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Uptake of iron by *Geotrichum candidum*, a non-siderophore producer

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Summary. *Geotrichum candidum* (isolate I-9) pathogenic on citrus fruits, appears to lack siderophore production. 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. Radioactive iron uptake from $^{59}FeCl_3$ showed an optimum at pH 6 and 35° C, and Michaelis-Menten kinetics (apparent $K_m = 3\ \mu M$, $V_{max} = 0.054$ nmol · mg^{-1} · min^{-1}). The maximal rate of Fe^{2+} uptake was higher than Fe^{3+} ($V_{max} = 0.25$ nmol · mg^{-1} · min^{-1}) but the K_m was identical. Reduction of ferric to ferrous iron prior to transport could not be detected. The ferrioxamine B system exhibits an optimum at pH 6 and 40° C and saturation kinetics ($K_m = 2\ \mu M$, $V_{max} = 0.22$ nmol · mg^{-1} · min^{-1}). 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. Mössbauer studies revealed that cells fed with either $^{57}FeCl_3$ or $^{57}FeCl_2$ accumulated unknown ferric and ferrous binding metabolites.

Key words: Iron — Ferrioxamine B — Transport — *Geotrichum candidum*

Introduction

In many microorganisms the uptake of iron under iron-deficient conditions is mediated by siderophores (Neilands 1981; Winkelmann et al. 1987). However, since iron is indispensable for life and microorganisms are known to inhabit numerous different microenvironments, it is reasonable to assume that a multitude of mechanisms for iron assimilation may exist, even in a single organism. Indeed, a few species of bacteria, which appear not to form a siderophore system and to utilize means other than siderophores for iron acquisition, have been detected (Norrod and Williams 1978; Reeves et al. 1983; Evans et al. 1986; Neilands et al. 1987). Among the fungi, only the common yeast, *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, have so far been reported to lack siderophore production (Neilands et al. 1987).

Geotrichum candidum Lk. ex Pers. is a versatile ubiquitous ascomycete fungus of considerable importance to man. It is a causal agent of sour rot, one of the major decays of citrus fruits (Eckert 1978) and responsible for a watery, soft decay in a wide range of fruits and vegetables (Butler 1960). *G. candidum* may also be pathogenic to man and animals (Emmons et al. 1970). It is associated with food spoilage and is found in various milk products (Butler 1960). The fungus is widespread in nature and a common inhabitant in citrus soils (Eckert 1978). Preliminary studies revealed that, although *G. candidum* has adapted to many different habitats, siderophore production was absent in all the citrus-pathogenic isolates which were examined by us. The present investigation was, therefore, intended to characterize mechanisms responsible for iron assimilation in *G. candidum*.

Materials and methods

Organism and growth conditions. The fungal strain of *G. candidum* (I-9) used in this study, was isolated from a lemon fruit

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infected with sour rot. Cultures were maintained on potato dextrose agar at 26° C, or stored at 5° C. 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 liquid medium. The medium was composed of the following compounds (g/l of double-distilled water): glucose, 20; asparagine 2; K_2HPO_4, 0.5; KH_2PO_4, 1; $MgSO_4$, 1; $ZnSO_4$, 0.02; $MnSO_4$, 0.02; pyridoxin, 0.005 and thiamin · HCl, 0.01. In some experiments, which were aimed at detection of siderophores, the medium was treated with 8-hydroxyquinoline (Donald et al. 1952) to remove traces of iron. Iron-sufficient medium contained up to 20 μM $FeCl_3$.

Assays for detection of siderophores. For detection of siderophores, *G. candidum* was grown on the glucose/asparagine medium, described earlier, or on glucose/glutamate medium (Manulis et al. 1987b). These media were also tested after treatment with 8-hydroxyquinoline for removal of iron traces. The cell-free supernatants obtained after filtration and extracts of the mycelia obtained after mycelial disruption in French press (Mor et al. 1984) were used for searching for the presence of extracellular and intracellular siderophores, respectively. The procedures developed by Schwyn and Neilands (1987) with the complexometric titration dye Chrom Azurol S (CAS) were employed as a major chemical probe for the presence of siderophores. Other procedures for extraction and detection of hydroxamate siderophores were according to Manulis et al. (1987a).

Assay of ^{59}Fe uptake. Mycelial cells and arthrospores were removed from the growth medium by suction-filtration on a Buchner funnel, with an Ederol filter paper (no. 15) and washed several times with deionized water. The cells were then resuspended (1.5 g fresh mass/100 ml) in 0.05 M phosphate buffer, pH 6, for 15 s with the lower speed of a Waring blender. The standard procedure for iron uptake was carried out in 100-ml-conical flasks, containing 10 ml cell suspension. The flasks were shaken at 35° C for 10 min prior to the addition of the radioactive iron, and during the experiment, on a reciprocal shaker. The reaction was started by a rapid addition of 0.1 ml of the radioactive iron solution. Samples of 3 ml were removed at various time intervals and immediately treated, as further described. In experiments aimed at siderophore-mediated iron uptake, samples were filtered through GF/C filter paper (2.5 cm diameter) and rinsed three times with 5 ml of 50 mM EDTA (ethylenediaminetetraacetic acid). The filters with the mycelial pads were tansferred to polyethylene tubes, dried for 1 h at 70° C and counted in a Packard gamma counter C (model 5166). Values were corrected for the measurements obtained at zero time. When the latter procedure was used for measuring uptake of $^{59}FeCl_3$ or $^{59}FeCl_2$, a significant adsorption of labelled iron on the cell surface was observed, in addition to the transported iron, as reported elsewhere (Manulis et al. 1987b). The cell-surface-bound iron was removed as follows. After incubation with labelled iron, the cells (3 ml) were promptly transferred into a vial (20 ml), containing 1 ml 400 mM EDTA and incubated for 1 h on a reciprocal shaker at 30° C. They were then filtered, washed with distilled water and the radioactivity measured, as previously described. Radioactive iron was given as either $^{59}FeCl_3$, $^{59}FeCl_2$ or chelated to desferrioxamine B, as described elsewhere (Manulis et al. 1987b). Ferrous ion was obtained by pre-incubation of $FeCl_3$ in the presence of excessive concentration (× 100) of L-ascorbic acid for at least 30 min and during the uptake experiment (Manulis et al. 1987b).

Mössbauer studies. Iron-uptake procedures for Mössbauer analyses were carried out as described earlier, except that the cells were enriched with $^{57}FeCl_3$ instead of $^{59}FeCl_3$. The $^{57}FeCl_3$ was obtained by dissolving 2 mg $^{57}Fe_2O_3$ in 1 ml 5 M HCl. Following iron uptake, the mycelia were immediately washed with 50 mM EDTA and further incubated in 100 mM EDTA for 30 min to remove cell-surface-bound iron. The mycelial suspension was filtered and rinsed with distilled water, peeled off the filter paper, frozen with liquid nitrogen and stored at −70° C. The mycelial samples were encapsulated in lucite holders. Measurements were carried out with a flow cryostat in the temperature range of 5–300 K. A 25-mCi $^{57}Co(Rh)$ source was used and kept at ambient temperature.

Chemicals. $^{59}FeCl_3$ (3–20 mCi/mg) was purchased from Amersham International (Amersham, UK). $^{57}Fe_2O_3$ was purchased from Oak Ridge National Laboratory; 4,7-bis(4-phenylsulphonate)-1,10-phenanthroline (BPDS), the disodium salt of 3-(2-pyridyl)-5,6-bis(4-phenylsulphonic acid) 1,2,4-triazine (ferrozine), 2,2′-dipyridyl (dipyridyl) and ethylenediamine-di(*o*-hydroxyphenylacetic acid) (EDDHA) were purchased from Sigma Chemical Co. Desferrioxamine B (desferal) was kindly provided by Ciba Laboratories.

Results

Lack of siderophore production by G. candidum

G. candidum was grown in glucose/asparagine media which were either deferrated with 8-hydroxyquinoline or after addition of various concentrations (1–10 μM) of ferric chloride. Iron-deprived medium could support only a limited growth, whereas the addition of 2 μM iron more than doubled the cell yield after 48 h. All cultures reached the stationary phase of growth within 48 h. The possibility of siderophore excretion was initially investigated by the addition of drops of a 1% ferric chloride solution to supernatants of low- and high-iron cultures. No apparent colour change was produced when the filtrates were examined at different growth periods of up to a week. Nor could we detect any colour changes after the filtrates were evaporated under vacuum at 35° C to about a tenth of the original volume and excess $FeCl_3$ was added. The concentrated filtrates were extracted with benzyl alcohol, subjected to paper electrophoresis and stained with Folin chiocalteus phenol reagent, as described elsewhere (Manulis et al. 1987a). However, the presence of siderophores, even in trace amounts, could not be confirmed.

For determination of siderophores by the CAS assay, the total phosphate concentration in the asparagine/glucose media was reduced to 0.3 g/l (Schwyn and Neilands 1987). Alternatively, benzyl alcohol extracts of the filtrates of the high-phosphate medium or of the glutamate/glucose medium were employed. Although the CAS assay is considered a universal method for detection of

siderophores, all our attempts to detect them by this procedure were unsuccessful. Neither could we detect cell-bound siderophores by any of the foregoing assays when mycelial extracts, obtained as described in Materials and methods were tested. Therefore, it was concluded that *G. candidum* did not form siderophores, at least under the growth conditions employed in this study.

Iron accumulation by mycelial cells

Results shown in Fig. 1 indicate that $^{59}FeCl_3$ accumulation by cells of *G. candidum* grown in iron-deficient medium was linear with time. Linearity was observed up to approximately 15 min (not shown in the plot). To distinguish between iron transported into the cells from iron bound to the cell's surface, the latter was removed by a high concentration of EDTA, as described earlier. The cell-surface-bound radioactive iron could account for about 34% of the total iron accumulated and the linear kinetics were retained after its removal (Fig.1). The EDTA treatment was, therefore, employed in all the uptake experiments with ferric or ferrous ion.

Properties of the Fe^{3+} uptake system

$^{59}FeCl_3$ transport, under conditions described in Fig. 1, showed pH dependence with an optimum at pH 6. The uptake of iron revealed a continuous increase of transport rates from 20° C to 35° C, followed by a loss of transport activity above

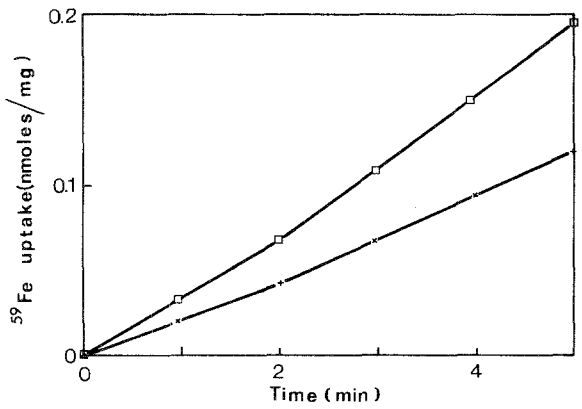

Fig. 1. Accumulation of iron by mycelial cells. Iron (1 μM) was given as $^{59}FeCl_3$. Experimental conditions for iron uptake and removal of cell-surface-bound iron by EDTA are described in Materials and methods. Iron uptake before (□) and after (×) the EDTA treatment

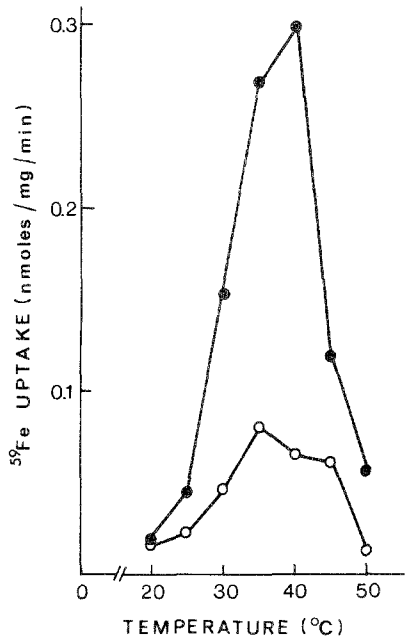

Fig. 2. Effect of temperature on iron uptake. The mycelial suspension was brought to the indicated temperature within 5 min prior to the addition of $^{59}FeCl_3$ (○) or ^{59}Fe-desferrioxamine B (●). Uptake was carried out as described in Materials and methods

35° C (Fig. 2). A Q_{10} value of 2.5 was calculated between 20° C and 30° C, suggesting the involvement of a biological system rather than diffusion. The energy of activation calculated from Arrhenius plot was 84 kJ (20 kcal).

The requirement for metabolic energy and functional membranes by the ferric ion transport system was demonstrated by high inhibitory effects of sodium azide or CCCP and nystatin, respectively (Table 1). Sulfhydryl groups were important in this system since inhibition was evident with *N*-ethylmaleimide and iodoacetamide. A plot

Table 1. Effect of inhibitors on $^{59}FeCl_3$ and ^{59}Fe-desferrioxamine uptake

Inhibitor	Inhibition by	
	$^{59}FeCl_3$ (%)	^{59}Fe-ferrioxamine B (%)
Sodium azide (1 mM)	95	99
CCCP (1 mM)	70	99
PMS (1 mM)	30	80
NEM (0.1 mM)	60	60
Iodoacetamide (1 mM)	45	50
Nystatin (0.1 mM)	83	85

CCCP = carbonyl cyanide *m*-chlorophenylhydrazone, PMS = phenazine methosulfate, NEM = *N*-ethylmaleimide

of transport rate versus Fe^{3+} concentration yielded Michaelis-Menten kinetics. The apparent K_m and V_{max} values extrapolated from a Lineweaver-Burk plot were 3 μM and 0.054 nmol · mg^{-1} · min^{-1}, respectively.

The expression of the ferric transport system was dependent on iron deficiency. A reduction of approximately 80% in Fe^{3+} transport was observed when *G. candidum* was grown in the presence of 2 μM $FeCl_3$ (Fig. 3).

Specificity of the Fe^{3+} transport system

The valence form in which iron was transported into the cells was investigated. Results from Lineweaver-Burk plot indicate that reduction of ferric into ferrous ion by L-ascorbate increased the maximal uptake rate by more than four fold (V_{max} = 0.25 nmol · mg^{-1} · min^{-1}), whereas the K_m values for ferric and ferrous ions were identical (3 μM). Thus, both ferric and ferrous ions could be readily transported into the cells of *G. candidum*.

Further experiments were designed to determine whether changes in the valence form of iron occur by mycelial cells prior to its transport. Consequently, the effect of ferric- and ferrous-specific chelators on iron uptake was investigated. Results given in Fig. 4 indicate that the ferric-specific chelator EDDHA caused 60% inhibition of Fe^{3+} transport, whereas the Fe^{2+} trapping reagents,

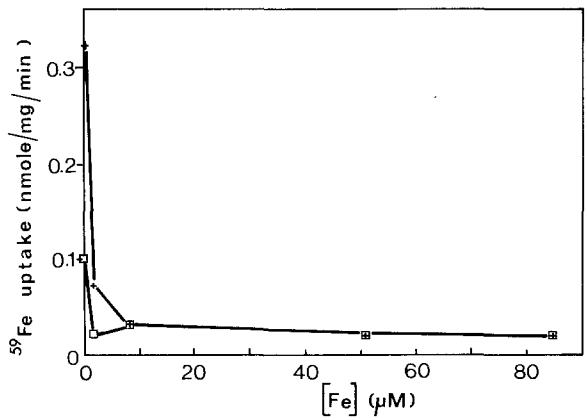

Fig. 3. Uptake of $^{59}FeCl_3$ and ^{59}Fe-desferrioxamine B as a function of iron concentration in the growth medium. *G. candidum* was grown for 48 h in the presence of various $FeCl_3$ concentrations. Transport measurements of $FeCl_3$ (□) and Fe-desferrioxamine B (+) were performed as described in Materials and methods

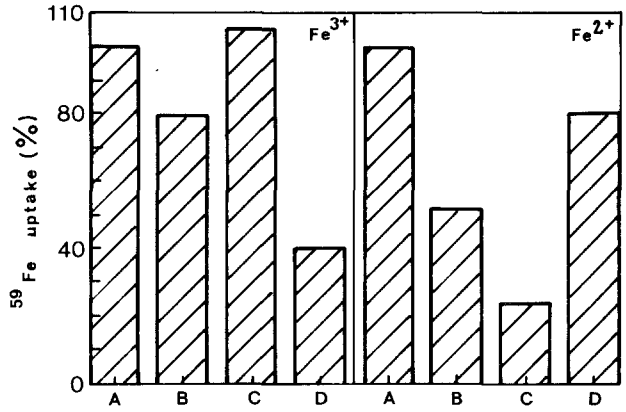

Fig. 4. Effect of various chelates on ferric and ferrous ion uptake. Uptake conditions were as in Fig. 1, except that iron concentration was 6 μM. The concentration of ferrozine, dipyridyl and EDDHA was 60 μM each. Inhibitors were added at zero time. (A) Control; (B) dipyridyl; (C) ferrozine; (D) EDDHA

ferrozine and dipyridyl, were either noneffective or caused only slight inhibition. No inhibition of Fe^{3+} transport could be detected even when ferrozine concentration was increased to 1.2 mM (not shown). When the Fe^{2+} transport was examined (Fig. 4), ferrozine and dipyridyl caused 80% and 50% inhibition respectively, whereas EDDHA was only slightly inhibitory (about 20%). The failure of the ferrous-specific chelators to exert a significant inhibition on the ferric ion transport suggests that the latter may not be reduced prior to its penetration into the cells, at least during the transport period. The latter conclusion was further supproted by our unsuccessful attempts to reduce ferric chloride with cells of *G. candidum* using BPDS as an indicator for ferrous ion formation (Manulis et al. 1987b; Lesuise et al. 1987) and longer incubation periods. The merely partial inhibition of Fe^{3+} by EDDHA or of Fe^{2+} by dipyridyl, could be accounted for by competition between the binding sites on the mycelial surface with the relevant chelators for iron.

The possibility that the iron transport system is shared by other cations was investigated by competition experiments. Ferric ion transport was measured as described in Fig. 1 in the presence of 1 mM Mg^{2+}, Ni^{2+}, Mn^{2+}, Co^{2+}, Al^{3+} and Zn^{2+}. Only Zn^{2+} and Al^{3+} caused a significant inhibition (about 60%). However, when the concentration of these cations was reduced to 50 μM, which was still 25-fold higher than the $^{59}FeCl_3$ (2 μM), inhibition could not be detected. It appears that the investigated system is quite specific to ferric and ferrous ions.

Characterization of ferrioxamine B mediated iron uptake G. candidum

Although *G. candidum* is unable to produce any siderophores, it was found capable of utilizing iron effectively from exogenous hydroxamate siderophores of various structural classes (Mor and Barash, unpublished results). The uptake of iron via ferrioxamine B, one of the more efficient siderophores for iron utilization by this fungus, was further characterized. This transport system exhibited a pH optimum at about pH 6 and temperature optima at 40° C (Fig. 2). The calculated Q_{10} between 20° C and 30° C was 6.2 and the energy of activation as calculated from Arrhenius plot was 138 kJ (33 kcal). Kinetic studies of initial uptake versus siderophore concentration exhibited Michaelis-Menten kinetics with apparent K_m of 2 μM and V_{max} of 0.22 nmol · mg^{-1} · min^{-1} (Fig. 5). The requirement for energy, sulfhydryl groups, is evident by the significant inhibitory effect of CCCP, NaN_3 or PMS and *N*-ethylmaleimide or iodoacetamide, respectively (Table 1). The ferrioxamine-mediated iron transport was almost completely repressed in the presence of 2 μM $FeCl_3$ in the medium (Fig. 3).

Since the ferrioxamine and the $FeCl_3$ systems show many similar properties (e.g. Table 1, Fig. 3 and others), it was suspected that the two systems may share a common membrane-mediated carrier. However, a competition experiment described in Table 2 indicates that neither $FeCl_3$ nor Fe-desferrioxamine B could compete with ^{59}Fe-desferrioxamine and $^{59}FeCl_3$ transport, respectively, when given at equimolar concentrations.

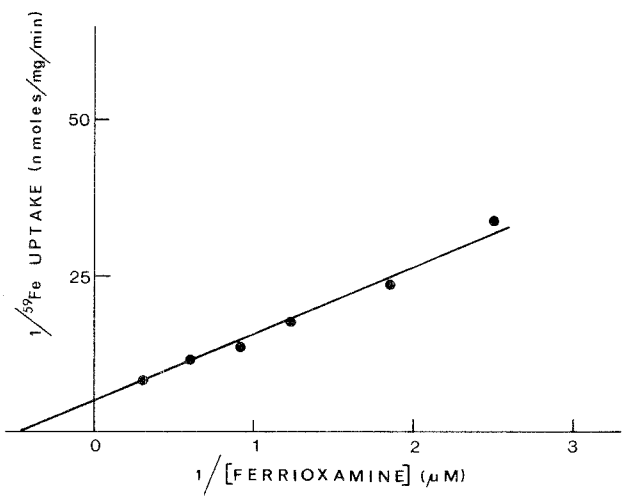

Fig. 5. Lineweaver-Burk plot of ^{59}Fe-desferrioxamine-B-mediated iron uptake by *G. candidum*. Transport conditions are described in Materials and methods

Table 2. Competition between $^{59}FeCl_3$ and Fe-desferrioxamine B or between ^{59}Fe-desferrioxamine B and $FeCl_3$

Substrate[a]	^{59}Fe uptake at	
	2 min (nmol · mg^{-1} · min^{-1})	5 min
$^{59}FeCl_3$	0.04	0.11
$^{59}FeCl_3$ + Fe-desferr. B	0.05	0.11
^{59}Fe-desferr. B	0.15	0.32
^{59}Fe-desferr. B + $FeCl_3$	0.17	0.32

[a] Each substrate was added at a concentration of 4 μM

Mössbauer study of iron assimilation

Cells of *G. candidum* grown under iron deficiency were incubated with either $^{57}FeCl_3$ (6 mM) or $^{57}FeCl_2$ (i.e. 6 mM $FeCl_3$ in the presence of 300 mM L-ascorbate) for 30, 60 or 120 min. Following the incubation period, the cells were treated as described in Materials and methods and used for Mössbauer measurements. A typical spectrum of the freeze-dried cells at room temperature after a 30-min incubation is shown in Fig. 6. A least-squares fit was attempted by assuming two quadrupole split components and an equal line width for both. Measurements done at this temperature range have not shown any magnetic interaction. This involves the existence of very fast relaxation times of high-spin Fe^{2+} and Fe^{3+} species. The solid line through the experimental points is the theoretical spectrum resulting from the computer fitting.

After uptake of $FeCl_2$, the reduced hyperfine constants for Fe^{2+} were $\delta = 1.37(1)$ mm/s and $\Delta Q = 3.02(4)$ mm/s, whereas for the Fe^{3+}, $\delta = 0.48(1)$ mm/s and $\Delta Q = 0.61(2)$ mm/s. After uptake of $FeCl_3$ the hyperfine constants for Fe^{2+} were, $\delta = 1.34(8)$ mm/s and $\Delta Q = 3.08(1)$ mm/s, whereas for Fe^{3+}, $\delta = 0.487(6)$ mm/s and $\Delta Q = 0.586(9)$ mm/s. δ and ΔQ stand for isomer shift with respect to iron and quadruple splitting, respectively. The half-line width (Γ) for both components was 0.25(1) mm/s, indicating a possible existence of site distribution. From the spectra we calculated the percentages of Fe^{2+}, namely 20% and 39% following the uptake of $FeCl_2$ and $FeCl_3$, respectively. These results suggest that iron is stored within the cells as both ferric and ferrous metabolites, regardless of the valence in which the iron enters the cell. The ferric iron species might represent the predominant fraction, under the experimental conditions used, and its relative amount is not affected by the valence of iron sup-

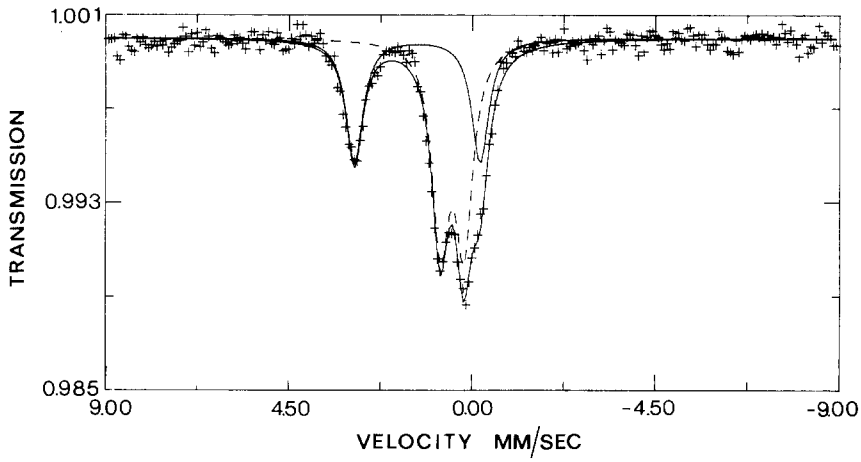

Fig. 6. Mössbauer spectrum at room temperature of freeze-dried cells of *G. candidum* after incorporation of $^{57}FeCl_3$ for 30 min. The spectrum is composed of two components corresponding to Fe^{3+} (---) and Fe^{2+}; (——) theoretical spectrum obtained by a least-squares fitting

plied to the cells. No significant changes in the pattern of stored iron was observed during longer incubation periods.

Discussion

Results of the present study have shown that *G. candidum* lacks siderophore production and its iron uptake is mediated by at least two distinct active transport systems. One of the systems is specific to ferric and ferrous ions, whereas the other displays specificity towards ferrioxamine B and, presumably, other hydroxamate siderophores. Although the two systems exhibit many similar properties, e.g. energy- and temperature-dependence, pH optima at 6, requirement for sulfhydryl groups, repression by iron (Fig. 3) and saturation kinetics, the possibility that they share the same recognition or transport system may be excluded on the basis of the following observations: (a) reciprocal competition experiments between $FeCl_3$ and ferrioxamine B gave negative results (Table 2); (b) the ferrioxamine B system is significantly more sensitive to temperature elevation than the Fe^{3+} system, as expressed by the distinctive Q_{10} values and energies of activation (Fig. 2); (c) the differential inhibitory effect of the electron acceptor PMS on the two systems (Table 1) may imply that the ferrioxamine-B-mediated iron uptake demands more cell-generated energy than ferric iron uptake. The latter conclusion may also be supported by the significantly higher sensitivity of the ferrioxamine system towards the uncoupler CCCP. It is also noteworthy that, in contrast to the excessive adsorption of iron on the cell-surface during Fe^{3+} uptake (Fig. 1), the iron adsorption by the ferrioxamine system was negligible.

Although the possibility that iron is being transported across the cell membrane in the ferric form by an energy-dependent saturable process has not been reported in fungi, a similar transport system was detected in *Yersiniae* (Perry and Brubaker 1979). Several cases have been reported in fungi (Manulis et al. 1987b; Rodriguez et al. 1984; Lesuisse et al. 1987) and bacteria (Evans et al. 1986; Cowart and Foster 1985), which indicate that iron is transported across the cell membrane only in the ferrous form. Reduction of ferric iron in the foregoing transport systems is achieved by either external reductants (Manulis et al. 1987b; Cowart and Foster 1985; Rodriguez et al. 1984; Lesuisse et al. 1987) and/or by a membrane-associated enzymatic process (Rodriguez et al. 1984; Lesuisse et al. 1987). Reduction of ferric iron prior to its transport does not seem to occur in *G. candidum*. The latter conclusion is supported by our failure to inhibit Fe^{3+} uptake with ferrous specific chelates (Fig. 4). Neither could we detect formation of Fe^{2+} when cells were incubated for a long period with ferric iron in the presence of the ferrous iron indicator BPDS as reported by Manulis et al. (1987b) for *Stemphylium botryosum* or Lesuisse et al. (1987) for yeast. It appears therefore, that the ferric iron might be directly transported also in the oxidized form.

Ferrous iron could be readily transported into cells of *G. candidum* with an identical Michaelis constant as the ferric iron, but with higher V_{max}. Fe^{2+} in the present study was obtained by reduction with L-ascorbate. Therefore, it might be difficult to assign the higher V_{max} merely to ferrous iron, since ascorbate also maintained the sulfhydryl groups of the cell surface in the reduced form. The latter appeared significant for the function of the present transport system (Table 1). Studies on iron assimilation through Mössbauer

spectroscopy indicate that iron is being stored as both ferric and ferrous metabolites. The ratio between the former and the latter iron forms appears not to be affected by the valence of the iron incorporated into the cells. The Mössbauer spectra for the Fe^{2+} and Fe^{3+} species illustrated in Fig. 6 are quite similar to spectra of yet unidentified metabolites reported by Matzanke et al. (1987) in *Neurospora crassa*. They assumed that the Fe^{2+} species represents a novel internal iron compound, whereas the Fe^{3+} species might correspond to an iron-storage compound, similar to bacterioferritin.

The absence of siderophore formation by citrus-pathogenic isolates of *G. candidum* is quite interesting, since this fungus maintains the capacity to transport iron via different hydroxamate siderophores. The ability of *G. candidum* to utilize iron efficiently from ferrioxamine B, an hydroxamate siderophore produced by *Streptomyces* spp. has also been observed in yeast (Lesuisse et al. 1987). However, the latter siderophore was not recognized by other fungi, such as *Stemphylium botryosum* (Manulis et al. 1987a) and *Verticillium dahliae* (Cordova and Barash, unpublished results). It should also be remembered that *G. candidum* comprises a complex of asexual fungi with different sexual stages (Butler and Peterson 1972). Thus, the possibility that isolates of *G. candidum* which maintain the production of siderophores will be found, cannot be entirely excluded.

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