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Siderophore Production by Pathogenic *Mucorales* and Uptake of Deferoxamine B

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Abstract Clinical reports have established that mucormycosis, mainly caused by *Rhizopus* spp., frequently occurs in patients treated with deferoxamine B (DFO, Desferal®) which is misappropriated by these fungi. Siderophore production by twenty mucoralean isolates was therefore investigated using a commercial iron-depleted culture medium. Siderophore production was detected for most of the isolates. Our experiments confirmed that feroxamine B (iron chelate of DFO) promoted in vitro growth of *Rhizopus arrhizus*. Electrophoretic analysis of somatic extracts revealed iron-regulated proteins of 60 and 32 kDa which were lacking in iron-depleted culture conditions. Using a fluorescent derivative of deferoxamine B, we showed by fluorescence microscopy the entry of the siderophore within the fungal cells, thus suggesting a shuttle mechanism encompassing the uptake of the entire siderophore-ion complex into the cell. This useful tool renders possible a better understanding of iron metabolism in *Mucorales* which could lead to the development of new diagnostic method or new antifungal therapy using siderophores as imaging contrast agents or active drug vectors.

Keywords *Mucorales* · *Rhizopus arrhizus* · Siderophore · Fluorescent derivative of deferoxamine B

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Introduction

Iron is essential for growth of most living organisms. Although it is abundant on Earth, iron is essentially unavailable because the aerobic atmosphere has converted it to oxyhydroxide polymers of very sparing solubility. To overcome this problem, microorganisms excrete low molecular weight ferric iron-specific chelators called siderophores. These molecules which solubilize environmental iron are widely distributed among bacteria and fungi. To recover the metal from these ferrisiderophore complexes, microorganisms present specific uptake systems which remarkably operate, not only for native siderophores, but also for chelates synthesized by other microorganisms [1–3].

For example, the bacterial siderophore called deferoxamine B (DFO) can be misappropriated by various human pathogens and stimulate their growth [4]. This metal chelating agent is commercially available as Desferal® to treat iron overload in thalassemic patients or either iron or combined aluminum and iron overload in dialysis patients. Clinical studies and animal models have shown that its use increases the risk of fungal infections, particularly of mucormycosis which is caused by fungi of the order *Mucorales* [5]. *Rhizopus arrhizus* is by far the most common agent of these infections, but other species such as *Rhizopus microsporus* var. *rhizopodiformis*, *Lichtheimia corymbifera*, *Rhizomucor pusillus* and *Cunninghamella bertholletiae* [6] may also determine rhinocerebral, pulmonary or disseminated mucormycosis. These life-threatening fungal infections are on the rise since two decades, because of the increasing frequency of predisposing factors such as diabetic ketoacidosis, severe neutropenia, broad spectrum antibiotics, immunosuppression and prolonged deferoxamine therapy [7, 8]. When mucormycosis is associated with deferoxamine therapy, it is often fatal with mortality reaching approximately 80 % [6]. Therefore, other iron chelators have been developed such as deferiprone which presents the advantage not to deliver iron to *R. arrhizus* [9, 10]. Moreover, associated with an antifungal, it inhibits efficiently the growth of *R. arrhizus,* and this combination may constitute a novel therapy for refractory mucormycosis infections [10].

Additionally, susceptibility profiles against antifungal drugs in the *Mucorales* reveal a considerable variation among and within genera and species [8]. *Rhizopus arrhizus* exhibits a low susceptibility to amphotericin B; high doses are required which frequently result in nephrotoxicity. Although the success rate of posaconazole therapy was reported to be 79 % [11], this antifungal shows reduced activity against *Mucor* and *Cunninghamella* isolates. Voriconazole and echinocandins have little or no activity in vitro against most *Mucorales*. Because of this limited therapeutic efficacy, there is an urgent need for a better knowledge on the pathogenic mechanisms of these fungi and for the development of improved strategies to treat and prevent invasive mucormycosis. Such strategies can be facilitated by clear understanding of iron acquisition which constitutes a key step in all infectious processes. Siderophores are increasingly considered virulence factors, suggesting that siderophores or analogs can be used for iron transport-mediated drug delivery in antifungal therapy [3]. The aim of the present study therefore was to analyze the production of siderophores in twenty isolates of human pathogenic *Mucorales* using a commercial iron-depleted liquid medium, to investigate the uptake of feroxamine B by *R. arrhizus* and finally to test the ability of this fungus to internalize this ferrisiderophore using a fluorescent derivative of deferoxamine B.

Materials and Methods

Organisms and Growth Conditions

Twenty fungal isolates belonging to four different genera of the order *Mucorales* (presented in Table 1) were obtained from our hospital laboratory (Laboratory of Parasitology-Mycology of Angers –LMA– University Hospital, France) or from the IHEM (Institute of Hygiene and Epidemiology-Mycology section, Scientific Institute of Public Health, Brussels, Belgium) or IP (Pasteur Institute, Paris, France) culture collections. Isolates were maintained on yeast extract–peptone–dextrose agar plates containing 0.5 % chloramphenicol with incubation at 37 °C for 10–15 days.

For some experiments, the isolates were cultivated in 200-ml flasks containing each 50 ml of modified Czapek-Dox liquid medium where iron was omitted. The mycelial matt was collected from culture medium and washed three times (1,500*g*, 5 min) in sterile ultrapure water (Milli-Q Plus, $R = 18.2$ MΩ cm at 25 °C, Millipore). The final pellet was resuspended in sterile water and fragmented with a ground-glass grinder. The poterized mycelial suspension was standardized in sterile water by adjusting the cell density spectrophotometrically to an absorbance of 0.6 at 450 nm (which corresponds to 5×10^4–2.5×10^5 CFU/ml).

Rhizopus arrhizus IP 1553.84, already used in our laboratory in a previous study [12], was also cultivated on modified Czapek-Dox agar plates without iron. After incubation for 5 days at 37 °C, the mycelium was scraped off the agar plates in sterile water. The suspension was then filtered through a 30-µm pore size-nylon Monyl membrane (Scrynel NY 30 HC Nitex, Züricher Beuteltuchfabrik AG, Rüschlikon/Switzerland) to remove the sporangiophores and the hyphal fragments. After centrifugation at 1,200*g* for

Table 1 Detection of siderophore production by the CAS-YMA plate assay

Species	Isolate	CAS reaction rate[a]
Lichtheimia corymbifera	IHEM 3809	2.7
	IHEM 10339	3.1
	IHEM 10462	2.7
	IHEM 14056	2.2
	IHEM 14734	2.5
Cunninghamella bertholletiae	IHEM 6390	1.2
Rhizomucor pusillus	IHEM 4897	3.7
	IHEM 10343	4.2
	IHEM 13322	1.5
	IP 1127-75	3.3
	LMA 70000035	4.2
Rhizopus microsporus var. *rhizopodiformis*	IHEM 5208	2.6
	IHEM 5234	1.8
	IHEM 7978	1.8
	IHEM 9836	2.1
	IHEM 15210	1.6
Rhizopus arrhizus	IHEM 5215	1.6
	IHEM 5233	1.6
	IHEM 6017	1.4
	IP 1553-84	1.7

[a] Values correspond to the ratio between the diameters of the red–orange halo and of the fungal colony on the CAS-YMA blue agar plates

5 min, spores were washed three times and then resuspended in sterile water. After being quantified by hemacytometer counts, density of the spore suspension was finally adjusted to 0.5–2.5 $\times$ 10^6 spores/ml.

Siderophore Detection

A modification of the universal chemical assay of Schwyn and Neilands [13] was used to detect siderophore production in the agar media. The inoculum consisted of 100 µl of the poterized mycelial suspension spotted in the middle of chrome azurol S (CAS, Sigma Chemical) agar plates, composed of 1/10 CAS indicator solution and 9/10 Yeast Morphology Agar (YMA, Difco Laboratories). A positive reaction results in a color change of CAS reagent from blue to orange that means a clear visible red–orange halo around the fungal colony after a 48-h incubation at 37 °C. The CAS reaction rate was determined by measuring the diameters of the red–orange halo and of the fungal colony. Siderophore production was expressed as the ratio between these two diameters.

Siderophore Production

For study of the kinetics of siderophore production by *R. arrhizus* IP 1553.84, 1-ml aliquots of the poterized mycelial suspension was inoculated in 200-ml flasks containing each 50 ml of basal liquid medium (BLM) consisting in copper- and iron-depleted yeast nitrogen base (YNB)-glucose 2 % (Bio 101, Inc.) supplemented with amino acids (complete supplement mixture, CSM; Bio 101, Inc.) and adjusted to pH 4, 7 or 8. After incubation at 37 °C for 2–7 days, cultures were centrifuged at 50,000*g* for 30 min at 4 °C. The resulting supernatants were freeze-dried and solubilized in 1 ml of ultra-pure water for nonspecific siderophore assay by the ferric perchlorate test [14]. Fungal pellets were treated for mycelial dry weight determinations. Experiments were performed in triplicate and results correspond to mean values (±SD). Statistical analysis was performed using the Student's *t* test with significance at $p < 0.05$.

For screening the productive isolates, triplicate cultures were done in BLM adjusted to pH 7. After incubation for 4 days at 37 °C, culture supernatants and mycelia were recovered for siderophore chemical assays and mycelial dry weight determinations, respectively.

Chemical Assays for Siderophores

Chemical assays were performed on concentrated culture supernatants. For nonspecific assay, the ferric perchlorate test [14] was used, whereas production of siderophores belonging to catecholate and hydroxamate types was investigated by the Arnow test [15] and the periodate oxidation assay [16], respectively. Catechol and deferoxamine B mesylate (DFO) were obtained from Sigma. Catechol was used at 1 mM final concentration as control, and, to quantify siderophore production, standard curve was obtained with DFO using serial twofold dilutions ranging from 0.25 to 0.004 mM. For nonspecific assay, siderophore concentration was normalized as DFO equivalent. Results are expressed in µmol of DFO equivalent per g of fungal biomass.

Influence of Iron Sources on Fungal Growth

Rhizopus arrhizus IP 1553.84 was grown in 30-ml loosely capped tubes containing each 10 ml of BLM. Various iron sources, such as $FeSO_4$, $FeCl_3$ and iron citrate or ferrisiderophores (i. e. feroxamine B (FO), catecholate and 2,3-dihydroxybenzoate) generated by combining deferrisiderophores and iron citrate in stoichiometric quantities, were added to the basal medium at 1 μM final concentration. Media were inoculated with the spore suspension at a final cell density of 5×10^5 spores/ml. Cultures were performed in triplicate and incubated for 7 days at 37 °C. Thereafter, the fungal mass was pelleted by centrifugation at 50,000*g* for 30 min at 4 °C. The resulting pellets were washed in ultra-pure water and finally lyophilized for determination of mycelial dry weight. Statistical analysis was performed using the Student's *t* test with significance at $p < 0.01$.

Analysis of Iron-Regulated Proteins

For protein extraction, *R. arrhizus* IP 1553.84 was grown in 200 ml of BLM supplemented either with 1 mM iron citrate or with 1 μM FO. After 7 days of incubation at 37 °C, the fungal elements were harvested by filtration, washed with water and disrupted in a MSK cell homogenizer (B. Braun, Melsungen, Germany) by using a mix of 1- and 0.25- to 0.30-mm-diameter glass beads and cooling with CO_2. Glass beads and insoluble cell fraction were removed by successive centrifugations (1,000*g* for 5 min, followed by 50,000*g* for 30 min) at 4 °C. The clear supernatant which corresponded to the cytosolic fraction was collected. Proteins were precipitated by acetone and finally resuspended in a minimum volume of electrophoresis sample buffer. Total protein content of the extracts was determined by the Bio-Rad protein assay (Bio-Rad Laboratories). Samples (100 μg of proteins per lane) were analyzed under reducing conditions by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) on a 12 % polyacrylamide gel according to the method of Laemmli [17]. Gels were stained with Coomassie brilliant blue R 250, and molecular mass of separated proteins was determined with low-molecular-mass electrophoresis calibration kit (Amersham Biosciences).

Uptake of Fluorescent DFO Derivative by Fungal Cells

A fluorescent derivative of DFO, 7-nitrobenz-2-oxa-1,3-diazole-deferoxamine B (NBD-DFO), was synthesized as previously described [18]. Germ tubes were obtained by inoculation of spores of *R. arrhizus* IP 1553.84 in medium 199 with Earle's salts (Sigma) supplemented with L-glutamine 0.01 %, $NaHCO_3$ 0.02 % and MOPS 5 mM, pH 6.7 [12]. After 5 h of incubation at 37 °C, fungal elements were washed in BLM (12,000*g*, 5 min), resuspended in BLM containing 10 or 100 μM final concentration of NBD-DFO and incubated at 37 °C for 3 or 24 h. Control was performed by incubation in BLM containing DFO. After washing in BLM (12,000*g*, 5 min), fungal elements were dropped on glass slides and observed using an Olympus microscope equipped with epifluorescence. Same experiment was carried out with dormant spores which were incubated with NBD-DFO for 30 min.

Results

Siderophore Detection

Universal chrome azurol S-Yeast Morphology Agar (CAS-YMA) blue agar plate assay was used to screen the mucoralean isolates searching for siderophore production revealed by a clear red–orange halo around the colonies (Fig. 1). All the fungal strains tested produced extracellular siderophores on this medium with quantitative variations according to the strains (Table 1). Siderophore production, expressed as the ratio between the diameters of the red–orange halo and of the colony, did not differ significantly among isolates of a same fungal species, except for *Rhizomucor pusillus* IHEM 13322 which presented a weak ratio of 1.5, whereas it was higher (3.8) for the other *R. pusillus* isolates. Isolates of *Lichtheimia corymbifera* showed high ratios with a mean of 2.6. Siderophore production was lower for isolates of *Rhizopus microsporus var. rhizopodiformis* and *R. arrhizus* with mean ratios of 2 and 1.6, respectively. The weakest siderophore production was detected for *Cunninghamella bertholletiae* IHEM 6390, whereas the best producers were *R. pusillus* IHEM 10343 and LMA 70000035.

Fig. 1 Culture of *R. arrhizus* on CAS-YMA blue agar plate. Siderophore production, revealed by a clear visible halo around the colony, was evaluated by determining the ratio between diameters of the halo and of the colony

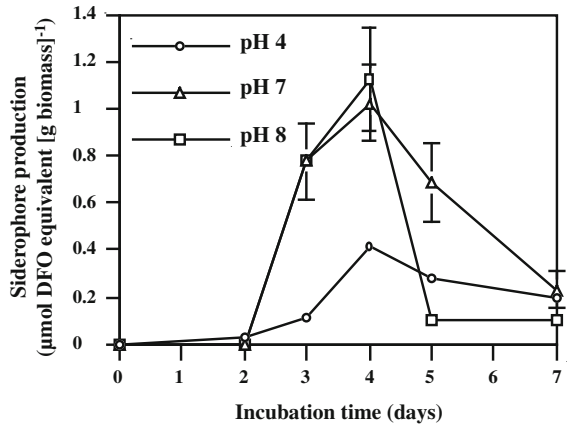

Fig. 2 Study of the kinetics of siderophore production by *R. arrhizus* in a commercial iron-depleted basal liquid medium buffered at pH 4 (*circle*), 7 (*triangle*) or 8 (*square*). Data are means of triplicate experiments. *Bars* indicate standard deviations. $p < 0.01$ for pH 7 and 8 versus pH 4 by Student's *t* test

Study of the Kinetics of Siderophore Production by *R. arrhizus* at Different pH

Siderophore production by *R. arrhizus* was investigated at three distinct pH and monitored every day during 1 week using a nonspecific assay. The highest production rates were obtained after 96 h of incubation, whatever the pH of the culture medium (Fig. 2). However, the maximal rate was obtained at pH 8. After 4 days of incubation at 37 °C, siderophore production was 2.5 and threefold higher at pH 7 and pH 8, respectively, compared with pH 4 ($p < 0.01$ for both). Then, it decreased drastically and reached a weak level, almost equivalent for the three pH, after 7 days of incubation.

Quantitative Assays for Siderophore Detection

All fungal isolates, except *L. corymbifera* IHEM 14734, gave a positive reaction for the nonspecific assay (Table 2). The highest values were obtained for isolates of the *Rhizopus* genus. Isolates IP 1553-84 and IHEM 5215 gave ratios of 0.39 and 0.68 µmol/g, respectively, and isolate IHEM 6017 produced the highest ratio (0.97 µmol/g). Likewise, *R. microsporus* var. *rhizopodiformis* isolates IHEM 5208 and 5234 gave high values of 0.25 and 0.30 µmol/g, respectively. Weak values (lower than 0.18 µmol/g) were obtained for all the other isolates.

No catecholate- or hydroxamate-type siderophores were detected for all the isolates, except for *R. pusillus* LMA 70000035 and *R. microsporus* var. *rhizopodiformis* IHEM 15210 which seemed to produce catecholates as suggested by a positive Arnow test (data not shown).

Negative and positive controls (catechol and DFO) produced appropriate reactions for each specific assay (data not shown).

Effect of Iron Sources on *R. arrhizus* Growth

Figure 3 shows the influence of mineral or organic iron sources on growth of *R. arrhizus*. Results showed that feroxamine B (FO, iron chelate of DFO) strongly stimulated the fungal growth which was eightfold and about 3.5-fold higher than in control medium and in medium containing catecholate, respectively ($p < 0.01$ for both, Fig. 3). The fungal growth was slightly enhanced by iron citrate and catecholate ($p < 0.05$ and 0.01, respectively, Fig. 3). In contrast, fungal growth with ferrous sulfate and 2,3-dihydroxybenzoate was equivalent to that observed in the control medium.

Protein Patterns of Cytosolic Extracts

Rhizopus arrhizus IP 1553-84 was grown on a commercial BLM supplemented with 1 µM FO or

Table 2 Nonspecific assay for siderophores

Species	Isolate	µmol of DFO equivalent per g of fungal biomass[a]
Lichtheimia corymbifera	IHEM 3809	0.12 ± 0.01
	IHEM 10339	0.11 ± 0.01
	IHEM 10462	0.05 ± 0.01
	IHEM 14056	0.06 ± 0.02
	IHEM 14734	0.02 ± 0.01
Cunninghamella bertholletiae	IHEM 6390	0.10 ± 0.01
Rhizomucor pusillus	IHEM 4897	0.13 ± 0.01
	IHEM 10343	0.03 ± 0.01
	IHEM 13322	0.04 ± 0.01
	IP 1127-75	0.18 ± 0.01
	LMA 70000035	0.16 ± 0.01
Rhizopus microsporus var. *rhizopodiformis*	IHEM 5208	0.25 ± 0.02
	IHEM 5234	0.30 ± 0.01
	IHEM 7978	0.05 ± 0.01
	IHEM 9836	0.15 ± 0.01
	IHEM 15210	0.11 ± 0.01
Rhizopus arrhizus	IHEM 5215	0.68 ± 0.05
	IHEM 5233	0.16 ± 0.04
	IHEM 6017	0.97 ± 0.01
	IP 1553-84	0.39 ± 0.05

[a] Siderophore levels were quantified by measuring the absorbance of ferric perchlorate complexes at 425 nm

1 mM iron citrate. SDS-PAGE analysis of cytosolic proteins, performed under reducing conditions, revealed six major proteins of 48, 35, 34, 30, 27 and 25 kDa synthesized in both conditions (Fig. 4). However, protein patterns differed by the presence of a minor band of 60 kDa and a major band of 32 kDa which were both detected in cytosolic extract from culture grown in 1 mM iron citrate-supplemented medium (Fig. 4, lane 1).

Uptake of Fluorescent DFO Derivative by *R. arrhizus*

Siderophore uptake by dormant conidia and hyphae of *R. arrhizus* IP 1553-84 was investigated using a fluorescent derivative of DFO, NBD-DFO. No labeling was observed for dormant spores (data not shown). In contrast, a bright and uniform fluorescence was

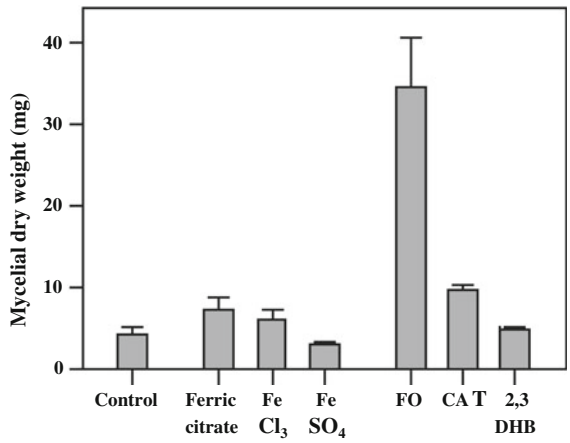

Fig. 3 Effect of various mineral and organic iron sources, feroxamine B (FO), catecholate (CAT) and 2,3-dihydroxybenzoate (2,3-DHB), on the growth of *R. arrhizus*. Data are means of triplicate experiments. *Bars* indicate standard deviations. $p < 0.01$ for FO versus other iron sources by Student's *t* test

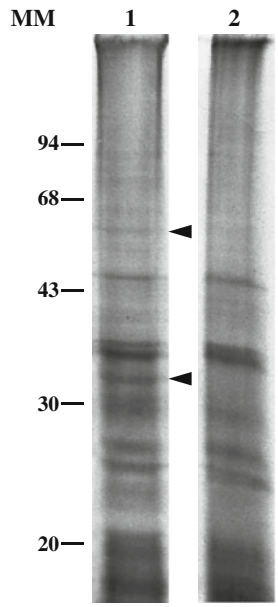

Fig. 4 SDS-PAGE analysis under reducing conditions of cytosolic extracts from *R. arrhizus*. The fungus was grown for 7 days at 37 °C on BLM + 1 mM iron citrate (*lane 1*) or + 1 µM FO (*lane 2*). Separated proteins were stained with Coomassie brilliant blue R 250, and their molecular masses were determined from the migration of standard proteins mentioned in kDa. *Arrows* indicate differences in protein patterns

detected in mother cells of germ tubes incubated for 3 h with 10 µM NBD-DFO (Fig. 5a), whereas the hyphal part of germ tubes was weakly and heterogeneously

labeled. After an incubation of 24 h, thin and long hyphae were observed which exhibited a patchy distribution of the labeling (Fig. 5c). Controls using DFO were free of any labeling.

Discussion

Patients treated with the iron chelator deferoxamine are known to be at high risk for mucormycosis. Indeed, while deferoxamine B (Desferal®) has clinical applications as an iron chelator in hematological diseases or in dialysis patients with iron overload, it actually serves as a siderophore, delivering free iron to *R. arrhizus*, the major cause of mucormycosis [5]. Therefore, iron chelators that cannot be utilized by the fungus to scavenge iron from the host have been developed such as deferiprone and deferasirox [10, 19].

A large number of pathogenic fungi have been shown to use siderophores, even though they cannot synthesize them. In nature, the misappropriation of siderophores synthesized by other microorganisms belongs to a survival strategy. *Rhizopus arrhizus* can make both. It secretes rhizoferrin, a polycarboxylate siderophore which supplies it with iron through a receptor-mediated, energy-dependent process [20] while being inefficient in obtaining iron from serum [5, 21]. However, *R. arrhizus* is also able to use xenosiderophores, such as deferoxamine, which is more efficient in obtaining iron from the host.

In this work, disposing of a fluorescent derivative of deferoxamine, we tested the hypothesis that deferoxamine B may be a vector for iron transport-mediated drug delivery to pathogenic mucoralean fungi. *Rhizopus arrhizus* is responsible of 70 % of all cases of mucormycosis, but other genera of the order Mucorales such as *Rhizomucor*, *Cunninghamella* or *Lichtheimia* may also cause human diseases [22, 23]. Therefore, siderophore production by 20 mucoralean isolates was first investigated, and we showed that this iron uptake mechanism is widely used in the order *Mucorales*. Siderophore production was evidenced on CAS-YMA plates, and a characteristic orange halo around the colonies was detected for all the isolates tested. A commercial iron-depleted liquid culture medium was then used to study the kinetics of siderophore production by *R. arrhizus*. In our experimental conditions, maximal rate of siderophore production was reached after an incubation of only

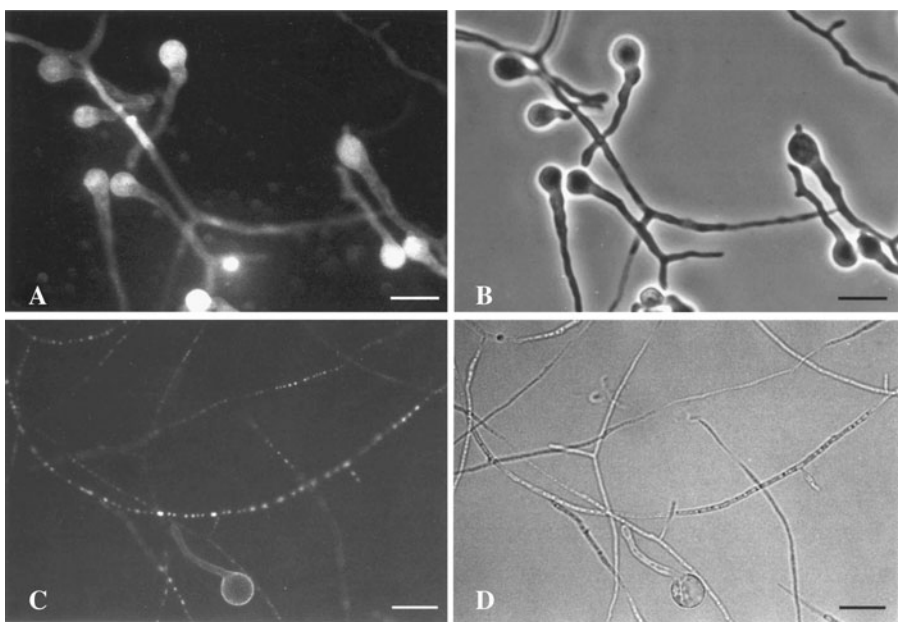

Fig. 5 Fluorescence (**a** and **c**) and phase-contrast (**b** and **d**) microscopies of *R. arrhizus* cells incubated with 10 µM NBD-DFO for 3 h (**a** and **b**) or 24 h (**c** and **d**). Mother cells of germ tubes presented a bright and uniform fluorescence after a 3-h incubation, whereas a heterogeneous labeling was detected along hyphae. Note the patchy distribution of the labeling inside the hyphae when incubation time was prolonged up to 24 h. *Bars* 10 µm

4 days at 37 °C and neutral pH. Prolonging the incubation time up to 7 days resulted in a drastic reduction in siderophore production whatever the pH of the culture medium. Surprisingly, much longer incubation time such as 1 or 2 months is usually recommended in hand-made prepared iron-depleted media [24, 25].

At pH 4, a low rate of siderophore production was observed which may be explained by an increased availability of the residual iron in the medium, leading to a subsequent repression of siderophore production. Lowering the pH favors iron assimilation by reduction in ferric to ferrous molecular species which are more soluble and therefore more easily assimilated. This alternative mode of iron supply has already been suggested for some fungi such as *Neurospora crassa*, *Saccharomyces cerevisiae* and *Candida albicans* [26, 27]. Our results are consistent with the fact that among the modes of iron acquisition, a reductive mechanism also may be used by *R. arrhizus* [21]. Conversely, at neutral or alkaline pH, higher rates of siderophore production were observed, probably due to much lower iron availability for fungal cells. It is known that genes involved in the synthesis of siderophore are derepressed in the presence of low iron levels [3].

Previous studies reported the detection of a wide range of siderophores in fungi [1, 24, 25, 28], but no phenolates–catecholates were identified [28]. Surprisingly, in our study, a positive catecholate reaction was obtained for one isolate of *R. pusillus* and of *R. microsporus* var. *rhizopodiformis*. The nonspecific assay gave positive results for most of the fungal isolates, but results were sometimes conflicting with those obtained on CAS-YMA plates. This may be due to the limits of the CAS-YMA plate assay which lacks both specificity and sensitivity and which was used here only as a rapid screening assay. Moreover, this medium requires the addition of a toxic detergent, hexadecyltrimethylammonium bromide, which renders it less favorable for growth of the fungi tested.

Hydroxamates which are the most common siderophores in fungi [1] have been reported once in *Mucorales* [24], but this observation has never been confirmed. Consistently, none of our isolates gave a positive reaction in the specific assay for hydroxamates, suggesting that siderophores rather belong to another type. Besides, a siderophore called rhizoferrin has been isolated from *R. microsporus* var. *rhizopodiformis* and is considered the representative of the third type of siderophores, called polycarboxylates which seemed to be largely distributed among the order *Mucorales* [20, 28, 29].

Whether rhizoferrin releases the iron extracellularly or is first internalized before to release iron in the cytoplasm remains to be defined, but studies have shown that rhizoferrin is not efficient in obtaining iron from serum [5, 21]. To survive, *R. arrhizus* may utilize other siderophores belonging to other types which may be present in its human host, particularly during chelation therapy. Our experiments confirmed the misappropriation in vitro of a hydroxamate-type iron-chelating drug by *R. arrhizus*. Among the different iron sources tested, FO was by far the most efficient to promote fungal development. Literature in this field is controversial since some authors did not observe a direct growth-promoting effect of DFO and its iron chelate on *Mucorales* isolates [30], whereas others found an enhanced fungal growth in their presence [31, 32].

Although siderophore production seems to be common in pathogenic fungi, little is known about their synthesis and that of iron-regulated proteins. In this study, the effect of iron source added in the culture medium on protein patterns of *R. arrhizus* was investigated by SDS-PAGE. The medium was supplemented with 1 mM iron citrate, a condition which led to the acquisition of the metal according to a reductive mechanism, or with 1 μM of FO which justified an iron uptake by means of siderophores. Electrophoretic patterns of cytosolic proteins obtained under these culture conditions disclosed very few differences. Two polypeptides of 60 and 32 kDa were detected for the fungus grown in the presence of 1 mM iron citrate, and one may speculate their involvement in the reductive uptake system [21].

Uptake of DFO was further investigated by fluorescence microscopy using a fluorescent derivative (NBD-DFO). Labeling was detected in mother cells and along the hyphal part of germ tubes, suggesting that *R. arrhizus* is capable to use this iron chelator during germination and hyphal growth. When incubation time was prolonged up to 24 h, a patchy distribution of the labeling was seen within hyphae. Besides, the absence of labeling in dormant conidia confirmed that uptake of this siderophore requires metabolically active cells. A similar observation has been reported for *Ustilago maydis* [33]. For this fungus, fluorescence was found intracellularly, clearly

concentrated in vesicles within the cell after a 16-h incubation with a fluorescent derivative of ferrichrome (B9-LRB).

Although a reductive uptake mechanism without entry of DFO within the cells of *Rhizopus microsporus* var. *rhizopodiformis* has been evidenced [21] and though a high affinity iron permease has been characterized in *R. arrhizus* [34], our results suggest that NBD-DFO was taken up as a whole by live active germlings. This may be due to an increased permeation across cell membranes due to the NBD group which renders DFO more lipophilic [18], but study of iron uptake in *U. maydis* using a similar fluorescent compound did not support this hypothesis [33]. In this study, no labeling was observed intracellularly using the fluorescent feroxamine B analog CAT18. On the contrary, another fluorescently labeled ferrichrome analog, B9-lissamine rhodamine B (B9-LRB), was found intracellularly after 4 h of incubation. Thus, two distinct iron uptake mechanisms co-exist in *U. maydis*: a siderophore transporter or shuttle mechanism (B9-LRB) and a reductive or taxicab mechanism (CAT18) [33]. Other examples of such duality of iron-regulated transport systems have been reported for other fungi including *Geotrichum candidum*, *Candida albicans* and *Saccharomyces cerevisiae* [35–39]. Moreover, for this last yeast species, authors have evidenced an iron uptake by both pathways from the same siderophore, feroxamine B [40]. Recently, a genome-sequencing project of *R. arrhizus* was initiated, and 13 possible siderophore permeases were identified that might act as receptors for siderophores, including rhizoferrin and deferoxamine [41].

The logical extension of recognizing the roles of key virulence factors, such as iron uptake by *R. arrhizus*, is to develop therapeutic strategies. One of these strategies may consist in iron transport-mediated drug delivery using mixed-ligand siderophore-antimicrobial conjugates. In mycology, promising results have been obtained against *Candida albicans*, *Cryptococcus neoformans* and *Aspergillus fumigatus* with a 5-fluorouridine-conjugated hydroxamate siderophore [42]. Recently, desketoneoenactin-siderophore conjugates showed an inhibitory activity against *Candida* spp. via a siderophore transporter [43]. Siderophores have also significant potential for the development of imaging contrast agents for pathogen-selective detection [44, 45].

Our work shows the interest of synthesized fluorescent derivatives of siderophores which constitute suitable tools for experiments on transport and iron uptake processes. The unique spectroscopic properties of our iron chelator offer the possibility to assess the intracellular pathways of iron metabolism in various pathogenic microorganisms or parasites [18]. Elucidation of the mode of iron acquisition by these pathogens may also lead to the development of new diagnostic and therapeutic strategies. In this way, our fluorescent iron chelator may be considered as an appropriate model for vectorization of radioactive, toxic or antifungal agents into the fungal cell. From that, structural analogs of siderophores may be conjugated to such agents and used to target pathogenic *Mucorales* according the "Trojan Horse" approach [46].

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