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Ferricrocin - an ectomycorrhizal siderophore of *Cenococcum geophilum*

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Abstract

The ectomycorrhizal fungus *Cenococcum geophilum* was grown in low-iron medium and the excreted siderophores were extracted, purified and analyzed by HPLC. The principal hydroxamate siderophore produced, was identified as ferricrocin as confirmed by analytical HPLC, FAB-mass spectrometry and ^{1}H- and ^{13}C-NMR spectra. Although the occurrence of ferricrocin has been shown earlier to occur in the ericoid mycorrhizal fungi, this is the first report of ferricrocin in a true ectomycorrhizal fungus which is taxonomically related to the ascomycetes.

Introduction

Mycorrhizal symbiosis is a common phenomenon in all terrestrial plant communities. One of the major types of mycorrhiza is the ectomycorrhiza, typically formed by almost all tree species in temperate forests. Mycorrhizal infection affects the mineral nutrition of plants, including micronutrient uptake (Haselwandter & Bowen 1996; Smith & Read 1997). One of the most important mineral nutrients is iron being scavenged by excreted siderophores in nearly all bacterial and most fungal genera (Drechsel & Winkelmann 1997). So far, the chemical structure of the main siderophores released has only been described for three ericoid mycorrhizal fungal species and the ectendomycorrhizal fungus *Wilcoxina* (Haselwandter *et al.* 1992; Haselwandter 1995; Prabhu *et al.* 1996). *Cenococcum geophilum* covers an enormous ecological amplitude and appears to be adapted to extreme site conditions (Bledsoe *et al.* 1990; Haselwandter & Read 1980; Theodorou 1978; Trappe 1988). In its natural environment this fungus forms only sterile mycelia, thus rendering it difficult to study. In addition to typical ectomycorrhizal root tips (Agerer & Gronbach 1988), *C. geophilum* produces occasionally sclerotia which can amount to a biomass of something like 2785 kg per ha in a Western Oregon forest ecosystem (Fogel & Hunt 1979). The build-up of all that fungal biomass requires adequate amounts of iron.

Application of a bioassay involving *Microbacterium* previously named *Arthrobacter* or *Aureobacterium*) *flavescens* JG-9 has revealed that a range of different ectomycorrhizal fungi has the potential of releasing hydroxamate siderophores (Haselwandter 1995). A mixture of unidentified hydroxamate siderophores isolated from the ectomycorrhizal fungus *Boletus edulis* was shown to have a significant influence on Fe solubility (Cline *et al.* 1982). In addtion to thirteen other strains of ectomycorrhizal fungi, *C. geophilum* proved to release significant quantities of unidentified hydroxamates as determined by the *M. flavescens* JG-9 bioassay (Szaniszlo *et al.* 1981). The iron metabolism of *Cenococcum* was discussed by Rodriguez *et al.* (1984) without structural elucidation of the excreted siderophores.

The ectomycorrhizal fungus *C. geophilum* is known to represent an ecologically important ectomycorrhizal fungus with a global distribution and a broad host range. In this paper we report on the isolation and chemical structure of the main siderophore released by *C. geophilum* under iron limiting conditions in vitro. To our knowledge, this is the first report of the chemical nature of an hydroxamate siderophore released by an ectomycorrhizal fungus.

Materials and methods

We used strain Cegeo1 (culture collection of the Department of Microbiology, University of Innsbruck, Austria) of *Cenococcum geophilum* Fr., kindly supplied by C. Plassard, INRA, F-34060 Montpellier, France. The isolate was sub-cultured three times in 100 ml low iron medium (LIM-1 medium with 1 g l^{-1} of each, L-proline and L-ornithine.HCl; Szaniszlo *et al.* 1981) in 250 ml Erlenmeyer flasks on a gyratory shaker at 150 rev min^{-1} and 25 °C in order to reduce possible iron storage by the fungal mycelium. Furthermore, the low iron medium was deferrated with Chelex 100 according to Haselwandter & Winkelmann (1998). The siderophores were extracted from 730 ml of supernatant of final cultures after 22 days of incubation. At that time the siderophore concentration of the culture filtrate as determined by the CAS assay (Schwyn & Neilands 1987) was equivalent to 335 μM ferrioxamine B.

Isolation of siderophores

The hydroxamate siderophores were extracted from the culture filtrate following the procedure of Haselwandter & Winkelmann (1998). The siderophores were adsorbed onto XAD-16 (Sigma, Munich, Germany), washed with three volumes of distilled water and desorbed with one volume of methanol, followed by further purification using Biogel P2 (Biorad, Munich, Germany). After evaporation of the solvent under vacuum and lyophilisation, the residue was re-dissolved in a known quantity of distilled water, filtered through a 0.2 μm Minisart RC4 filter (Sartorius, Göttingen, Germany) and analyzed by HPLC. For structural formulas of ferrichrome-type siderophores the reader is referred to a previous paper (Heymann *et al.* 2000) where the transport of ferrichromes via MFS transporters was studied in *Saccharomyces cerevisiae*.

Identification of siderophores

HPLC

The extract was separated on a Nucleosil C_{18} column (5 μm, 4.6 mm × 250 mm) fitted with an adequate pre-column using a gradient of distilled water + 0.1% formic acid or TFA acetonitrile + 0.1% formic acid or TFA. Gradient: 6–15% acetonitrile in 10 min, 15–60% in 15 min, 60% for 5 min. Flow rate: 0.87 ml/min; injected volume: 20 μl. HPLC separations were performed with a Shimadzu LC10 or a Hewlett Packard Series 1100 equipped with diode-array-detector. Absorption was measured at 435 nm.

Mass spectrometry

Fast-atom-bombardment (FAB) spectra were recorded on a Varian MAT 711 instrument, equipped with a SS 200 data system. FAB spectra were measured from a matrix of glycerol/p-toluenesulfonic acid. The ion source temperature was 323 K.

NMR spectra

^{1}H and ^{13}C NMR spectra were recorded at 250 and 400 MHz (Bruker AC 250/AMX 400), respectively, at 305 K using iron-free ferricrocin, deferrated by the hydroxyquinoline method (Wiebe & Winkelmann 1975) in D_2O solution (c = 10 mg ml^{-1}, 303 K). Resonances were assigned by chemical shift correlation with known siderophores (Jalal & van der Helm 1991).

Results

As the iron concentration was claimed to represent the overriding factor regulating the production of hydroxamate siderophores by ectomycorrhizal fungi (Szaniszlo *et al.* 1981), *C. geophilum* was grown in a deferrated nutrient medium. Under such conditions the isolated siderophores showed one main peak (Figure 1, No. 4) in the HPLC chromatogram possessing a retention time equivalent to ferricrocin. The identity could be confirmed by simultaneous injection (spiking) of authentic ferricrocin together with the isolated sample into the HPLC column (data not shown). The chromatogram also contains some minor hydroxamate compounds as evidenced by additional peaks (No. 3, 5, 6, 7), detectable at 435 nm and achieving peak areas much smaller than ferricrocin. Due to the low amount of these additional siderophores produced in the culture, a structural identification was not performed in this investigation. However, a comparison with known retention times of our siderophore collection revealed that the retention time of No. 3 is similar to that of the fusarinine monomer, that of No. 5 corresponds to ferrichrome and No. 6 and 7 are equivalent to fusigen and coprogen, respectively. Although these are tentative results, they show that ferricrocin is not the only hydroxamate siderophore produced and that additional hydroxamate siderophores occur as found in other siderophore producing fungi.

Evidence of the structural identity of ferricrocin was obtained by mass spectrometry and NMR analy-

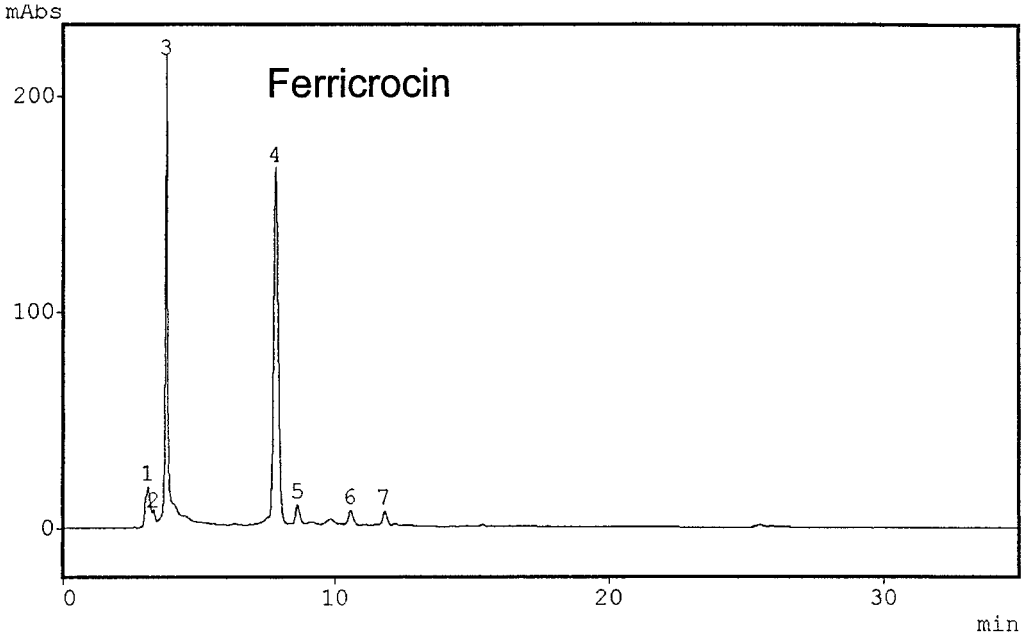

Fig. 1. HPLC separation of the isolated siderophores from culture filtrates of *C. geophilum* grown in low iron medium (LIM-1). Analytical column Nucleosil C_{18}, 5 μm, 4.6 × 250 mm, sample volume 20 μl, photometric detection at 435 nm. Gradient of distilled water /acetonitrile with 0.1% TFA, 6–60%. Peak No.4 = Ferricrocin, peak No.3. 5, 6, 7 are unidentified siderophores.

sis. FAB mass spectrometry (Figure 2) of the iron-containing complex revealed a molecular mass of m/z = 771.0 (MH^+) and an additional ion peak at m/z = 717.8 corresponding to M-Fe+3H. Loss of oxygen resulted in m/z = 701.8. These results provide clear evidence for the presence of ferricrocin (MW 770), a common siderophore of ascomycetous fungi including *Aspergillus* and *Neurospora* (Van der Helm & Winkelmann 1994).

Further structural proof for the presence of ferricrocin was obtained from ^{1}H NMR and ^{13}C NMR spectra. The chemical shifts of the recorded ^{13}C NMR spectrum of the isolated ferricrocin are given in Table 1 which could be assigned by comparison with known data (Jalal & van der Helm 1991). ^{1}H NMR data were also in accordance with the proposed ferricrocin structure (data not shown). Taken together, HPLC, mass spectra and NMR data clearly showed that ferricrocin is produced as the major siderophore by the mycorrhizal fungus *C. geophilum* when grown under iron limitation.

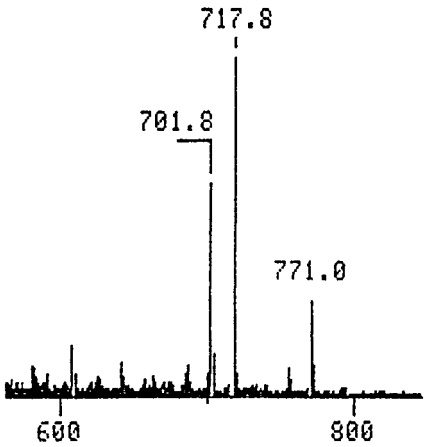

Fig. 2. FAB mass spectrum (600–800 m/z) of ferricrocin isolated from *Cenococcum geophilum*. The iron-containing complex gave a molecular mass of m/z = 771.0 (MH^+) and an additional ion peak at m/z = 717.8 (M-Fe+3H) corresponding to the iron-free siderophore. Loss of oxygen resulted in m/z = 701.8.

Discussion

C. geophilum has undoubtedly an enormous geographic distribution covering a wide range of ecologically quite different habitats. In a Norway spruce forest in south Sweden *Cenococcum* ectomycorrhizae accounted for 18% of the mycorrhizal abundance of the mycorrhizal root tips examined in a 100-year-old Norway spruce forest (Dahlberg *et al.* 1997). Ectomycorrhizae formed by *Cenococcum* belong also to those which are most abundant on naturally regen-

Table 1. ^{13}C NMR chemical shifts of the isolated desferriform of ferricrocin.

Chemical shift (ppm)	assigned structural group
20.2	-CO-$\underline{C}H_3$
24.0	orn $CH_2\gamma$
24.2	orn $CH_2\gamma$
24.4	orn $CH_2\gamma$
28.4	orn $CH_2\beta$
30.1	orn $CH_2\beta$
30.2	orn $CH_2\beta$
43.8	gly $CH_2\alpha$
44.4	gly $CH_2\alpha$
54.5	orn CH α
55.0	orn CH α
56.0	orn CH α
56.7	ser CH α
62.3	ser $CH_2\beta$
169.9	hydroxamic C=O
171.8	gly C=O
172.5	gly C=O
173.7	ser C=O
174.2	orn C=O
174.3	orn C=O
174.5	orn C=O

erated western hemlock across a range of different forest openings (Kranabetter & Wylie 1998). In jack pine stands *C. geophilum* was shown to represent one of the multi-stage species in ectomycorrhizal fungal succession (Visser 1995). ITS-RFLP analysis of the spatial distribution of mycorrhizae and propagules in soil has revealed that *Cenococcum* propagules can be localized, thus exhibiting a patchy distribution over the soil profile (Taylor & Bruns 1999). The distribution of *C. geophilum* ectomycorrhizae may be affected by the qualitiy and quantitiy of litter (Walker *et al.* 1999). On the other hand, the relative abundance of *Cenococcum* ectomycorrhizae appeared to be stable, even when nitrogen was added to an oligotrophic Norway spruce forest in SW Sweden (Jonsson *et al.* 2000). As *C. geophilum* is so widespread both in terms of geographic distribution and the host range this species forms mycorrhizae with, it seems feasible to select this fungus as first example of ectomycorrhizal fungi for determination of their potential with regard to siderophore synthesis, and for elucidation of the chemical structure of the main compound released. The implication of siderophores in microbial metabolism, iron nutrition in particular, is reviewed for example by Winkelmann (2001).

As *C. geophilum* lacks any significant morphological variability, its taxonomy and phylogeny have until recently remained unclear (Hawksworth *et al.* 1995). Shinohara *et al.* (1999) constructed a molecular phylogeny based on the nuclear ribosomal DNA (internal transcribed spacers ITS1 and ITS2) of 69 isolates from various habitats and hosts. It appears that the DNA sequence in the ITS regions is highly conserved despite the wide geographic distribution and the broad host range of this ectomycorrhizal fungus. In addition, *C. geophilum* has only very short ITS regions, thus limiting the number of mutable sites and the variability of ITS regions. Apparently, according to this study *C. geophilum* is a single taxonomic entity and extremely adaptable to different ecological conditions. Farmer & Sylvia (1998) have described some intraspecific variation within *C. geophilum* on basis of ITS-RFLP analysis of a set of 19 isolates.

Phylogenetic analysis of nucleotide sequence data from the nuclear small subunit (18S) ribosomal RNA positioned *C. geophilum* as a basal, intermediate lineage between the two Loculascomycete orders, the Pleosporales and the Dothideales (Lobuglio *et al.*1996). This is of particular interest as ferricrocin is regarded as a siderophore typical for Ascomycetes (van der Helm & Winkelmann 1994). Many strains of *Aspergillus* and *Penicillium* are known to produce ferrichrome-type siderophores among which ferricrocin is often predominant (van der Helm & Winkelmann 1994). Ferricrocin is a prominent siderophore of the *A. fumigatus* group (Diekmann & Krezdorn 1975; Wiebe & Winkelmann 1975), of some dermatophytic fungi (Bentley *et al.* 1986; Mor *et al.* 1992), and also occurring as an intracellular siderophore in *Neurospora crassa* (Matzanke *et al.* 1987, 1988). Hence, our finding that *C. geophilum* produces ferricrocin as main siderophore can be regarded as additional evidence corroborating the (hypo)thesis that this species is a teleomorph of an ascomyceteous fungus.

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