CURRENT MICROBIOLOGY Vol. 31 (1995), pp. 220–223

Current Microbiology
An International Journal

Studies on Siderophore Production and Effect of Iron Deprivation on the Outer Membrane Proteins of *Madurella mycetomatis*

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Abstract. The purpose of this investigation was to determine whether *Madurella mycetomatis*, the most frequent agent of eumycotic mycetomas, produces siderophores and synthesizes new outer membrane proteins under iron-starvation conditions. Siderophore production, only of the hydroxamate type, was demonstrated in all nine strains tested. It was regulated by extracellular iron concentrations. Under iron-restricted conditions, *M. mycetomatis* expressed various outer membrane iron-regulated proteins, particularly of 24-kilodalton, that may participate in iron metabolism.

Iron is vital for the survival and proliferation of almost all living cells [19]. Iron is necessary for important enzymatic functions, such as the transport and storage of oxygen, and as a catalyst in electron transport processes [17]. However, the concentration of free iron in living tissues is far below the level required to support microbial growth [4]. Therefore, pathogenic organisms must be able to develop mechanisms for obtaining iron from the host to overcome this nutritional deficiency and establish an infection [3]. The most widespread mechanism involves the excretion of low-molecular-weight, high-affinity iron chelators (siderophores) in conditions of iron restriction and the co-regulated expression of iron-regulated proteins acting as cell surface receptors specific for each siderophore-iron complex [18].

Little is known about the siderophores and iron-regulated proteins of fungi that cause disease in humans, although siderophore production appears to be common in fungi that are either pathogenic [5, 6, 10, 12] or not [2, 9]. Such iron chelators have never been identified in the pathogenic fungus *Madurella mycetomatis*, the most frequent agent of eumycotic mycetomas [15]. The purpose of this investigation was to determine whether *M. mycetomatis* produces siderophores and synthesizes new outer membrane proteins (OMPs) under iron-starvation conditions.

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Materials and Methods

Organisms and reagents. Nine *Madurella mycetomatis* strains, all isolated from human mycetomas, were obtained from several culture collections (Table 1). The strains were maintained at 25°C on Sabouraud agar slant tubes. For experiments, each strain was subcultured in Petri dishes at 37°C on Sabouraud agar covered with a sterile acetate filter membrane (HAWP 04700, Millipore Corp., Bedford, Massachusetts) for 10–15 days. Then the fungal growth was scraped off the membrane, washed three times with sterile deionized water, weighed wet, and 50 mg was suspended in 1 ml 0.9% saline. The suspension was fragmented for 10 min in an Ultra-Turrax tissue grinder (Pellet, Poly Labo, Strasbourg, France). The suspension was standardized in a low-iron medium (LIM) by adjusting the density spectrophotometrically to an absorbance of 0.6 at a wavelength of 450 nm, and quantified in colony forming units (CFU) per ml by plating graded dilutions of the suspension on Sabouraud agar.

The LIM preparations and iron depletion of KH_2PO_4 through a column of Chelex™ 100 resin (Bio-Rad Laboratories, Richmond, California) were performed as described by Holzberg and Artis [8]. The preparations were then stored at 4°C in 50-ml plastic tubes (Falcon 2098, Becton Dickinson and Co., Paramus, New Jersey) until used. The iron concentrations of LIM were checked colorimetrically (Fer-Kit, bioMérieux, Marcy-l'Etoile, France). They were determined to be lower than 0.125 μg/ml (2.2 μM).

Nonspecific assay for siderophores. A modification of the universal chemical assay of Schwyn and Neilands [21] was used to detect siderophore production in the agar media. The inoculum consisted of 100 μl of the fragmented mycelium (adjusted to 500 CFU/ml) spotted in the middle of 20-ml-containing chrome azurol S (CAS, Sigma Chemical Co., St Louis, Missouri) blue plates, constituted with 1/10 CAS indicator solution and 9/10 Yeast Morphology Agar (YMA, Difco Laboratories, Detroit, Michigan). The CAS-YMA medium contained 0.200 μg/ml (3.5 μM) of iron. A clear visible red-orange halo around the fungal colony was assessed as CAS-positive after 10 days' incubation at 37°C.

Fungal cultures and collection of cells and supernatants. The *M. mycetomatis* strains were cultured in 10 ml of LIM contained within 50-ml, loosely capped tubes (Falcon 2098), with or without supple-

Table 1. Source and origin of *Madurella mycetomatis* strains

Strain	Collection[a]	Geographical origin
1175.79	CIP	Cameroon
1767.88	CIP	Senegal
0491.94	NRCMAA	Mali
201.38	CBS	Indonesia
206.47	CBS	West Indies (The Netherlands)
592.74	CBS	Marocco
599.60	CBS	Djibouti
13140	ATCC	Soudan
66167	ATCC	USA

[a] CIP, Collection of Institut Pasteur, Institut Pasteur, Paris, France; NRCMAA, National Reference Centre for Mycosis and Antifungal Agents, Institut Pasteur; CBS, Centraalbureau voor Schimmelcultures, Baarn, The Netherlands; ATCC, American Type Culture Collection, Rockville, Maryland, USA.

mentation with $FeSO_4$ or EDDA. Growth was initiated with 1 ml of the fragmented mycelium (adjusted to 500 CFU/ml). The cultures were incubated for 1 month at 37°C for isolation of OMPs from cells and for specific determination of siderophores in the supernatants. Culture fluid was separated from the fungal mass by centrifugation at 9000 rpm for 20 min at 4°C. The resulting supernatant was removed, filtered through a 0.45-μm membrane filter, and assayed immediately for siderophore determination. Pelleted cells were further analyzed for the presence of OMPs.

Specific detection of siderophores. The presence of catechol, 2,3-dihydroxybenzoate and/or hydroxamic acids was determined from cultures in LIM by the Arnow test [1], absorption at 318 nm [23], and absorption at 264 nm after oxidation [9] respectively. Catechol (174 μM, Sigma), 2,3-dihydroxybenzoic acid (200 μM, Sigma), and deferoxamine (1.56 mM, Laboratories CIBA-GEIGY, Rueil-Malmaison, France) were used either as positive or negative controls.

In other experiments, hydroxamate-type siderophore production, as well as iron concentrations, was measured in LIM supplemented with either 0.0125, 0.025, 0.050, and 0.100 mM $FeSO_4$, or 0.14, 0.28, 0.56, and 1.12 mM ethylenediamine dihydroxyphenylacetic acid (EDDA) after 15 days' incubation at 37°C.

Analysis of iron-regulated proteins. The pellets, obtained from cultures in LIM or LIM supplemented with $FeSO_4$ (0.025 mM) or EDDA (0.28 mM), were washed twice and resuspended in 1.5 ml of PBS. The cell suspensions were fragmented in tissue grinders for 10 min at 4°C. Undisrupted cells and large fragments were removed by centrifugation at 9000 *g* for 10 min at 4°C. The supernatant was then centrifuged at 53,000 *g* for 60 min at 4°C to produce a gelatinous membrane pellet. Outer membrane proteins were suspended in 20 μl Laemmli solubilization buffer [16] and heated at 100°C for 5 min. After centrifugation at 5000 *g* for 5 min, 10 μl of each sample was submitted to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) by using a 4% stacking gel and a 10% separation gel at a constant current of 50 mA. The gels were then silver stained (Bio-Rad).

Results

Nonspecific assay for siderophores. A clear visible red-orange halo around the fungal colony indicated siderophore production. All the *M. mycetomatis* strains tested produced extracellular siderophores on the detection medium with quantitative variations among the different strains (results not shown).

Specific detection of siderophore. None of the culture supernatants of the strains gave a yellow to orange-red color change when the mixture was made basic, or produced an absorbance band at 318 nm. Therefore, none of the *M. mycetomatis* strains was positive for catechol and dihydroxybenzoate, whereas the pure catechol and 2,3-dihydroxybenzoic acid standards were positive in each test. The negative control hydroxamate siderophore deferoxamine always produced negative reactions.

All of the culture supernatants produced a strong absorption band at 264 nm after the formation of a nitrosodimer. All except the deferoxamine standards, as well as LIM, showed no reaction. Strain 1175.79, which gave the highest level of siderophore production and the highest rapidity of growth, was chosen for OMPs analysis.

Figure 1 shows the inhibition and stimulation of hydroxamate-type siderophore production of strain 1175–79 when concentrations of iron were increased ($FeSO_4$) or decreased (EDDA) respectively, with a direct dose-response relationship.

Analysis of outer membrane proteins. The OMPs profile of *M. mycetomatis* 1175.79 grown on iron-limiting medium (LIM and LIM + EDDA) was examined and compared with the non-iron-limited control (LIM + $FeSO_4$) (Fig. 2). A reproducible appearance of a major 24-kilodalton (kDa) protein band was observed in iron-deficient medium after 30 days of incubation. Five other proteins of ~105-, 82-, 31-, ~16.5-, and ~15.5-kDa were also synthesized in reduced amounts. Moreover, the expression of three proteins of 46-, 37-, and 12.5-kDa was amplified in comparison with their expression under iron-sufficient conditions.

Discussion

The presence of siderophores in nine strains of *M. mycetomatis* was first detected by observing the development of the characteristic yellow-orange color reaction in CAS-YMA. This result was not unexpected since, following the first indication of siderophores produced by the fungi *Histoplasma capsulatum* [6], siderophores have been isolated from numerous pathogenic fungi, including *Aspergillus* spp., *Blastomyces dermatitidis*, *Candida albicans*, *Paracoccidioides*

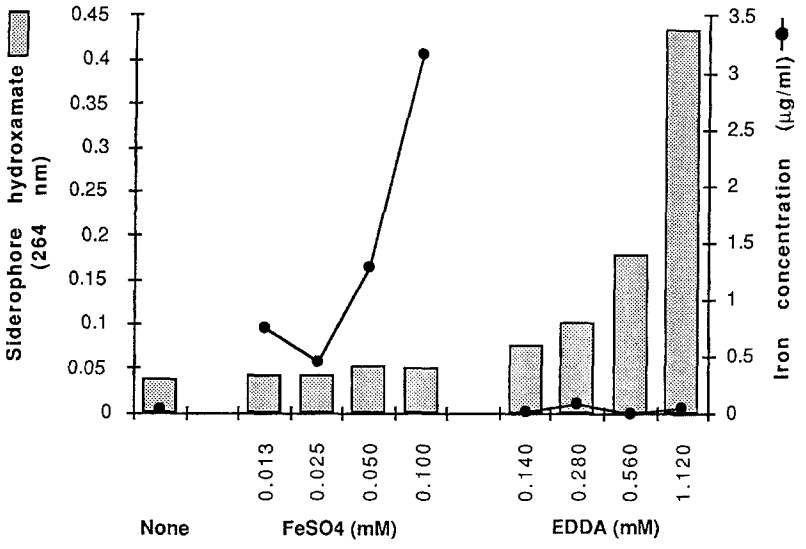

Fig. 1. Influence of extracellular iron concentration on siderophore production. Level of hydroxamate-type siderophore of strain 1175–79 was measured by absorption at 264 nm after oxidation in LIM supplemented with either $FeSO_4$ or EDDA after 15 days' incubation.

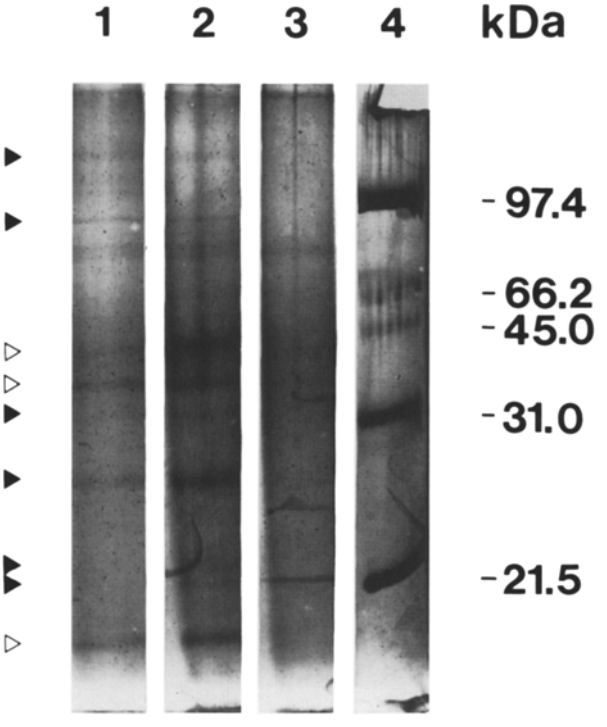

Fig. 2. SDS-PAGE of outer membrane proteins from *Madurella mycetomatis* 1175-79. This strain of *M. mycetomatis* was grown for 30 days on LIM (lane 1), LIM + EDDA (lane 2), and LIM + $FeSO_4$ (lane 3). Phosphorylase B (97.4-kDa), bovine serum albumin (66.2-kDa), ovalbumin (45.0-kDa), bovine carbonic anhydrase (31.0-kDa), and soybean trypsin inhibitor (21.5-kDa) were used as calibration proteins (lane 4). White arrows show the amplified proteins, and black arrows the neo-synthesized proteins in iron-deficient media.

brasiliensis, *Rhizopus* spp., *Sporothrix schenckii*, and *Trichophyton mentagrophytes* [7, 10, 12, 14]. One exception of apparently siderophore nonproducing pathogenic fungi concerns *Cryptococcus neoformans*, which seems able to reduce extracellular ferric ion before it can be internalized without the participation of either a siderophore or a cell surface receptor [13].

Specific determination of the siderophores produced indicated that they belonged only to the hydroxamate class. This would seem to be consistent with the observations of other investigators who reported that fungi, either pathogenic or not, produced siderophores only of the hydroxamate type [22]. The known exception concerns rhizoferrin, a polyhydroxycarboxylamid-type siderophore produced by *Rhizopus microsporus* var. *rhizopodiformis* [8], and probably phenolate-type siderophore production by approximately 40% of *C. albicans* isolates, simultaneously with production of hydroxamate-type siderophores [11], both siderophores being equally effective in promoting growth of this yeast [12].

At least one OMP of 24-kDa was found to be induced under iron-deficient conditions and repressed under iron-sufficient conditions. Although iron-regulated protein expression has been reported in several bacteria [20], our observation is the first to describe such OMP in fungi. This protein may participate in iron metabolism by direct binding of the traces of iron available in the medium and thus could be the surface receptor permitting the siderophore-facilitated iron translocation through the outer membrane. Whether the 24-kDa OMP (or one of the other neo- or overexpressed OMP) of *M. mycetomatis* might be acting as ferrous siderophore receptors is under investigation.

It has not been determined whether *M. mycetomatis* produces siderophores under physiological conditions in vitro or in vivo and whether possession of an iron-uptake system is a determinant of virulence.

ACKNOWLEDGMENT

We thank Dr. Françoise Dromer (Institut Pasteur) for her help in the preparation of the manuscript.

Literature Cited

1. Arnow LE (1937) Colorimetric determination of the components of 3,4-dihydroxyphenylalanine-tyrosine mixtures. J Biol Chem 118:531–537
2. Atkin CL, Neilands JB, Phaff HJ (1970) Rhodotorulic acid from species of *Leucosporidium*, *Rhodosporidium*, *Sporidiobolus*, and *Sporobolomyces* and a new alanine-containing ferrichrome from *Cryptococcus melibiosum*. J Bacteriol 103:722–733
3. Bagg A, Neilands JB (1987) Molecular mechanism of regulation of siderophore-mediated iron assimilation. Microbiol Rev 51:509–518
4. Bullen JJ, Rogers HJ, Griffiths E (1978) Role of iron in bacterial infection. Curr Top Microbiol Immunol 80:1–35
5. Burt WR (1982) Identification of coprogen B and its breakdown products from *Histoplasma capsulatum*. Infect Immun 35:990–996
6. Burt WR, Underwood AL, Appleton GL (1981) Hydroxamic acid from *Histoplasma capsulatum* that displays growth factor activity. Appl Environ Microbiol 42:560–563
7. Castaneda E, Brummer E, Perlman AM, McEwen JG, Stevens DA (1988) A culture medium for *Paracoccidioides brasiliensis* with plating efficiency, and the effect of siderophores. J Med Vet Mycol 26:351–358
8. Drechsel H, Metzger J, Freund S, Jung G, Boelaert JR, Winkelmann G (1991) Rhizoferrin, a novel siderophore from the fungus *Rhizopus microsporus* var. *rhizopodiformis*. BioMetals 4:238–243
9. Emery T (1974) Biosynthesis and mechanism of action of hydroxamate-type siderochromes. In: Neilands JB (ed) Microbial iron metabolism. New York: Academic Press, Inc., pp 107–123
10. Holzberg M, Artis WM (1983) Hydroxamate siderophore production by opportunistic and systemic fungal pathogens. Infect Immun 40:1134–1139
11. Ismail A, Lupan DM (1986) Utilization of siderophores by *Candida albicans*. Mycopathologia 96:109–113
12. Ismail A, Bedell GW, Lupan DM (1985) Siderophore production by the pathogenic yeast, *Candida albicans*. Biochem Biophys Res Commun 130:885–891
13. Jacobson ES, Vartivarian SE (1992) Iron assimilation in *Cryptococcus neoformans*. J Med Vet Mycol 30:443–450
14. Jalal MAF, Mocharla R, Barnes CL, Hossain MB, Powell DR, Eng-Wilmot DL, Grayson SL, Benson BA, Van der Helm D (1984) Extracellular siderophores from *Aspergillus ochraceus*. J Bacteriol 158:683–688
15. Kwon-Chung KJ, Bennett JE (1992) Mycetoma (Madura foot, maduromycosis). In: Kwon-Chung KJ, Bennett JE, Medical mycology. Philadelphia: Lee & Febiger, pp 560–593
16. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685
17. Litwin CM, Calderwood SB (1993) Role of iron in regulation of virulence genes. Clin Microbiol Rev 6:137–149
18. Martinez JL, Delgado-Iribarren A, Baquero F (1990) Mechanisms of iron acquisition and bacterial virulence. FEMS Microbiol Rev 75:45–56
19. Neilands JB (1972) Evolution of biological iron binding centers. Struct Bonding 11:145–170.
20. Schryvers AB, Lee BC (1993) Analysis of bacterial receptors for host iron-binding proteins. J Microbiol Methods 18:255–266
21. Schwyn BC, Neilands JB (1987) Universal chemical assay for the detection and determination of siderophores. Anal Biochem 160:47–56
22. Winkelmann G (1991) Specificity of iron transport in bacteria and fungi. In: Winkelmann G (ed), Handbook of microbial iron chelates. Boca Raton, Florida: CRC Press, pp 65–105
23. Young IG, Cox GB, Gibson F (1967) 2,3-Dihydroxybenzoate as a bacterial growth factor and its route of biosynthesis. Biochim Biophys Acta 141:319–331