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IDENTIFICATION OF SIDEROPHORES AND SIDEROPHORE-MEDIATED UPTAKE OF IRON IN *STEMPHYLIUM BOTRYOSUM*

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Key Word Index—*Stemphylium botryosum*; siderophores; iron uptake.

Abstract—Under iron-deficient conditions *Stemphylium botryosum* f. sp. *lycopersici* produces three major siderophores; dimerum acid, coprogen B and an unidentified monohydroxamate siderophore designated as A. The system of siderophores mediating uptake of iron was characterized. It exhibits active transport, saturation kinetics and an optimum at pH 6 and 30°. The rate of iron uptake via dimerum acid and coprogen B was four times higher than siderophore A. *S. botryosum* was capable of taking up iron from hydroxamate siderophores produced by other fungi, e.g. ferrichrome, fusigen, rhodotorulic acid but not ferrioxamine B. Double labelling experiments suggest that ferric coprogen B accumulates in mycelial cells as an intact chelate.

INTRODUCTION

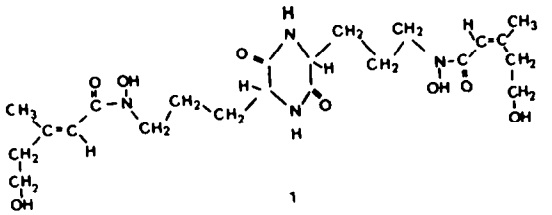
The uptake of iron in fungi and other microorganisms under iron deficient conditions, is generally mediated by siderophores [1, 2]. Siderophores are low-molecular-weight ferric chelating agents which supply the metal to the cell by binding to specific receptors in the cell membrane. An array of hydroxamate siderophores have been isolated from a small number of fungi belonging to the Basidiomycetes, Ascomycetes and Deuteromycetes [2, 3].

Stemphylium botryosum Walr. f. sp. *lycopersici* is the causal agent of leaf spot and foliage blight disease of tomato [4]. The capacity of an invading pathogen to obtain iron from living tissue may emerge as a critical element in the host–pathogen interaction. Evidence in support of the latter hypothesis has been adduced in bacterial and fungal diseases of animals [1] and could be involved in virulence of plant pathogens as well. *S. botryosum* was reported to secrete two novel phytotoxic chelates of ferric ion designated as stemphyloxin I and stemphyloxin II [4–6]. Stemphyloxins differ from siderophore compounds in their dependence on iron for optimal biosynthesis and a distinctively lower affinity for ferric ion [6]. They have been recently implicated in the acquisition of iron by *S. botryosum* under conditions of iron sufficiency [7]. The present study was undertaken to characterize the siderophore mediated absorption of iron by the phytopathogenic fungus *S. botryosum*.

RESULTS AND DISCUSSION

Isolation and identification of siderophores

Siderophores were extracted from filtrates of 16-day-old cultures and purified as described in the Experimental. Three major components eluting from the Bio-Gel P-2 column were designated as A, B and C according to their migration towards the cathode on paper electrophoresis. Siderophore C was a highly cationic compound whereas B and A were only slightly cationic in pyridine–acetate buffer, pH 5.2. Further purification was achieved by chromatography on a Cellex CM column which exhibited differential adsorption towards the three siderophores. Thus siderophores A and B were eluted with distilled water whereas siderophore C was removed only with pyridine–acetate buffer, pH 5.2. Each siderophore was finally brought to homogeneity by employing preparative



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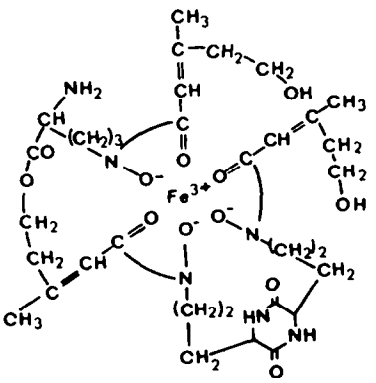

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paper electrophoresis and separation on Sephadex LH-20 (see Experimental).

Initial identification of siderophore B as dimerum acid (1) and C as coprogen B (2) was carried out by cochromatography with authentic samples in silica gel TLC (Table 1). Identical migration rates of siderophore B with dimerum acid and siderophore C with coprogen B were also demonstrated with high voltage electrophoresis. Comparative ^{1}H NMR spectra and IR spectra, with literature values [8–10] also supported the identity of siderophore B as dimerum acid and siderophore C as coprogen B. The structure of siderophore A has not yet been determined. However, based on its stability towards dissociation in acid solution and decolourization in the presence of 0.02 M EDTA it could be identified as a monohydroxamate [11]. Thus the maximal absorbance of siderophore A was shifted from 440 nm at pH 7 to 460 nm at pH 4.6 and was associated with a decrease in absorbance. The production of dimerum acid has previously been reported in *Fusarium dimerum* [8] and *Verticillium dahliae* [9] whereas coprogen B has been found in *Fusarium* sp. and *Myrothecium* sp. [8].

Production of siderophores

The secretion of siderophores by *S. botryosum* was dependent on iron deficiency and was completely repressed in the presence of 2 μM iron (Fig. 1). Production of siderophores was correlated with mycelial growth and reached maxima during the stationary growth phase after approximately 16 days. A sharp decrease in siderophore concentration, presumably due to degradation, was observed after 20 days. The total amount of siderophores secreted by *S. botryosum* was approximately 125 mg/l. The relative amounts of siderophore A, dimerum acid and coprogen B after 16 days were 2.2:1:1.5, respectively.

Characterization of siderophore-iron uptake by S. botryosum

Uptake of ^{59}Fe-labelled coprogen B, dimerum acid and siderophore A was found to be linear within 2 hr (Fig. 2). The transport rate of ^{59}Fe-siderophore A was four times lower than with the two other siderophores. Iron uptake was completely inhibited by 1 mM sodium azide. All three

Table 1. R_f values of siderophores in four solvents

Siderophore*	R_f in solvent†			
	1	2	3	4
A	0.68	0.19	0.25	0.37
B	0.72	0.10	0.13	0.34
C	0.54	0.07	0.11	0.49
Dimerum acid	0.72	0.10	0.13	0.34
Coprogen B	0.54	0.07	0.11	0.49
Rhodotorulic acid	0.61	0.05	0.07	—
Fusigen	0.66	0.03	0.06	—

*The siderophores were taken to dryness and dissolved in MeOH prior to their application on TLC.

†TLC in: (1) dioxan–0.33 N HOAc (2:1); (2) *n*-BuOH–HOAc–H_2O (4:1:1); (3) *n*-PrOH–HOAc–H_2O (4:1:1); (4) *n*-PrOH–NH_4OH (14:1).

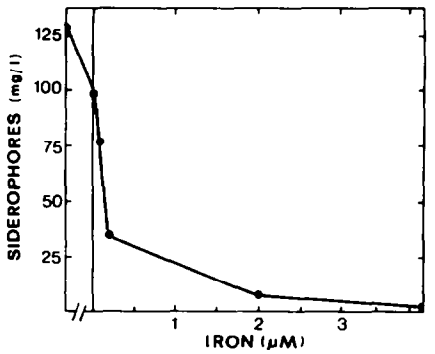

Fig. 1. Production of siderophores by *S. botryosum* as a function of iron concentration. Siderophore concentration was determined after 16 days. Value below zero was obtained by growing the fungus in a medium from which traces of iron were removed with 8-hydroxyquinoline [18].

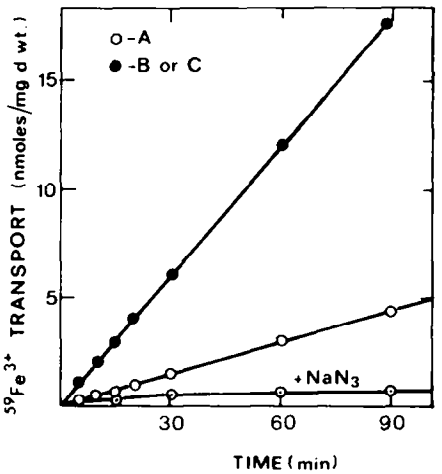

Fig. 2. Effect of siderophores and sodium azide on iron uptake. Initial uptake was determined as described in the Experimental. The labelled chelate was added to mycelium grown in an iron-deficient culture. The final iron concentration was 1 μM (0.2 μCi/ml). Sodium azide concentration was 1 mM. A—Siderophore A, B—dimerum acid and C—coprogen B.

siderophores exhibited pH optima at *ca* pH 6 and temperature optima at 30°. Kinetic studies of initial uptake versus siderophore concentration exhibited saturation kinetic with apparent K_m of 2.8 μM for coprogen B and 2.2 μM for dimerum acid. The foregoing results indicate that the siderophore-mediated iron transport in *S. botryosum* exhibits many features similar to those found in other fungal systems [1, 3].

The specificity of ferric ion transport system by various ligands in *S. botryosum* is shown in Table 2. In addition to its own siderophores, this fungus was capable of utilizing effectively exogenous siderophores which are naturally produced by other fungi, such as rhodotorulic acid, fusigen and ferrichrome. On the other hand, ferrioxamine B was ineffective. Rhodotorulic acid is structurally related to dimerum acid but fusigen and ferrichrome represent different structural classes of hydroxamate siderophore

Table 2. Relative uptake of chelated siderophores by *S. botryosum**

Siderophore	Relative uptake (%)
Coprogen B	100
Dimerum acid	100
Siderophore A	25
Rhodotorulic acid	100
Ferrichrome	75
Fusigen	83
Ferrioxamine B	8

*Conditions were the same as those described for Fig. 2.

[2, 11]. Various species of *Aspergillus* were able to utilize iron from siderophores of the ferrichrome-type family irrespective of the type of ferrichrome which these species normally produced. However, they were not able to take iron from an exogenous chelate of a different class, such as coprogen [12]. *Neurospora crassa* was reported to accumulate iron by its own chelate coprogen as well as by structurally unrelated siderophores such as ferricrocin and ferrichrysin [13].

Results shown in Fig. 3 indicate that iron uptake via the siderophores was four times higher with siderophore A and eight times higher with both coprogen B and dimerum acid in mycelium grown under iron starvation as compared to mycelium grown under iron-sufficient conditions. These results together with those illustrated in Fig. 1 suggest simultaneous regulation of the biosynthesis of siderophores and their transport processes by iron.

The fate of the ferric-coprogen B complex during uptake was followed by comparing the kinetics of the tritiated labelled ligand (Fig. 4) with that of the ^{59}Fe-labelled siderophore (Fig. 2). In both cases the iron complex was taken up quantitatively for at least 90 min through an energy dependent transport system as indicated by the sensitivity to sodium azide. Double labelled experiments with both the iron and the ligand revealed two basic mechanisms for iron uptake from ferric siderophore [14]. Mechanism 1 known as the iron shuttle mechanism consists of uptake of the iron and the ligand at identical rates. After internal release of the iron the free ligand reappears in the medium and may serve for another round of iron transport. The latter mechanism is exemplified by ferrichrome uptake in *Ustilago sphaerogena* [15]. Mechanism 2, known as the iron taxi mechanism [14] involves the donation of iron to the cell without penetration of the complex or the ligand. Iron uptake from rhodotorulic acid in *Rhodotorula pilimanae* [16] or ferrichrome A in *U. sphaerogena* [14] occurs by this mechanism. An additional mode of uptake was demonstrated in *Neurospora crassa* where ferric coprogen was taken up as an intact chelate molecule without being decomplexed in appreciable amounts [17]. Results presented for ferric-coprogen B in *S. botryosum* are in accordance with the latter pattern, namely the uptake and accumulation of the iron–ligand complex without subsequent release of the ligand back into the medium. These results support the view that siderophores such as coprogen or coprogen B may have a storage function for iron

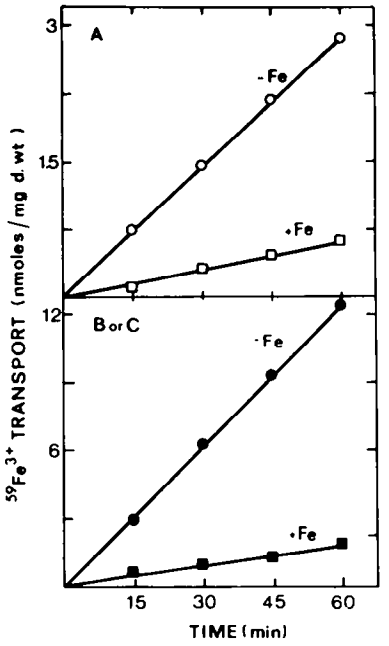

Fig. 3. Comparative uptake of ^{59}Fe-siderophores by iron-sufficient and iron-deficient mycelium. Uptake conditions are as described in Fig. 2. A—iderophore A, B—dimerum acid, C—coprogen B.

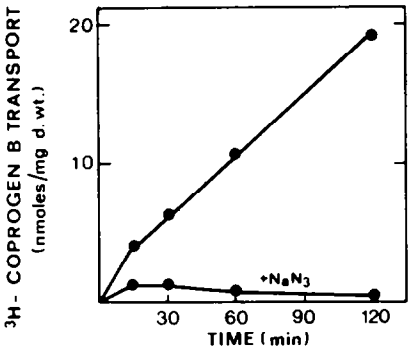

Fig. 4. Uptake of tritiated ferric coprogen B. Transport conditions were as described in Fig. 2.

in addition to their role in solubilization and transport [17].

Results presented here and our previous study [7] indicate that *S. botryosum* possesses mechanisms for iron acquisition under iron-sufficient and iron-deficient conditions. The role of these systems in pathogenicity of this fungus remain to be investigated.

EXPERIMENTAL

Culturing. The fungal strain of *S. botryosum* f. sp. *lycopersici* used in this study and culturing conditions have been previously described [6]. The fungus was grown for 16 days in 1-l. Roux bottles containing 100 ml of a defined sucrose–glutamate medium, composed of the following compounds (g/l. of double

distilled water): sucrose, 20; monosodium glutamate, 9; $MgCl_2 \cdot 6H_2O$, 0.5; KH_2PO_4, 0.3; thiamine HCl, 0.005; and pyridoxine, 0.005. All the chemicals were of analytical grade. This low-iron medium contains *ca* 0.015 mg Fe/l. (*ca* 3×10^{-7} M), as contamination from other ingredients. In some experiments the medium was treated with 8-hydroxyquinoline [18] to remove any traces of iron. Measurement of growth was performed by harvesting the mycelia by filtration through Whatman no. 1 paper and drying to constant weight at 60°.

Extraction and purification of siderophores. At the end of the growth period the mycelia were removed by filtration through Whatman no. 1 paper and the culture filtrates (1500 ml) were collected. The filtrate was evaporated under vacuum at 40–45° to about 1/5 of the original volume and excess $FeCl_3$ was added to convert siderophores to iron chelates. The reddish solution was saturated with $(NH_4)_2SO_4$ and left overnight at 4°. The filtrate was then centrifuged at 10000 *g* for 10 min and the clear supernatant was extracted with benzoyl alcohol according to the method of Neilands [19]. The dark-reddish organic layer was mixed with 2 vol. Et_2O and extracted with 0.1 vol. distilled water. The coloured aq. layer was washed with Et_2O (0.5 vol × 3) and reduced to dryness *in vacuo*.

The siderophores were dissolved in 5 ml water and initially purified by gel filtration through a column of Bio-Gel P-2 (2.5 × 115 cm). Fractions of 5 ml were collected and the amount of siderophores in each fraction was determined by absorbance at 440 nm. Individual components of the extract were then separated, concentrated *in vacuo* and chromatographed on Cellex CM (Bio-Rad) column (3 × 5 cm). The column was rinsed with water to remove neutral siderophores and the cationic siderophores were eluted with pyridine–HOAc–H_2O (14:10:930) pH 5.2. Fractions containing individual components were concentrated and applied to preparative paper electrophoresis (17 × 23 cm Whatman 3 MM) with pyridine buffer, pH 5.2 at 200 V for 2 hr. The siderophores were detected by their reddish colour and were eluted from the paper with water. Final purification was carried out with Sephadex LH-20 (Pharmacia) column (2.5 × 25 cm) using MeOH as an eluting solvent.

Analytical methods. The amount of total siderophores was measured according to Subramanian *et al.* [20]. Purity of the compounds was established by high voltage electrophoresis [21] and silica gel thin layer chromatography [4, 7]. For facilitation of structure determination the siderophores were deferrated with 8-hydroxyquinoline according to Wiebe and Winkelmann [12]. ^{1}H NMR spectra were recorded on Bruker WH-270 spectrometer and IR spectra as previously described [6].

^{59}Fe uptake assay conditions. Five-day-old mycelium was removed from the growth medium by suction filtration with an Ederol filter paper (no. 15) and washed several times with deionized water. The mycelium was then resuspended (4 g fr. wt per 100 ml) in 0.05 M phosphate buffer, pH 6 for 30 sec in a blender. The homogenous suspension of the fungal cells obtained by the latter procedure contained negligible amounts of broken cells which did not interfere with the uptake assay.

The standard procedure for iron uptake was carried out in 100 ml flasks, containing 20 ml of cell suspension. The flasks were shaken for 10 min prior to and after addition of the siderophore on a reciprocal shaker at 30°. The reaction was started by a rapid addition of the ^{59}Fe-chelated siderophore solution. Samples of 3 ml were removed at various time intervals and immediately filtered through GF/C Whatman filter paper (2.5 cm diameter) and rinsed × 3 with 5 ml 50 mM EDTA (ethylene diaminetetraacetic acid). The filters with the mycelial pads were transferred into 5 ml polyethylene vials, dried for 1 hr at 70° and counted in a Packard gamma counter C (model 5166). Counts were corrected for the readings obtained at zero time.

The ^{59}Fe-labelled siderophores were prepared by addition of $^{59}FeCl_3$ (specific activity 3–20 mCi/mg Fe in 0.05 N HCl) to an approximately 10% excess of ligand in water to give a stock solution of 0.5 mM (0.1 mCi/ml) in complex. The molar ratio between the ferric ion and the ligand was 2:3 for dimerum acid, 1:1 for coprogen B and 1:3 for siderophore A. The ligand was preincubated for at least 1 hr at 25° prior to the experiment to allow complete formation of the complex.

Tritiated coprogen B was prepared at the Radiochemistry department of the Institute for Atomic Research, Negev. The tritiated coprogen B was repurified by paper electrophoresis and Sephadex LH-20 column. High voltage electrophoresis showed that approximately 90% of the radioactivity was associated with the spot of coprogen B. Stock solutions (0.5 mM) of the ^{3}H-labelled ferric coprogen B (specific activity 0.1 mCi/ml siderophore) were prepared as above, using unlabelled ferric chloride. The uptake experiments were as described earlier except that the filter papers containing the mycelial pads were transferred into counting vials containing 0.5 ml of 60% perchloric acid–30% H_2O_2, 2:1) and incubated at 70° for 1 hr. Ten millilitres of hydroluma scintilation counting solution was then added and the vials were counted in Packard liquid scintilation counter model 3380.

Chemicals. Rhodotorulic acid was obtained from Dr. B. Hemming, Monsanto Co. St. Louis; dimerum acid, from Dr. J. B. Neilands, University of California, Berkeley, fusigen and coprogen B, from Dr. G. Winkelmann, University of Tubingen, Tubingen. Isotopic ferric chloride was purchased from the Radiochemical Centre, Amersham.

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