severity of lesions did not increase from 48 h to 72 h (data not shown). These results clearly demonstrate that (i) the ΔhcsA strain shows a reduced germination rate within infected tissues, indicating a lysine deficiency at early times of infection; (ii) although strongly delayed, some conidia of the mutant may start to grow within the tissue; and (iii) supplementation of the drinking water of mice with lysine increases the tissue lysine pool, allowing restoration of virulence of the ΔhcsA strain.

**Reconstitution of virulence by addition of lysine to drinking water.** The time-response study implied that lysine supplementation might restore virulence of the ΔhcsA strain. To confirm this hypothesis, we designed an experiment to study the effect of lysine supplementation in animals infected with 1 × 10⁶ conidia of the ΔhcsA mutant. Cohorts of 10 mice were given either drinking water with glucose only, drinking water with the addition of lysine from day −3 to day 1, or supplementation with lysine for the whole duration of the experiment.

Upon infection with 1 × 10⁶ conidia of the ΔhcsA mutant, 9 of 10 mice without lysine supplementation developed clinical symptoms. However, only four animals succumbed to infection, including three on day 3 and one on day 7. Similarly, all mice supplemented with lysine from day −3 to day 1 became clinically ill, and four of them died or were euthanized (two on day 3, one on day 4, and one on day 11). In contrast, eight mice supplemented with lysine for the whole duration died or were euthanized. Three of these succumbed on day 2, three on day 3, one on day 4, and one on day 6 (Fig. 6B). Histopathology revealed more-severe inflammatory lesions in the mice given lysine for the whole duration of the experiment, indicating the larger number of conidia rapidly germinating within the lung tissue (Fig. 8). From this experiment, we concluded that a continuous supply of a high lysine concentration is required to build up a lysine pool in the lung tissue that lasts sufficiently long to allow normal virulence of the mutant. However, even without lysine supplementation, some mice developed invasive aspergillosis due to the increased conidium concentration used in this experiment. Lastly, depending on the lysine content of the drinking water, mice infected with the ΔhcsA strain showed a slight difference in survival rate compared to mice infected with the wild-type strain. The survival curve for mice infected with 1 × 10⁶ conidia of the ΔhcsA strain is shown in panel A of Figure 6, and the survival curves for mice infected with 1 × 10⁵ conidia are shown in panels B and C of Figure 6.
FIG. 7. Histological analyses of mice infected intranasally with either $1 \times 10^5$ (A) or $1 \times 10^6$ (B) conidia. (A) Sections from the survival experiment shown in Fig. 6A. (B) Sections from a time-response study. WT, wild type; ΔhcsA, homocitrate synthase deletion mutant; comp, complemented mutant; PBS, uninfected control mice. (A) Hematoxylin-eosin (HE) and silver staining (silver) of lung sections representative of the respective cohort. For the ΔhcsA mutant, an additional series is shown for the single mouse which died on day 8 (ΔhcsA † d 8). (Top) HE staining. Magnification, ×20. (Middle) Same area with silver staining. (Bottom) Enlargement of the areas indicated with red squares in the middle row. Magnification, ×63. No inflammatory lesions were visible for mice infected with the ΔhcsA mutant and surviving the observation period. Additionally, no fungal cells were detected. The wild type and the complemented mutant caused severe inflammation with massive fungal burdens. The single mouse infected with the ΔhcsA mutant showed mild inflammation and some foci with fungal cells. (B) Lung tissue sections from mice euthanized 8, 24, and 48 h after infection with $1 \times 10^6$ conidia of the respective strains. For the ΔhcsA mutant, mice were either fed 100 mM lysine via the drinking water or kept without lysine addition. The wild type and the complemented mutant, as well as the ΔhcsA mutant with lysine supplementation, showed rapid germination and mycelium formation in the tissues. For the mutant, only a few germinated conidia were detected.
the diet (that is, supplementation via the drinking water for the whole duration of the experiment), the tissue lysine pool can indeed increase to a level allowing the development of severe invasive aspergillosis by the \textit{hcsA} mutant.

**Virulence of homocitrate synthase mutants after intravenous infection.** Our infection studies showed that a homocitrate synthase mutant possesses the general ability to cause bronchopulmonary aspergillosis if sufficient free lysine is supplied. In addition, our \textit{in vitro} growth experiments revealed that once mycelium is formed, the mutant is able to acquire lysine from proteins by secreting proteases to the surrounding medium. Furthermore, growth of the mutant on agar plates containing whole blood or serum as the sole nutrient source was indistinguishable from that of the wild type (Fig. 3A). Therefore, we assumed that a homocitrate synthase mutant might display full virulence when tested in a murine model of disseminated aspergillosis. By injecting conidia directly into the bloodstream, we expected a sufficient lysine supply for rapid swelling and germination of \textit{hcsA} conidia and expected that hyphae, once entering the tissue of inner organs, might produce proteases to satisfy their further need for lysine by protein degradation. To test this assumption, mice were infected with $1 \times 10^5$ conidia via the tail vein, and survival was monitored over a period of 8 days. As shown in the survival curves in Fig. 6C, not only the wild type and the complemented mutant but also the homocitrate synthase mutant caused high mortality rates, and comparison of the survival curves revealed no significant differences between the three strains. Histological analyses of liver and kidneys additionally showed similar developmental stages for all strains, indicating that germination and growth of the mutant were not delayed (see Fig. S3 in the supplemental material). Therefore, it can be concluded that the \textit{de novo} synthesis of lysine is dispensable during disseminated infection, because blood contains sufficient amounts of lysine to support growth of the deletion mutant.

**DISCUSSION**

In this study, we identified and biochemically characterized the homocitrate synthase HcsA from \textit{A. fumigatus} and confirmed that the enzyme is an essential component of the fungal \textit{\alpha}-aminoadipate pathway for lysine biosynthesis. An \textit{hcsA} deletion mutant requires lysine for growth, but supplementation completely restores the growth defect of the mutant. Therefore, HcsA causes the same growth defect as that observed for homoaconitase deletions from \textit{Aspergillus} strains (30, 61), confirming that the \textit{\alpha}-aminoadipate pathway is the only biosynthetic path for lysine biosynthesis in \textit{Aspergillus}.

Although homocitrate synthases have previously been characterized from \textit{S. cerevisiae} and \textit{P. chrysogenum}, several differences concerning the existence of isoenzymes, subcellular localization, expression, and biochemical properties were observed. In contrast to \textit{A. fumigatus} and \textit{P. chrysogenum}, the yeast \textit{S. cerevisiae} contains two homocitrate synthase isoenzymes, called Lys20p and Lys21p, which share 89% sequence identity. Lys21p contributes mainly to homocitrate synthesis...
during respiratory growth on ethanol (47), but under fermentative conditions, both enzymes serve for homocitrinate synthesis (47, 49). In addition, the activity of Lys21p is strongly inhibited by lysine, and that of Lys20p is only weakly inhibited (20, 47). Several independent studies have shown that both enzymes localize to the nucleus (13, 20), although the conversion of homocitrinate to α-ketoacidopate was assumed to take place within the mitochondria (8). Therefore, the mechanism of substrate and product shuttling between compartments remains unclear, but a nuclear localization allows the enzymes to act as transcriptional regulators in response to the intracellular lysine concentration. This is in agreement with a reduction of homocitrinate synthase gene expression in the presence of high intracellular lysine concentrations. Therefore, lysine counteracts the activating function of α-aminoacidopate semialdehyde, which is the inducer of the transcriptional activator Lys14p in S. cerevisiae (19, 20). Lys14p is a GAL4-like Zn$_2$Cys$_6$ transcription factor, and although transcription factors of this type are also present in A. fumigatus, BLAST searches against its genome revealed no Lys14p homologue, implying different regulatory mechanisms between these fungi. This was confirmed by qRT-PCR analyses of cells cultivated in the presence or absence of lysine. Transcript levels did not decline in the presence of lysine, but rather increased, implying that there is no direct lysine-dependent feedback inhibition in A. fumigatus. Whether the intron detected in the 5′-untranslated region of A. fumigatus hcsA additionally contributes to gene expression needs a more detailed analysis. Preliminary investigations on promoter-lacZ fusions showed no significant differences in gene expression between promoters with the intron always removed and those with the intron always maintained (data not shown). However, an explanation for the increased hcsA transcript levels in the presence of lysine as the sole nitrogen source and after 2 h of exposure to lysine after a shift from a medium without lysine might derive from lysine degradation via the α-aminoacidopate pathway. Our growth analysis (Fig. 3) showed that biomass formation was less pronounced when lysine was given as the sole nitrogen source than that in a medium containing lysine and nitrate or with nitrate as the sole nitrogen source. This indicates that lysine is not a preferred nitrogen source for A. fumigatus, which might be due either to a low degradation rate or to the accumulation of toxic intermediates, as shown for S. cerevisiae (62, 67). Culture supernatants of Schizosaccharomyces pombe, C. albicans, and Filobasidiella neoformans showed an accumulation of α-ketoacidopate in the growth medium when the organisms were cultivated on lysine as the sole nitrogen source (65). This indicates that the reactions of the α-aminoacidopate pathway leading from α-ketoacidopate to lysine (see Fig. S1 in the supplemental material) are completely reversible and may be used for lysine degradation. Assuming that in A. fumigatus, which is not a penicillin producer, all enzymes of the α-aminoacidopate pathway are placed under the same transcriptional control, the upregulation of hcsA expression in the presence of lysine may result from an increased expression of the complete pathway during lysine degradation.

In contrast to the lack of negative feedback regulation in A. fumigatus, some feedback inhibition of the lysI homocitrinate synthase gene from the penicillin producer P. chrysogenum has been described (35), although this inhibition seemed only slightly dependent on the ability to produce large amounts of penicillin (2, 3). Therefore, S. cerevisiae, A. fumigatus, and P. chrysogenum developed different sensitivities to the regulation of homocitrinate synthase expression in response to extra- and intracellular lysine.

In contrast to the nuclear localization of S. cerevisiae homocitrinate synthases, subcellular fractionation performed on P. chrysogenum showed that the main homocitrinate synthase activity derived from the cytoplasm and that only a minor proportion of enzymatic activity was present within mitochondria (approximately 25% of total activity) (26). Further analysis of a GFP-tagged homocitrinate synthase showed fluorescence only in the cytoplasm (4), and no indications were given for a mitochondrial or nuclear localization. These results are in perfect agreement with our observations on A. fumigatus, because a GFP fusion strain revealed an exclusively cytoplasmic localization. Therefore, a direct role of homocitrinate synthases from filamentous fungi in regulation of gene expression seems unlikely.

Another difference between homocitrinate synthases was derived from the comparison of inhibition of enzymatic activity mediated by lysine. As described above, different sensitivities were observed for the two S. cerevisiae enzymes. The P. chrysogenum enzyme also seems to be inhibited directly by lysine, but in a strain-dependent manner. One strain tested showed 50% inhibition at 50 μM lysine, whereas the enzyme from another strain required approximately 500 μM lysine (26). However, the A. fumigatus enzyme was at least 10 times less sensitive to lysine inhibition, since at least 5 mM lysine was required to inhibit the enzymatic activity by 50%. Therefore, it appears unlikely that lysine has a direct regulatory effect on A. fumigatus homocitrinate synthase activity under in vivo conditions.

Concerning other biochemical properties, the A. fumigatus HcsA protein behaved like a mixture of the P. chrysogenum and S. cerevisiae enzymes. Although for P. chrysogenum a Mg$^{2+}$ dependency was first described by activity determinations in cell extracts (37), this was not confirmed by investigations on the purified enzyme, which revealed 30% inhibition at 10 mM MgSO$_4$ (26). In agreement, we did not find a stimulatory effect of any bivalent metal ion on the A. fumigatus enzyme. Although the addition of EDTA had an irreversibly inactivating effect (97% inhibition at 5 mM), activity could not be restored by the addition of divalent cations, implying that effects other than simple metal chelation caused EDTA inhibition. Interestingly, the EDTA sensitivity of the P. chrysogenum enzyme was much less pronounced, and the enzyme showed only 30% inhibition at 5 mM EDTA (26). Although S. cerevisiae homocitrinate synthase is also Mg independent, it has been assumed to be a Zn-containing metalloenzyme, and the Zn$^{2+}$ might be involved directly in catalysis (46). Since A. fumigatus HcsA was 25% inhibited in the presence of 1 μM ZnSO$_4$, a Zn$^{2+}$ requirement of A. fumigatus HcsA appears unlikely. Furthermore, the yeast enzyme was also strongly sensitive to inhibition by the metal chelators 2,2′-dipyridyl and 1,10-phenanthroline (>80% inhibition at 0.01 mM) (22), whereas these chelators only slightly affected the A. fumigatus enzyme.

Concerning substrate affinity, Lys20p from S. cerevisiae showed $K_m$ values for acetyl-CoA and α-ketoglutarate of 42 μM and 140 μM, respectively (46), which are similar to the values determined for A. fumigatus HcsA (acetyl-CoA $K_m$ = 17...
µM: α-ketoglutarate $K_m$ = 261 µM). In contrast, the $K_m$ value for acetyl-CoA of 41 µM for the P. chrysogenum enzyme was similar to those for S. cerevisiae and A. fumigatus, but the $K_m$ value for α-ketoglutarate was significantly higher (2.2 mM) (26). Therefore, concerning the regulation of expression and biochemical properties, we concluded that (i) the homocitrate synthases from filamentous fungi seem to be located in the cytoplasm, not in the nucleus; (ii) lysine causes only low-level or no feedback inhibition in filamentous fungi; and (iii) all of the enzymes are independent of Mg$^{2+}$ ions, and only the yeast enzyme seems to be Zn dependent.

Another major focus of this work was the reevaluation of lysine biosynthesis as a suitable antifungal drug target. Former investigations of a homoaconitase mutant of A. fumigatus in a neutropenic murine infection model implied an essential contribution of the α-amino adipate pathway to virulence (27). Studies of other fungi confirmed this assumption. Aspergillus nidulans mutants defective in the lysA gene, coding for the saccharopine dehydrogenase, showed attenuated virulence in murine models of bronchopulmonary aspergillosis. These mutants additionally showed a growth defect when incubated in human or murine serum. This implied that lysine was not sufficiently available within the bloodstream to support growth of lysine-auxotrophic A. nidulans mutants (59). In agreement, a lysine-auxotrophic mutant of C. albicans was strongly attenuated in virulence after intravenous infection of mice (54). However, a more recent investigation of an S. cerevisiae lys9 mutant, also defective in saccharopine dehydrogenase, showed no reduced survival rate after intravenous infection. This indicates that at least some microorganisms seem to find sufficient lysine within the bloodstream to overcome their auxotrophy (27). To either confirm or reject the assumption of an essential contribution of lysine biosynthesis to fungal virulence, we extended this investigation by (i) using a corticosteroid-based model, (ii) adding excess lysine to the drinking water of mice, (iii) performing time-response studies to follow the fate of inhaled conidia, and (iv) applying an intravenous infection to follow the importance of the de novo synthesis of lysine during disseminated infection. Additionally, we correlated the in vitro studies with several in vivo growth experiments.

The idea for using a corticosteroid-based immunosuppression regimen rather than a neutropenic model derived from different considerations. Both neutropenic patients and patients treated with high doses of corticosteroids belong to the high-risk group for acquiring invasive fungal infection (38, 55). Therefore, the corticosteroid-based immunosuppression regimen and a cyclophosphamide-based model have been well established for the study of A. fumigatus virulence. Comparative virulence studies showed that attenuation in virulence may be observed in one but not the other model system (28, 58), and therefore the use of both model systems seems justified. Previous virulence studies on an A. fumigatus homoaconitase (lysF) mutant revealed a completely avirulent phenotype in a neutropenic infection model (30). However, neutrophils recruited to the site of infection are assumed to play a major role in combating A. fumigatus infections (39). Neutrophils produce strong oxidative bursts and release proteolytic enzymes during degranulation (17). These activities not only contribute to killing of pathogens but also may cause severe tissue damage. This tissue destruction can release nutrients that feed A. fumigatus conidia during germination. Therefore, differences in the immunosuppression regimen might alter the outcome of bronchopulmonary aspergillosis when investigating metabolic mutants.

Our results showed that an A. fumigatus hcsA mutant was clearly attenuated in virulence in a corticosteroid-based murine infection model of bronchopulmonary aspergillosis under normal feeding conditions. The supply of excess lysine via the drinking water at least partially restored virulence, implying that lysine auxotrophy is most likely the only factor that attenuates the virulence of this mutant. However, the attenuation in virulence of the hcsA mutant without lysine supplementation appeared less pronounced than the attenuation observed for the lysF mutant in the neutropenic infection model. This difference may indeed be due to the proteolytic activity of degranulating neutrophils, which might release lysine from surrounding proteins and enhance the germination rate of lysine-auxotrophic conidia. This difference between the corticosteroid and cyclophosphamide immunosuppression regimens is also in agreement with our in vitro studies. These studies showed that conidia of lysine-auxotrophic mutants are unable to produce sufficient proteases to release lysine from proteins, whereas the mycelium of such a mutant, once germinated, is able to overcome its auxotrophy in the presence of proteins. Externally added proteases, which may derive from neutrophils under the corticosteroid regimen, allowed germination of mutant conidia in the presence of proteins as the sole nutrient source. These results are in agreement with the lethal infection of some mice infected intranasally with mutant conidia (one mouse infected with $1 \times 10^5$ conidia and four mice infected with $1 \times 10^6$ conidia) (Fig. 6A and B). Death of these mice generally occurred later than that of mice infected with either the wild type or the mutant with lysine supplementation and might be explained by the delayed germination and decreased growth speed of the mutant. Therefore, blocking lysine biosynthesis strongly delays but does not definitely prevent the development of invasive bronchopulmonary aspergillosis after corticosteroid treatment. Furthermore, a homocitrate synthase mutant was not attenuated in a murine model of disseminated aspergillosis. In contrast to the results for the lysA mutant of A. nidulans described above (59), our in vitro growth analyses with porcine serum and sheep blood indicated that lysine is sufficiently available within the bloodstream of mammals to support growth of the A. fumigatus hcsA mutant and that the ∆hcsA strain retained full virulence when tested in an intravenous infection model.

From these studies, we conclude that the de novo synthesis of lysine has a strong impact on the development of bronchopulmonary aspergillosis but not on disseminated aspergillosis. Antifungals targeting lysine biosynthesis may therefore appear attractive only as prophylactic drugs to prevent the acquisition of invasive bronchopulmonary aspergillosis in high-risk patients. Once an infection has been established, the need for de novo synthesis of lysine is strongly reduced and such drugs are assumed to be ineffective. Additionally, our studies show that it is rather difficult to identify metabolic pathways which appear suitable for the development of new antifungals. Therefore, further investigations will be required to understand the metabolic processes essential for a broad spectrum of pathogenic fungi to cause and establish infection.
sential contribution of divalent metal ions in catalysis of the EDTA treatment, because dialysis or the addition of different thase showed that the enzyme is irreversibly inhibited by Okada, T. Tomita, A. P. Wulandari, T. Kuzuyama, and M. R. C. Trievel, J. Biol. Chem., 284: 35769–35780, 2009; S. L. Bulfer, E. M. Scott, L. Pillus, and R. C. Trievel, J. Biol. Chem., 285: 70446–70453, 2010; and T. Okada, T. Tomita, A. P. Wulandari, T. Kuzuyama, and M. Nishiyama, J. Biol. Chem. 285: 4195–4205, 2010) show an essential contribution of diveral metal ions in catalysis of the mixed aldol Claisen condensation during homocitrinate synthesis. Our investigations on the A. fumigatus homocitrate synthase showed that the enzyme is irreversibly inhibited by EDTA treatment, because dialysis or the addition of different divalent cations, such as Mg$$^{2+}$$, Cu$$^{2+}$$, or Zn$$^{2+}$$, did not restore enzymatic activity. In contrast, EDTA-treated homocitrate synthase from T. thermophilus was inactive, but addition of Mg$$^{2+}$$, Mn$$^{2+}$$, or Co$$^{2+}$$ reactivated the enzyme (Okada et al., 2010). Although EDTA-treated S. pombe homocitrate synthase was not tested for its specific activity, purification via a Zn(II)-loaded metal chelate column led to an active enzyme with a Zn(II) content of 87% ± 5%. In agreement with this result, analyses of the crystal structure showed that the divalent metal ion is coordinated by a glutamate and two histidine residues, which seem conserved in all homocitrate synthases (Bulfer et al., 2009). This metal ion additionally interacts with the C1 carboxylate and the C2-oxo carbonyl group of 2-oxoglutarate and a water molecule, which is essential for the hydrolysis of the thioester bond of homocitryl-coenzyme A in the proposed reaction mechanism (Bulfer et al., 2009). Furthermore, analyses on the crystal structures of the T. thermophilus and S. pombe homocitrate synthases in the presence of the competitive inhibitor lysine showed that the divalent metal ion can also interact with the α-amine and the carboxylic groups of lysine, leading to a competitive inhibition with respect to 2-oxoglutarate binding (Bulfer et al., 2010; Okada et al., 2010). Although lysine was only an inhibitor of A. fumigatus homocitrate synthase when added in millimolar concentrations, the conservation of the active-site residues among homocitrate synthases from different species implies that divalent metal ions may play a general essential role in the homocitrate synthesis reaction (Bulfer et al., 2009). However, removal of this metal ion in A. fumigatus seems to be accompanied by an irreversible denaturation of the enzyme, which prohibits the investigation of the exact metal ion contributing to A. fumigatus homocitrate synthase activity.
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