Fungal siderophore biosynthesis is partially localized in peroxisomes

Introduction
Iron is an essential nutrient for all eukaryotes and nearly all prokaryotes (Kaplan and Kaplan, 2009). Moreover, the control over iron access is one of the central battlefields during infection as pathogens have to ‘steal’ the iron from the host – in particular as the mammalian innate immune system restricts iron access to pathogens via a variety of mechanisms (Ganz, 2009; Weinberg, 2009).

Aspergillus fumigatus is a ubiquitous saprophytic fungus, which has become the most common air-borne fungal pathogen of humans (Tekaya and Latge, 2005). Clinical manifestations range from allergic reactions to life-threatening invasive disease, termed aspergillosis, particularly in immunocompromised patients. The identification and functional characterization of more than 20 genes that are involved in iron homeostasis maintenance in A. fumigatus and/or its less pathogenic relative Aspergillus nidulans have made them models for iron metabolism in filamentous fungi (Haas, 2012). Both Aspergillus species employ low-affinity ferrous iron acquisition as well as siderophore-assisted iron uptake, a high-affinity ferric iron uptake system (Eisendle et al., 2003; Schrettl et al., 2004). In contrast to A. nidulans, A. fumigatus possesses a second high-affinity iron uptake system, termed reductive iron assimilation. Interestingly, the fungal model system Saccharomyces cerevisiae differs from most other fungi due to the lack of siderophore biosynthesis and employment of iron regulators that are not conserved in most other fungal species (Haas et al., 2008). Both Aspergillus species excrete the fusarinine-type siderophore triacetylfusarinine C (TAFC) to mobilize extracellular iron. Iron-chelated TAFC is taken up by siderophore-iron transporters (Haas et al., 2003; 2008; Philpott and Protchenko, 2008). Intracellular release of iron from TAFC involves hydrolysis of the siderophore backbone catalysed in part by the esterase EstB (Kragl et al., 2007). Furthermore, both Aspergillus species produce the intracellular siderophore ferrococin (FC) for hyphal iron storage and distribution (Schrettl et al., 2007; Wallner et al., 2009; Blatzer et al., 2011).

TAFC consists of three N²-acetyl-N⁵-anhydromevalonyl-N²-hydroxymorinine residues cyclically linked by ester bonds; FC is a cyclic hexapeptide with the structure Gly–Ser–Gly–(N²-acetyl-N⁵-hydroxymorinine)₉ (Haas

Summary
Siderophores play a central role in iron metabolism and virulence of most fungi. Both Aspergillus fumigatus and Aspergillus nidulans excrete the siderophore triacetylfusarinine C (TAFC) for iron acquisition. In A. fumigatus, green fluorescence protein-tagging revealed peroxisomal localization of the TAFC biosynthetic enzymes SidI (mevalonyl-CoA ligase), SidH (mevalonyl-CoA hydratase) and SidF (anhydromevalonyl-CoA transferase), while elimination of the peroxisomal targeting signal (PTS) impaired both, peroxisomal SidH-targeting and TAFC biosynthesis. The analysis of A. nidulans mutants deficient in peroxisomal biogenesis, ATP import or protein import revealed that cytosolic mislocalization of one or two but, interestingly, not all three enzymes impairs TAFC production during iron starvation. The PTS motifs are conserved in fungal orthologues of SidF, SidH and SidI. In agreement with the evolutionary conservation of the partial peroxisomal compartmentalization of fungal siderophore biosynthesis, the SidI orthologue of coprogen-type siderophore-producing Neurospora crassa was confirmed to be peroxisomal. Taken together, this study identified and characterized a novel, evolutionary conserved metabolic function of peroxisomes.

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Introducing the use of peroxisomes
Several prokaryotic species produce siderophores, which are proteins or peptides, with the ability to bind iron with high affinity. In eukaryotic organisms, such as fungi, siderophores are also produced and play a role in iron uptake and distribution. In this study, the authors investigated the role of peroxisomes in siderophore biosynthesis in fungal species, specifically Aspergillus fumigatus and Aspergillus nidulans. They found that the siderophore triacetylfusarinine C (TAFC) is partially localized in peroxisomes, and that this localization is crucial for its production during iron starvation. The authors used green fluorescence protein-tagging to confirm the localization of the siderophore biosynthetic enzymes SidI, SidH, and SidF to peroxisomes. They also showed that the elimination of the peroxisomal targeting signal (PTS) impaired both, peroxisomal SidH-targeting and TAFC biosynthesis. This study suggests that peroxisomes play a key role in siderophore biosynthesis and iron uptake in fungi.
et al., 2008). The siderophore biosynthetic pathway is shown in Fig. 1. The first committed step in the biosynthesis of these siderophores is the hydroxylation of ornithine catalysed by the ornithine monooxygenase SidA (Eisendle et al., 2003; Schrettl et al., 2004; Olucha et al., 2011). Subsequently, the pathways for biosynthesis of extra- and intracellular siderophores split. For biosynthesis of extracellular siderophores, the transacylase SidF transfers anhydromevalonyl to \( N^\delta \)-hydroxoyornithine (Schrettl et al., 2007). The required anhydromevalonyl-CoA moiety is derived from mevalonate by CoA ligation and dehydration catalysed by SidI and SidH respectively (Yasmin et al., 2011). The acetylation of \( N^\delta \)-hydroxoyornithine for FC biosynthesis involves two transacetylases, the constitutively expressed SidL (Blatzer et al., 2011) and an unidentified enzyme, the activity of which is upregulated by iron starvation. Assembly of Fusaririne C (FsC) and FC is catalysed by two different non-ribosomal peptide synthetases (NRPS), SidD and SidC respectively. Subsequently, SidG catalyses \( N^\gamma \)-acetylation of FsC for forming TAFc. As typical for NRPS, SidD and SidC depend on activation by 4′-phosphopantethenyl transferase (Oberegger et al., 2003). Both extra- and intracellular siderophores are crucial for growth during iron limitation in \( A. \) fumigatus and \( A. \) nidulans (Eisendle et al., 2003; Schrettl et al., 2004). Elimination of the entire siderophore biosynthesis (\( \Delta \)sidA mutant) results in absolute avirulence of \( A. \) fumigatus in a murine model of invasive pulmonary aspergillosis (Schrettl et al., 2004; Hissen et al., 2005), while deficiency in either extracellular (\( \Delta \)sidI, \( \Delta \)sidH, \( \Delta \)sidF or \( \Delta \)sidD mutants) or intracellular siderophores (\( \Delta \)sidC mutants) causes partial attenuation of virulence (Schrettl et al., 2007; Yasmin et al., 2011).

Peroxisomes are single membrane organelles, which compartmentalize a wide range of metabolic functions in eukaryotic cells. Important functions of peroxisomes include fatty acid \( \beta \)-oxidation, the glyoxylate cycle, metabolisms of cholesterol and reactive oxygen species, as well as methanol oxidation (Schrader and Fahimi, 2008). Due to the diversity of metabolic pathways in peroxisomes, their content varies depending on the species, cell or tissue type, as well as on environmental conditions (Brown and Baker, 2008). In contrast to mitochondria, peroxisomes are devoid of DNA and protein synthesis machinery. Therefore, all peroxisomal proteins are encoded by nuclear genes and are post-translationally imported into peroxisomes. In \( S. \) cerevisiae, more than 60 peroxisomal proteins have been identified including the peroxins (Pex), which are essential for peroxisomal biogenesis and maintenance as well as matrix protein import (Schrader and Fahimi, 2008).

This study revealed peroxisomal targeting signal of type one (PTS1) or type two (PTS2) in SidI, SidH and SidF of \( Aspergillus \) spp. and their orthologues in other Ascomycetes. Peroxisomal localization of all three enzymes was confirmed by green fluorescence protein (GFP) tagging and by PTS1 mutation of SidH in \( A. \) fumigatus. The analysis of \( A. \) nidulans mutants deficient in peroxisomal bio-

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genesis, ATP import and protein import depending on either PTS1, PTS2, or both, indicated that cytosolic mislocalization of individual enzymes but not of the entire TAFc biosynthetic machinery impairs TAFc production during iron starvation.

Results

A. fumigatus SidI, SidH and SidF carry PTS and localize to peroxisomes

The majority of peroxisomal matrix proteins are post-translationally directed to the lumen of the organelle by peroxisomal targeting signals (PTS) and two different motifs have been characterized (Olivier and Krisans, 2000). PTS1 is a tri-peptide with the consensus sequence (S/A/C)(K/H/R)(L/M) located at the extreme C-terminus, whereas PTS2 is a nona-peptide of the consensus sequence (R/K)(L/V/I)X5(H/Q)(L/A) located near the N-terminus of a matrix protein. Sequence analysis of the A. fumigatus TAFc biosynthetic enzymes revealed the presence of the putative PTS1 motifs SKL and AKL in SidH and SidF, respectively, and a putative PTS2, RLQTLSQHL, localized at amino acid 6–14 in SidI.

To confirm the peroxisomal localization of SidH and SidF, ΔsidH and ΔsidF mutant strains were complemented with respective N-terminally GFP-tagged versions as described in Experimental procedures. SidI was C-terminally GFP-tagged in a wild-type (wt) strain via integration of the GFP-encoding gene at the sidI locus. Consequently, the respective mutant produces only GFP-tagged SidI. Consistent with the predicted peroxisomal localization, the GFP-tagged versions of all three enzymes localized to punctate dots in the cytoplasm (Fig. 2A). ΔsidH and ΔsidF mutant strains lack TAFc production (Schrettl et al., 2007), while the expression of the respective GFP-fusion proteins increased TAFc production to 86 ± 11% and 91 ± 8%, respectively, of the wild type (wt). Similarly, the SidI–GFP carrying strain displayed 95 ± 19% of the wt TAFc production. These data indicate correct enzymatic activity and subcellular localization of the GFP-tagged protein versions. Truncation of the putative PTS1 motif of GFP–SidH (GFP–SidH<sup>PTS1</sup>) caused cytosolic localization, which strongly suggests that the C-terminus of SidH is indeed a targeting sequence required for peroxisomal localization (Fig. 2A). In contrast to GFP–SidH, GFP–SidH<sup>PTS1</sup> did not support TAFc synthesis, i.e. TAFc biosynthesis was not detectable in the respective mutant strain (data not show), indicating that peroxisomal localization of SidH is essential for TAFc biosynthesis.

Recently, red fluorescence protein (RFP) C-terminally tagged with a PTS1 motif (RFP–PTS1) has been shown to localize to peroxisomes in A. fumigatus (Beck and Ebel, 2013). In a strain producing both GFP–SidH and RFP–PTS1, we found virtually perfect colocalization (Fig. 2B). These data underline the peroxisomal localization of SidH.

Peroxisomal targeting signals are conserved in fungal SidI, SidH and SidF orthologues

With few exceptions such as S. cerevisiae, Candida albicans or Cryptococcus neoformans, most fungi produce siderophores and encode respective genes (Yasmin et al., 2011). Genome mining by BLAST searches and sequence analysis revealed that putative SidI, SidH and SidF orthologues from various Ascomycetes carry putative PTS motifs. Table 1 lists the closest homologues to SidI, SidH and SidF, which were identified by BLAST searches. Genes involved in siderophore biosynthesis (including sidI, sidH and sidF) tend to be organized in gene clusters (Schrettl et al., 2008). The orthologues of sidI, sidH and sidF listed in Table 1 are colocalized in the genome with other putative siderophore biosynthetic gene (Table S1), which emphasizes that these genes are indeed the orthologues. Besides these orthologues, all analysed species possess additional homologues (see below). All identified SidF and SidH orthologues possess PTS1 motifs. Importantly, SidI orthologues from Sordariomycetes such as Neurospora crassa possess a PTS1 in contrast to the PTS2 found in other Ascomycetes.

A. fumigatus SidH and SidI are targeted to peroxisomes via their corresponding PTS1 and PTS2 receptors PexE and PexG, respectively, in A. nidulans

Aspergillus fumigatus and A. nidulans produce the same siderophores and the proteins involved in their biosynthesis are highly conserved (Haas et al., 2008; Schrettl et al., 2008). The amino acid sequence identities of the corresponding TAFc biosynthetic enzymes of A. fumigatus and A. nidulans are given in Table S2. Moreover, the PTS are perfectly conserved (Table 1). To investigate the peroxisomal localization of TAFc biosynthesis in more detail, we switched from A. fumigatus to A. nidulans, because the peroxisomal system of the latter has been characterized in great detail and a number of respective peroxin mutants, described in Table S3, is available (Hynes et al., 2008). Peroxins are proteins required for the assembly and function of peroxisomes. These are highly conserved among fungal species (Kiel et al., 2006); a comparison of relevant peroins of A. fumigatus and A. nidulans is given in Table S3 (Hynes et al., 2008).

In a first step, localization of A. fumigatus GFP–SidH was heterologously studied in A. nidulans wt and mutants lacking peroxisomes (pexC::bar), PTS1-dependent peroxisomal import (ΔpexE, missing the PTS1 receptor PexE) or PTS2-dependent peroxisomal targeting (pexG14, lacking a functional PTS2 receptor PexG). In perfect agreement with PTS1 receptor-mediated peroxi-
Localization, GFP–SidH localized in cytosolic spots in *A. nidulans* wt and *pexG14* strains but mislocalized to the cytosol in ∆*pexE* and *pexC::bar* mutants (Fig. 3). The peroxisomal localization of GFP–SidF in *A. fumigatus* together with its evolutionary conserved PTS1 (Fig. 2, Table 1) strongly suggests that SidF is directed to peroxisomes in the same manner.

In contrast to GFP–SidH, SidI–GFP was mislocalized to the cytosol in a *pexG14* strain missing the PTS2 receptor, which demonstrates a PTS2-dependent peroxisomal targeting of SidI (Fig. 4).

These data confirm that PTS1 and PTS2 receptors function independently in filamentous fungi, which contrasts with the situation in mammals and plants (Kiel et al., 2006; Hynes et al., 2008). Moreover, the data suggest that inactivation of the PTS1 receptor mislocalizes SidH and SidF, while inactivation of the PTS2 receptor mislocalizes SidI to the cytosol.
Table 1. Peroxisomal targeting signals in orthologues of SidI, SidH and SidF.

| Organism            | Accession | PTS      | Score (PTS1) | Accession | PTS      | Score (PTS1) | Accession | PTS      | Score (PTS1) |
|---------------------|-----------|----------|--------------|-----------|----------|--------------|-----------|----------|--------------|
| **Eurotiomycetes**  |           |          |              |           |          |              |           |          |              |
| Aspergillus fumigatus | XP_753087.1 | RLQQTLSHL | –            | XP_748661.1 | SKL      | 10.8         | XP_748660.1 | AKL      | 7.5          |
| Neosartorya fischeri       | XP_001264038.1 | RLQQTLSHL | –            | XP_001259092.1 | SKL      | 10.5         | XP_001259091.1 | AKL      | 7.5          |
| Aspergillus niger         | XP_001390954.1 | RLQQTLSHF | –            | XP_001390237.1 | SKL      | 9.6          | XP_001390236.1 | AKL      | 6.9          |
| Aspergillus clavatus      | XP_001268556.1 | RLQQTLSHL | –            | XP_001273567.1 | SNL      | 9.3          | XP_001273568.1 | AKL      | 7.3          |
| Aspergillus oryzae        | XP_001821069.1 | RLQQTLSHI | –            | XP_001826764.1 | SNL      | 5.4          | XP_001826765.1 | AKL      | 5.2          |
| Penicillium chrysogenum   | XP_002569340.1 | RLQQTLSHV | –            | XP_002565937.1 | SKL      | 8.1          | XP_002565938.1 | AKL      | 6.9          |
| Aspergillus nidulans      | XP_658213.1  | RLQQTLSHL | –            | XP_663839.1  | SNL       | 5.9          | XP_663838.1  | AKL      | 7.7          |
| Ajellomyces capsulatus    | EEE02760.1  | RLQQTINHI | –            | EEE07298.1  | SKL       | 12.4         | XP_001536540.1 | SKL      | 14.0         |
| Talaromyces stipitatus    | XP_002484364 | RLQQTQRHI | –            | XP_002486020.1 | SKL      | 8.4          | XP_002486021.1 | AKL      | 6.9          |
| **Leotiomycetes**         |           |          |              |           |          |              |           |          |              |
| Botryotinia fuckeliana    | XP_001546797.1 | PKL\(^a\) | 13.5         | XP_001551006 | SKL      | 9.1          | XP_001551005.1 | PKL\(^a\) | 6.5          |
| Sclerotinia sclerotiorum  | XP_001585101.1 | PKL       | 12.7         | XP_001594442.1 | SKL\(^b\) | 9.1          | XP_001594441.1 | AKL      | 6.9          |
| **Sordariomycetes**       |           |          |              |           |          |              |           |          |              |
| Neurospora crassa         | XP_959826.1  | SKL       | 6.2          | XP_962600.1  | SKL      | 6.3          | XP_959825.1  | PKL      | 6.5          |
| Gibberella zeae           | XP_384509.1  | SKL       | 3.7          | XP_383922.1  | SKL      | 10.4         | XP_383921.1  | PKL      | 2.2          |
| Podospora anserina        | XP_001905332.1 | AKL      | 12.6         | XP_003437360.1 | SKL      | 8.6          | XP_001905331.1 | AKL      | 8.0          |
| Chaetomium globosum       | XP_001226170.1 | AKL      | 0.0          | XP_001227391.1 | SKL      | 8.6          | XP_001227400.1 | AKL      | 6.9          |
| Magnaporthe oryzae        | EHA55987    | AKL      | 10.1         | EHA47011.1  | SKL      | 9.3          | XP_362759.1  | PKL      | 8.2          |
| **Dothideomycetes**       |           |          |              |           |          |              |           |          |              |
| Phaeosphaeria nodorum     | XP_001804551.1 | RLQNTLLQ\(^f\) | –            | XP_001790987.1 | SKL      | 11.7         | XP_001804552.1 | ARL      | 8.5          |
| Basidiomycota             | XP_760950.1  | –         | –            | XP_757580.1  | Fer5      | –            | XP_757579.1  | Fer5      | –            |

\(^{a}\) Updated genome data from Botrytis cinerea strain B05.10 with the locus tag B0510_2726 (SidI) and B0510_7543 (SidF) reveal gene products containing a PTS1 (PKL). http://www.broadinstitute.org/annotation/genome/botrytis_cinerea/FeatureSearch

\(^{b}\) The annotated gene is organized in four exons, whereas all other orthologues have two. Manual reannotating of the intron/exon structure increased the sequence similarity to its orthologues and led to a PTS1-containing C-terminus (EWYPRLVKSPNFAEGIQAYVDKRPPKWVNSKL). Variants of the classical PTS1 SKL sequence such as –ARL, –AKL or –PKL were shown to be functional peroxisomal targeting signals in human, yeast and Penicillium chrysogenum (Amery et al., 1998; Kiel et al., 2009). The PTS1 scores of proteins were obtained using the PTS1-predictor program http://mendel.imp.ac.at/mendeljsp/sat/pts1/PTS1predictor.jsp (Neuberger et al., 2003).

Positive scores indicate high probability of peroxisomal targeting, sequences with scores < -10 are unlikely to function as PTS1, and motifs with scores in between have unclear function. PTS2 motifs were identified using the PTS2 finder http://www.peroxisomedb.org/diy_PTS2.html, which does not provide reliability scores.
Deficiency in either PTS1- or PTS2-dependent peroxisomal protein import or AntA-mediated peroxisomal ATP import decreases TAFC production in *A. nidulans*

To characterize the role of peroxisomes in siderophore biosynthesis at the metabolite level, production of TAFC and FC was analysed in the *A. nidulans* wt and mutants affected in peroxisomal import (ΔpexE, pexG14, pexF23, ΔpexE/pexG14), peroxisomal proliferation (ΔpexK), peroxisomal biogenesis (pexC::bar) or peroxisomal ATP import (antA14) respectively (Fig. 5).

In accordance with the importance of PexE-mediated peroxisomal transport of SidH and SidF, the TAFC production of the ΔpexE deletion mutant (ΔpexE) was decreased to 12% of the wt during iron starvation (Fig. 5). The decreased TAFC production of ΔpexE is most likely the reason for the 37% reduced biomass production compared with wt (Fig. 5) because the lack of siderophore production has been shown to decrease growth during iron limitation (Schrettl et al., 2004; 2007). The TAFC and biomass production defects during iron starvation are cured by *pexE* gene complementation in strain *pexE*<sup>+</sup> (Fig. 5). Inactivation of *PexG*-mediated peroxisomal import (strain *pexG14*) reduced TAFC production to 51% of the wt, which suggests a crucial role for PTS2-dependent peroxisomal import of Sidl in TAFC biosynthesis. In agreement with the higher TAFC production compared with Δ*pexE*, *pexG14* biomass production was also higher. Taken together, these data indicate that cytosolic mislocalization of SidF and SidH or of Sidl via inactivation of PTS1 or PTS2 receptors, respectively, impairs TAFC biosynthesis in *A. nidulans*.

The peroxisomal membrane is impermeable to ATP (Palmieri et al., 2001; van Roermund et al., 2001; Hynes et al., 2008). In *A. nidulans*, inactivation of the peroxisomal ATP-importer AntA in the *antA15* strain reduces growth on fatty acids (Hynes et al., 2008) because ATP is required to fuel peroxisomal β-oxidation by activation of fatty acyl-CoA synthetases, which belong to the acyl-CoA-ligase family. Sidl belongs also to the acyl-CoA ligase protein family and converts mevalonate to mevalonyl-CoA in an ATP-dependent manner (Yasmin et al., 2011). Consistent with the requirement for ATP within peroxisomes for Sidl activity, TAFC production was reduced to 39% of wt in *antA15*, a similar reduction to that observed for *pexG14*. Both β-oxidation and TAFC biosynthesis were not blocked completely in *antA15* suggesting an AntA-

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**Fig. 3.** In *A. nidulans*, peroxisomal localization of *A. fumigatus* GFP–SidH is blocked by inactivation of PexC (pexC::bar) or PexE (ΔpexE) but not PexG (pexG14). Fungal strains were grown in iron-depleted minimal medium for 18 h at 37°C. Upper panel, bright-field image; mid-panel, confocal fluorescence microscopy; lower panel, overlay. Scale bar, 10 μm.
independent peroxisomal ATP delivery system. In this respect it can be noted that glycolytic/gluconeogenic enzymes have recently been shown to have dual localization in fungi, i.e. in the cytoplasm and peroxisomes (Freitag et al., 2012). This metabolic network may function in redox/ATP shuttling or as a buffer system to cope with perturbations of redox/ATP equivalents (Freitag et al., 2012).

Remarkably, neither blocking of peroxisome biogenesis (pexC::bar) nor inactivation of entire peroxisomal protein import by simultaneous inactivation of both PexE and PexG (ΔpexE/pexG14), or inactivation of pexF (pexF26) impaired TAFC production (Fig. 5). Remarkably, the TAFC production of pexF26, which lacks PexF and consequently both PTS1- and PTS2-dependent peroxisomal protein targeting (Erdmann and Schliebs, 2005; Koek et al., 2007; Hynes et al., 2008), was even 31% increased compared with wt (Fig. 5). Peroxisomes are generated either de novo from the endoplasmatic reticulum, which requires PexC, or by PexK-dependent peroxisomal division of the novo-generated peroxisomes (Hoepfner et al., 2005; Hettema and Motley, 2009). PexK deficiency (ΔpexK) also did not affect TAFC production (Fig. 5).

The FC content of ΔpexE, pexF23, pexC::bar and ΔpexK was between 85% and 105% of the wt indicating that FC biosynthesis does not rely on peroxisomes (Fig. 5). In agreement, SidI, SidH and SidF are not involved in FC biosynthesis (Fig. 1). Interestingly, FC production was increased between 19% and 35% compared with wt in pexG14, ΔpexE/pexG14 and antA15.

**Fig. 4.** In A. nidulans, peroxisomal localization of A. fumigatus Sidi-GFP is blocked by inactivation of PexG (pexG14). Fungal strains were grown in iron-depleted minimal medium containing 200 μM BPS to generate harsh iron starvation for 24 h at 37°C. Upper panel, bright-field image; mid-panel, confocal fluorescence microscopy; lower panel, overlay. Scale bar, 10 μm.

**Fig. 5.** Inactivation of PexE, PexG or AntA but not of PexC, PexK or both PexE and PexG impairs TAFC biosynthesis. Production of TAFC and FC was measured after growth for 24 h in iron-depleted minimal medium. The values represent the means ± STD of three experiments normalized to biomass and wt (100%).
Inactivation of PexE, PexG or AntA impairs growth under iron-depleted conditions

Consistent with the extremely reduced TAFC production and biomass production in liquid culture (Fig. 5), PexE deficiency dramatically decreased growth on plates under iron starvation but not iron sufficiency (Fig. 6). All mutants lacking peroxisomes or PTS1-dependent peroxisomal protein import display reduced conidiation (Hynes et al., 2008). However, deficiency in PexG or AntA completely blocked sporulation during iron starvation, most likely due to the decreased TAFC production because siderophore-mediated iron supply has previously been shown to be crucial for sporulation (Schrettl et al., 2007; Wallner et al., 2009). Consistently, siderophore cross-feeding from wt increased growth and conidiation of ΔpexE and pexG14 (Fig. 7).

The N. crassa SidI orthologue localizes to peroxisomes in Aspergillus spp. and assists TAFC biosynthesis

In contrast to A. fumigatus and A. nidulans, N. crassa secretes the siderophore coprogen instead of TAFC, but shares the same hyphal siderophore FC (Matzanke et al., 1987). Similar to TAFC, coprogen contains anhydromevalonyl residues (Haas et al., 2008). Therefore, it is not surprising that N. crassa possesses orthologues to SidH, SidF and SidI. The SidI orthologues from A. fumigatus and N. crassa share 60% identity at the amino acid sequence level, but in contrast to the PTS2-carrying A. fumigatus SidI, N. crassa SidI harbours a PTS1 motif (Table 1). To confirm its role in siderophore biosynthesis and its peroxisomal localization, the N. crassa sidI was expressed as an N-terminal GFP-tagged protein in A. nidulans wt and the A. nidulans ΔpexE strain (Fig. 8). The N. crassa GFP–SidI localized to peroxisomes in A. nidulans wt, which emphasizes conservation of peroxisomal localization. In accordance with the PTS1 motif, it was mislocalized to the cytoplasm in A. nidulans ΔpexE. Remarkably, the expression of N. crassa GFP–SidI increased TAFC production from 11% of the parental ΔpexE strain to 86% of the wt. In this strain, GFP–SidI, SidH and SidF are now all cytosolic, which clearly shows that colocalization of all three enzymes, either in peroxisomes or in the cytosol, is essential for efficient TAFC production.

Discussion

This study has revealed peroxisomal localization of the three TAFC biosynthetic enzymes from A. fumigatus:

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Peroxisomal localization of A. nidulans have been shown to be cytosolic (Kragl et al., 2011). Cytosolic mislocalization of one to two of the siderophore precursors mevalonate ($M_r = 148$) and $N_5$-hydroxyornithine ($M_r = 148$) can enter and the SidF product $N^2$-anhydromevalonyl-$N^5$-hydroxyornithine ($M_r = 260$) can freely pass the peroxisomal membrane. In contrast, the CoA-intermediates mevalonol-CoA ($M_r = 916$) and anhydromevalonyl-CoA ($M_r = 898$) are unlikely to be able to freely exit peroxisomes. This feature of the peroxisomal membrane is expected to increase the local concentration of the CoA-intermediates of TAFC biosynthesis, which might enhance the efficiency of the involved enzymatic reactions and consequently provides a rational for the localization in peroxisomes. The decrease in TAFC biosynthesis varied between the different ways of cytosolic mislocalization and can be explained by the different, in part pleiotropic, effects: SidH PTS1 truncation mislocalizes only SidH, PTS1 inactivation mislocalizes SidH and SidF together with all other PTS1-dependent peroxisomal proteins, and PTS2 inactivation mislocalizes SidI together with all PTS2-dependent peroxisomal proteins. Moreover, differences in peroxisomal import and export efficiency of $N^5$-hydroxyornithine and $N^2$-anhydromevalonyl-$N^5$-hydroxyornithine might play a role. In this respect it is interesting to note that the vast majority of peroxisomal matrix proteins are PTS1 receptor-dependent and SidI is one of the few exceptions (Kiel et al., 2006). Despite the clear peroxisomal localization of SidI, SidH and SidF, it cannot be excluded that low levels of these enzymes are present and operate in the cytosol.

In agreement with the dramatically reduced TAFC production, the PexE-deficient mutant displayed reduced growth during iron starvation on solid and in liquid growth media (Figs 5 and 6). However, growth of mutants lacking PexC or PexF was also somehow reduced despite their TAFC production not being reduced. These data indicate that siderophore-independent peroxisomal functions are additionally important for adaptation to iron starvation.

Orthologues of SidI, SidH and SidF are found in numerous Ascomycetes as these enzymes are not only required for fusarinine-type but also coprogen-type siderophores, which also contain anhydromevalonyl moieties (Haas et al., 2008). Genome mining indicated PTS motif conservation in SidI, SidH and SidF orthologues of numerous Ascomycetes (Table 1). In agreement, the SidF ortho-

![Fig. 8. Peroxisomal localization of N. crassa GFP–SidI in A. nidulans is PexE-dependent. Fungal strains were grown in iron-depleted minimal medium for 18 h at 37°C. Upper panel, bright-field image; mid-panel, confocal fluorescence microscopy; lower panel, overlay. Scale bar, 10 μm.](image)
logue of the coprogen producer *Penicillium chrysogenum* was recently identified as a peroxisomal matrix protein in a proteomic approach (Kiel et al., 2009). Interestingly, SidI orthologues of *Sordariomycetes* possess a PTS1 in contrast to the PTS2 motif found in other *Ascomycetes* and the PTS1 motif of the SidI orthologue from coprogen-producing *N. crassa* was confirmed to be functional here. Therefore, all three siderophore enzymes in *Sordariomycetes* rely on PexE-dependent peroxisomal import only.

The enoyl-CoA hydratase Fer4 (UM01433) and the SidF orthologue Fer5 (UM01432.1), which are essential for ferrichrome A biosynthesis in the *Basidiomycete Ustilago maydis* (Winterberg et al., 2010), lack PTS indicating non-peroxisomal localization (Table 1). Due to the differences in siderophore structure and biosynthesis, *U. maydis* lacks a SidI orthologue. A BlastP search (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi?organism=fungi) identified SidF homologues in various *Basidiomycetes* besides *U. maydis* (Table S7; *Malassezia globosa, Serpula lacrymans, Schizopyllum commune, Puccinia graminis, Melampsora larici-populina*) but none of these proteins contained PTS1 or PTS2 motifs, which suggests non-peroxisomal localization of siderophore biosynthesis in *Basidiomycetes*. Moreover, BlastP searches (http://fungidb.org/fungidb/) failed to identify homologues to SidF or SidA in *A. oryzae*. A BlastP search (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi?organism=fungi) failed to identify homologues to SidF or SidA in *A. oryzae*.

Nevertheless, the evolutionary conservation of peroxisomal localization of siderophore biosynthetic enzymes in *Ascomycetes* indicates its importance. Therefore, it is remarkable that cytosolic mislocalization in *A. nidulans* of all three TFeC biosynthetic enzymes by (i) inactivation of peroxisomal biogenesis (strain pexC::bar), (ii) simultaneous inactivation of both PTS1- and PTS2-dependent transport (strains pexG14/pexE and pexF), or (iii) expression of PTS1-containing Sidl from *N. crassa* in a mutant lacking PTS1-dependent peroxisomal protein import (strain GFP–SidlΔpexE) did not decrease TFeC biosynthesis.

Taken together these data suggest that siderophore biosynthesis can efficiently work in both peroxisomes and the cytosol as long as Sidl, SidH and SidF share the same compartment. In contrast, peroxisomes are essential for biotin biosynthesis in *A. nidulans*, because it has originally been shown that pex mutants defective in PTS1 protein import were found to be auxotrophic for biotin due to an inability to synthesize the intermediate pimelic acid (Hynes et al., 2008). Further studies confirmed peroxisomal localization of BioF, a biotin synthetic enzyme in *A. nidulans* and *A. oryzae* (Magliano et al., 2011; Tanabe et al., 2011). Peroxisomes are indispensable for Ak toxin biosynthesis in the fungal plant pathogen *Alternaria alternata* (Imazaki et al., 2010) and β-oxidation in *A. nidulans* (Hynes et al., 2008). Similarly, cytosolic mislocalization of the single peroxisomal penicillin biosynthetic enzyme completely blocks penicillin biosynthesis in *P. chrysogenum* (Muller et al., 1992). Nevertheless, there are known pathways that are localized in peroxisomes but also work outside. For example, the acyl-CoA transferase IAT (containing a PTS1 motif), which is involved in penicillin biosynthesis, works better in peroxisome but is still functional in the cytosol in *A. nidulans* (Sprote et al., 2009). Moreover, in the glyoxylate cycle both malate synthase (using acetyl-coA and glyoxylate) and isocitrate lyase (producing glyoxylate and succinate) normally operate in peroxisomes, but cytoplasmic localization of both allows growth on acetate (Hynes et al., 2008). These data raise the general question for the rationale of peroxisomal localization of pathways that are also functional outside. A possible explanation is that all the mentioned pathways include CoA-ligands and peroxisomes appear to provide the optimal conditions for this, which might be favoured by the distinct permeability feature of the peroxisomal membrane mentioned above and the high local CoA concentration. Another possible explanation for the siderophore biosynthetic enzymes could be their homology to and evolution from peroxisomal enzymes involved in β-oxidation, e.g. Sidl and SidH display significant similarity to acyl-CoA synthase and enoyl-CoA hydratase (Figs S1 and S2).

Peroxisomes are generated either *de novo* from the endoplasmatic reticulum or by PexK-dependent peroxisomal division (Hettema and Motley, 2009). PexK deficiency (strain ΔpexK) did not affect TAFc production (Fig. 5) which indicates that *de novo* peroxisome biogenesis from the endoplasmatic reticulum is sufficient for TAFc biosynthesis. In contrast, PexK is required for the increase of peroxisomes during growth on fatty acids and optimal β-oxidation (Valenciano et al., 1996) (Hynes et al., 2008). Deficiency in peroxisomal biogenesis, protein import or ATP import did not decrease FC production (strains ΔpexE, ΔpexF23, ΔpexC::bar and ΔpexK). Intriguingly, all investigated deficiencies impairing Sidl localization or activity (pexG14, ΔpexE/pexG14 and antA15) displayed increased FC contents (Fig. 5). Inactivation of Sidl blocks mevalonate consumption for siderophore biosynthesis (Yasmin et al., 2011). It is likely that N-hydroxyornithine, the common precursor for TAFc and FC, and the mevalonate precursor acetyl-CoA is redirected to FC biosynthesis.

Taken together, this study has demonstrated for the first time that particular siderophore biosynthetic enzymes are localized in peroxisomes and that this compartmentalization is evolutionary conserved. Remarkably, the peroxisomal part of siderophore biosynthesis is an...
example of a metabolic pathway that functions as long as all three components are localized in the same compartment independent of peroxisomes, which is indicated by the fact that siderophore production was impaired by cytoplasmic mislocalization of individual enzymes but not the complete loss of peroxisomes. The importance of iron acquisition in the pathogenicity of both plant and animal fungal pathogens has been shown in many studies (Haas et al., 2008). Our results indicate that peroxisomes play a crucial role in the production of virulence-determining siderophores.

Experimental procedures

Strains, oligonucleotides, plasmids, growth conditions

The fungal strains, plasmids and oligonucleotides used in this study are listed in Table S4, Table S5 and Table S6. Gener-ally, A. fumigatus and A. nidulans strains were grown at 37°C in Aspergillus minimal medium according to Pontecorvo et al. (1953) containing 1% glucose as the carbon source and 20 mM glutamine as the nitrogen source. Iron-replete media (+Fe) contained 30 μM FeSO4. For iron-depleted conditions (–Fe), addition of iron was omitted. The final concentration for required supplements was 1 mg l–1 biotin, 4 mg l–1 p-amino benzoic acid, 2.5 mg l–1 pyridoxine, 2.5 mg l–1 riboflavin. The bathophenanthroline disulphonate (BPS) concentration was omitted.

DNA manipulations

For extraction of genomic DNA, mycelia were homogenized and DNA was isolated according to Sambrook et al. (1989). For general DNA propagations Escherichia coli DH5α strain was used as a host.

Generation of fungal strains expressing GFP-tagged versions of the studied enzymes

The sidH (AFUA_3g03410) and sidF (AFUA_3g03400) coding regions including introns were amplified from cosmid psidD-COS as template using primers osidHgfp1 and osidHgfp3 with add-on BgII and NotI sites for sidH and ogfpsidF3 and osidHgfp2 with add-on BamHI and NotI sites for sidF respectively. The PCR products were cloned in frame with the 5-prime preceding GFP-encoding region into the BgII/NotI restriction sites of the plasmid pCAME703-AoHapX-full, replacing the original hapX fusion (Goda et al., 2005). This expression vector for the GFP-fusion protein is driven by a constitutive XAH promoter from Chaetomium gracile. A PTS1 lacking version of SidH was constructed with the primers osidHgfp1 and osidHgfp2 in the same manner. In order to express the sidl orthologue from N. crassa, RNA from mycelia grown for 72 h under iron starvation was transcribed into cDNA (SuperScript II Kit, Invitrogen). This cDNA was then used as a template for PCR-amplification of the N. crassa sidl coding region excluding introns using the primers oNcSidl1-oNcSidl2 containing add-on BgII and /NotI sites. The resulting fragment was cloned into the BgII/NotI sites of the plasmid pCAME703-AoHapX-full resulting in plasmid pGFP–NcSidl. The resulting plasmids pGFP–SidH, pGFP–SidF, pGFP–sidH–PGST1 and pGFP–NcSidl were then used to transform the respective A. fumigatus and A. nidulans strains. Transformation of Aspergillus spp. was carried out as described by Tilburn et al. (1995). The selection was ensured by co-transformation with the plasmid pSK275, which carries the pyrithiamine resistance gene ptrA using 0.1 μg ml–1 pyrithiamine (Sigma). The screening for transformants was performed by PCR (ogfp1/ oAf538RAC1-f for gfp-sidlH and ogfp4/oAf538-AT1-r for gfp-sidF), TAFC production and GFP-fluorescence.

To visualize localization of Sidl, a sidl–gfp fusion encoding a fusion of Sidl C-terminus with the enhanced green fluorescent protein (EGFP) was constructed. Therefore, a 1.2 kb fragment encoding the C-terminal region of Sidl was amplified using oligonucleotides oAfSidl1 and oAfSidl2, thereby replacing the sidl stop codon with a BamHI site. A 2.2 kb Smal–Sacl GFP-encoding fragment was sub-cloned from plasmid pUCGH (Langfelder et al., 2001) into the compatible EcoRv–SacI sites of plasmid pGEM-5z (+) (Promega), yielding plasmid pGfp. The pGfp and the PCR fragment were digested with Sphl and BamHI respectively. Both the insert and the vector were made blunt ended using Klenow fragment and ligated to give plasmid pSidl–Gfp. A 2.2 kb BssHII fragment of ptrA gene from pks275 was inserted into the BssHII site of pGEM-sidl, yielding pSPGfp. pSPGfp was introduced into the wt strain by protoplast transformation. Pyrithiamine-resistant transformants containing the Sidl–GFP in-frame fusion of the sidl and EGFP-encoding genes (sidlgfp strain) were selected and used for the subcellular localization of Sidl. Single homologous recombination was confirmed by Southern blot analysis of Xhol digested DNA.

To gain a plasmid carrying the gene plus promoter encoding the entire Sidl–GFP fusion protein, a 5 kb fragment was amplified from genomic DNA of the sidl–gfp strain by PCR with oligonucleotides oAfSidlF1 and oAfSidlR2 containing add-on EcoRV and ClaI sites respectively. The PCR product was cloned into the EcoRv/ClaI site of the plasmid pphleo. The resulting plasmid pGFP–SidH, pGFP–SidF, pGFP–sidH–PGST1 and pGFP–NcSidl were then used to transform the respective A. fumigatus and A. nidulans strains. Phleomycin-resistant transformants containing the Sidl–GFP in-frame fusion of the sidl and EGFP-encoding genes were selected and used for the subcellular localization of Sidl.

For the colocalization studies of GFP–SidH and RFP–PTS1, the RFP–PTS1-encoding plasmid pSK379–RFP–PTS1 was integrated into the GFP–SidH producing A. fumigatus mutant by co-transformation with the plasmid pphleo, which confers phleomycin resistance. Transformants were screened via resistance to pyrithiamine and phleomycin.

Analysis of siderophores

Analysis of siderophore was carried out by reversed phase HPLC as described previously (Oberegger et al., 2001). To

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quantify extracellular or intracellular siderophores, culture supermutants or cellular extracts were saturated with FeSO₄, and siderophores were extracted with 0.2 volumes of phenol. The phenol phase was separated and subsequent to addition of five volumes of diethyl ether and one volume of water, the siderophore concentration of the aqueous phase was measured photometrically using a molar extinction factor of 2996/440 nm (M⁻¹cm⁻¹) for TAF and 2460/434 nm (M⁻¹cm⁻¹) for FC.

**Fluorescence microscopy**

For confocal microscopy strains were grown in glass bottom dishes (MatTec) with supplemented minimal media for 17 h at 37°C. Images were taken on a confocal laser scanning microscope (SP5, Leica) equipped with a 63×/1.40 oil immersion objective. Z series of optical sections were obtained and projected along the z axis to obtain a general view of the specimen. The acquisition software was LAS AF software (Leica Microsystems). Images were processed using ImageJ (http://rsbweb.nih.gov/ij/).

**Computational analysis**

The following databases were used for gene comparisons, NCBI: http://www.ncbi.nlm.nih.gov/; Broad Institute: http://www.broadinstitute.org/. PTS1 predictions were performed using the general function of the PTS1 Predictor (http://arst.med.mpg.de/pts1/pts1.cgi). PTS2 motifs were identified using a PTS2 finder (http://www.peroxisomedb.org/diy_PTS2.html).

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**Supporting information**

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