Iron uptake in *Ustilago maydis*: studies with fluorescent ferrichrome analogues

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Iron uptake by the phytopathogenic fungus *Ustilago maydis* was studied using synthetic biomimetic ferrichrome analogues and their fluorescently labelled derivatives as structural and dynamic probes, respectively. The use of structurally distinct analogues enabled determination of the structural requirements for recognition by the fungal iron-uptake system. The application of fluorescently labelled derivatives which convert from a non-fluorescent to a fluorescent state upon iron (III) release enabled monitoring of iron uptake in real time both fluorimetrically and microscopically. Different rates of 55Fe uptake were found for two structurally distinct synthetic analogues, B9 and B5, which differ in their amino acid building blocks. B9 mediated uptake of 55Fe at a higher rate than B5. The behaviour of the fluorescent derivatives B9-Ant (anthracene-labelled B9) and B5-Ant (anthracene-labelled B5) paralleled that of their non-labelled precursors. Exposure of fungal cells to B9-Ant led to a higher increase of fluorescence in the medium than exposure to B5-Ant, indicating a more effective iron uptake from B9-Ant. By using fluorescence microscopy it was possible to trace the label of B9-Ant. Fluorescence was localized in regularly shaped vesicles in the treated cells. The rate of fluorescence appearance within the cells lagged behind the rate of iron uptake, suggesting use of the siderophores for iron storage.

**Keywords**: fungal iron uptake, ferrichrome, fluorescent biomimetic siderophores, fluorescence microscopy

INTRODUCTION

Although iron is the fourth most abundant element in the earth’s crust, under aerobic conditions at near-neutral pH its solubility and bioavailability are very low. To scavenge iron from the environment under conditions of iron stress, micro-organisms produce low molecular mass compounds, termed siderophores, which scavenge the scarcely available iron and deliver it to their cells (Nie Lands, 1981). The first siderophore discovered was ferrichrome, produced by the fungus *Ustilago sphaerogena* (Nie Lands, 1952). A large number of siderophores of various chemical structures have been isolated from different bacteria and fungi (Winkelm ann, 1991b). Ferric siderophores are transported into cells via specific transport systems (Winkelm ann & Huschka, 1987). However, the mechanism of interaction between the siderophore and its recognition and transport sites in fungi is largely unknown (Winkelm ann, 1993), although there is evidence that the metal centre and its surrounding residues are likewise crucial for siderophore recognition and transport in fungi (Huschka et al., 1986; Winkelm ann & Huschka, 1987). The phytopathogenic fungus *Ustilago maydis* produces the siderophores ferrichrome and ferrichrome A under iron-limiting conditions (Budde & Leong, 1989). Both siderophores are tripeodal molecules consisting of a hexapeptide anchor and three extending hydroxamate chains of three residues of glycine (ferrichrome) or two residues of serine and one of glycine (ferrichrome A).

**Abbreviations**: B5-Ant, anthracene-labelled B5; B9-Ant, anthracene-labelled B9.

§Dr Jacqueline Libman passed away on 30 March 1997, while this article was being prepared. This article is dedicated to her memory.
(Emery & Neilands, 1961). Ferrichrome–iron uptake in U. sphaerogeta has been found to involve an iron-shuttle mechanism (Ecker et al., 1982a, b; Emery, 1971). Using double-labelling experiments with $^{55}$Fe, $^{14}$C/ferrichrome, it was demonstrated that iron and siderophore are taken up at identical rates. Yet, while iron accumulates gradually, the siderophore reaches a maximum within the cell and is thereafter excreted. The fungus thus scavenges iron intracellularly from the iron-loaded siderophore, and then excretes the free siderophore to the medium to sequester additional iron for another transport cycle.

Mutants of U. maydis defective in siderophore biosynthesis have been isolated and characterized (Mei et al., 1993). Such non-siderophore-producing mutants are an invaluable tool in the study of siderophore recognition and uptake by micro-organisms because there is no interaction (e.g. ligand exchange) between the native siderophore and the one(s) being evaluated.

We have recently synthesized siderophore analogues that possess fluorescent markers at a site remote from the iron (III)-binding domain and receptor-recognition sites (Weizman et al., 1996). These derivatives act as structural probes, by tracing the structural requirements for recognition by the fungal iron-uptake system, and as dynamic probes for iron-exchange processes, by converting from a non-fluorescent to a fluorescent state upon iron (III) release. Such biomimetic analogues may provide a useful tool for the study of siderophore-uptake mechanisms because they allow the tracing of the ligand in real time and the tracing of the fate and localization of the free ligand. These fluorescent biomimetic analogues match their non-labelled precursors as microbial iron (III) carriers in iron-uptake studies in the Gram-negative bacterium Pseudomonas putida.

In this article, we describe the ferrichrome-uptake system in U. maydis using biomimetic ferrichrome analogues as probes. We test the structural requirements for siderophore activity by examining fungal $^{55}$Fe uptake with structurally distinct synthetic ferrichrome analogues. Using kinetic measurements as well as competition tests between the synthetic analogues and natural ferrichrome, we examine the involvement of a common transport system. We further distinguish between alternative siderophore–iron-uptake processes by applying fluorescent ferrichrome analogues, and evaluate the possible role of siderophores in iron storage within the fungal cells.

### METHODS

**Fungal growth and maintenance.** Ustilago maydis Sidmutant S023 (Sally Leong, Madison, WI, USA) was grown in Ustilago medium as described previously by Garibaldi & Neilands (1955), with the minor modification of a lower phosphate concentration ($1 \text{ g l}^{-1}$) to reduce iron contamination in the medium. This mutant belongs to the class II mutants, which are blocked in the biosynthesis of $\delta$-N-hydroxyornithine (Mei et al., 1993).

**Siderophores.** Ferrichrome was obtained from Sigma. The L-alanine-based analogue B5 and the glycine-based analogue B9 were synthesized as described previously by Dayan et al. (1993) and Jurkevitch et al. (1992). The anthracene fluorescent derivatives of B5 and B9, B5-Ant and B9-Ant, respectively, were synthesized as described previously (Weizman et al., 1996). Siderophores were complexed with Fe$^{3+}$ using FeCl$_3$ to 90% saturation, equilibrated for 24 h and then filtered through 0.2-μm filters. Stock solutions were kept frozen and in the dark.

**$^{55}$Fe-uptake studies.** For the $^{55}$Fe-uptake studies, 2-3-d-old cultures grown at 30 °C and 150 r.p.m. were centrifuged for 15 min at 3000 g, resuspended in fresh half-strength standard succinate medium (Meyer & Abdallah, 1978) to a final OD$_{595}$ of 1.5 (HP-8452A diode spectrophotometer), and incubated for 60 min in a water-bath at 30 °C. When specified, sodium azide was added to a final concentration of 5 mM 30 min prior to the addition of the siderophores. The labelled $^{55}$Fe (Amersham) complex was added to a final concentration of 1 μM. Aliquots (0.5 ml) were taken in duplicate, layered onto a mixture of dibutyl phthalate/octyl phthalate (1:1), v/v; Sigma) and centrifuged. The supernatant was discarded and the Eppendorf tube tips were cut. Radioactivity in the tips of the tubes containing the fungal cells was measured on a Beckman LS1801 counter using Insta Gel II as the scintillation mixture.

**Fluorometry.** For fluorescence studies, the cells were grown and prepared as described for the $^{55}$Fe-uptake studies. The iron and fluorescently labelled ferrichrome analogue complex was added to the fungal suspension to a final concentration of 5 μM. Aliquots (1 ml) were taken in duplicate, centrifuged and the supernatant was collected and measured for fluorescence with an SLM Instruments fluorometer (model 4800) with excitation at 375 nm and emission measured at 415 nm. Results are given in arbitrary fluorescence intensity units.

**Fluorescence microscopy.** For fluorescence microscopy, the siderophore was added to a 2 ml fungal suspension to a final concentration of 50 μM, and samples were taken at various time intervals. Cells were observed using a Carl Zeiss epifluorescence microscope (Jena), with a × 63 oil-immersion objective. The fluorescence filters used were Carl Zeiss G365 excitation, FT395 beam splitter and LP420 emission barrier. Photographs were taken with a Zeiss MC100 camera and Fujichrome 100 film. Control images were captured using an AppleTech MSU 700L video camera and NIH Image 1.57 software on a Power Macintosh computer.

**Reproducibility of results.** All experiments were performed at least three times in duplicate. Standard deviations were all within 10% of the mean value within an experiment. However, between separate experiments a maximum variation of 15% was observed without any change in the overall pattern of results.

### RESULTS

**$^{55}$Fe-uptake studies**

To determine the availability of iron from the synthetic siderophores to the fungus, and to test the structural requirements for siderophoric activity of the fungal ferrichrome–iron uptake system, we studied $^{55}$Fe uptake mediated by two structurally distinct synthetic analogues, B9 and B5. B9 is a glycine-based analogue, whereas B5 is an L-alanine derivative, displaying a Λ-cis
concentrations ranging between 0 and 50 pM in the incubation medium. The fungal cells were incubated for 20 min, and then measured for radioactivity. Kinetic uptake experiment was set up, with 55Fe-siderophore indicating the uptake system's higher affinity for natural ferrichrome than for the analogues.

To compare the uptake behaviour of the natural siderophore ferrichrome, a concentration-dependent 55Fe-ferrichrome was added to an incubation medium amended with 55Fe-B9 or 55Fe-B5. An equimolar concentration of iron-ferrichrome (1 μM) inhibited 55Fe uptake mediated by B9 by 70% (Fig. 1a). However, iron-ferrichrome did not compete with the B5 analogue (Fig. 1b). When uptake of 55Fe-ferrichrome was studied in the presence of iron-B5 or iron-B9, even at levels of up to 20 times the concentration of 55Fe-ferrichrome, no competition or inhibitory effect was found. These data indicate the uptake system's higher affinity for natural ferrichrome than for the analogues.

To compare the uptake behaviour of the natural siderophore ferrichrome with that of the two ferrichrome analogues, a concentration-dependent 55Fe-uptake experiment was set up, with 55Fe-siderophore concentrations ranging between 0 and 50 μM in the incubation medium. The fungal cells were incubated for 20 min, and then measured for radioactivity. Kinetic measurements of radioactive iron uptake from 55Fe-ferrichrome, 55Fe-B9 and 55Fe-B5 (data not shown) demonstrated a similar trend of saturation kinetics, indicative of a receptor-mediated uptake process (Ecker et al., 1982a; Winkelmann, 1991b). The affinity of the uptake system for the iron-complexed siderophores was similar for all three siderophores, as determined by the respective K_m values obtained in this experiment. However, the maximum rate of uptake (V_max) was significantly higher for the natural compound, ferrichrome, than for the synthetic analogues (Table 1).

**Table 1. Kinetic parameters of 55Fe transport mediated by ferrichrome and its synthetic analogues B5 and B9**

<table>
<thead>
<tr>
<th>Siderophore</th>
<th>K_m (μM)</th>
<th>V_max (nmol min⁻¹)</th>
</tr>
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<tbody>
<tr>
<td>Ferrichrome</td>
<td>2.86 ± 0.96</td>
<td>0.066 ± 0.004</td>
</tr>
<tr>
<td>B5</td>
<td>6.85 ± 0.81</td>
<td>0.017 ± 0.002</td>
</tr>
<tr>
<td>B9</td>
<td>7.80 ± 0.92</td>
<td>0.018 ± 0.002</td>
</tr>
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</table>

Configuration as iron (III) complex. B9 (Fig. 1a) mediated 55Fe uptake at a higher rate than B5 (Fig. 1b), totalling 0.24 nmol 55Fe and 0.07 nmol 55Fe, respectively, after 20 min incubation. The preferential uptake of iron from the two analogues was inhibited by the respiratory inhibitor sodium azide, suggesting an active uptake mechanism.

To establish whether the analogues share a common component with the uptake system of natural ferrichrome, competition experiments were conducted. Iron-ferrichrome was added to an incubation medium amended with 55Fe-B9 or 55Fe-B5. An equimolar concentration of iron-ferrichrome (1 μM) inhibited 55Fe uptake mediated by B9 by 70% (Fig. 1a). However, iron-ferrichrome did not compete with the B5 analogue (Fig. 1b). When uptake of 55Fe-ferrichrome was studied in the presence of iron-B5 or iron-B9, even at levels of up to 20 times the concentration of 55Fe-ferrichrome, no competition or inhibitory effect was found. These data indicate the uptake system's higher affinity for natural ferrichrome than for the analogues.

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**Fluorescence studies**

Anthracene-labelled ferrichrome analogues were employed as dynamic probes to follow iron utilization by the fungus. In this type of study, fungal cells are incubated with ferrated siderophores which are non-fluorescent when loaded with iron (III). Once iron is removed from the siderophore, the fluorescence increases. In our experiments, fluorescence in fungal filtrates after incubation with the iron complex was monitored for 3-5 h. Since these compounds are stable under standard experimental conditions, the increase of fluorescence in the medium is derived from fungal utilization of the iron.

Incubation of U. maydis S023 cells with the ferric B9-Ant, at a concentration of 5 μM (Fig. 2), resulted in an increase of fluorescence in the culture filtrate. The rate of increase was constant during the 3-5 h incubation. B5-Ant, however, showed a lower rate of fluorescence increase in the medium, suggesting a lower rate of iron utilization by the fungus. To test the chemical intactness of the siderophore in the spent medium, FeCl_3 was added to the filtered medium. This experiment resulted in a sharp decrease in fluorescence, thus proving the intactness of the siderophore (data not shown).

To determine whether the two analogues share a common component(s) in the iron-uptake system of the fungus, we incubated the fungal culture with B9-Ant in the presence of the non-fluorescent B5 analogue. This experiment resulted in a 54% inhibition of the fluorescence increase obtained when using B9-Ant alone.
Fig. 2. Fluorescence increase in cultures of U. maydis S023 amended with 5 μM of the fluorescent ferrichrome analogues iron–89-Ant (■), iron–85-Ant (△) and iron–89-Ant in the presence of equimolar concentrations of the non-fluorescent derivative of iron–85-Ant, iron–85, as competitor (○). Mean values from three experiments are shown (SD up to 5%).

This inhibition in iron utilization by the fungus suggests possible interactions between the B5 analogue and the B9 iron-uptake system. Incubation of the fungal cells with increasing concentrations (0–20 μM) of either of the two analogues, iron–89-Ant and iron–85-Ant, for 120 min resulted in increased fluorescence, which displayed saturation kinetics for B9-Ant and B5-Ant (Fig. 3).

Fluorescence microscopy

U. maydis cells incubated with iron–89-Ant were also monitored by fluorescence microscopy. Fungal cells were incubated with 50 μM iron–89-Ant, washed and mounted on microscopic slides. Fluorescence appeared in the cells after 16 h incubation. The fluorescence was located in regularly shaped vesicles in the treated cells (Fig. 4a). After shorter incubation periods (4 and 8 h), only slight fluorescence was apparent, and its location in the fungal cells was diffuse and hard to determine. To capture control samples, much longer integration times were applied for the video camera. Nevertheless, no fluorescence was detected in the cells (Fig. 4b). These results suggest that the iron–siderophore complex is taken up by the fungal cell with consecutive iron removal from the siderophore occurring inside the cell.

**DISCUSSION**

In this report, the use of fluorescently labelled biomimetic ferrichrome analogues to study the ferrichrome-uptake system in U. maydis has been described. The use of these analogues allowed the mechanism and time course of iron acquisition from iron–siderophores by the fungus to be followed. Iron uptake, as evidenced by the accumulation of the radioactive label in the cells, was very rapid. The 55Fe label was found in the cells within minutes, following the addition of the ferric siderophores to the incubation medium. Chelated iron was then removed by the cell and the ligand was excrated, as evidenced by the appearance of the fluorescently labelled biomimetic siderophores in the medium. This egress of the desferri-siderophore was a slower process, lagging behind the uptake of the chelated iron and lasting for hours after the start of iron uptake. The appearance of the fluorescent ligand was also followed in the fungal cells by fluorescence microscopy. Fluorescence was seen clearly only 16 h after the start of incubation, suggesting long-term storage of chelated iron inside the cells.

Several mechanisms of siderophore-mediated iron transport in fungi have been suggested (Winkelmann, 1993): (i) a shuttle mechanism, in which the iron–siderophore complex enters the cells, and after cellular removal of the iron, the ligand exits the cell to chelate another iron atom (Emery, 1971); (ii) hydrolysis of the ligand after iron removal inside the cell without its recycling; (iii) a taxi mechanism, in which the iron enters the cell, while the ligand (the taxi) remains extracellular. In this latter mechanism, the ferric ion is either reduced and exchanged at the cell surface with a membrane-bound chelating agent that completes the active transport of iron into the cell (Ecker & Emery, 1983), or exchanged via a non-reductive mechanism, as observed in Rhodotorula species (Carrano & Raymond, 1978).

The pathways of iron metabolism and the intracellular role of siderophores are much less understood (Matzanke et al., 1987), as is the mechanism of the interaction between siderophores and fungal membrane proteins (Winkelmann, 1993).

The first step in iron uptake by micro-organisms has to be specific recognition between some components of the iron-uptake system and the iron complex. There is evidence that in fungi, as in bacteria, recognition of siderophores by the iron-uptake system is both enantio-
and stereoselective, with both the chirality of the metal centre and the structure of the terminal groups of the metal octahedron being important (Huschka et al., 1985; Winkelmann, 1979, 1991c). Studies with retrohydroxamate ferrichrome, where the directionality of the hydroxamic acid moieties are inverted but the A-cis configuration of the iron centre is retained, revealed comparable activity of the retro-isomer and the natural compound in the ferrichrome-producing U. sphaerogena (Emery et al., 1984). A des(methyl)-retrohydroxamate ferrichrome, lacking the three methyl groups around the iron centre, exhibited significantly lower transport rates (Emery et al., 1984).

Our results with biomimetic ferrichrome analogues concur with these findings, in that they demonstrate differential uptake from structurally different siderophores. $^{55}\text{Fe}$-uptake studies with the two analogues B5 and B9, which differ in their amino acid skeleton and chirality, demonstrated different transport rates. Whereas B9 is glycine-based, the B5 analogue is composed of L-alanine amino acids and is optically active, with a metal centre exhibiting A chirality (Jurkevitch et al., 1992).

The observed saturation kinetics for $^{55}\text{Fe}$ uptake by the natural ferrichrome and its analogues B9 and B5 demonstrate the involvement of specific iron-uptake systems in U. maydis. Whereas the glycol derivative B9 and ferrichrome share a component or a common transport system, as evidenced by the inhibition of iron uptake from B9 by ferrichrome, the alanyl derivative B5 seems to make use of a different uptake system. This distinction between the two structurally different derivatives demonstrates the sensitivity of the ferrichrome-iron-uptake system to subtle structural modifications.

Ferrichrome and ferrichrome A biosynthesis in U. maydis has been studied and characterized (Budde & Leong, 1989; Mei et al., 1993; Voisard et al., 1993). Emery (1971) used double-labelled [$^{55}\text{Fe},^{14}\text{C}$]ferrichrome to study ferrichrome uptake in cultures of U. sphaerogena. These studies revealed rapid removal of iron from $^{55}\text{Fe}$-labelled ferrichrome by the fungus. When $^{14}\text{C}$-labelled ferrichrome ligand was used, an initial uptake of label was followed by its exit into the medium. These observations led to the conclusion that the desferri-ferrichrome acts as a shuttle to bring iron into the cell. After removal of the metal in the cell, the fungus ‘recycles’ the ligand by secreting it into the medium, where it can re-form iron-ferrichrome and re-enter the cell.

Iron-uptake studies in micro-organisms are mostly performed with isotope-labelled siderophores, and with atomic absorption, while ESR and Mössbauer spectroscopic measurements serve as complementary methods (Winkelmann, 1991b). Double-labelling of the iron-siderophore complex (i.e. $^{55}\text{Fe}-^{14}\text{C}$-labelled siderophores) enables investigation of the siderophore-transport mechanism via simultaneous measurement of both labels. An analysis of double-labelled siderophore kinetics is thus the method of choice for investigating the mechanism of siderophore-mediated iron transport (Winkelmann, 1991b). Double-labelling with one radioactive label, $^{55}\text{Fe}$, and one fluorescent label bound to the siderophore, as applied here, has the additional ad-
vantage of enabling differentiation between the iron-loaded siderophore and the free ligand. The latter possibility provides a means of localizing iron-exchange processes in real time.

Our results with the fluorescent analogues B9-Ant and B5-Ant are analogous to those obtained in $^{59}$Fe-uptake studies using their non-fluorescent conjugates B9 and B5. These data suggest a mechanism in U. maydis similar to that reported for iron-ferrichrome uptake in U. sphaerogena. The appearance of fluorescent label in the fungal medium soon after the start of incubation with the ferrated siderophore is due to the movement of the iron from the iron complex and egress of the now fluorescent desferri-siderophore. The uptake of iron-ferrichrome by the fungus has been found to be pH- and temperature-dependent, and to be inhibited by various inhibitors, mainly respiratory ones (Emery, 1971). The application of sodium azide in this study (Fig. 1) revealed a similar inhibition of the iron-uptake system, indicating the involvement of an active iron-uptake process. In U. sphaerogena, desferri-ferrichrome is not taken up by the cells, nor does it diffuse passively into them, even in highly concentrated cell suspensions (13%). Our results with fluorescence microscopy are in agreement with this. Uptake of the desferri-fluorescent B9-Ant into the cells was not seen, nor did we find a decrease in fluorescence when the desferri compound was monitored in culture filtrates (data not shown).

Ecker et al. (1982a) compared the rate of $^{59}$Fe-ferrichrome transport measured as radioactivity, and the rate of reduction of siderophore iron (III) to iron (II) in the cell suspension by electron paramagnetic resonance spectroscopy. These results demonstrated that iron reduction lags behind the rate of iron uptake, indicating that the complex is taken up without dissociation. Ecker & Emery (1983) suggested storage as another role for ferrichrome in U. sphaerogena and isolated ferrichrome from cells grown in iron-rich media (iron concentrations of up to 50 μM, which far exceeded cell requirements). Matzanke et al. (1990) also suggested a ferritin-substituting function for ferrichrome in U. sphaerogena. The use of siderophores for storage has also been described in the fungus Neurospora crassa (Matzanke et al., 1987; Winkelman, 1991a).

Our results with fluorescence microscopy in U. maydis support the suggestions put forward by Ecker et al. (1982a) and Ecker & Emery (1983). The appearance of the fluorescent label in the fungal cells lagged behind iron uptake by the fungus, being clearly visible in the cells after 16 h incubation, in vesicles that may serve for storage and subsequent reduction of the metal by the fungus.

Together these observations suggest an iron-uptake system in U. maydis which involves a shuttle mechanism, where the iron-loaded siderophore complex enters the cell, and upon iron removal from the complex, the siderophore is egressed back to the medium. Fluorescent-labelling experiments, rather than radioactive double-labelling, were used in the present work. The fluorescent labels introduced here are advantageous in that they facilitate analysis and enable localization of the iron-exchange process, when used in conjunction with fluorescence microscopy. Experiments to test the scope and limitations of fluorescent siderophore analogues for the imaging of iron-exchange processes in real time are currently in progress.

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