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Characterization of siderophore-mediated iron transport in *Geotrichum candidum*, a non-siderophore producer

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Summary. *Geotrichum candidum* is capable of utilizing iron from hydroxamate siderophores of different structural classes. The relative rates of iron transport for ferrichrome, ferrichrysin, ferrioxamine B, fusigen, ferrichrome A, rhodotorulic acid, coprogen B, dimerium acid and ferrirhodin were 100%, 98%, 74%, 59%, 49%, 35%, 24%, 12% and 11% respectively. Ferrichrome, ferrichrysine and ferrichrome A inhibited [^{59}Fe]ferrioxamine-B-mediated iron transport by 71%, 68% and 28% respectively when added at equimolar concentrations to the radioactive complex. The inhibitory mechanism of [^{59}Fe]ferrioxamine B uptake by ferrichrome was non-competitive (K_i 2.4 μM), suggesting that the two siderophores do not share a common transport system. Uptake of [^{59}Fe]ferrichrome, [^{59}Fe]rhodotorulic acid and [^{59}Fe]fusigen was unaffected by competition with the other two siderophores or with ferrioxamine B. Thus, *G. candidum* may possess independent transport systems for siderophores of different structural classes. The uptake rates of [^{14}C]ferrioxamine B and ^{67}Ga-desferrioxamine B were 30% and 60% respectively, as compared to [^{59}Fe]ferrioxamine B. The specific ferrous chelates, dipyridyl and ferrozine at 6 mM, caused 65% and 35% inhibition of [^{59}Fe]ferrioxamine uptake. From these results we conclude that, although about 70% of the iron is apparently removed from the complex by reduction prior to being transported across the cellular membrane, a significant portion of the chelated ligand may enter the cell intact. The former and latter mechanisms seem not to be mutually exclusive.

Key words: Iron – Siderophores – Transport – *Geotrichum candidum*

Introduction

Geotrichum candidum Lk. ex Pers. is a versatile ubiquitous ascomycete fungus of considerable importance to man. It is responsible for a watery, soft decay in a wide range of fruits and vegetables (Butler 1960) and may also become pathogenic to man and animals (Emmons et al. 1970). The fungus is widespread in nature and a common inhabitant of soils (Eckert 1978). Although *G. candidum* has adapted to many different habitats, it is incapable of siderophore production (Mor et al. 1988).

It has previously been shown (Mor et al. 1988) that iron uptake by *G. candidum* is mediated by two distinct iron-regulated, energy- and temperature-dependent transport systems that require sulfhydryl groups. One system exhibits specificity for either ferric or ferrous iron, whereas the other exhibits specificity for ferrioxamine-B-mediated iron uptake and, presumably, other hydroxamate siderophores. The two systems were distinguished as two separate entities by negative reciprocal competition and, on the basis of differential response, to temperature and phenazine methosulfate (Mor et al. 1988). The present study was undertaken to characterize the siderophore-mediated transport system in *G. candidum*.

Materials and methods

Culturing. The fungal strain of *G. candidum* (I-9) used in this study and the culturing conditions have previously been described (Mor et la. 1988). For uptake experiments the fungus was grown for 2 days at 26° C on a rotary shaker at 200 rpm in 1-l conical flasks containing 200 ml of an iron-deficient glucose/asparagine liquid medium (Mor et al. 1988).

Transport assays. The preparation of cell suspension and experimental conditions for siderophore-mediated iron uptake were as described (Mor et al. 1988). Samples were filtered through GF/C filter paper (2.5 cm diameter) and rinsed there times with 5 ml 50 mM EDTA. The filters with the mcyelial pads were transferred to polyethylene tubes, dried for 1 h at 70° C and counted in a Packard gamma counter C (model 5166).

Labelled compounds. The radioactive gallium complex of desferrioxamine B was prepared by adding $^{67}GaCl_3$ (2.5 mCi/mg) to desferrioxamine B to a final concentration of 4 μM with 50% excess ligand. The absence of free gallium in the chelate solution

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was confirmed by chromatography on Whatman no. 1 paper (10 cm) in two solvent systems consisting of methanol/water (85:15, by vol.) and saline solution. The chelate complex had an $R_f = 1$ in both solvents, wheras the R_f of the Ga salt was close to zero. [^{14}C]Ferrioxamine B was prepared by adding [^{14}C]desferrioxamine B (≈ 0.35 μCi/mg) to $FeCl_3$ solution to a final Fe concentration of 4 μM with 50% excess ligand. Experimental conditions were as described earlier but with the following modifications. The samples were filtered through nitrocellulose membrane filters (0.45 μm, 2.5 cm diameter), rinsed with 50 mM EDTA and dried for 1 h at 70° C. The filters with the mycelial pads were counted in 5 ml Liquiscint scintillation fluid with a Tri-Carb Packard liquid scintillation counter (model 1500).

Chemicals. ^{59}Fe (3–20 mCi/mg) was purchased from Amersham International (Amersham, UK). [^{14}C]Desferrioxamine B (≈ 0.35 μCi/mg) was a gift from Dr H. H. Peter (Ciba-Geigy Ltd, Basel). [^{67}Ga]Gallium citrate (Neoscan) was obtained from Med-Physics Incorp. (Richmond, California). The 1,2,4-triazine (ferrozine) and 2,2′-dipyridyl (dipyridyl) were purchased from Sigma Chemicals. Desferrioxamine B (desferal) was kindly provided by Ciba Laboratories. Siderophores were kindly provided by Prof. van der Helm and Prof. Winkelmann.

Results and discussion

Siderophore-mediated iron uptake

Results shown in Fig. 1 indicate that *G. candidum* can utilize iron from an array of different hydroxamate siderophores in a differential manner. The highest rate of uptake was recorded with ferrichrome and ferrichrysin (98–100%) followed by ferrioxamine B (76%), fusigen (61%), ferrichrome A (51%), rhodotorulic acid (35%) and coprogen B (24%). The rate of $^{59}FeCl^3$ uptake was considerably lower (26%) than that of ferrichrome (Fig. 1). The ability of *G. candidum* to transport iron differentially from siderophores of various structural classes may be analyzed in the light of two major hypotheses.

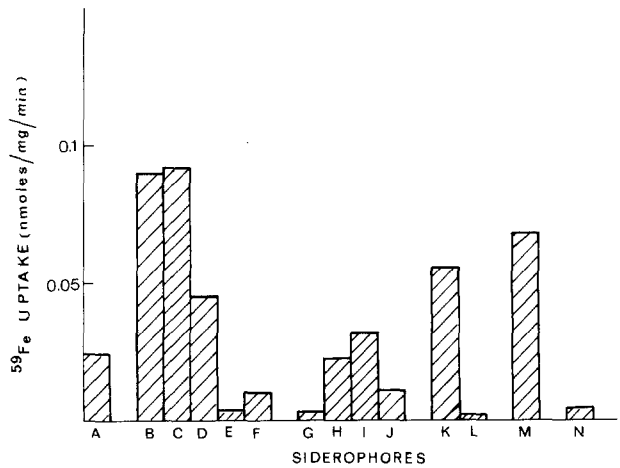

Fig. 1. Comparative uptake of ^{59}Fe-siderophores by *G. candidum*. Uptake conditions are as described in Materials and Methods. Conditions for $^{59}FeCl_3$ uptake were as described elsewhere (Mor et al. 1988). **(A)** $FeCl_3$; **(B)** ferrichrysin; **(C)** ferrichrome; **(D)** ferrichrome A; **(E)** ferrirubin; **(F)** ferrirhodin; **(G)** coprogen H; **(H)** coprogen B; **(I)** rhodotorulic acid; **(J)** dimerum acid; **(K)** fusigen; **(L)** triacetyl fusarinine; **(M)** ferrioxamine B; **(N)** aerobactin

(a) Iron is first removed by a membrane-bound siderophore reductase and then taken up by the cells via the ferrous transport system described earlier (Mor et al. 1988). The broad range of soderophores utilized could be attributed to a low specificity of this enzyme which, nevertheless, exhibits differential affinity towards the various siderophores. (b) Siderophore-mediated iron transport is performed via receptors as described for other fungi (Winkelmann and Huschka 1987).

When the variation of iron uptake within each siderophore class is analyzed according to structure/activity relationship, a rather unique interaction emerges which could favour the receptor hypothesis. Among the ferrichrome class of compounds (Fig. 1B–F) the transport rate seems to be significantly affected by the *N*-acyl residues. Thus ferrichrome and ferrichrysine, which showed the highest transport activity, contain three ornithyl *N*-acetyl residues. Ferrichrome A, which showed significantly lower activity, contains three *trans*-β-methylglutaconyls, whereas ferrirubin and ferrihodin, which exhibited the lowest activity, possess three anhydromevalonyl residues in the *trans* and *cis* configuration, respectively.

The foregoing results are in agreement with the structural requirements of the ferrichrome receptor in *Neurospora crassa* (Winkelmann and Huschka 1987) and *Aspergillus ochraceus* (Jalal et al. 1984). Using different ferrichrome-type siderophores including asperchromes, it was demonstrated that in both fungi the efficiency of siderophores seem to increase when various *N*-acyl residues in the ornithines are gradually replaced by acetyls. A similar phenomenon was also observed with two siderophores of the coprogen class. Thus, rhodotorulic acid, which was twice as active as dimerum acid (Fig. 1, I–J), contains two ornithyl *N*-acetyl residues, whereas the *N*-acyls of the latter are composed of *trans*-anhydromevalonyls.

Results in Fig. 1, G, H indicate that coprogen B is an effective iron transport molecule in *G. candidum*, whereas coprogen (acetylated coprogen B) is not. These results suggest that the positive charge of the free amino group supports recognition and transport. In contrast to *G. candidum*, coprogen was the effective and coprogen B the non-effective siderophore in *N. crassa* (Ernst and Winkelmann 1974). In the latter study, substitution of the *N*-acetyl group in coprogen by *N*-propyl or *N*-butyryl residues produced a significant increase in uptake, suggesting that this part of the molecule has to be non-polar for *N. crassa*. The necessity of a positive charge for an effective uptake was also observed with fusigen in *G. candidum* (Fig. 1 K, L). Acetylation of the free ornithyl amino groups of fusigen and formation of triacetyl fusigen almost completely retarded iron uptake. The effect of a positive charge on uptake rate was also demonstrated with ferrioxamines in *Streptomyces pilosus*, a natural producer of these siderophores (Müller and Raymond 1984). The lack of a positive charge in ferrioxamine D_1 reduced the uptake rate by 40% as compared to ferrioxamine B, which has identical architecture but the amino group remained unacetylated.

Table 1. Inhibition of [^{59}Fe]ferrioxamine-B-mediated iron uptake in the presence of various siderophores

Siderophore	Inhibition (%)
Ferrioxamine B	40
Ferrichrome	68
Ferrichrysin	71
Ferrichrome A	28
Fusigen	8
Rhodotorulic acid	7
$FeCl_3$	6

The concentration of [^{59}Fe]ferrioxamine B and each of the added siderophores was 10 μM. Uptake conditions are as described in Materials and methods

Specificity of siderophore transport systems

The question as to whether different transport systems might be involved in siderophore-mediated iron uptake of *G. candidum* was investigated by competition studies. Results shown in Table 1 reveal that [^{59}Fe]ferrioxamine B uptake is highly inhibited in the presence of equimolar concentrations of ferrichrome or ferrichrysin, whereas fusigen, rhodotorulic acid or $FeCl_3$ showed only negligible inhibition. Interestingly, the inhibition rate by the ferrichrome-type compounds was significantly higher (68–71%) than the control with ferrioxamine (40%). Nevertheless, the apparent K_m for ferrichrome and ferrioxamine B, as calculated from Lineweaver-Burk plots (Fig. 2), was identical (2 μM), suggesting that the affinity of each compound for its own membrane carrier is similar.

The nature of inhibition of [^{59}Fe]ferrioxamine B by ferrichrome was examined by using the Dixon plot. When the reciprocal velocities of two [^{59}Fe]ferrioxamine concentrations were tested with increasing ferrichrome concentrations, the two lines coincided on the x axis (Fig. 3), indicating a non-competitive inhibition mechanism. The apparent inhibition constant (K_i) obtained for ferrichrome was 2.4 μM. These results imply that the two structurally different siderophores may not share a common receptor. The effective inhibitory action of ferrichrome could result from its binding to the ferrioxamine B receptor and interfering with iron transport by causing either conformational changes of the receptor protein or another mechanism. Alternatively, it is possible that the receptors of ferrioxamine B and ferrichrome are closely situated within the membrane and may, under certain conditions, interact with each other. Thus, the binding of the ferrichrome to its own receptor could lead to changes in the ferrioxamine B receptor protein which adversely affect the binding of its specific siderophore. It is noteworthy that [^{59}Fe]ferrichrome-mediated iron uptake was also inhibited by the presence of ferrioxamine B (30% inhibition), suggesting that the interaction between the two siderophores is reciprocal. This finding might favour the latter hypothesis.

The possibility that other siderophores recognized by *G. candidum* (Fig. 1) may share common transport systems was further investigated. Results shown in Fig. 4 indicate that neither ferrichrome nor rhodotorulic acid, nor fusigen could inhibit siderophores of the different structural classes. Similar results were obtained

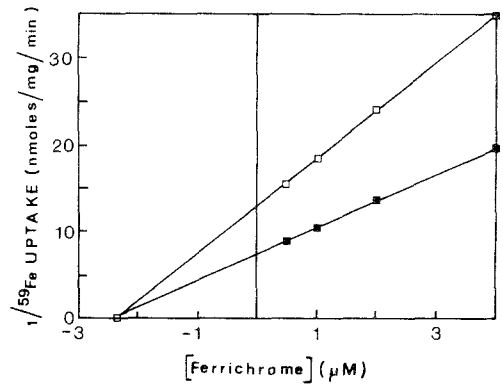

Fig. 3. The Dixon plot of [^{59}Fe]ferrioxamine-B-mediated iron uptake with ferrichrome as the inhibitor. Uptake was measured at two different concentrations of [^{59}Fe]ferrioxamine B (i.e. 1 and 4 μM). Each line was obtained by plotting $1/v$ of [^{59}Fe]ferrioxamine B versus concentration of ferrichrome. Transport conditions are as in Fig. 1

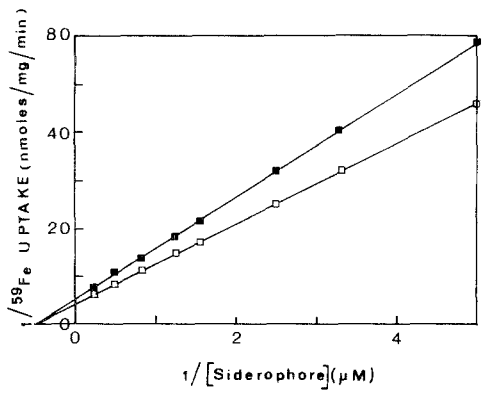

Fig. 2. Lineweaver-Burk plot of [^{59}Fe]ferrichrome- and [^{59}Fe]ferrioxamine-B-mediated iron uptake. Transport conditions are as in Fig. 1

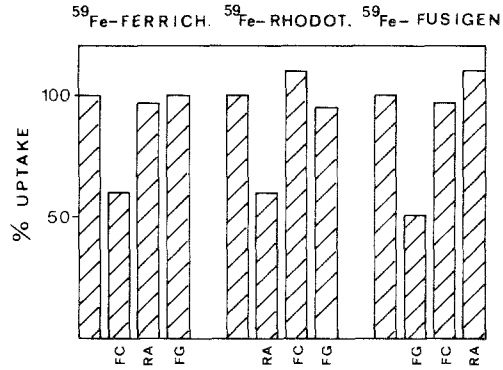

Fig. 4. Iron uptake mediated by [^{59}Fe]ferrichrome, [^{59}Fe]rhodotorulic acid and [^{59}Fe]fusigen in the presence of siderophores from other structural classes. The radioactive and unlabelled siderophores were given in equimolar concentrations (6 μM). Transport conditions are as in Fig. 1

when iron uptake via these siderophores was performed in the presence of ferrioxamine B (not shown). The foregoing data suggest that *G. candidum* possesses independent transport systems for the different classes of siderophores.

Müller et al. (1984) have shown that the kinetically inert chromic complex of desferrioxamine B was a strong inhibitor of iron uptake mediated by ferrichrome and ferric-*enantio*-rhodotorulic acid in *S. pilosus*. These inhibition experiments may indicate that, in contrast to *G. candidum*, iron from these exogenous siderophores is transported by the same uptake system as ferrioxamine B. Since the ligands have no structural similarity to ferrioxamine B, except the presence of three hydroxamate groups, it was concluded that only the hydroxamate iron centre and its direct surroundings are important for recognition and uptake (Müller et al. 1984). However, due to lack of information on the mechanism of inhibition, the possibility that these siderophores compete for the same transport system cannot be conclusive. A shared transport system for siderophores of different structural types (i.e. ferrichrome and coprogen) was demonstrated in *N. crassa* (Huschka et al. 1985).

Mechanism of ferrioxamine-B-mediated iron transport

Further experiments were designed to distinguish between two basic mechanisms for iron uptake via siderophores: (a) the iron chelate is transported through the cytoplasmic membrane as an intact complex; (b) removal of the iron from the siderophore is a prerequisite for transfer of the metal across the cellular membrane, whereas the ligand remains extracellular. To distinguish between the former and latter possibilities, the transport of [^{59}Fe]ferrioxamine B was compared with [^{14}C]ferrioxamine B and ^{67}Ga-desferrioxamine B. The uptake of the ^{14}-labelled siderophore allows the fate of the ligand to be closely followed during transport. Since Ga^{3+} cannot be reduced by reductase, it can be used as a probe for the uptake of the intact complex

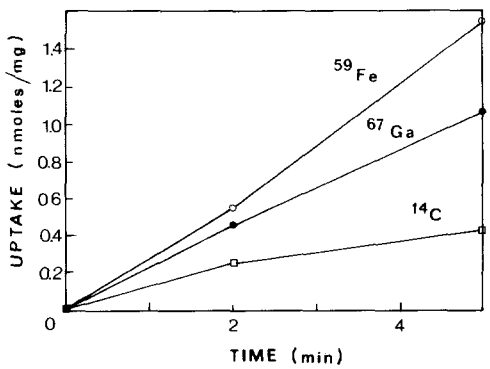

Fig. 5. Uptake of [^{59}Fe]ferrioxamine B, [^{14}C]ferrioxamine B and ^{67}Ga-desferrioxamine B. The metal complexes were added at 4 μM concentration. Conditions for transport and preparation of the labelled siderophores are described in Materials and Methods

Table 2. Effect of ferrous-specific chelates on [^{59}Fe]ferrioxamine-B-mediated iron uptake

Chelate added	^{59}Fe uptake (%)
None	100
2,2′-Dipyridyl	65
Ferrozine	35

Chelate concentration was 6 mM

and/or the ability of the cells' surface to remove the metal by an exchange mechanism rather than reduction.

Results shown in Fig. 5 indicate that the uptake rates of [^{14}C]ferrioxamine B and ^{67}Ga-desferrioxamine B were 30% and 60% respectively, as compared to the [^{59}Fe]ferrioxamine B. The reduced uptake of the ^{14}C-labelled ligand suggests that most of the iron ($\approx$70%) is removed from the complex prior to being transported into the cell. Nevertheless, a significant portion of the ligand ($\approx$30%) can be transported across the cellular membrane. The observation that ^{67}Ga uptake was 30% higher than the ^{14}C-labelled ligand (Fig. 5) suggests that this metal can drag a higher proportion of the ligand into the cell than can iron. The latter conclusion is based on the assumption that reduction, rather than exchange, is the only extracellular mechanism for decomplexation of iron from ferrioxamine B in *G. candidum*. In *S. pilosus*, both ^{67}Ga-desferrioxamine B and Cr-desferrioxamine B were transported at rates similar to [^{59}Fe]ferrioxamine B (Müller and Raymond 1984). This implies that decomplexation of the metal does not take place as in *S. pilosus*.

Evidence for reduction of ferrioxamine B by the fungal cells is given in Table 2. Dipyridyl and ferrozine, two effective and specific chelators of Fe^{2+}, inhibited iron uptake of [^{59}Fe]ferrioxamine B by 65% and 35% respectively, at 6 mM concentration. Dipyridyl, at an identical concentration, completely inhibited ferrichrome A uptake by *Ustilago sphaerogena* (Emery 1987), whereas ferrozine at 1 mM caused >80% inhibition of ferrioxamine B transport in *Saccharomyces cerevisiae* (Lesuisse et al. 1987). The former and latter siderophores have been shown to mediate iron transport by the so-called 'taxi mechanism', where the ligand remains extracellular. The observation that only partial inhibition of [^{59}Fe]ferrioxamine B was obtained in the presence of dipyridyl or ferrozine (Table 2) further indicates that the two mechanisms exist in *G. candidum* for ferrioxamine-B-mediated iron uptake and that they are not mutually exclusive. It is of interest to note that [^{59}Fe]ferrioxamine B is taken up intact by *S. pilosus* (Müller et al. 1984), completely decomplexed by reduction prior to transport by *S. cerevisiae* (Lesuisse et al. 1987) and both occur in *G. candidum*.

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References

Butler EE (1960) Pathogenicity and taxonomy of *Geotrichum candidum*. Phytopathology 50:665–672

Eckert JW (1978) Postharvest disease of citrus fruits. Outlook Agric 9:225–259

Emery T (1987) Reductive mechanisms of iron assimilation. In: Winkelmann G, van der Helm D, Neilands JB (eds) Iron transport in microbes, plants and animals. VCH Verlagsgesellschaft, Weinheim, pp 317–336

Emmons CW, Binford CH, Utz JP (1970) Medical mycology. Lea & Febiger, Philadelphia

Ernst J, Winkelmann G (1974) Metabolic products of microorganisms. Uptake of iron by *Neurospora crassa*. Arch Microbiol 100:271–282

Huschka H, Naegeli HU, Leuenberger-Ryf H, Keller-Schierlein W, Winkelmann G (1985) Evidence for a common siderophore transport system but different siderophore receptors in *Neurospora crassa*. J Bacteriol 162:715–721

Jalal MAF, Mocharla R, Barnes C, Hossain MB, Powell DR, Engwilmot DL, Grayson SL, Benson BA, van der Helm D (1984) Extracellular siderophores from *Aspergillus ochraceous*. J Bacteriol 158:683–688

Lesuisse F, Raguzzi F, Crichton RR (1987) Iron uptake by the yeast *Saccharomyces cerevisiae:* involvement of a reduction step. J Gen Microbiol 133:3229–3236

Mor H, Paternak M, Barash I (1988) Uptake of iron by *Geotrichum candidum*, a non-siderophore producer. Biol Metals 1:99–105

Müller G, Raymond KN (1984) Specificity and mechanism of ferrioxamine-B-mediatd iron transport in *Streptomyces pilosus*. J Bacteriol 160:304–312

Müller G, Matzanke BF, Raymond KN (1984) Iron transport in *Streptomyces pilosus* mediated by ferrichrome siderophores, rhodotorulic acid and emantio-rhodotorulic acid. J Bacteriol 160:313–318

Winkelmann G, Huschka H (1987) Molecular recognition and transport of siderophores in fungi. In: Winkelmann G, van der Helm D, Neilands JB (eds) Iron transport in microbes, plants and animals. VCH Verlagsgesellschaft, Weinheim, pp 317–336