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Characterization of siderophores produced by different species of the dermatophytic fungi *Microsporum* and *Trichophyton*

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The dermatophytic fungi *Trichophyton* spp and *Microsporum* spp secrete ferrichrome type siderophores under low iron conditions. Three different species of *Microsporum*, i.e. *M. qypseum*, *M. canis* and *M. audouinii*, as well as *T. rubrum* produce ferrichrome C and ferricrocin, whereas *T. mentagrophytes* and *T. tonsurans* produce only ferrichrome. The identification of the siderophores was established by means of thin layer chromatography, high performance liquid chromatography and mass spectroscopy.

Keywords: *Microsporum*, siderophores, *Trichophyton*

Introduction

The availability of iron has been shown to be a critical factor in pathogenicity of microorganisms invading living hosts (Bullen & Griffiths 1987, Weinberg 1989). The human body has evolved elaborate defense mechanisms to deprive the invading microorganisms of iron. The amount of free iron which might be available to the invading pathogen within the host is extremely low. Most of the intracellular iron is bound in ferritin, hemosiderin or heme whereas the extracellular iron in normal body fluids, i.e. plasma and mucosal secretions, is chelated to high affinity iron-binding glycoproteins such as transferrin and lactoferrin. Consequently, pathogenic microbes must be endowed with effective mechanisms for iron assimilation in order to survive and function in iron restricted environments (Winkelmann 1991).

Dermatophytes are a group of fungi capable of causing superficial infections in humans or animals (Emmons *et al.* 1963). These fungi are physiologically adapted for growth on keratin and their infections are usually limited to the epidermis and its appendages. The inability of dermatophytes to invade deeper tissues has also been attributed to the inhibitory activity of the serum (Carlisle *et al.* 1974). King *et al.* (1975) identified the serum inhibition factor for dermatophyte growth as transferrin and demonstrated that addition of iron, but not other metals, reduced the inhibitory capacity of the serum. These results focused attention on the ability of dermatophytic fungi to compete for iron by means of siderophores (Artis *et al.* 1984). With the exception of ferricrocin, which has been identified as the principal siderophore of *Microsporum gypseum* (Bentley *et al.* 1986), no information is available on the nature and distribution of siderophores among the dermatophytes. The present study was undertaken to characterize the siderophores produced by six different species of *Microsporum* and *Trichophyton* which constitute the two major genera of fungi causing dermatophytoses.

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Materials and methods

Organisms and growth conditions

Clinical isolates of *T. mentagrophytes*, *T. tonsurans*, *T. rubrum*, *M. gypseum*, *M. canis* and *M. audouinii* were obtained from the department of Human Microbiology at Tel Aviv University and from different hospitals in Israel. Cultures were maintained on potato dextrose agar at 26 °C or stored at 5 °C. For siderophore production the *Trichophyton* isolates were grown for 22 days in 1 l Roux bottles containing 100 ml of a medium composed of the following compounds ($g l^{-1}$ of double distilled water): glucose, 20;

asparagine, 2; K_2HPO_4, 0.75; KH_2PO_4, 0.75; $MgSO_4$, l: $ZnSO_4$, 0.02; $MnSO_4$, 0.02; pyridoxin, 0.005; thiamine–HCl, 0.01. The *Microsporum* isolates were grown for 14 days at 26 °C on a rotary shaker at 200 r.p.m. in 1 l conical flasks containing 200 ml of the same liquid medium.

Extraction and purification of siderophores

At the end of the growth period the mycelia were removed by filtration through Ederol no. 15 filter paper and the culture filtrates (1500 ml) were evaporated under vacuum at 30 °C to 1/10 of the original volume. Excess $FeCl_3$ was added, and the reddish solution was saturated with $(NH_4)_2SO_4$ and left overnight at 4 °C. The filtrate was then centrifuged at 10 000 × *g* for 10 min and the clear supernatant was extracted with benzoyl alcohol as described previously. The colored aqueous solution was reduced to dryness *in vacuo* and dissolved in 50 ml methanol. The undissolved compounds were removed by centrifugation, and the supernatant was taken to dryness and dissolved in 3 ml distilled water.

The aqueous siderophore solution was initially chromatagraphed on a Whatman anion exchange column DE52 (2.5 × 15 cm) and rinsed with 50 ml distilled water to elute neutral or positively charged siderophores. No detectable siderophores were retained by the column. The water eluant was reduced to dryness *in vacuo*, dissolved in chloroform:ethanol:water (80:19:1) and chromatographed on a silica gel 60H column (2 × 20 cm). Siderophores were eluted with chloroform:ethanol:water (80:19:1) by employing the technique of vacuum liquid chromatography (Targett *et al.* 1979). The siderophores were further separated by high performance liquid chromatography (HPLC) on a RP-C_{18} (5 μm) using a gradient of 6% acetonitrile/94% ammonium acetate, pH 3 (6–40%) as described by Knoetschny-Rapp *et al.* (1988).

Fast atom bombardment (FAB) mass spectrometry

FAB spectra were recorded with a Varian MAT 711 A instrument combined with an SS 200 data system with glycerol as solvent and a xenon ionizing beam produced with a saddle-field primary atom gun (Ion Tech, UK) at an ion source temperature of 25 °C.

Ion spray mass spectrometry

Ion spray mass spectra were recorded via direct injection of siderophore solutions on a Sciex API III triple-quadrupole mass spectrometer with 2400 Da mass range equipped with an ion spray ion source (Sciex, Toronto, Canada), as described in a previous paper (Haselwandter *et al.* 1992).

Analytical procedures

For structural determination, the siderophores were deferrated with 8-hydroxyquinoline (Wiébe & Winkelmann 1975). The amount of siderophores was estimated according to Subramanian *et al.* (1965). Thin layer chromatography (TLC) with various solvents was performed as previously described (Manulis *et al.* 1987). ^{1}H-NMR and ^{13}C-NMR spectra were recorded on a Bruker WM-360 spectrometer.

Results and discussion

All the species of *Microsporum* and *Trichophyton* studied in the present investigation were found to produce hydroxamate siderophores during growth under iron limitation. The amounts of siderophores produced by *T. mentagrophytes* and *M. gypseum* at 22 days were 16 and 52 mg, respectively, per gram fresh weight of mycelium. Siderophores were extracted and purified from 22 day old cultures of *Trichophyton* sp. and 14 day old cultures of *Microsporum* sp. When the crude siderophore extracts were analyzed by TLC, two orange spots with identical R_f values of 0.46 and 0.18 were observed in *M. gypseum, M. canis, M. audouini* and *T. rubrum,* whereas a single spot with an R_f of 0.26 was observed in *T. mentagrophytes* and *T. tonsurans* (Figure 1). The fast and slow moving siderophores in the four former species were designated as I and II, respectively, whereas the single siderophore detected in the latter two species was designated as III.

Preparative purification of the siderophores from *M. gypseum, T. rubrum* and *T. mentagrophytes* was achieved by a silica gel column as described earlier. Depending on the fungal species used, either siderophore I, II or III was eluted according to the orange band observed. Each siderophore was subjected to HPLC analysis on a C_{18} reversed-phase column under conditions described earlier. Results shown in Figure 2 indicate that siderophore I of *T. rubrum* was eluted at a retention time of 12.53 min, which

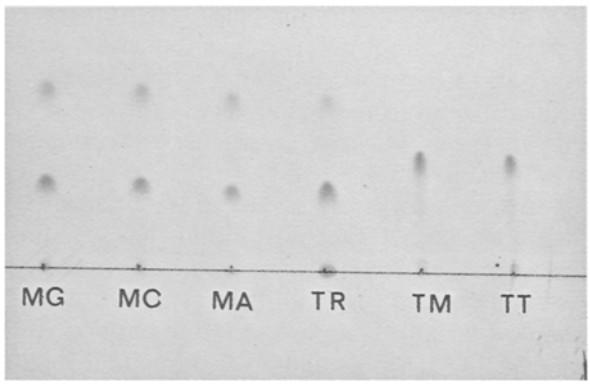

Figure 1. TLC of crude siderophore preparations from various dermatophytes. Siderophores were separated with chloroform:ethanol:water (80:19:1, v/v) as solvent. TM, *T. mentagrophytes*; TT, *T. tonsurans*; TR, *T. rubrum*; MG, *M. gypseum*; MC, *M. canis*; and MA, *M. audouini*.

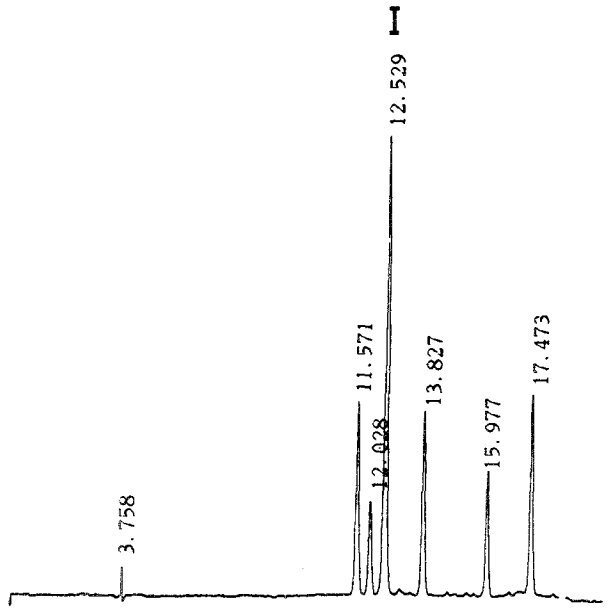

Figure 2. HPLC separation of siderophore I from *T. rubrum*. Chromatography was performed on a C_{18} reversed-phase column as described in Materials and methods. A standard mixture of reference siderophores was co-injected with the unknown as described in Knoetschny-Rapp *et al.* (1988). The standard siderophores and their retention time (min) were as follows: ferricrocin (11.57), ferrichrome (12.03), ferrichrome C (12.53), Coprogen (13.83), ferrirubin (15.98) and ferrirhodin (17.47). I, unknown siderophore.

corresponds to ferrichrome C. The same retention time was obtained with siderophore I of *M. gypseum*. The retention time for siderophore II from either *T. rubrum* or *M. gypseum* was 11.56 min, which corresponds to ferricrocin, whereas a retention time of 12.03 min, corresponding to ferrichrome, was obtained with siderophore III of *T. mentagrophytes* (results not shown).

The identity of the three siderophores was confirmed by either FAB or ion spray mass spectroscopy. The molecular mass peaks $[M + H]^+$ for siderophores I, II and III were found at *m/z* 755, 771 and 741, respectively (Figure 3), corresponding to molecular weights of 754, 770 and 740. The obtained molecular weights and mass spectra of siderophores I, II and III resembled those of authentic ferrichrome C, ferricrocin and ferrichrome, respectively. The type and number of hydroxamate siderophores produced by the different dermatophytic fungi are summarized in Table 1. In order to confirm that siderophores I and II were identical in all three *Microsporum* spp and *T. rubrum*, and that siderophore III was identical in *T. mentagrophytes* and *T. tonsurans*, they were subjected to TLC analysis with two additional solvents: chloroform:methanol:water (35:12:2, v/v) and dioxane:ethanol (8:2, v/v). Identical R_f values were obtained with all of the corresponding siderophores.

Results presented indicate that siderophore production is characteristic of dermatophytic fungi. Ferricrocin, ferrichrome C and ferrichrome have been shown to be also prevalent among other ascomycetous fungi such as *Aspergillus* spp, *Penicillium* spp and *Neurospora crassa* (Diekmann 1975, Horowitz *et al.* 1976, Winkelmann 1987). It has been previously postulated that siderophore identity may prove to be a useful trait in fungal taxonomy (Charlang *et al.* 1981). This assumption could be

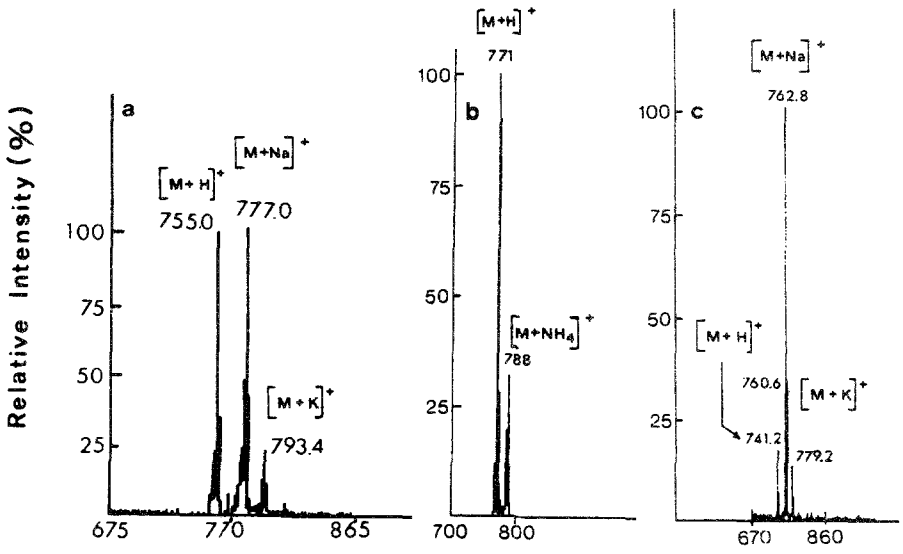

Figure 3. FAB or ion spray mass spectra of (**a**) siderophores I of *M. gypseum*, (**b**) siderophore II of *T. rubrum* and (**c**) siderophore III of *T. mentagrophytes*.

Table 1. A summary of siderophores produced by different species of *Microsporum* and *Trichophyton*

Fungus	Ferrichrome C	Ferricrocin	Ferrichrome
M. gypseum	+	+	−
M. canis	+	+	−
M. audouinii	+	+	−
T. rubrum	+	+	−
T. mentagrophytes	−	−	+
T. tonsurans	−	−	+

supported by the identity of the two siderophores produced by all three Microsporum spp (Table 1). However, the striking similarity between siderophores produced by *T. rubrum* and the *Microsporum* spp as opposed to the two other *Trichophyton* spp contradicts this hypothesis. It appears that the dermatophytic fungi exhibit a capacity for siderophore production similar to that of other fungi (Winkelmann & Huschka 1987). Nevertheless it might be possible that their secretion rate or other properties are insufficient to antagonize transferrin's antifungal activity and host defense.

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