Structural Requirements for the Activity of the MirB Ferrisiderophore Transporter of *Aspergillus fumigatus*

Isabelle Raymond-Bouchard, Cassandra S. Carroll, Jason R. Nesbitt, Kevin A. Henry, Linda J. Pinto, Mina Moinzadeh, Jamie K. Scott, and Margo M. Moore

Departments of Biological Sciences and Molecular Biology and Biochemistry and Faculty of Health Sciences, Simon Fraser University, Burnaby, BC, Canada

Siderophores have been identified as virulence factors in the opportunistic fungal pathogen *Aspergillus fumigatus*. The 14-pass transmembrane protein MirB is postulated to function as a siderophore transporter, responsible for uptake of the hydroxamate siderophore *N*,*N*,*N*-triacetylfusarinine C (TAFC). Our aim was to identify amino acids of *A. fumigatus* MirB that are crucial for uptake of TAFC. Site-directed mutagenesis was used to create MirB mutants. Expression of wild-type and mutant proteins in the *Saccharomyces cerevisiae* strain PHY14, which lacks endogenous siderophore transporters, was confirmed by Western blotting. TAFC transport assays using *55*Fe-labeled TAFC and growth assays with Fe-TAFC as the sole iron source identified alanine 125, tyrosine 577, loop 3, and the second half of loop 7 (Loop7Del2) as crucial for function, since their substitution or deletion abrogated uptake completely. Wild-type MirB transported ferriferrocron and coprogen as well as TAFC but not ferrichrysin. MirB was localized by fluorescence microscopy using antisera raised against a MirB extracellular loop peptide. Immunofluorescence microscopy showed that in yeast, wild-type MirB had a punctate distribution under the plasma membrane, as did the A125D and Y577A strains, indicating that the defect in transport of these mutants was unlikely to be due to mislocalization or degradation. MirB immunolocalization in *A. fumigatus* showed that the transporter was found in vesicles which cycled between the cytoplasm and the plasma membrane and was concentrated at the hyphal tips. The location of MirB was not influenced by the presence of the siderophore TAFC but was sensitive to internal iron stores.

Over the past few decades, as the number of immunosuppressed individuals has increased, so too has the number of individuals worldwide infected by fungal pathogens (38). *Aspergillus* spp. are filamentous fungi that are responsible for a variety of respiratory diseases, collectively termed aspergillosis. *Aspergillus fumigatus* is the most common causative agent of human infections linked to *Aspergillus* spp., followed by *A. flavus*, *A. terreus*, *A. niger*, and *A. nidulans* (13).

Aspergillosis can be classified as allergic, saprophytic, or invasive (3, 54). The primary route of infection is through the inhalation of fungal spores. The immune status of the host and the virulence of the strain determine which disease is likely to occur. Allergic *Aspergillus* sinusitis, allergic bronchopulmonary aspergillosis (ABPA), and hypersensitivity pneumonias generally occur in asthma and cystic fibrosis patients and are a result of hypersensitivity to inhaled spores or germlings (3, 13, 55). Saprophytic infections, commonly called aspergillosomas, occur when the fungus invades preexisting cavities in the lungs and forms clumps of mycelia that secrete toxins and allergens (55). Invasive aspergillosis, in which hyphae breach epithelial and endothelial barriers and spread via the bloodstream, occurs in severely immunocompromised individuals, including leukemia patients, solid-organ and bone marrow transplant patients, individuals with genetic immunodeficiencies (such as chronic granulomatous disease), patients with AIDS, and cancer patients undergoing cytotoxic chemotherapy (10, 13).

A number of studies have focused on the role that iron plays in the virulence of *A. fumigatus*. *A. fumigatus* employs both siderophore secretion and ferric reductase activity for iron assimilation; however, it has been shown that survival of *A. fumigatus* in human serum is accompanied by the removal by siderophores of iron from host iron-binding molecules (27). Further studies showed that biosynthesis of siderophores, but not reductive iron assimilation, was required for virulence of *A. fumigatus* in a mouse model of invasive aspergillosis (28, 49). Therefore, in the mammalian host, siderophore secretion is the main mechanism by which *A. fumigatus* obtains the iron required for its growth and survival.

*Aspergillus fumigatus* is known to secrete several hydroxamate siderophores, including ferriferrocron, ferrichrome, ferrichrome C, and *N*,*N*,*N*-triacetylfusarinine C (TAFC). TAFC constitutes 90% of the dry weight of siderophores produced by *A. fumigatus* grown in serum-containing medium (27). Newly synthesized siderophores are secreted in an apo form outside cells, where they can bind ferric iron. Ferrisiderophores are transported back into cells via membrane proteins called siderophore-transporters/receptors. Many bacteria and fungi also possess mechanisms for uptake of exogenous siderophores (“xenosiderophores”) that they themselves do not synthesize. While bacteria and fungi have developed separate and distinct systems for siderophore-iron uptake, less is known about siderophore transport in fungi. In fungi, siderophores are transported across the membrane via transmembrane transporters belonging to the unknown major facilitator (UMF) family, recently renamed the siderophore iron transporter (SIT) family, a subgroup within the major facilitator superfamily (MFS) (19, 46).

Much of our knowledge about fungal siderophore-iron trans-
porter comes from work that has been done with the yeast *Saccharomyces cerevisiae*. *S. cerevisiae* does not synthesize siderophores but is able to take up exogenous xenosiderophores through the use of membrane siderophore-iron transporters. Four siderophore transporters have been characterized in *S. cerevisiae*, two of which have high substrate specificity (19). *Enb1p/Arn4p* is specific for enterobactin, while *Taf1p/Arn3p* transports TAFc (61). The two other siderophore transporters are the ferroxamine and ferrichrome transporters *Sit1p/Arn3p* (40) and *Arn1p*, which transport a number of siderophores (23, 61). *Candida albicans* possesses only one siderophore transporter, *CaArn1p/CaSit1p*, a close homologue of the *S. cerevisiae Sit1p/Arn3p* transporter, responsible for the uptake of several siderophores, including ferrichrome, TAFc, and ferricrocin (24). In the *Neurospora crassa* genome, several homologs of *S. cerevisiae* siderophore transporters have been found (19).

In *Aspergillus nidulans*, MirA, MirB, and MirC have been characterized as siderophore transporters. In a strain of *Saccharomyces cerevisiae* deficient in high-affinity iron uptake and lacking endogenous siderophore transporters, investigation of expression of MirA, MirB, and MirC by use of 55Fe-labeled siderophore uptake assays revealed that in *N. nidulans*, MirA and MirB are enterobactin and TAFc transporters, respectively. Both transporters are highly specific and are functionally similar to the siderophore transporters in *Neurospora crassa* (20). Although MirC shows a high degree of conservation with other siderophore transporters, an exact role in siderophore uptake has yet to be found (20). In *A. nidulans*, expression of siderophore transporter-encoding genes, as with siderophore biosynthesis, is regulated by the transcription factors SreA and HapX (20, 45, 52).

To date, no studies have examined siderophore transporters in *Aspergillus fumigatus*, and very little is known about the steps of ferrated siderophore transport in *A. fumigatus*. The genome of *A. fumigatus* contains seven putative orthologues of siderophore transporter-encoding genes, including *mirB*, *mirC*, *sit1*, and some as yet uncharacterized transporters. Based on their work with *A. nidulans*, Haas et al. (21) hypothesized that MirB mediates siderophore uptake in *Aspergillus fumigatus*; however, this has not been confirmed experimentally to date.

The overall aim of our research was to functionally and structurally characterize siderophore-iron uptake by the transporter MirB in *A. fumigatus*. Specifically, our goals were to identify amino acids that are present in the extracellular loops of MirB and are involved in the transport of ferrated TAFc and to determine how mutations involving selected amino acids affect the function and localization of MirB expressed in yeast.

**MATERIALS AND METHODS**

**Bioinformatic analyses.** Bioinformatic analyses were conducted using the genomic sequence from *A. fumigatus* strain ATCC 13073 acquired from the Entrez gene database (GenEId 3505886, locus AFUA_3G03640) or the protein sequence, acquired from NCBI’s PubMed (PMID 16372009, accession number XP_748684). Information about the genomic sequence of *mirB* was obtained from the *mirB* entry in the Entrez gene database (41). Introns and exons were predicted using GeneWise (6). The ProtParam tool (17) was used to determine the molecular weight and theoretical isoelectric point of MirB. WoLF PSORT (29) was used to predict the cellular localization of MirB and to detect the presence of signal peptides. HMMTOP 2.0 (50) and SOSUI 1.11 (25) were used to model the expected membrane topology and two-dimensional (2D) structure of MirB.

Conserved domains in MirB were predicted using the Conserved Domain Database (CDD) (42) and search tool from the NCBI (39). Default settings were used (max hit = 100, e-value = 0.01, with low-complexity filtering). Functional information about the Gene Ontology website (4) using the MirB protein sequence page from the NCBI database (accession number XP_748684). Selected programs were also used to identify possible glycosylation sites (NetNGLyc, OPAL, NetOGlyc, and YinOYang) (16, 18, 33).

Multiple sequence alignments were carried out using annotated fungal siderophore transporters from *Aspergillus nidulans* (MirB, MirA, MirC), *Schizosaccharomyces pombe* (Str1 to -3), *Saccharomyces cerevisiae* (Arn1 to -4), and *Candida albicans* (Arn1); putative TAFc transporters in *Aspergillus clavatus*, *Aspergillus terreus*, and *Neosartorya fischeri*; and other putative siderophore transporters in *Coccidioides immitis*, *Neosartorya fischeri*, *Aspergillus clavatus*, *Neurospora crassa*, and *Cryptococcus neoformans*. All sequences were obtained from the NCBI, and alignments were carried out using ClustalX 1.83 (12).

**Growth conditions.** *Saccharomyces cerevisiae* strain PHY14 (gift of Joachim F. Ernst, Heinrich-Heine-Universität, Düsseldorf, Germany) and strain DEY1394 (gift of David Eide University of Wisconsin, Madison) were cultured on YPAD (0.0075% adenine hemisulfate, 1% yeast extract, 2% Bacto agar, dhO4) at 30°C. DEY1394 is the parent strain of PHY14-URA, which is deficient in endogenous and xenosiderophore transporters as well as iron permease. Transform- phy14 and DEY1394 strains were cultured on synthetic dextrose (SD) agar (0.67% yeast nitrogen base without amino acids, 2% dextrose, 1.3 g amino acid dropout powder without uracil, 2% Bacto agar, 1 liter dhO4) at 30°C. Uracil was omitted for selection of transformants. Liquid cultures were grown in synthetic raffinose (SR) (0.67% yeast nitrogen base without amino acids, 2% raffinose, 1.3 g amino acid dropout powder without uracil, 2% Bacto agar, 1 liter dhO4) or SR supplemented with 0.5% galactose (SR+gal) to induce expression of the recombinant protein. For a description of the *S. cerevisiae* strains used in this study, refer to Table 1.

*Aspergillus fumigatus* strain ATCC 13073 (wild type), and the non-siderophore-producing mutant ATCC 13073 ΔsidA were cultured on YM broth (3 g malt extract, 3 g yeast extract, 5 g Bacto peptone, 10 g glucose, 1 liter H2O), potato dextrose agar (PDA), or PDA spread with a solution of ferricrocin (10 pmol/10-cm-diameter plate) at 37°C until sporulation occurred. Conidiospores were harvested into phosphate-buffered saline (PBS) (0.05%) Tween 20, washed with water and stored at −20°C until needed. Liquid cultures used for RNA extraction were grown in modified Grimm–Allen medium (47) without iron, overnight at 37°C; cultures used for immunofluorescent microscopy were grown in the same medium supplemented with FeSO4, as described below.

**Construction of pESC-mirB and creation of mirB mutant sequences.** RNA was extracted from wild-type *A. fumigatus* using the RNeasy plant extraction kit (Qiagen), and cDNA was synthesized with Superscript II reverse transcriptase (RT) (Invitrogen) and the primer Spel-EscmirBrev. mirB was amplified by Accuprime Taq high fidelity (Invitrogen) using NotI-EscmirBfor and Spel-EscmirBrev primers (Table 2). The PCR product was cloned into the yeast expression vector pESC-URA (Agilent Technologies) under the control of the GAL10 promoter and in frame with the C-terminal FLAG epitope. The resulting pESC-mirB vector was transformed into competent *Escherichia coli*, and the presence of the *mirB* insert was confirmed by sequencing (Macrogen, South Korea).

The QuikChange Lightning mutagenesis kit (Agilent Technologies) was used for site-directed mutagenesis of the MirB wild-type gene (MirBWT gene). Primers were designed using the QuikChange primer design program (Agilent Technologies) (Table 2). Yeast codon usage bias was taken into account when we selected the appropriate codons. Mutated plasmids were transformed into *Escherichia coli* DH5α and confirmed by sequencing.

**Transformation of Saccharomyces cerevisiae.** All wild-type and mutant pESC-mirB vectors were transformed into PHY14, a strain of *Saccha-
**TABLE 1 Saccharomyces cerevisiae** strains and plasmids used in this study

| Strain or plasmid | Genotype or description | Reference |
|------------------|-------------------------|-----------|
| pESC-URA         | Yeast expression vector with Gal10 promoter and FLAG epitope | Stratagene |
| pESC-mirBWT      | 1.8-kb NotI-SpeI mirB cDNA fragment in pESC-URA | This study |
| pESC-mirB/N-termDel | N-terminal deletion of first 12 amino acids (following Met) | This study |
| pESC-mirB/A125D  | Alanine to aspartic acid at position 125, loop 1 | This study |
| pESC-mirB/F129D  | Phenylalanine to aspartic acid substitution at position 129, loop 1 | This study |
| pESC-mirB/W251/253A | Tryptophan to alanine double substitution at positions 251 and 253, loop 3 | This study |
| pESC-mirB/Loop3Del | Loop 3 deletion of amino acids 247–256 | This study |
| pESC-mirB/Y398A  | Tyrosine toalanine substitution at position 398, loop 5 | This study |
| pESC-mirB/Loop6Del | Loop 6 deletion of amino acids 467–474 | This study |
| pESC-mirB/R570A  | Arginine to alanine substitution at position 570, loop 7 | This study |
| pESC-mirB/Y577A  | Tyrosine to alanine substitution at position 577, loop 7 | This study |
| pESC-mirB/Loop7Del1 | Loop 7 deletion of amino acids 533–544 | This study |
| pESC-mirB/Loop7Del2 | Loop 7 deletion of amino acids 566–580 | This study |
| pESC-mirB/C-termDel | C-terminal deletion of last 5 amino acids | This study |

**TABLE 2 Primers used for the creation of pESC-mirB and mutant plasmids**

| Primer name | Primer sequence (5’ to 3’) |
|-------------|----------------------------|
| NotIp-ESCmirBfor | ATTGGGCCCCATGTCTTCCAGCTTTATTCGAG |
| SpeIp-ESCmirBrev | CCGGAGTAGCCACATATTCTGGTG |
| mirR-R570AFor | GTCGGAGATGCTACCGCTACCGCATGCGGTAG |
| mirR-R570ARev | CTCCACCGTGAGTGATAGCACTCCAGAC |
| mirY577AFor | CGCCACATCGTGAGGAGCCAGGGTGATAGCC |
| mirY577ARev | GGGCCATACCGTCCGAGGCGGTAGGAGG |
| mirA125DFor | CAAGCTTGAACGCTTATGACACAAGTTCTTTCCTCGG |
| mirA125DRev | CCGAGGAAAAGAATCCTGTGTAACAGGTTGTCAGTTGGT |
| mirY398AFor | CTCACTACTGTATGCGCCAGGCTTTCGAGTGGAC |
| mirY398ARev | CTGCAAGAACGCAGGCGGCGGCGCTGGAGTGGCCAGG |
| mirW251AFor | GTCTGAGCGCCGCGGGCGGCTGGAGTGGCCAGG |
| mirW251ARev | GGGGCGGCGATGGCGGTATTCGAG |
| mirN-termDelF | CGCAGTAACAGGCGGAGCGGGAG |
| mirN-termDelR | CTGAAAGACGCACTGACAGGCGGAGG |
| mirC-termDelF | GTGAAGAACGTACGTAGCAAGGGCGAGG |
| mirC-termDelR | GTGAAAGGGTCGAGGCACTGACAGGCGGAGG |
| MirRF129D-F | TGGCGGTGGTCACATGCGGATAGCGAGGC |
| MirRF129D-R | GGGGCGGCGATGGCGGTATTCGAG |
| MirR-loop3Del-F | GATGGCGCCGACATCGCAGGAGAGAGCGAG |
| MirR-loop3Del-R | GTTCCACCTTGTGGAGGAGAGAGAGCGAG |
| MirR-loop6Del-F | AGAAGAAAAAGTCTGAGGCGGAGG |
| MirR-loop6Del-R | GTTCCACCTTGTGGAGGAGAGAGAGCGAG |
| MirR-loop7-1597Del-F | GCCGAGGAAAAGAATCCTGTGTAACAGGTTGTCAGTTGGT |
| MirR-loop7-1597Del-R | GTGAAAGACGCACTGACAGGCGGAGG |
| MirR-loop7-1696Del-F | GTGAAAGAAAAGTCTGAGGCACTGACAGGCGGAGG |
| MirR-loop7-1696Del-R | GTGAAAGAAAAGTCTGAGGCACTGACAGGCGGAGG |
type (WT) A. fumigatus grown in modified Grimm-Allen medium with- out iron. The supernatants were centrifuged where appropriate, extracted with Amberlite 16 (Supelco), and purified by silica gel chromatography (27). Coprogen and ferrichrysin were gifts from Alison Hadwin, Simon Fraser University, Burnaby, Canada. All siderophores were in their fer- rated forms unless otherwise stated. **Growth assays; yeast expressing MirB constructs.** Induced MirBWT gene-transformed yeast cells (MirBWT) were used to inoculate a 96-well plate containing SR-gal (0.25%) plus 250 μM bathophenanthroline sul- fonate (BPS). Siderophores (TAFc, ferricrocin, ferrichrysin, and copro- gen; purity, >95%) were added to a final concentration of 10 μM. Inoc- ulum was added to an optical density at 620 nm (OD620) of 0.2. Controls included wells with no additions, wells with 10 μM FeCl3, and wells with- out BPS. Plates were incubated at 30°C with gentle shaking for 17.5 h, and absorbance at 620 nm was recorded.

Growth rate comparisons were done for the mutant strains relative to MirBWT in the presence and absence of TAFc. Yeast strains were grown overnight in SD, and cells were counted with a hemocytometer and di- luted to 109, 108, 107, and 106 cells/ml in 96-well plates. Cells were trans- ferred to agar plates containing SR-gal with or without BPS (500 μM) using a 48-pin transfer tool. To test the ability to use TAFc as an iron source, SR-gal plus BPS plates were prepared with a 5-mL overlay con- taining 150 μM TAFc.

**[55Fe]TAFc uptake assays.** [55Fe]TAFc was prepared by incubating 41 nmol of [55Fe]Cl3 with 50 nmol desferri-TAFc at room temperature for 2 h. Formation of [55Fe]TAFc was confirmed by radio-thin-layer chromatography (mobile phase: 80% methanol in D2O). For the [55Fe]TAFc uptake assay, 2 μM [55Fe]TAFc was added to induced yeast cells, followed by incubation at 30°C for 60 min in SR in the presence of 100 μM BPS. Cells were collected by filtration onto glass microfiber filters (Ahlstrom) and washed 4 times with assay buffers 1 (2% raffinose, 50 mM sodium citrate, 10 μM nonradioactive TAFc) and 2 (2% raffinose, 50 mM sodium citrate, 200 μM BPS, 5% BSA), and the bound radioactivity was determined by liquid scintillation counting (Beckman Coulter model LS6500). Statistical analysis was conducted using GraphPad Prism 5.

**Immunogen preparation and animal immunizations.** A peptide cor- responding to the N terminus of the MirB loop 7 (peptide 7a; acetyl [Ac]-NH2-STFLSLRNLNPSAMPDLCLCONH2) (see Fig. S1 in the supplemental material) was synthesized (EZBioLab, Carmel, IN); the peptide was >95% pure. The peptide was conjugated to a Δ3 filamentous bacteriophage (57) using the heterobifunctional cross-linking agent N-sucinimidyl-3-(2-pyridyldithio)propionate (SPDP) (Pierce, Rock- ford, IL). Briefly, 7.08 × 1013 particles were added to 1 mM EDTA. The reaction was quenched with 2 ml 1 M Tris-HCl, pH 8.3, and then phage were pelleted by ultracentrifugation at 556,200 × g for 4 h, washed to remove unreacted cross-linker, and resuspended in 10 ml PBS containing 1 mM EDTA. Peptide 7a (1.16 mg) was reacted with the SPDP-acti- vated phage for 2 days at 4°C with tumbbling, and the final conjugate was puriﬁed by two rounds of polyethylene glycol (PEG)/NaCl precipitation (7) followed by ultracentrifugation. Conjugation was assessed by moni- toring shifts in the electrophoretic mobility of the phage major coat pro- tein by SDS-PAGE and Western blotting with antiphage antibody (data not shown).

Groups of 6- to 8-week-old BALB/c mice (n = 5; Charles River, Saint- Constant, Quebec, Canada) were immunized subcutaneously in the hind flank with 100 μl PBS containing 3.4 × 1011 phage particles (conjugated or nonconjugated) (10 μg phage protein). Immunizations and blood col- lection via the saphenous vein were performed at weeks 0, 2, 4, 6, 8, 10, and 12; final bleeds and euthanasia were performed at week 14 by cardiac puncture under CO2 anesthesia. Three days before euthanasia, animals were given a final boosting immunization. Blood was allowed to clot at room temperature for 24 h, and then sera were collected by centrifugation and stored at −20°C. All protocols involving mice were approved by the Simon Fraser University (SFU) Animal Care Committee and were in compliance with the Canadian Council on Animal Care.

Enzyme-linked immunosorbent assays (ELISAs) were carried out by coating wells of microtiter plates overnight at 4°C with 2 × 1011 phage particles or 200 ng peptide in 35 μl TBS. After blocking at 37°C for 1 h with 200 μl TBS containing 2% weight per volume (wt/vol) BSA, wells were washed 3 times with TBSB, and then 5 μl of diluted sera was added for 2 h at room temperature. Wells were washed 6 times with TBSB, incubated with 35 μl HRP-conjugated goat anti-mouse IgG (diluted 1:1,500) for 45 min at room temperature, and then detected with 35 μl citrate-buffer solution containing ABTS and H2O2. The OD405/690 of each well was recorded, and data from each titration were analyzed to give the dilution factor corresponding to the half-maximal OD reading. All sera and anti- bodies were diluted in TBSB containing 1% BSA (see Fig. S2 in the supplemental material).

**Dot blot analysis of antipeptide antibodies.** TCA precipitates were prepared from the yeast strains MirBWT and PHY14-URA grown in SR overnight followed by MirB induction in SR-gal for 5 h. Total yeast protein (35 μg) or peptide 7a (10 μg) was spotted onto a nitrocellulose membrane and dried for 30 min at room temperature. Blots were blocked in 3% BSA in TBSB for 2 h and incubated overnight at room temperature with primary antibody (1:2,500 or 1:5,000 dilution of final bleed serum from mouse 3 immunized with Δ3/7a conjugate). Preimmune serum was used as a control. Blots were washed 3 times in TBSB and incubated at room temperature with a 1:10,000 dilution of a goat anti-mouse secondary antibody conjugated to HRP (Jackson Immunoresearch Laborato- ries, Inc.). Unbound secondary antibody was removed by washing 3 times in TBSB, followed by addition of SuperSignal West Pico and exposure to film (described above) (see Fig. S3 in the supplemental material).

**Immunofluorescence microscopy.** (i) *S. cerevisiae*. Slides for immu- nofluorescence microscopy were prepared as described in reference 53 with some modifications. Induced cells were centrifuged at 800 g, washed, resuspended in SR-gal medium, and fixed with 4% formalde- hyde for 30 min. Fixed cells were washed with 0.1 mM potassium phosphate (pH 6.5) and then with P solution (0.1 M potassium phosphate [pH 6.5], 1.2 M sorbitol), and spheroplasts were made by adding 15 μl of 10 mg/ml zymolyase (US Biological) in P solution. Spheroplast formation was fol- lowed microscopically. Spheroplasts were washed with P solution and applied to 0.1% poly-l-lysine-coated slides, and the adherent spheroplasts were immersed in ice-cold methanol for 5 min followed by 30 s in ice-cold acetone. Slides were washed once with PBS-BSA followed by overnight incubation with mouse anti-peptide 7a serum, diluted 1:500 in PBS- BSA. In some samples, preimmune serum from the same mouse (1:500) was used as a control. Slides were washed with PBS-BSA, followed by addition of Alexa Fluor 488-conjugated anti-mouse antibodies (Invitro- gen), diluted 1:1,000 in PBS-BSA. After incubation for 3 h in the dark, slides were washed 3 times in PBS-BSA and once with PBS. DNA was stained by adding DAPI (4′,6-diamidino-2-phenylindole) (1 μg/ml in PBS) for 5 min. Slides were washed with PBS and mounted in ProLong Gold antifade reagent (Invitrogen). Negative controls consisted of PHY14-URA and MirBWT incubated without primary antibody. Images were collected using a WaveFX spinning disc confocal system (Quorum Technologies) and a Zeiss Axio Observer inverted microscope. Image acqui- sition and processing were performed using Velocity software (PerkinElmer). All fluorescence images were collected using the same ex- posure and sensitivity settings.

(ii) **A. fumigatus.** Wild-type *A. fumigatus conidia* (106) were seeded onto sterile cover slips in 5 ml liquid modiﬁed Grimm-Allen medium (47) with 0 or 500 μM FeSO4 and grown to germings at 37°C for 8 or 11 h. Spores from the ΔsidA strain were grown for 15 h at 37°C in modiﬁed Grimm-Allen medium with 10 μM FeSO4, at which time various combina- tions of FeSO4 (to 500 μM) or TAFc (to 0.1 μM) were added. After 2 h of further incubation, cover slips were removed and ﬁxed. Alternatively, the ΔsidA strain was grown on PDA with an overlay of ferrirocinn (10 pmol/100 mm plate) to produce spores preloaded with ferrirocinn and
grown as above. All coverslips were processed according to Kaminskyj (34) using a PIPES [piperazine-N,N'-bis(2-ethanesulfonic acid)]-based buffer containing EGTA to preserve microtubule ultrastructure. Briefly, cells were fixed in freshly prepared 3.7% paraformaldehyde for 40 min and washed 3 times with buffer. The cell walls were partially solubilized with 50 mg/ml Vinotaste (Gusmer Enterprises) plus 50 mg/ml BSA for 15 min at 30°C. The cell membranes were permeabilized with 0.01% Triton X-100 and blocked with 5% BSA plus 2% normal goat serum plus 0.1% Tween 20 in buffer. Primary antibody, identical to that used for the yeast immunofluorescence experiments, was diluted 1:300 in blocking buffer and incubated with the cells for 2 h at 20°C, and the coverslips were washed in buffer containing 0.1% Tween 20. The secondary antibody, Alexa Fluor 594 goat anti-mouse IgG (Invitrogen), was used at a dilution of 1:500 in 10% blocking buffer. Coverslips were mounted in Prolong Gold (Invitrogen) and stored in the dark at 4°C. Slides were examined on a Zeiss Axioskop 2 plus microscope equipped with a Colibri light-emitting diode (LED) fluorescent light source at 590 nm. Images were recorded on an AxioCam MRm camera using AxioVision 4.7.1 software.

RESULTS

MirB bioinformatics analysis. The Entrez gene database entry for A. fumigatus mirB reports that the gene is 2,079 bp, is located on chromosome 3, and contains 3 introns and 4 exons. GeneWise (6) confirmed the presence and locations of these introns: 291 to 389 bp (99 bp), 661 to 721 bp (61 bp), and 1,068 to 1,147 bp (80 bp). No untranslated regions at either the 3’ or 5’ end were identified. Two possible SREA binding sites (GATA sites) were found in the 855-bp promoter region of mirB (at −845 bp and −34 bp, respectively, from the start codon). We used ProtParam (17) to calculate the molecular mass of MirB (612 amino acids) as 66.85 kDa and found the theoretical pI to be 7.14. WoLF PSORT (29) predicted the localization of MirB to be in the plasma membrane. No known signal peptides were found using WoLF PSORT, although the C-terminus does contain a KXXX-like motif (TKGNg), a possible endoplasmic reticulum (ER) membrane retention signal (58).

Membrane topology analysis carried out by HMMTOP (56) and SOSUI (26) supported the membrane prediction of WoLF PSORT. Both programs predicted MirB to be a transmembrane protein with 14 transmembrane α-helices connected by loops, with cytosolic N and C termini; no β-strands were identified. Figure 1 was created by SOSUI and shows the 2D structure and predicted 2D membrane topology of A. fumigatus MirB.

Four different conserved domains were found using an NCBI conserved domain search, TRI12, MFS-1, KOG0254, and KOG0255; all four correspond to transporters that are members of the major facilitator superfamily (MFS). The function of MirB as suggested by gene ontology is that of a membrane-bound siderochrome-iron (ferrioxamine) uptake transporter. Such proteins operate as symporters, catalyzing the transfer of a solute from one side of a membrane to the other using a proton gradient. Multiple sequence alignment of the C-terminal region of MirB with various characterized or putative fungal siderophore transporters revealed numerous conserved amino acids (see Fig. S1 in the supplemental material).

The data gathered from the bioinformatics analysis of MirB, including the predicted membrane localization with 14 transmembrane domains, the presence of conserved domains belonging to members of the MFS, and the similarity to A. nidulans MirB, support the hypothesis that A. fumigatus MirB functions as a siderophore transporter. Because of its close relation to MirB from A. nidulans, it is also likely that A. fumigatus MirB is responsible for uptake of the ferrated siderophore TAFc.

The model of MirB has seven extracellular loops, which contain several conserved amino acids determined from alignment of selected fungal siderophore transporters (Fig. 1; see also Fig. S1 in the supplemental material). We hypothesized that these residues have a role in recognition and binding of ferrated TAFc; therefore, we targeted seven conserved amino acids for mutation. In addition, to study the importance of the loops themselves in uptake, we also created deletion mutants, targeting 7 to 14 amino acids, of loops 3, 6, and 7 (Fig. 1). Due to the length of the seventh loop, two different deletion mutants were created, targeting opposite ends of the loop. For all of the loop deletion mutants, care was taken to leave a few amino acids in the loop so as to, in theory, allow for proper looping and reinsertion in the membrane. We also created N- and C-terminal deletion mutants to identify potential sorting signals. Figure 1 and Tables 1 and 2 list the mutant strains that were constructed.

Creation of the pESC-mirB vector and mirB mutants. Sequencing of the mirB insert showed differences in the amplified cDNA sequences (from strain ATCC 13073) compared to the published putative mRNA sequence from the Af293 strain. Plas-
mids isolated from several colonies were sequenced, and in each case, mirB was found to contain an additional 18 bases (GTACG CAATTCTAGTGCT) starting at position 291. In the current annotated putative mRNA sequence, these bases are predicted to be part of the first intron. However, based on the sequencing results, these bases appear to be a part of the mRNA sequence of mirB, and result in an extra 6 amino acids in the protein sequence (YAILVL). Sequencing was repeated with newly synthesized cDNA to ensure that this was not the result of an error during the original reverse transcription (RT)-PCR. The same result was obtained. Hence, we concluded that, at least in strain ATCC 13073, MirB comprises 618 amino acids, not 612.

In addition, the mirB gene from A. fumigatus strain ATCC 13073 contains five strain-specific base changes from that of the published sequence, obtained from strain Af293; however, these base changes do not result in any amino acid changes (data not shown).

Expression of mirB in S. cerevisiae. All MirB proteins were C-terminally tagged with the FLAG protein epitope; therefore, to test for the presence of the MirB proteins, Western blotting of membrane proteins was performed using anti-FLAG antibodies. Lysates from MirBWT and all mutant strains but not from PHY14-URA showed bands of the expected size of ~69 kDa (Fig. 2). Cells transformed with pESC-URA containing MirBWT and the A125D, Y398A, W251/253A, R570A, Y577A, and C-terminal and N-terminal deletion mutants all had comparable expression levels, whereas the expression levels of F129D and the remaining deletion mutants appeared to be somewhat lower. Nevertheless, for all constructs, a protein of the expected size was detected in yeast lysates.

Growth assays on iron-limited and TACF-supplemented media. First, we determined the ability of yeast expressing MirBWT to grow in iron-limited medium supplemented with one of four different hydroxamate siderophores. In addition to N,N’N’-triacetylflusarinine C, the major siderophore secreted by A. fumigatus, we compared the growth levels of yeast in medium supplemented with ferricrocin and ferrichrysin or coprogen. Only TACF and ferricrocin have been detected in A. fumigatus. Figures 3A and B show that in addition to TACF, growth was stimulated by the addition of ferricrocin and coprogen but not ferrichrysin, suggesting that MirB is capable of transporting hydroxamate siderophores other than TACF.

Results from inoculation of control plates can be found in Fig. 4B and C. All strains showed approximately equal levels of growth on SR+gal- only plates (Fig. 4B) and, as expected, no growth on medium with low iron containing SR+gal with 500 μM BPS (Fig. 4C). Figure 4A shows that on SR+gal-plus-BPS medium containing Fe-TACF, the C-termDel, F129D, Y398A, N-terminal deletion (N-termDel), and R570A mutants showed growth comparable to MirBWT, whereas the A125D, loop 7 deletion (Loop7Del2), and Loop3Del mutants showed growth only at the highest cell concent-

FIG 2 Western blot of yeast lysates from cells expressing the MirB-FLAG constructs. For each yeast sample, the same amount of protein was loaded on the gel (40 μg). Strain labels are described in Table 1. The mock-transformed control (PHY14-URA) and the FLAG-tagged control protein (BAP) are also shown. MirB-FLAG is 69 kDa, and FLAG-BAP is 49 kDa.

FIG 3 Various hydroxamate siderophores support the growth of PHY14-URA expressing the MirBWT gene. Yeast cells were grown and induced with galactose as described in Materials and Methods. Yeast cells were inoculated into SR+gal with or without a ferrous iron chelator (BPS). Ferric iron was added to samples as ferrated TACF, ferricrocin, ferrichrysin, or coprogen (purity, >95%) or as FeCl₃ in a final concentration of 10 μM, and growth was monitored by absorbance at 620 nm. (A) One-way ANOVA showed significant variation among the means, and all pairs were compared using the Tukey-Kramer honestly significant difference test (HSD) in JMP8. Samples with different letters were significantly different at a P value of <0.05. (B) One-way ANOVA showed significant variation among the means; all pairs were compared using Tukey-Kramer HSD. Groups were significantly different at a P value of <0.01.
The Loop7Del1 mutant grew as well as MirBWT, while the Y577A mutant did not show growth above that of PHY14-URA. The W251/253A and Loop6Del mutants had growth levels between those of PHY14-URA and MirBWT. These strains grew at $10^6$ cells/ml, unlike PHY14-URA, but growth at this concentration was less pronounced than that of MirBWT.

**Iron uptake assays.** $[^55Fe]$TAFC uptake assays were performed for MirBWT and all MirB mutant strains, as well as for PHY14-URA (Fig. 5). A one-way analysis of variance (ANOVA) conducted using rates from all MirB strains and PHY14-URA showed the means to be significantly different ($P < 0.05$). Using Dunnett’s multiple comparison test ($P < 0.05$), we determined statistically significant differences between groups. The C-termDel and F129D mutants were found to have uptake rates similar to those for MirBWT. Uptake rates for the Y398A mutant were signifi-
cantly different from those for both PHY14-URA and MirBWT. Because of the high variation observed in N-termDel and F129D mutant counts, these strains were not included in the statistical analysis. However, it is clear from the results that the uptake rate of the N-termDel strain was higher than that of the wild-type MirB. None of the other strains differed significantly from the negative control, PHY14-URA (Fig. 5).

**Immunofluorescence localization of \textit{A. fumigatus} MirB expressed in yeast.** To localize MirB within \textit{S. cerevisiae}, immunofluorescence microscopy was performed. Figure 6 shows the punctate staining of wild-type MirB under the plasma membrane in recombinant yeast cells, which was not present in the negative-control samples, PHY14-URA, or in yeast cells expressing MirBWT incubated with preimmune serum (Fig. 6).

The expression and localization of MirB in selected mutant strains were also studied, and Fig. 6 shows that the A125D and Y577 mutants had a distribution and intensity of staining comparable to strains expressing WT MirB. In contrast, the N-terminal deletion mutant had a similar intensity but a different pattern of staining, with a larger number of smaller vesicles than that of the wild-type MirB. Although the distribution of green fluorescence in the Loop7Del2 mutant and loop 3 deletion mutants had punctate features similar to the wild-type MirB, the stain intensity was lower and the amount of diffuse cytoplasmic staining appeared to be greater, particularly in the yeast expressing the loop 3 deletion mutant.

**Immunofluorescence localization of endogenous MirB in \textit{Aspergillus fumigatus}.** Using the anti-peptide 7a antisera raised in mice, we also examined the distribution of endogenous MirB in \textit{A. fumigatus} during different developmental stages. Figure 7 shows that in low-iron medium, MirB was expressed in conidia and that the protein is found at or near the plasma membrane in vesicles or aggregates (Fig. 8B and inset). In germlings, the staining is increased at the hyphal tips. As hyphae developed in low-iron conditions (11 h postinoculation), MirB was concentrated at the hyphal tips, although the protein was distributed along the hyphal length at a lower concentration (Fig. 8C and inset). As expected, addition of FeSO$_4$ (500 μM) to the growth medium decreased the intensity of staining, although a few hyphal tips were still positive (Fig. 8D).

To establish whether the presence of TAFC influenced the distribution of MirB, we used a siderophore-deficient strain of \textit{A. fumigatus} that has a mutation in \textit{sidA}, the gene encoding the first step of TAFC and ferricrocin biosynthesis (28). Addition of TAFC to the Δ\textit{sidA} mutant incubated in low iron (10 μM) had little
membrane and hyphal tips than in the parental strain (Fig. 8B and C). The expression level of MirB in the \( \Delta \text{sidA} \) mutant was significantly higher for all treatments than in the wild type. When we used identical imaging procedures, exposure times had to be reduced 5-fold to prevent overexposure of the fluorescent images. In panel A, cells were grown and processed under the same conditions as in panel E except that samples were incubated with preimmune serum.

effect on growth (Fig. 8B versus C), whereas TAFC addition in high-iron concentrations (500 \( \mu \)M) markedly stimulated hyphal growth (Fig. 8D versus E). The expression level of MirB in the \( \Delta \text{sidA} \) mutant was significantly higher for all treatments than in the wild type. When we used identical imaging procedures, exposure times had to be reduced 5-fold to prevent overexposure of the fluorescent pictures for the \( \Delta \text{sidA} \) strain. Interestingly, in the samples incubated in 10 \( \mu \)M iron, MirB was more uniformly distributed in the cytoplasm and less concentrated in the plasma membrane and hyphal tips than in the parental strain (Fig. 8B and C compared with Fig. 7B and C), but addition of TAFC had no apparent effect on MirB distribution. When the \( \Delta \text{sidA} \) strain was incubated for 2 h in high iron (500 \( \mu \)M), the amount of MirB was reduced but the cytosolic distribution remained. Addition of TAFC had no dramatic effect (Fig. 8).

The \( \Delta \text{sidA} \) mutant is not only TAFC deficient, it is also unable to synthesize ferricrocin, a siderophore that binds intracellular iron. The absence of ferricrocin biosynthesis is known to increase sensitivity to oxidative challenge, likely due to higher free iron levels in the cytoplasm (15). To determine whether the more diffuse pattern of MirB staining in the \( \Delta \text{sidA} \) mutant was due to higher intracellular iron levels, we grew the \( \Delta \text{sidA} \) mutant in iron-free modified Grimm-Allen medium and grown for 15 h at 37°C. Cultures were then incubated for an additional 2 h in the presence or absence of TAFC (100 nM) or 500 \( \mu \)M FeSO\(_4\). Cultures were harvested at 17 h of incubation and coverslips were fixed and stained. MirB was localized similarly to the parent strain, i.e., primarily in the plasma membrane during conidial swelling and concentrated at hyphal tips at later stages of growth. Addition of 500 \( \mu \)M iron completely suppressed any signal from MirB. In conclusion, we found that MirB was localized at the plasma membrane when intracellular iron levels were low (high iron storage capacity) and iron levels in the medium were low. No effect of TAFC on the distribution of MirB was observed with either the parental or \( \Delta \text{sidA} \) strains.

**DISCUSSION**

MFS permeases transport a wide variety of compounds, such as organic anions/cations, simple sugars, oligosaccharides, amino acids, and nucleosides. SIT transporters typically have 14 transmembrane \( \alpha \)-helices and are believed to function as substrate symporters, using energy derived from the plasma membrane potential to transport the siderophore-iron complex (59). While no crystal structures of fungal siderophore transporters exist, crystal structures of other MFS transporters have been determined, including the *E. coli* lactose symporter LacY and the glycero-3-phosphate antiporter, among others (1, 14, 25, 31, 60). All of these proteins share a remarkably similar structure regardless of their individual functions as symporters, uniporters, or antiporters (39).

Two previously characterized siderophore transporters, *A. niger* MirB and *S. cerevisiae* Arn2p, transport TAFC exclusively (20, 61). Based on the results of our study, MirB from *Aspergillus fumigatus* is capable of transporting coprogen and ferricrocin as well as TAFC. The 3D structure of TAFC is composed of three identical molecules of \( N^2 \)-acetyl-\( N^5 \)-cis-anhydromevalonyl-\( N^4 \)hydroxy-L-ornithine bound together via ester bonds (30). The iron-chelating center has three five-membered rings formed by

---

**FIG 8** Immunolocalization of MirB in a siderophore-deficient strain of *A. fumigatus*: effect of TAFC. *A. fumigatus* ATCC 13073 \( \Delta \text{sidA} \) was grown on modified Grimm-Allen medium plus 10 \( \mu \)M FeSO\(_4\) for 15 h at 37°C followed by 2 h of incubation with or without TAFC (0.1 \( \mu \)M) in low (10 \( \mu \)M) or high (500 \( \mu \)M) FeSO\(_4\). MirB was detected using preimmune sera (A) or antisera raised in mouse against peptide 7a (B through E). Insets in panels B through E show details of the fluorescent images. In panel A, cells were grown and processed under the same conditions as in panel E except that samples were incubated with preimmune serum.

**FIG 9** Addition of the iron storage siderophore, ferricrocin, to the \( \Delta \text{sidA} \) strain results in wild-type MirB localization. *A. fumigatus* \( \Delta \text{sidA} \) was grown on PDA supplemented with ferricrocin, and conidia were transferred to iron-free modified Grimm-Allen medium and grown for 15 h at 37°C. Cultures were then incubated for an additional 2 h in the presence or absence of TAFC (100 nM) or 500 \( \mu \)M FeSO\(_4\). Cultures were harvested at 17 h of incubation and coverslips were fixed and stained.
the hydroxamate groups of the \(N^2\)-acyl-\(N^2\)-hydroxy-L-ornithines. The three ester and acetyl groups extend out toward the outside of the molecule and are likely to be involved in hydrogen bonding with more polar amino acids. However, with the exception of these groups, the remainder of the molecule is hydrophobic. Both TAFC and ferricrocin share the same basic core structure of \(N^2\)-acyl-\(N^2\)-hydroxyornithine, and both are secreted by \textit{A. fumigatus} in iron-limiting conditions (27), although ferricrocin functions primarily as an iron storage molecule (15, 50). Like ferric-TAFC, ferricropin is an ester-linked trihydroxamate siderophore that in solution has primarily a cis-conformation around the iron center. However, Adjimani and Emery (2) showed that only the A-isomer of TAFC was actively taken up by the fungus \textit{Mycella sterilis}. Ferricrocin has a predominantly A-cis configuration (32). Broad substrate specificity of fungal siderophore receptors has been found in the yeast \textit{S. cerevisiae}, where both \textit{Arn1p} and \textit{Arn3p} have been shown to transport coprogen as well as ferrichromes (61). In \textit{Neurospora crassa}, competitive inhibition of coprogen uptake by ferrichrome siderophores was demonstrated by Huschka et al. (32). These data suggest that receptor recognition does not depend on the amino acid linkages; TAFC and coprogen are ester linked whereas ferricrocin has peptide bonds. The data also suggest that the receptor predominantly recognizes a A-cis molecular structure around the iron center.

\textit{MirBWT}-transformed yeast cells (\textit{MirBWT}) were able to grow in iron-limited medium supplemented with Fe-TAFC. While the \[^{55}\text{Fe}\]TAFC uptake rates observed in \textit{MirBWT} were lower than those of \textit{DEY1394}, they were significantly higher than those for \textit{PHY14-URA}, the negative-control strain that contains no endogenous siderophore transporters, and were similar to published rates for \textit{A. nidulans MirB} (20). \textit{DEY1394}, the parent strain of PHY14-URA, possesses not only the endogenous \textit{S. cerevisiae} TAFC transporter \textit{Arn2p} but all other \textit{S. cerevisiae} iron uptake permeases and xenosiderophore transporters, including \textit{Arn1p} (various siderophores), \textit{Arn3p} (ferrioxamine and ferrichrome), and \textit{Arn4p} (enterobactin) (23, 40, 61).

It is not known at this time if the GATA sites found in the promoter region of \textit{mirB} are true binding sites for transcription factors. However, since siderophore biosynthesis- and siderophore transporter-encoding genes, including \textit{mirB} in \textit{Aspergillus nidulans} and \textit{sidA} in both \textit{A. nidulans} and \textit{Aspergillus fumigatus}, are all regulated by SreA (20, 45, 51), it is probable that SreA is also a regulator of \textit{mirB}. In an \textit{A. nidulans} SreA mutant, \textit{mirB} was found to be only partially derepressed (20), but more recent studies have pointed to a major role of the CCAAT-binding transcription factor HapX in the regulation of \textit{A. fumigatus mirB}. Schrettl et al. (52) showed that \textit{hapX} deletion dramatically reduced \textit{mirB} transcript levels during iron limitation.

We compared the abilities of the various \textit{A. fumigatus} \textit{MirB} mutants to transport \[^{55}\text{Fe}\]TAFC and/or use TAFC as a source of iron for growth. The substitutions of alanine 125 and tyrosine 577 and the deletions of loop 3 and the second half of loop 7 had the most severe impact, abrogating function completely. Indeed, the inability of these mutants to both transport \[^{55}\text{Fe}\]TAFC and use TAFC as a source of iron suggests that these amino acids and loops are crucial for proper functioning of the transporter. Immunofluorescence microscopy showed that the complete loss-of-function seen in the absence of loop 3 and the second half of loop 7 is likely due to misfolding/mislocalization of the protein. Whether the lack of uptake seen in the Loop7Del2 mutant is due solely to the absence of tyrosine 577, one of the deleted amino acids, or there are other functionally crucial amino acids in this region of loop 7 is unknown.

Like Loop7Del2, Loop7Del1 showed no significant uptake of \[^{55}\text{Fe}\]TAFC, but TAFC supplementation supported the growth of the Loop7Del1 mutant on solid medium. We hypothesize that the uptake rate was too low to reach significance in the \[^{55}\text{Fe}\] test (assay time of 60 min) and that the longer incubation period on agar revealed that even a low rate of iron uptake from TAFC supported growth.

In \textit{Arn1p}, the \textit{S. cerevisiae} ferrichrome transporter, mutations of numerous amino acids in the seventh loop, including four tyrosine residues, resulted in significant or complete loss of transport and binding of the siderophore by the transporter (36). One of these \textit{Arn1p} mutants, QRYR-A, contained mutations in several amino acids in the seventh loop, including the conserved tyrosine (577) and arginine (570) residues mutated in our study. It would be interesting to see if mutation of this conserved tyrosine residue in \textit{S. cerevisiae} (amino acid 558) would result in the same phenotype as seen in this study. The mutagenesis data from \textit{Arn1p}, combined with our data from \textit{A. fumigatus MirB}, point to the seventh loop as having a fundamental and conserved role in fungal siderophore uptake. More specifically, the loss-of-function observed in the seventh-loop tyrosine mutants, both in this study (tyrosine 577 in \textit{MirB}) and in studies by Kim et al. (36) (tyrosine 534 and 538 in \textit{Arn1p}) and Nevitt and Thiele (44) (tyrosine 575 in \textit{Candida glabrata} \textit{Sit1}, which corresponds to Y577 in \textit{A. fumigatus MirB}), suggests that tyrosine may be a key amino acid in the uptake of fungal siderophores.

Kim et al. (36) found that mutations in the first loop of \textit{Arn1p}, although they were able to dramatically reduce iron uptake, were not enough to prevent binding of the siderophore or growth of those mutant strains on ferricrocin. In contrast, we identified a crucial mutation in the first loop of MirB, A125D, which had one of the most pronounced loss-of-function phenotypes despite an expression level and localization similar to \textit{MirBWT}. In contrast, the other first-loop mutation, F129D, did not have any effect on iron transport.

The lower iron uptake seen in all of the loop deletion mutants cannot be explained solely by the lower expression levels observed with Western blotting. For example, the F129D mutant, whose expression was comparable to or even lower than the deletion mutants, exhibited transport rates that were similar to those of \textit{MirBWT} and was able to utilize TAFC efficiently for growth.

The three remaining mutations, C-termDel, N-termDel, and Y398A, did not show any appreciable defects in growth on TAFC compared to \textit{MirBWT}. Transport of \[^{55}\text{Fe}\]TAFC in the Y398A mutant was reduced but still significantly above background, whereas no reduction in \[^{55}\text{Fe}\]TAFC uptake was observed in the N- or C-terminal deletion mutants. In fact, deletion of the five N-terminal amino acids increased \[^{55}\text{Fe}\]TAFC uptake. While there was a large variance in the rate of uptake observed in this mutant, N-terminal deletion may have increased the levels of MirB protein in the plasma membrane, which could explain the higher rates of uptake, but this was not evident from the immunolocalization in yeast. Yeast proteins Kex2p and dipetidyl aminopeptidase (DPAP) A, an \(\alpha\)-factor-processing enzyme and a dipetidyl aminopeptidase, respectively, both of which are targeted to the endosomal pathway, contain signals for retention in the late Golgi (8, 9, 11). Removal of these signal sequences, which, in the
case of DPAP A, are contained in the N-terminal region, lead to an accelerated relocation to the late endosome. While such a mechanism could help explain the increased uptake seen in the N-term Del mutant, the presence of a retention signal at the N terminus of MirB is still purely speculative.

All of the MFS crystal structures determined to date are from bacteria, and we have yet to discover if eukaryotic MFS permeases adopt a similar structure. Furthermore, evidence suggests that fungal siderophore transporters are taken up along with their substrates through endocytosis of the entire complex (48). This is in contrast to bacterial MFS permeases, which act to shuttle their substrates across the membrane through the transporter to the cytoplasm (39). Another key difference between MFS transporters and Arn1p, and possibly other fungal siderophore transporters as well, is the presence of two substrate binding sites in Arn1p (43) instead of one, as is the case in other MFS members. It appears that development of this second binding site was possible in part because of the presence of the additional transmembrane domains, 13 and 14 (36).

Several different mechanisms of siderophore-mediated iron transport across the membrane have been proposed to occur in fungi. In S. cerevisiae, one model holds that transporters for enterobactin and TAFC are not located in the plasma membrane at all times; instead, they are kept in endosome-like vesicles near the plasma membrane and are localized to the plasma membrane when cells are exposed to siderophores (37). Furthermore, it is believed that siderophores in S. cerevisiae are not simply transported across the plasma membrane but instead are taken up by endocytosis of the transporter after it binds to its specific siderophores (37). Once in the endosome, the transporter would shuttle the siderophore-iron complex across the endosomal membrane to the cytosol. In our study, MirB was found in the plasma membrane as well as in small vesicles that were concentrated at the hyphal tips during active growth. The presence of TAFC did not influence the pattern of MirB distribution; however, when intracellular levels of iron were elevated (in the sldA mutant strain grown in the absence of ferricrocin), MirB-containing vesicles were diffusely distributed throughout the cytoplasm. Hence, we propose that low intracellular iron levels, and not TAFC, cause MirB to shift to the plasma membrane. Transport of secretory vesicles to hyphal tips is a well-known feature of polarized growth. In our study, MirB-containing secretory vesicles were likely distributed to the hyphal tip along microtubules and distributed to the plasma membrane via a microfilament network organized by the Spitzkörper, an apical body that acts as a vesicle-organizing center for hyphal morphogenesis (22). Further research is required to unravel the cytoskeletal elements required for MirB transport.

The results of this study have provided important insight into siderophore uptake in A. fumigatus and the structural requirements of siderophore transporters in both A. fumigatus and S. cerevisiae and likely fungal transporters in general. Further research is necessary to elucidate exactly how siderophore transporters recognize and transport ferrated siderophores in Aspergillus fumigatus. It is critical that we better understand the mechanism by which A. fumigatus obtains TAFC, and consequently iron, since this is the key to its survival in the human host. Moreover, the synthesis and transport of siderophores present possible target areas in the development of new drugs as these biochemical pathways do not exist in humans.

ACKNOWLEDGMENTS

We gratefully acknowledge the assistance of Jessica Holland with the phage conjugation experiments and M. Dearden and K. Buettner for care and assistance with the mice used in this study.

We thank the Natural Sciences and Engineering Research Council of Canada for financial support (M.M.M.).

REFERENCES

1. Abramson J, et al. 2003. Structure and mechanism of the lactose permease of Escherichia coli. Science 301:610–615.
2. Adjiman JP, Emery T. 1987. Iron uptake in Mycelia sterilia EP-76. J. Bacteriol. 169:3664–3668.
3. Agarwal R. 2009. Allergic bronchopulmonary aspergillosis. Chest 135: 805–826.
4. Ashburner M, et al. 2000. Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. Nat. Genet. 25:25–29.
5. Askwith G, et al. 1994. The FET3 gene of S. cerevisiae encodes a multi-copper oxidase required for ferrous iron uptake. Cell 76:403–410.
6. Birney E, Clamp M, Durbin R. 2004. GeneWise and Genomewise. Genome Res. 14:988–995.
7. Bonnycastle LLC, Mehroke JS, Rashed M, Gong X, Scott JK. 1996. Probing the basis of antibody reactivity with a panel of constrained peptide libraries displayed by filamentous phage. J. Mol. Biol. 258:747–762.
8. Bowers K, Stevens TH. 2005. Protein transport from the late Golgi to the vacuole in the yeast Saccharomyces cerevisiae. Biochim. Biophys. Acta 1744:438–454.
9. Brickner JH, Fuller RS. 1997. SO1 encodes a novel, conserved protein that promotes TGN-endosomal cycling of Kex2p and other membrane proteins by modulating the function of two TGN localization signals. J. Cell Biol. 139:23–36.
10. Brookman J, Denning DW. 2000. Molecular genetics of iron uptake and homeostasis in Aspergillus fumigatus. Fungal Genet. Biol. 3/48–474.
11. Bryant NJ, Stevens TH. 1997. Two separate signals act independently to localize a yeast late Golgi membrane protein through a combination of retrieval and retention. J. Cell Biol. 136:287–297.
12. Chenna R, et al. 2003. Multiple sequence alignment with the Clustal series of programs, Nucleic Acids Res. 31:3497–3455.
13. Dagenais TRT, Keller NP. 2009. Pathogenesis of Aspergillus fumigatus in invasive aspergillosis. Clin. Microbiol. Rev. 22:447–465.
14. Dang SY, et al. 2010. Structure of a fucose transporter in an outward-open conformation. Nature 467:734–738.
15. Eisendle M, et al. 2006. The intracellular siderophore ferricrocin is involved in iron storage, oxidative stress resistance, germination and sexual development in Aspergillus nidulans. Eukaryot. Cell 5:1396–1603.
16. Frishman D, Argos P. 1996. Incorporation of non-local interactions in protein secondary structure prediction from the amino acid sequence. Protein Eng. 9:133–142.
17. Gasteiger E, et al. 2005. Protein identification and analysis tools on the ExpASy server, p 571–607. In Walker JM (ed), The proteomics protocols handbook. Humana Press, Totowa, NJ.
18. Gupta R, Brunak S. 2002. Prediction of glycosylation across the human proteome and the correlation to protein function. Pac. Symp. Biocomput. 7:310–322.
19. Haas H. 2004. Molecular genetics of iron uptake and homeostasis in fungi, p 1–31. In Marzluf GA, Brambl R (ed), The mycota III: biochemistry and molecular biology. Springer, Berlin, Germany.
20. Haas H, et al. 2003. Characterization of the Aspergillus nidulans transporters for the siderophores enterobactin and triacyltetraarsine C. Biochem. J. 371:505–513.
21. Haas H, Eisendle M, Turgeon BG. 2008. Siderophores in fungal physiology and virulence. Annul. Rev. Phytopathol. 46:149–187.
22. Harris SD, et al. 2005. Polarisome meets Spitzkörper: microscopy, genetics, and genomics converge. Eukaryot. Cell 4:225–229.
23. Heymann P, Ernst JF, Winkelmann G. 2000. Identification and substrate specificity of a ferrichrome-type siderophore transporter (Arn1p) in Saccharomyces cerevisiae. FEMS Microbiol. Lett. 186:221–227.
24. Heymann P, et al. 2002. The siderophore iron transporter of Candida albicans (Sit1p/Arn1p) mediates uptake of ferrichrome-type siderophores and is required for epithelial invasion. Infect. Immun. 70:5246–5255.
25. Hirai T, Subramaniam S. 2004. Structure and transport mechanism of the bacterial oxalate transporter OxdT. Biophys. J. 87:3600–3607.
26. Hirokawa T, Boom-Chieng S, Mitaku S. 1998. SOSUI: classification and
secondary structure prediction system for membrane proteins. Bioinformatics 14(4):378–379.

27. Hissen AHT, Chow JMT, Pinto LJ, Moore MM. 2004. Survival of Aspergillus fumigatus in serum involves removal of iron from transferrin: the role of siderophores. Infect. Immun. 72:1402–1408.

28. Hissen AHT, Wan ANC, Warwas ML, Pinto LJ, Moore MM. 2005. The Aspergillus fumigatus siderophore biosynthetic gene sidA, encoding t-oritannate N-O-oxynase, is required for virulence. Infect. Immun. 73:5493–5501.

29. Horton P, et al. 2007. WoLF PSORT: protein localization predictor. Nucleic Acids Res. 35:W585–W587.

30. Hossain MB, Engelmut DL, Loghrity RA, Vanderham D. 1980. Circular dichroism, crystal structure, and absolute configuration of the siderophore ferric N,N',N'-triacetylfusarinine, FeC_{30}H_{33}N_{6}O_{6}. J. Am. Chem. Soc. 102:5766–5773.

31. Hissen AHT, Lemieux MJ, Song J, Auer M, Wang DN. 2003. Structure and mechanism of the glycerol-3-phosphate transporter from Escherichia coli. Science 301:616–620.

32. Huschka H, Mølgaard A, Gupta R, Brunak S. 2005. Prediction, conservation analysis, and structural characterization of mammalian mucin-type O-glycosylation sites. Glycobiology 15:153–164.

33. Kaminský SGW. 2001. Fundamentals of growth, storage, genetics and mass spectrometry of Aspergillus nidulans. Fungal Genet. Newsl. 48:25–31.

34. Keogh M-C, et al. 2006. A phosphatase complex that dephosphorylates gamma H2AX regulates DNA damage checkpoint recovery. Nature 439:497–501.

35. Kim Y, Lampert SM, Philpott CC. 2005. A receptor domain controls the intracellular sorting of the ferrichrome transporter, Arn1p. EMBO J. 24:952–962.

36. Kim Y, Yun CW, Philpott CC. 2002. Ferrichrome induces endosome to plasma membrane cycling of the ferrichrome transporter, Arn1p, in Saccharomyces cerevisiae. EMBO J. 21:3632–3642.

37. Latgé J-P. 2001. The pathobiology of Aspergillus fumigatus. Trends Microbiol. 9:382–389.

38. Law CJ, Maloney PC, Wang DN. 2008. Ins and outs of major facilitator superfamily antiporters. Annu. Rev. Microbiol. 62:289–305.

39. Lesuisse E, Simon-Casteras M, Labbe P. 1998. Siderophore-mediated iron uptake in Saccharomyces cerevisiae: the STI1 gene encodes a ferrioxamine B permease that belongs to the major facilitator superfamily. Microbiology 144:3455–3462.

40. Maglott D, Ostell J, Pruitt KD, Tatusova T. 2005. Entrez Gene: gene-centered information at NCBI. Nucleic Acids Res. 33:D54–D58.

41. Marchler-Bauer A, Bryant SH. 2004. CD-Search: protein domain annotations on the fly. Nucleic Acids Res. 32:W327–W331.

42. Moore RE, Kim Y, Philpott CC. 2003. The mechanism of ferrichrome transport through Arn1p and its metabolism in Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. U. S. A. 100:5664–5669.

43. Nevitt T, Thiele DJ. 2011. Host iron withholding demands siderophore utilization for Candida glabrata to survive macrophage killing. PLoS Pathog. 7:e1001322. doi:10.1371/journal.ppat.1001322.

44. Obreger H, Schoerer M, Zadra I, Abt B, Haas H. 2001. SRE is involved in regulation of siderophore biosynthesis, utilization and uptake in Aspergillus nidulans. Mol. Microbiol. 41:1077–1089.

45. Pao SS, Paulsen IT, Saier MH. 1998. Major facilitator superfamily. Microbiol. Mol. Biol. Rev. 62:1–34.

46. Payne SM. 1994. Detection, isolation, and characterization of siderophores. Methods Enzymol. 235:329–344.

47. Philpott CC. 2006. Iron uptake in fungi: a system for every source. Biochim. Biophys. Acta 1763:636–645.

48. Schrettl M, et al. 2004. Siderophore biosynthesis but not reductive iron assimilation is essential for Aspergillus fumigatus virulence. J. Exp. Med. 200:1213–1219.

49. Schrettl M, et al. 2007. Distinct roles for intra- and extracellular siderophores during Aspergillus fumigatus infection. PLoS Pathog. 3:e128. doi:10.1371/journal.ppat.0010128.

50. Schrettl M, et al. 2008. SreA-mediated iron regulation in Aspergillus fumigatus. Mol. Microbiol. 70:27–43.

51. Schrettl M, et al. 2008. Detection, isolation, and characterization of siderophores during Aspergillus fumigatus infection. PLoS Pathog. 3:e128. doi:10.1371/journal.ppat.1001124.

52. Silver P. 2009. Indirect immunofluorescence labeling in the yeast Saccharomyces cerevisiae. Cold Spring Harbor Protoc. doi:10.1101/pdb.prot5317.

53. Soubani AO, Chandrasekar PH. 2002. The clinical spectrum of pulmonary aspergillosis. Chest 121:1988–1999.

54. Thornton CR. 2010. Detection of invasive aspergillosis. Adv. Appl. Microbiol. 70:187–216.

55. Tsunády G, Simon I. 1998. Principles governing amino acid composition of integral membrane proteins: application to topology prediction. J. Mol. Biol. 283:489–506.

56. van Houten NE, Henry KA, Smith GP, Scott JK. 2000. Siderophore-iron assimilation is essential for Aspergillus fumigatus virulence. J. Exp. Med. 200:1213–1219.

57. van Houten NE, Henry KA, Smith GP, Scott JK. 2010. Engineering filamentous phage carriers to improve focusing of antibody responses against peptides. Vaccine 28:2174–2185.

58. Vincent MJ, Martin AS, Compans RW. 1998. Function of the KXXX motif in endoplasmic reticulum retrieval of a transmembrane protein depends on the length and structure of the cytoplasmic domain. J. Biol. Chem. 273:950–956.

59. Winkelmann G. 2001. Siderophore transport in fungi, p 463–479. In Winkelmann G (ed), Microbial transport systems. Wiley-VCH, Weinheim, Germany.

60. Yin Y, He X, Szewczyk P, Nguyen T, Chang G. 2006. Structure of the multidrug transporter EmrD from Escherichia coli. Science 312:741–744.

61. Yun CW, Tiedeman JS, Moore RE, Philpott CC. 2000. Siderophore-iron uptake in Saccharomyces cerevisiae. J. Biol. Chem. 275:16354–16359.