EstB-Mediated Hydrolysis of the Siderophore Triacetylfusarinine C Optimizes Iron Uptake of *Aspergillus fumigatus*\(^\text{\textregistered}\)

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*Aspergillus fumigatus* excretes the fusaramine-type siderophore desferri-triacetylfusarinine C (DF-TafC) to mobilize iron. DF-TafC is a cyclic peptide consisting of three \(N^5\)-cis-anhydromevalonyl-\(N^2\)-hydroxy-\(N^2\)-acetyl-L-ornithine residues linked by ester bonds; these linkages are in contrast to peptide linkages found for ferrichrome-type siderophores. Subsequent to the binding of iron and uptake, triacetylfusarinine C (TafC) is hydrolyzed, the cleavage products are excreted, and the iron is transferred to the metabolism or to the intracellular siderophore desferri-ferricrocin (DF-FC) for iron storage. Here we report the identification and characterization of the TafC esterase EstB, the first eukaryotic siderophore-degrading enzyme to be characterized at the molecular level. The encoding gene, *estB*, was found to be located in an iron-regulated gene cluster, indicating a role in iron metabolism. Deletion of *estB* in *A. fumigatus* eliminated TafC esterase activity of cellular extracts and caused increased intracellular accumulation of TafC and TafC hydrolysis products in *vivo*. *Escherichia coli*-expressed EstB displayed specific TafC esterase activity but did not hydrolyze fusaramine C, which has the same core structure as TafC but lacks three \(N^2\)-acetyl residues. Localization of EstB via enhanced green fluorescent protein tagging suggested that TafC hydrolysis takes place in the cytoplasm. EstB abrogation reduced the intracellular transfer rate of iron from TafC to DF-FC and delayed iron sensing. Furthermore, EstB deficiency caused a decreased radial growth rate under iron-depleted but not iron-replete conditions. Taken together, these data suggest that EstB-mediated TafC hydrolysis optimizes but is not essential for TafC-mediated iron uptake in *A. fumigatus*.

*Aspergillus fumigatus* is a ubiquitous saprobic fungus, normally associated with decaying organic matter (3, 13). In addition, *A. fumigatus* has become the most important airborne fungal pathogen, causing life-threatening disease, particularly in immunocompromised patients (28).

The ability to acquire iron is essential for all fungi during both saprobic and pathogenic growth. While iron is one of the most abundant metals on earth, in aerobic environments it is present mostly as nearly insoluble compounds such as oxhydroxide polymers. Consequently, the concentration of ferric iron in solution at neutral pH is probably not greater than \(10^{-18}\) M (14). However, an excess of iron within cells can be deleterious, because of its potential to catalyze the generation of cell-damaging reactive oxygen species. Therefore, microbes have developed various highly regulated systems for the uptake and storage of iron (7, 23). *A. fumigatus* employs two high-affinity iron uptake systems, reductive iron assimilation and siderophore-assisted iron mobilization (27). Siderophores are low-molecular-mass ferric iron-specific chelators, and most fungi also utilize siderophores for intracellular iron storage (7). Recently, the siderophore system was shown to be essential for the virulence of *A. fumigatus* in a mouse model of invasive aspergillosis (27) as well as for the virulence of the plant pathogens *Cochliobolus heterosporus*, *Cochliobolus miyabeanus*, *Fusarium graminearum*, and *Alternaria brassicicola* on different hosts (17). These findings sparked new interest in this poorly characterized fungal iron homeostasis-maintaining system.

*A. fumigatus* produces two major hydroxamate-type siderophores: the fusaramine desferri-triacetylfusarinine C (DF-TafC) is excreted to mobilize extracellular iron, and the ferrichrome desferri-ferricrocin (DF-FC) is employed for intracellular iron storage (4, 5, 7, 16, 27). Production of both siderophores is up-regulated by iron starvation, and the *A. fumigatus* siderophore production resembles that of *Aspergillus nidulans* (16, 27). TafC is a cyclic tripeptide consisting of three \(N^5\)-cis-anhydromevalonyl-\(N^2\)-hydroxy-\(N^2\)-acetyl-L-ornithine residues linked by ester bonds; these linkages are in contrast to peptide linkages found for ferrichromes such as DF-FC (Fig. 1). DF-FC is a cyclic hexapeptide with the structure Gly-Ser-Gly-(\(N^2\)-acetyl-\(N^2\)-hydroxy-L-ornithine), (7). Subsequent to the binding of iron and its uptake by specific ferric siderophore transporters (7, 19, 30), the ester bonds of triacetylfusarinine C (TafC) are hydrolyzed, the cleavage products (fusaramines) are excreted, and the iron is transferred to the metabolism or to the intracellular siderophore DF-FC for iron storage (Fig. 1). Hydrolysis of cyclic fusaramines decreases the affinity of the siderophore to iron and was therefore proposed to be required for intracellular iron release (6). Corresponding enzyme activities have been identified for *Fusarium roseum*, *Penicillium chrysogenum*, *Mycelia sterilia* EP-76, and *A. nidulans* (1, 6, 16). Production of the hydrolyzing esterases was found to be repressed by iron, but the respective enzymes have not been further characterized so far.

Release of iron by intracellular degradation of the chelating
siderophore hydrolysis by A. fumigatus. EstB also has been shown to be involved in the hydrolysis of other siderophores such as enterobactin and salmochelin, which are produced by enteric bacteria such as Escherichia coli and Salmonella enterica. In this study, we report the molecular analysis of the TafC esterase EstB and its role in the siderophore system of A. fumigatus.

Materials and Methods

**Growth conditions.** Generally, A. fumigatus strains (Table 1) were grown at 37°C in Aspergillus minimal medium (AMM) according to the work of Pontecorvo et al. (20); AMM contained 1% glucose as the carbon source, 20 mM glutamine as the nitrogen source, and 10 μM FeSO₄ (÷ Fe). For iron-depleted conditions (÷ Fe), iron was omitted. Shake flask cultures were performed with 200 ml of medium in 1.0-liter Erlenmeyer flasks inoculated with 10⁸ conidia.

**Analysis of siderophore production.** Siderophores were isolated from A. fumigatus as described previously (16). Analysis of siderophore synthesis was performed by high-performance liquid chromatography (HPLC) analysis according to the work of Konetschny-Rapp et al. (9) as described previously (16).

**Northern analysis.** RNA was isolated using TRI reagent (Sigma-Aldrich). Ten micrograms of total RNA was electrophoresed on 1.2% agarose-2.2 M formaldehyde gels and blotted onto Hybond N membranes (Amersham Biosciences). The hybridization probes used in this study were generated by PCR using the oligonucleotides oAfmirB1me and oAfmirB2me for Afu3g03640, oAfAt1me and oAfAt2me for Afu3g03650, oEstAf1 and oEstAf2 for Afu3g03660 (estB), oAbc4-1me and oAbc4-2me for Afu3g03670, and oTubum1 and oTubum2 for Afu3g1940 (tubA, encoding β-tubulin). All oligonucleotides used in this study are listed in Table 2.

**Generation of A. fumigatus mutant strains.** A schematic presentation of the generation of the A. fumigatus mutant strains used in this study is given in Fig. 2.

**For the inactivation of estB**, a 3.9-kb fragment containing A. fumigatus estB was amplified from genomic DNA by PCR using primers oEstAf3 and oEstAf4. After cleavage with HindIII and SacI, the amplification product was inserted into the respective restriction enzyme site of the plasmid pBluescript-KS (Stratagene), yielding vector pEstB. An internal 0.35-kb NotI-Stul fragment was replaced by a 2.4-kb NotI-EcoRV fragment from pHpy (a 2.3-kb AvrII-XbaI fragment carrying hph from plasmid pAN7-1 subcloned into the compatible restriction enzyme site Spel from pBluescript), carrying the hygromycin B resistance marker gene hph (22). A gel-purified 5.8-kb SacI-HindIII fragment was used to transform the A. fumigatus wild type (WT) (Fig. 2A). Hygromycin-resistant estB-negative transformants (ΔestB/WT) strain were used for further analysis.

**For the reconstitution of the ΔestB strain with a functional estB copy**, a 3.8-kb AvrII-ChaI fragment of pEstB was subcloned into the compatible restriction enzyme sites XbaI and NalI of plasmid pAN7-1 carrying the phleomycin resistance marker gene ble (21). The resulting 9.7-kb plasmid pEstphleo was linearized with BamHI and used to transform A. fumigatus ΔestB protoplasts. Phleomycin-resistant transformants with homologous reconstitution of the estB gene (estB⁺ strain) were selected by PCR (data not shown) and Southern blot analysis. The hybridization probe for Southern blot analysis of ΔestB and estB⁺ strains was generated by PCR using the oligonucleotides oEstAf1 and oEstAf2 (Fig. 2B and Table 2).

**In order to subcellularly localize EstB, an in-frame estB-egfp (enhanced green fluorescence protein-encoding gene) fusion was generated.** Therefore, a 2.2-kb Smal-SacI egfp-encoding fragment was subcloned from plasmid pUCGH (10) into the compatible EcoRV-SacI sites of plasmid pGEM-5zf (Promega), yielding plasmid pGfp. The 5’-flanking region and the open reading frame of estB were PCR amplified using oligonucleotides oEstAf9 and oEstAf11 (including a restriction enzyme site for NcoI, which replaces the stop codon). After cleavage with Spel and NcoI, the amplification product was inserted into the respective restriction enzyme sites of pGfp, resulting in plasmid pEstBgfp. A 2.5-kb BssHII fragment carrying hph from pHpy was cloned into the compatible restriction enzyme site MluI from pEstBgfp, resulting in plasmid pEstBhphph. In a final step, the 5’-flanking region of estB was PCR amplified using oligonucleotides oEstAf12 and oEstAf13. After cleavage with HindIII and KpnI, the amplification product was inserted into the respective restriction enzyme sites of pBLGbhph. A gel-purified 9-kb DraI-KpnI fragment was used to transform A. fumigatus WT. Hygromycin-resistant transformants containing an in-frame fusion of the estB and EGFP-encoding genes (estB⁺ hph) strain were selected and used for the subcellular localization of EstB. The hybridization probe for Southern blot screening of the estB⁺ hph strain was generated by PCR using the oligonucleotides oEstAf1 and oEstAf11 (Fig. 2C and Table 2).

**TABLE 1. A. fumigatus strains used in this study**

| Strain | Genotype | Source |
|--------|----------|--------|
| WT | ATCC 46645 | American Type Culture Collection |
| ΔestB | ATCC 46645; estB::hph | This work |
| estBΔ | ΔestB; ΔestB::estB ble | This work |
| estBΔH | ATCC 46645; estB::estB-egfp hph | This work |

**FIG. 1. Siderophore metabolism in A. fumigatus (A) including structures of TafC, fusaricine C and FC (B).** For TafC, R is acetyl; for fusaricine C, R is H.
Transformation of *A. fumigatus* was carried out as described by Tilburn et al. (29). In order to obtain homokaryotic transformants, colonies from single homokaryotic spores were picked and single genomic integration was confirmed by PCR (data not shown) and Southern blot analysis (Fig. 2D). Plasmids were propagated in *E. coli* DH5α/H9251 (Life Technologies). Standard molecular techniques were performed as described by Sambrook et al. (26). *A. fumigatus* DNA was isolated as described previously (32).

Fluorescence microscopy. *A. fumigatus* strains (10⁵ conidia) were grown on eight-well chambered cover glasses in 200 μl of AMM with (+Fe) or without (−Fe) iron for 15 h. The intracellular localization of the expressed fusion protein was observed using a Leica DM6000 microscope.

### TABLE 2. Oligonucleotides used in this study

| Gene   | Oligonucleotide | Sequence (5’–3’) | Gene reference |
|--------|-----------------|------------------|----------------|
| Afu3g03640 | oAfmirB1me     | AAGCCGAGAAAAAGGGGG | 15             |
| Afu3g03650 | oAfmirB2me     | AACCAGATGGAAGCCAG |                |
| Afu3g03670 | oAfAt1me       | ACAATTCAAGGCCTAGCCC |                |
| Afu5g1940  | oAbc4–1me      | ACTGCGAGCTCTTTGCGTCG |                |
| Afu5g1940  | oAbc4–2me      | CCAGCCACTAATGAGATG |                |
| Afu5g1940  | oTubfum1       | ATATGTTCCTGGCACTTC |                |
| Afu3g03660 | oTubfum2       | CTTTACGGAAATGAGCA |                |
| Afu5g1940  | oEstAf1        | CCACCACCATGGGGACAGCG | 15            |
| Afu5g1940  | oEstAf2        | GGTGGAGATCTCCCCGACTGGGAATG | 2 |
| Afu5g1940  | oEstAf3        | CAACCACCTGGCCCTTC |                |
| Afu5g1940  | oEstAf4        | GTGCAAGGCGCTGCCC |                |
| Afu5g1940  | oEstAf9        | CAAGATGGAGCAGCCTG |                |
| Afu5g1940  | oEstAf11       | GTGACCATTGCCCGCACTGG | 1 |
| Afu5g1940  | oEstAf12       | CCATGGAGCTTGACTCCACCCGGTTTT | 4 |
| Afu5g1940  | oEstAf13       | GGTGGACACAGCGGAAAGAGGCCTG | 5 |

* Introduced restriction enzyme sites are underlined as follows: 1, NcoI; 2, BglII; 3, SacI; 4, HindIII; 5, KpnI.

*FIG. 2.* Generation of the *A. fumigatus* *ΔestB* (A), *estB*<sup>R</sup> (B), and *estB*<sup>fpo</sup> (C) strains and Southern blot analysis (D). The solid box represents the *estB* gene. *hph*, *ble*, and *egfp* are the hygromycin B resistance gene, the phleomycin resistance gene, and the *egfp* gene, respectively. The arrows indicate the transcriptional orientation, and the dashed crosses show regions involved in homologous recombination. The dashed line represents the plasmid backbone. Restriction sites used for cloning and Southern blot analysis are indicated as follows: AvrII, A; BamHI, B; BssHII, Bs; ClaI, C; HindIII, H; KpnI, K; MluI, M; NarI, N; NcoI, Nc; NotI, N; SacI, S; SpeI, Sp; StuI, St; and XbaI, X. The predicted fragments resulting from the Southern blot analysis are marked by double arrows. Cloning and Southern blot analyses were performed as described in Materials and Methods. (A) The *ΔestB* deletion resulted from homologous recombination (double crossover) between the WT *estB* locus and the deletion construct containing a replacement of part of *estB* by *hph*. (B) The predicted *estB*<sup>R</sup> allele resulted from homologous recombination (single crossover) between plasmid pEstphleo (linearized at the BamHI site) and the *ΔestB* mutant allele. (C) The *estB*<sup>fpo</sup> allele resulted from homologous recombination (double crossover) between the WT *estB* locus and the fragment containing the in-frame *estB*-*egfp* fusion and the *hph* gene. (D) Southern blot analysis of BamHI- or NcoI-digested genomic DNA of WT (lanes 3 and 5), *ΔestB* (lane 2), *estB*<sup>R</sup> (lane 1), and *estB*<sup>fpo</sup> (lane 4) strains.
in the fungal hyphae was analyzed by light and fluorescent confocal microscopy performed with a microlens-enhanced Nipkow disk-based confocal system UltraVIEW RS (Perkin Elmer) mounted on an Olympus IX-70 inverse microscope (Olympus). EGFP fluorescence was visualized using 488/568-nm excitation/emission. Images were acquired using the UltraVIEW RS software.

Production and purification of recombinant *A. fumigatus* EstB in *E. coli*. The coding sequence of *estB* from fungal cDNA was amplified using oligonucleotides oEstAf1 and oEstAf2. After cleavage with NcoI and BglII, the resulting fragment was inserted into the respective restriction enzyme sites of pQE-60 (QIAGEN). Subsequently, *E. coli* M15 cells were transformed with the generated recombinant expression construct. Induction with isopropyl-β-D-thiogalactopyranoside led to the expression of the recombinant EstB (rEstB) with a six-histidine affinity tag at the C terminus, allowing purification of the protein via Talon metal resin affinity chromatography using a 15 to 50 mM imidazole gradient for elution. Eluted proteins were dialyzed against 10 mM Tris, pH 8.0, and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Coomassie brilliant blue staining.

**TafC esterase assay.** A fusarinine esterase enzyme activity assay based on the fact that EDTA cannot dissociate ferric iron from trihydroxamic acids but can effectively compete with monohydroxamic acids and dihydroxamic acids has been described by Emery (6). We established a new assay which is faster and more reliable: due to the difference in hydrophobicities, TafC and its degradation products can be separated by chloroform extraction (Fig. 3A). *A. fumigatus* cellular extracts or *E. coli*-expressed rEstB was incubated with 0.3 mM TafC or DF-TafC, respectively, in 0.1 M Na-phosphate buffer, pH 6.5, in a total volume of 1 ml at 37°C. After incubation for the indicated periods of time, the assay solution was extracted with 0.5 ml chloroform to separate TafC (lower chloroform phase) and its degradation products (upper aqueous phase), which are both orange colored in the presence of iron. The absorption at 440 nm (A440) of the aqueous and chloroform phases from panel A. (C) Representative reversed-phase HPLC analysis of TafC and its degradation products (dTafC) in the chloroform and aqueous phases subsequent to extraction. To generate dTafC, TafC was partially digested by incubation for 10 min with 0.05 mg cellular extract of WT grown under iron-depleted conditions. The marked peaks are TafC (peak 2) and TafC degradation products (peaks 3, 4, and 5). The absorption at 220 nm is given in milliabsorption units (mAU). (D) A 1.0-μg portion of the purified rEstB protein was subjected to 16% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and stained with Coomassie blue. The molecular masses of marker proteins are indicated. The predicted molecular mass of rEstB is 32.7 kDa. The slightly increased molecular mass is presumably due to the attached six-histidine affinity tag.

**RESULTS**

**Molecular characterization of estB.** In filamentous fungi, genes encoding proteins which are involved in a common metabolic pathway tend to be genomically clustered. The genome sequence of *A. fumigatus* has recently been released (15), and screening of the genome for putative siderophore transporter-encoding genes combined with expression analysis of adjacent genes identified an iron-regulated gene cluster, which is localized on chromosome III (http://www.cadre.man.ac.uk) and potentially encodes a siderophore transporter (Afu3g03640), an acetyltrans-
ferase (Afu3g03650), an esterase (estB) (Afu3g03660), and an ABC transporter (Afu3g03670) (Fig. 4).

Taken together, these data indicated a role of the estB gene product in iron metabolism. The estB cDNA sequence was analyzed by reverse transcription-PCR using RNA isolated from iron-starved mycelia. The comparison of cDNA and genomic sequences revealed the presence of a single intron of 64 bp in length. The deduced EstB protein consists of 292-amino-acid residues with a predicted molecular mass of 32.7 kDa. EstB contains an alpha/beta hydrolase fold (COG2819) and belongs to the esterase protein superfamily. The alpha/beta hydrolase fold is common to several hydrolytic enzymes of widely differing phylogenetic origins and catalytic functions (18).

Putative EstB homologues are present in several fungal species (Table 3). However, other fungi, including Neurospora crassa, Saccharomyces cerevisiae, and Candida albicans, as well as all nonfungal eukaryotes, appear to lack EstB homologues. In contrast, limited similarity can be found to various bacterial proteins, including E. coli IroD, IroE, and Fes as well as S. enterica IroD and IroE (Table 3).

Disruption of estB. In order to characterize the function of EstB, the region encoding amino acid residues 71 to 187 was replaced by the hygromycin B resistance marker gene hph in A. fumigatus WT as described in Materials and Methods and as depicted in Fig. 2A. The two independently isolated disruption mutants (ΔestB strain) displayed the same phenotype in all assays performed (data not shown). To prove that the phenotypic changes of the ΔestB strain are a direct result of the loss of estB, a single estB copy was integrated at the estB locus in the ΔestB strain as described in Materials and Methods and as drafted in Fig. 2B. In all assays performed, the reconstituted strain, the estB<sup>R</sup> strain, was indistinguishable from the WT (data not shown).

**EstB is a TafC esterase.** Due to its sequence similarity to esterases, we analyzed the putative role of EstB in the degradation of TafC. In a first step, we established a new TafC esterase assay based on the fact that TafC and its degradation products can be separated by chloroform extraction due to their differing hydrophobicities (Fig. 3A to C). In this assay, the entire TafC content was hydrolyzed within 0.5 h by cellular extracts derived from WT mycelia grown for 24 h under iron-depleted conditions. In contrast, TafC esterase activity could not be detected in cellular extracts from the iron-starved ΔestB strain, even after incubation for 24 h. In agreement with the estB expression pattern, TafC degradation was reduced by 90% in cellular extracts derived from A. fumigatus grown under iron-replete conditions compared to what was seen for iron-depleted conditions (data not shown). These analyses demonstrate that TafC hydrolysis by A. fumigatus cell extracts is associated with the presence of EstB.

**E. coli-expressed rEstB specifically hydrolyzes TafC.** To further characterize the enzymatic activity of EstB, the encoding cDNA was expressed as a C-terminally His-tagged protein in E. coli, using the pQE system, and purified via Talon metal resin affinity chromatography as described in Materials and Methods (Fig. 3D). Consistent with the genetic data, purified rEstB displayed TafC esterase activity (Fig. 3A and B) and was therefore employed to characterize the enzymatic activity in more detail. rEstB-catalyzed TafC degradation followed Michaelis-Menten kinetics, resulting in a K<sub>m</sub> of 0.4 mM. rEstB

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**TABLE 3. Putative homologues of EstB in other species**

| Sequence ID | Sequence title | Organism      | E value |
|-------------|----------------|---------------|---------|
| gi 67901628 | Hypothetical protein AN7801.2 | A. nidulans | 2e-50   |
| gi 46111093 | Hypothetical protein FG02428.1 | Gibberella zeae | 7e-42   |
| gi 88179653 | Hypothetical protein CHGG_03740 | Chaetomium globosum | 3e-30   |
| gi 46115802 | Hypothetical protein FG023743.1 | G. zea | 2e-24   |
| gi 88184645 | Hypothetical protein CHGG_00348 | C. globosum | 4e-24   |
| gi 14594881 | IroE protein | E. coli | 2e-04   |
| gi 2738251 | IroE protein | S. enterica | 3e-04   |
| gi 10761558 | Putative ferric enterochelin esterase | S. enterica | 0.014 |
| gi 25987937 | IroD | E. coli | 0.052 |
| gi 16128568 | Ferric enterobactin esterase (Fes) | E. coli | 0.12 |
| gi 62179187 | Enterochelin esterase | S. enterica | 0.15 |

*Selected putative EstB homologues (derived by a BLASTP search at http://www.ncbi.nlm.nih.gov/BLAST/) of fungal species, E. coli, and S. enterica are listed.*
showed maximal enzyme activity at pH 6.0 to 7.0 and at 25°C to 37°C. TafC and DF-TafC were hydrolyzed with similar efficiencies (data not shown). Remarkably, rEstB was not able to hydrolyze fusarinine C, which is distinguished from TafC by the lack of three N\(^2\)-acetyl residues (data not shown).

**Deletion of estB increases intracellular accumulation of TafC.** In order to study the role of EstB in vivo, *A. fumigatus* WT and ΔestB strains were grown in liquid shake flask cultures for 22 h under iron-depleted conditions to activate siderophore uptake. Subsequently, the mycelia were harvested and shifted for 2 h into minimal medium containing 30 \(\mu\)M of TafC as the iron source. Determination of the TafC content of the culture supernatant at the end of the shift period demonstrated that both strains had taken up about 80% of the available TafC, indicating similar TafC uptake rates (data not shown). The intracellular accumulation of siderophores was analyzed before and after the shift by reversed-phase HPLC (Fig. 5).

Under iron-depleted conditions, WT and ΔestB strains contained minor amounts of DF-TafC. Notably, the ΔestB strain displayed a 1.8-fold increase in DF-TafC content under iron-depleted conditions. After 2 h of incubation with TafC, the DF-TafC was barely detectable in both WT and ΔestB strains, but TafC and its degradation products appeared. Reversed-phase HPLC analysis of intracellular in vivo TafC degradation in a ΔsidA strain, which is unable to produce siderophores but is still able to utilize TafC (27), confirmed that the detected products are indeed degradation products derived from TafC (data not shown). Remarkably, in the ΔestB strain, the TafC content was increased 6.0-fold, whereas the TafC degradation products were increased only 1.9- to 2.7-fold. These data suggest that EstB deficiency results in increased intracellular accumulation of TafC due to decreased hydrolysis, as the ratio between TafC and its degradation products largely increases in the ΔestB strain from what is seen for the WT. However, these results also indicate that *A. fumigatus* is able to hydrolyze TafC into the very same degradation products independent of EstB, although with less efficiency. In this respect, the in vivo analysis of TafC degradation differs from the in vitro analysis using cellular extracts. In the latter analysis, EstB-independent TafC hydrolysis was not evident (see above). This difference might indicate the inactivation of the EstB-independent mechanism by the preparation of cellular extracts.

**EstB deficiency delays iron sensing.** The HPLC analysis of the intracellular siderophore also revealed that *A. fumigatus* WT and ΔestB strains accumulate about the same amounts of DF-FC under iron-depleted conditions (Fig. 5). Two hours after the shift into 30 \(\mu\)M TafC, 85% of the DF-FC pool was converted to FC in the WT, but only 52% was converted in the ΔestB strain. These data indicate that the decreased TafC hydrolysis rate caused by EstB deficiency results in a slower transfer of iron from TafC to DF-FC.

In order to simulate the transfer of iron from TafC to DF-FC in vitro, we employed the established TafC esterase assay containing 0.6 mM DF-FC in addition to 0.3 mM TafC. After incubation for 4 h, cross-chelation of TafC-iron by DF-FC was not detected in the absence of EstB. In contrast, the entire amount of iron transferred from TafC and its degradation products to DF-FC in the presence of 1 \(\mu\)g rEstB (data not shown). These data emphasize that the hydrolysis of TafC enables the efficient transfer of iron from TafC to DF-FC and that the affinity of FC to iron is higher than that of the TafC hydrolysis products.

To further analyze the consequences of the reduced release of iron from TafC, expression levels of the iron-repressed gene *Afu3g03640*, a putative siderophore transporter gene, were compared for WT and ΔestB strains during the shift from iron-depleted to iron-replete conditions (Fig. 6). Consistent with the decreased release of iron from TafC, the down-regu-
lation of Afu3g0640 expression was delayed in the ΔestB strain compared to what was seen for the WT, suggesting that a deficiency in EstB causes a delay in the sensing of iron.

**EstB deficiency reduces the growth rate under iron-depleted conditions.** Comparison of the growth rates of ΔestB and WT strains displayed no difference under iron-replete conditions (data not shown). However, the growth rate of the ΔestB strain was slightly reduced under iron-depleted conditions (data not shown), and it was significantly reduced under iron-depleted conditions in the presence of 200 μM bathophenanthroline disulfonic acid (BPS) (Fig. 7). In the presence of BPS, the siderophore system constitutes the only functional iron uptake system in *A. fumigatus*, because BPS is a ferrous iron specific chelator and therefore inhibits reductive iron assimilation (27). Consequently, these data suggest that EstB is essential for the optimal function of TafC-mediated iron utilization.

**EstB is localized in the cytoplasm.** To determine the cellular localization of EstB, *estB* was fused in frame at the 3′ end with the EGFP-encoding gene, yielding the estBgfp strain (Fig. 2C). In vitro and in vivo analysis of TafC hydrolysis (data not shown) and determination of the growth rate (Fig. 7) of the estBgfp strain demonstrated that EGFP-tagged EstB is biologically functional. Fluorescence microscopy revealed that EstB is localized in the cytoplasm, and consistent with the Northern analysis EstB-EGFP was detectable for iron-depleted conditions but to a much lesser extent during growth under iron-replete growth (Fig. 8). These data provide indirect evidence that the hydrolysis of TafC occurs in the cytoplasm.

**FIG. 8.** EstB is localized in the cytoplasm. *A. fumigatus* WT and estBgfp strains were analyzed by light (upper panel) and fluorescence (lower panel) microscopy after growth under iron-depleted (−Fe) and iron-replete conditions (+Fe) as described in Materials and Methods. EGFP typical fluorescence was detected in the cytoplasm of the estBgfp strain, in particular during iron starvation.

**DISCUSSION**

In contrast to that in fungi, siderophore-mediated iron uptake in various bacteria has been studied in great detail (23). One of the best-studied siderophores is the catecholate enterobactin, a 2,3-dihydroxybenzoylserine macrotrilactone produced by enteric bacteria such as *E. coli* and *S. enterica* (25). It is the strongest Fe3+ ligand known, with an estimated equilibrium dissociation constant of 10^{−49} M (12). Hydrolysis of its ester bonds by the esterase Fes is essential for iron release, to make it available for general cell metabolism (2, 11, 12). Recently, the Fes homologues IroD and IroE have been shown to mediate the hydrolysis of salmochelins, which are glycosylated enterobactin derivatives (12, 33).

Fungi do not produce catecholates but do produce hydroxamic acids, including ferrichromes and fusarinines (7). Similar to those of catecholates, the subunits of cyclic fusarinines (fusarinine C and TafC) are connected by ester bonds. The hydrolysis of cyclic fusarinines was demonstrated for several fungi and has been postulated to be required for intracellular iron release, similar to what is seen for bacteria (6). In this study, we identified and characterized the TafC esterase EstB of *A. fumigatus*, the first eukaryotic siderophore-degrading enzyme to be characterized at the molecular level. The EstB protein displays limited sequence similarity to the bacterial enzymes Fes, IroD, and IroE. Similar to its bacterial homologues (12, 24, 33), estB is genomically clustered with other genes involved in siderophore metabolism.

We demonstrated TafC hydrolysis by EstB in vitro and in vivo. Nevertheless, an EstB-deficient strain was still able to hydrolyze TafC, although with less efficiency. As the *A. fumigatus* genome does not appear to encode a related enzyme, the EstB-independent TafC hydrolysis is probably mediated by a different enzyme class. Due to the EstB-independent TafC degradation mechanism, it remains to be shown if TafC cleavage is essential for the release of iron from this siderophore. The utilization of TafC as the iron source has also been demonstrated for two yeast species, *S. cerevisiae* and *C. albicans*, which are not able to produce siderophores (8, 31). Both spe-
cies appear to lack orthologues of EstB, supporting the hy-
pothesis that this enzymatic activity is not essential for the use of TaFC as the iron source. Nevertheless, the EstB-independent TaFC hydrolytic mechanism is not able to compensate the loss of EstB in A. fumigatus, because estB deficiency reduces the transfer rate of iron from TaFC to the intracellular siderophore DF-FC, delays iron sensing, and impairs growth on TaFC as the sole iron source. These data indicate that EstB optimizes the utilization of TaFC as the iron source. For DF-
TaFC-producing fungal species, TaFC is the major source of iron, which is probably the reason for the evolution of a mech-
nism to improve the efficiency of iron release from TaFC. Consistently, putative EstB orthologues can be found in the DF-TaFC producers A. nidulans and Gibberella zeae (Table 2).

Unprotected iron-free siderophores within cells are potentially hazardous due to their high affinity to iron (7). Subsequent to synthesis, DF-TaFC appears to be rapidly excreted or hydrolyzed, because only very low amounts can be detected intracellularly. As EstB hydrolyzes TaFC and DF-TaFC with equal efficiencies, its function might not be restricted to the release of iron from the siderophore but might also include the degradation of the iron-free chelator to protect cells. Localization of EstB by EGFP tagging suggests that the hydrolysis of TaFC takes place in the cytoplasm. Therefore, EstB represents the first localized intracel-
lular component of a eukaryotic siderophore system.

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