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Surface Iron Polymers and Hydroxy Acids. A Model of Iron Supply in Sideramine-Free Fungi

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Abstract. Biochemical and electron microscopic evidence is presented that sideramine-free fungi form iron hydroxide polymer layers on the cell surface when grown in an iron containing medium.

Iron hydroxide polymer formation on the cell surface is completely prevented in sideramine producing strains of *Neurospora crassa*. After feeding a sideramine-free mutant of *Neurospora crassa* with ornithine in order to restore the sideramine synthesis the iron hydroxide coat is gradually dissolved.

The addition of excess citrate and malate to the incubation medium also prevents iron polymer adsorption, suggesting that hydroxy acids may be involved in iron supply, when sideramine-free organisms are grown in iron containing media.

In order to study the interaction between iron hydroxide polymer deposition upon the cell surface and iron chelating acids in *Neurospora crassa*, the amount and the proportion of excreted acids was studied under various experimental conditions. Gas chromatographic analysis of the acids produced under iron deficient conditions revealed that succinate, malate and citrate were present within the cells in the early growth phase. The acids were sequentially excreted into the medium in the order succinate, malate and citrate. The amount of succinate decreased after 2 days of cultivation, whereas the amount of malate and citrate continually increased. Although citrate was present within the cells from the 1st day, excretion occurred very late, generally after the 3rd day.

It is suggested that sideramine-free fungi first adsorb iron as a hydroxide polymer on the cell surface, and that it is gradually solubilized by excreted hydroxy acids such as citrate or malate. Thus high local concentrations of iron chelated by hydroxy acids provide sideramine-free fungi with a continuous iron supply.

Key words: Iron hydroxide – Hydroxy acids – Iron transport *Neurospora crassa* – Iron deficiency – Iron (III).

Food-decomposing fungi such as moulds live in an environment where most if not all iron is bound to organic ligands. Survival of these organisms is strongly dependent on extracting iron out of various natural compounds. Several fungi are able to synthesize desferri-sideramines, which show extraordinarily high stability constants with trivalent iron (reviewed by Neilands, 1973). It has been shon that iron-containing sideramines are specifically taken up by various fungi, and this enables these organisms to compete effectively with other organisms under iron-limited conditions (Winkelmann, 1974; Wiebe and Winkelmann, 1975). This property seems to be an important advantage for saprophytic organisms. Details of the sideramine iron transport in *Neurospora crassa* have been published recently (Ernst and Winkelmann, 1977a, b; Winkelmann, 1979).

Many filamentous fungi produce additionally high amounts of organic acids (Lockwood, 1975; Miall, 1978), among which citric acid is known to be an excellent iron chelator with high stability (Sillen and Martell, 1964). We reported earlier two routes of iron uptake in the fungus *Neurospora crassa* based on uptake kinetics with a sideramine-free mutant (Winkelmann and Zähner, 1973). Contrary to the high coprogen iron uptake which revealed typical characteristics of an active transport system, for example, saturation kinetics, inhibition and competition, iron uptake mediated by citrate was comparatively low. Despite the observed low iron uptake by citrate,

Abbreviations. BSTFA = N,O-Bis(trimethylsilyl)-trifluoracetamide; GC = Gaschromatography; EGTA = Ethylenglykol-bis(2-aminoethylether) N,N′-tetraacetic acid; TMS = Trimethylsilyl

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sufficient growth was obtained using the sideramine-free mutant.

In addition to the low iron uptake, citrate iron uptake seemed to be dependent on the environmental pH and the amount of citrate present in the medium. Alkaline solutions were reported to form polymeric ferric citrate with extraordinarily high molecular weights, which depolymerize with increasing concentration of citrate, resulting in a ferric dicitrate anion $[Fe(Cit)_2]^{5-}$ at a concentration ratio citrate to iron of 20 : 1 (Spiro et al., 1967a, b). The requirement of a low pH value and an excess of citrate are, however, not generally fulfilled in the usual media.

The present investigation was intended to study the occurrence and the amount of citrate in *Neurospora crassa* in order to clarify it biological involvement during iron supply in fungi.

Materials and Methods

Organisms and Growth Conditions

The wild type *Neurospora crassa* 74A and the mutant *Neurospora crassa* arg-5 ota aga were gifts from Rowland H. Davies, Irvine, California. The organisms were cultivated on a complex medium containing agar (1.5%), yeast extract (0.4%), malt extract (1%) and glucose (0.4%).

Conidia were harvested after a 7 day growth at 27° C by the addition of 100 ml 0.9% NaCl solution containing 0.1% Tween 80. After repeated washing and sedimentation the conidia were stored at 4° C until use.

Iron-deficient conditions were obtained by submerged cultivation in minimal medium without iron containing asparagine (0.5%), KH_2PO_4 (0.1%), $MgSO_4 \cdot 7H_2O$ (0.1%), $CaCl_2 \cdot 2H_2 \cdot 2H_2O$ (0.05%) and glucose (0.2%), autoclaved separately. In addition biotine (10 µg/l), putrescine (100 mg/l) and arginine (50 mg/l) were necessary for optimal growth of the mutant.

Chemicals

Salts for media, L(−)asparagine, putrescine (1,4 diaminobutan-dihydrochloride), D(+)biotine, L(+)arginine, citric acid, succinic acid, formic acid (98 − 100%) and pyridine (0.01% H_2O), nitric acid (65%, Suprapur) were from Merck, Darmstadt. L-Malic acid, BSTFA (N,O Bis(trimethylsilyl)-trifluoracetamide) and DOWEX 50WX8, Dowex 2X8 were obtained from Serva, Heidelberg. Yeast extract, malt extract, and agar (purified) were from Difco Laboratory, Detroit, Michigan.

Radiochemicals

$^{55}FeCl_3$ in 1M HCl (carrier free) was obtained from Amersham, Buchler Braunschweig.

Atomic Absorption Measurements

The iron content of the mycelia was determined using an atomic absorption spectrophotometer, Varian, Model 1200, equipped with a carbon rod atomizer, Model 63. Dried mycelia were solubilized with nitric acid (65%, Suprapur). After the addition of an equal amount of distilled water, 5 µl of the solution was measured using the flameless method and quantitated with the aid of a calibration curve.

Isolation of Acids

The acids produced by the mycelia were isolated according to the method of Tokumitsu and Ui (1974). The culture filtrate was passed through a DOWEX 50WX8 column (8 × 150 mm, H-form) to remove the cations.

After adjusting the pH to 6.8 with dilute NH_4OH, the solution was passed through a DOWEX 2X8 column (8 × 150 mm, formate-form), to allow binding of all acid anions. The column was previously treated with concentrated formic acid (98 − 100%) and then with decoct, distilled water. Acids were eluted with 20 ml formic acid (60%).

Preparation of Silyl-Derivatives

The acid-containing solutions were evaporated to dryness and dissolved in 200 µl dried pyridine. 100 µl of the pyridine solution was placed in a vial sealed with a teflon cap and mixed with an equal amount of BSTFA at room temperature. 1 µl of the reaction micture gave a sufficiently high detector response in the range of 4 − 16 $\times 10^{-10}$ attenuation. For calibration, 10 mg of each acid was dissolved in 500 µl pyridine with the addition of 500 µl BSTFA. The area/peak ratio was corrected using molar correction factors established by a calibration chromatogram.

GC-Analysis

The separation of silylated acids was carried out with a Varian Aerograph, series 1200, equipped with a hydrogen flame ionization detector and a 6 foot column, packed with OV-1, 3% on Gas-Chrom Q (Applied Science Laboratory Inc., Serva, Heidelberg). The following conditions were employed: injector 250° C, detector 250° C, and column 100 − 200° C temperature program 4° C/min.

Mass Spectrometry

The peaks obtained after GC analysis were identified by mass spectrometry after chromatography of the TMS-derivatives. The molecular weights of TMS derivatives were as follows: peak 1 (MG 314) phosphate-TMS; peak 2 (MG 262) succinate-TMS; peak 3 (MG 350) malate-TMS and peak 4 (MG 480) citrate-TMS.

Electron Microscopy

After fixation with 1% glutaraldehyde, 1% OsO_4, 20 mM s-collidine and 2.5 mM EGTA at pH 6.6, the cells were dehydrated with ethanol and embedded in Epon 812 or Epon-Araldite. Thin sections were mounted on 300-mesh grids and stained with both uranyl acetate and lead citrate and examined with a Siemens Elmiskop 1 or 102.

Results

Iron Accumulation Experiments

When a sideramine-free mutant of *Neurospora crassa* is grown in the presence of iron, added as $FeCl_3$ to the medium, the amount of iron accumulated by the cells was extraordinarily high and exceeded that observed with the sideramine-producing wild type strain. Atomic absorption measurements revealed that iron accumulation by the mutant was not saturable indicating an uncontrolled uptake process (Fig. 1). Similar experi-

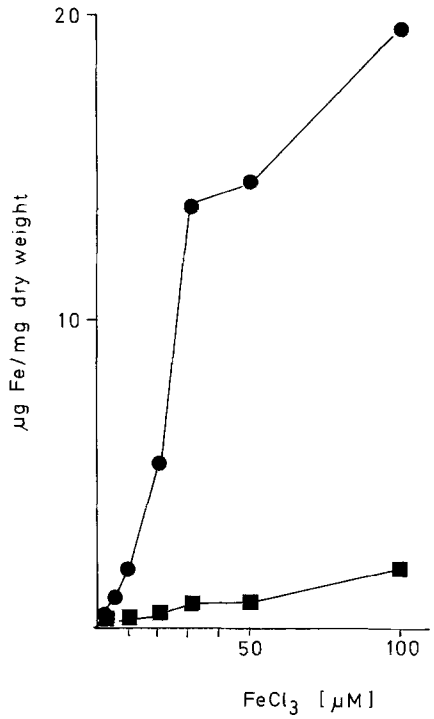

Fig. 1. Atomic absorption measurements of iron accumulated by the sideramine-free mutant and the wild type of *Neurospora crassa* after a 3 day growth in the presence of increasing amounts of $FeCl_3$ added to the cultivation medium. Mutant without sideramines (●), wild type (■)

ments with aluminum, added as $AlCl_3$, revealed very low uptake values not exceeding 1 µg per mg dry weight.

This unexpected high iron accumulation by sideramine-free organisms was extensively studied using the wild type strain and the mutant grown in the presence and absence of ornithine (Table 1). Ornithine is a component of the sideramines produced by *Neurospora*. As shown in Table 1, the absence of sideramines led to high iron accumulation whereas the presence of sideramines in the wild type as well as in the mutant led to comparatively low iron values.

Localization of Accumulated Iron

In order to determine whether iron accumulated by the sideramine-free mutant was actually taken up by the cells or bound at the cell surface, the cells were incubated with an iron chelating agent, desferrioxamine B, which had been shown earlier to be relatively impermeable in the iron containing form in *Neurospora crassa*.

After thoroughly washing, the mycelia were incubated with a desferrioxamine B solution and the amount of solubilized iron was determined spectrophotometrically at 436nm. The results are shown in Table 2. Large amounts of iron could be released from mycelia of the sideramine-free mutant previously incubated with $FeCl_3$.

Table 1. Iron content of mycelia grown in the presence and absence of sideramines in a medium containing 20 µM $FeCl_3$[a]

No.	Mutant (−Fe)	Mutant (+20 µM Fe)	Mutant (+20 µM Fe + ornithine)	Wild type (+20 µM Fe)
1	0.55	13.43	2.64	1.82
2	0.73	10.71	3.12	1.80
3	0.49	17.41	3.32	1.48
4	1.09	10.84	2.52	1.69
5	0.91	10.02	2.84	1.68
Mean	0.76 (±0.25)	12.41 (±2.91)	2.87 (±0.33)	1.69 (±0.13)

[a] Values are expressed as nmol per mg dry weight. Iron was added as $FeCl_3$ together with the inoculum (10^8 spores/100 ml medium). The iron content of the mycelia was determined with an atomic absorption spectrophotometer after 24 h of submerged cultivation

Table 2. Amount of surface bound iron that could be released with desferrioxamine B

Strain	Concentration of added $FeCl_3$ (µM)	Incubation time	Iron(III) released from mycelia (nmol/mg)	Iron(III) remaining in the culture-filtrate (nmol/mg)
Mutant	50	3 days[a]	2.20	–
	100	3 days	6.07	–
	500	3 days[b]	3.00	2.23
Wilde type	100	3 days	–	nd[c]
	500	3 h	–	nd

[a] Iron was added together with the inoculum
[b] Iron was added to a culture grown for 3 days under iron deficient conditions. The pH of the incubation medium was adjusted to pH 5.5 after addition of $FeCl_3$
[c] Not determined

Electron Microscopic Studies

Iron accumulation by the sideramine-free mutant could be visualized on electron micrographs (Fig. 2). Thin sections of the mycelia incubated with iron(III) for 3 h showed an electron dense layer of dark material on the cell surface which was regarded as an iron hydroxide polymer. Also other parts of the cell wall and even the cytoplasmic membrane showed an intensive dark colour.

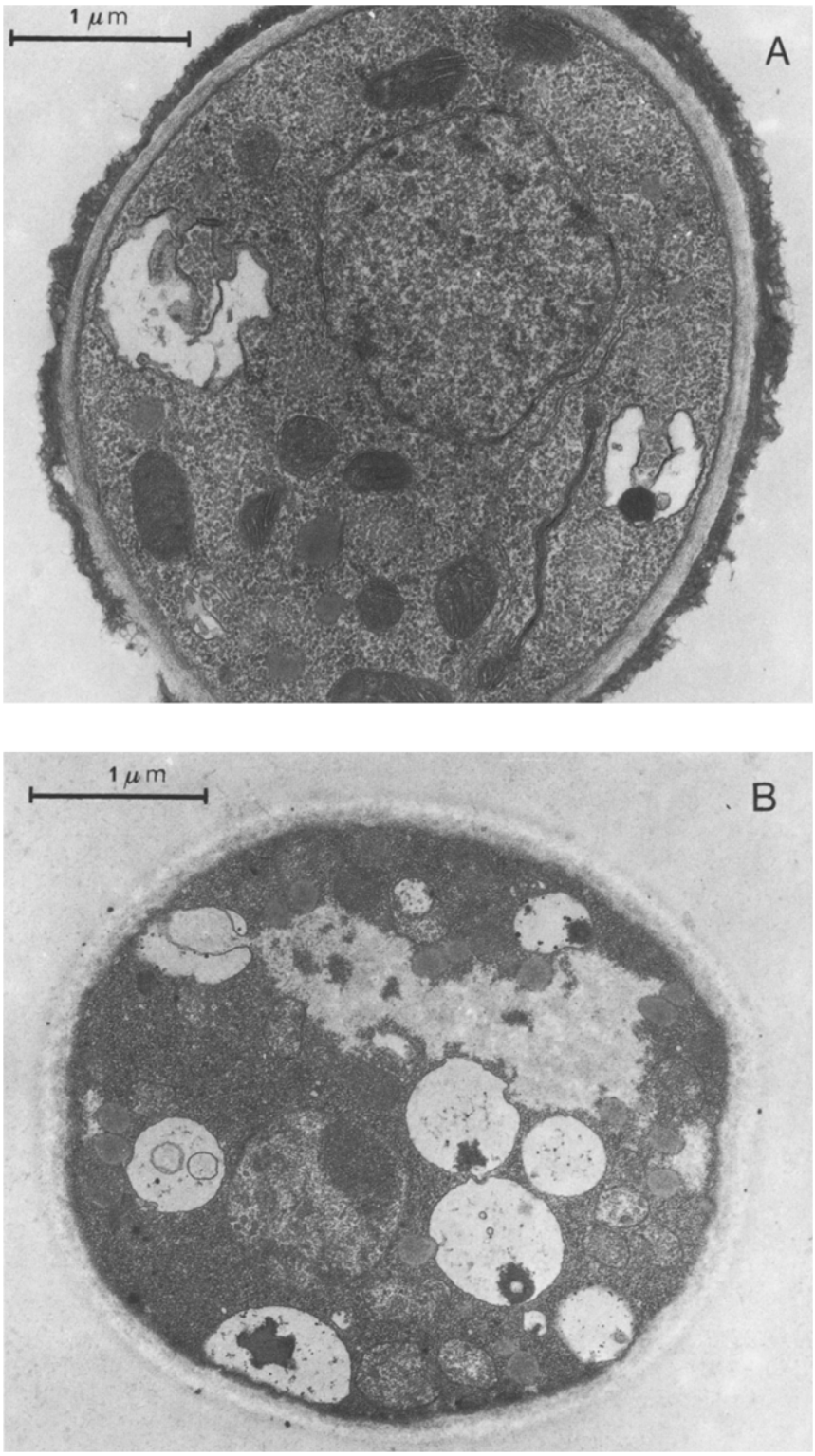

Fig. 2
Electron micrographs (*thin sections*) showing mycelia of the sideramine-free mutant incubated in the presence (**A**) and in the absence of $FeCl_3$ (**B**). Note the black iron hydroxide polymer layer on the cell surface and the pronounced dark colour of the other part of the cell wall material and the cytoplasmic membrane of the cells incubated with iron. Final magnification: 25,000

Table 3. Iron content of mycelia incubated with 50 µM $FeCl_3$ for 1 h at different pH[a]

	pH 3	pH 4	pH 5	pH 6	pH 7	pH 8
Mutant	0.150	0.325	1.300	1.475	2.100	2.350
Wild type	0.750	1.225	1.150	1.075	0.850	0.750

[a] Mycelia grown for 1 day in submerged culture were harvested, washed with cold NaCl-solution (0.9 %) and incubated in minimal medium containing 50 µM $FeCl_3$ adjusted to the pH values indicated in the table. Values are expressed as µg/mg dry weight

pH-Dependence of Iron Hydroxide Polymer Adsorption

As shown in Table 3, mutant and wild type differed in the amount of accumulated iron during increasing pH values. Iron polymer formation upon the cell surface of the mutant was strongly dependent on the prevailing pH, whereas the wild type showed an optimum of iron uptake. Although the absolute amount of iron adsorbed to the cell surface increased with increasing pH, the molecular size of iron hydroxide polymers and their integration into the cell surface may be optimal at a more acidic pH.

pH Changes during Submerged Cultivation

Alkaline pH values are generally not observed during early growth of fungal mycelia. In most instances a characteristic initial decrease of the pH in the medium is observed. This pH decrease may be due to a selective withdrawal of basic compounds or to an active extrusion of protons.

When the changes in pH of submerged cultures of *Neurospora crassa* were followed, wild type and mutant showed a corresponding initial decrease with a subsequent increase after several days of cultivation (Fig. 3). The observed pH decrease was more pronounced in the mutant. Furthermore, pH differences resulting from iron deficiency could not be observed prior to the 2nd day. thus the initial pH decrease seemed to be unaffected by the presence of iron. After the 2nd day, however, the iron supply resulted in a steep increase of the pH in the mutant indicating that the level of intracellular iron was critical. In contrast to the mutant, the wild type revealed a parallel development of pH changes irrespective of the presence of iron. Despite a rapid acidification during the 1st days, iron polymer adsorption to the mycelia of the sideramine-free mutant was observed, indicating that the decrease of pH did not prevent iron hydroxide polymer adsorption to the cell surface.

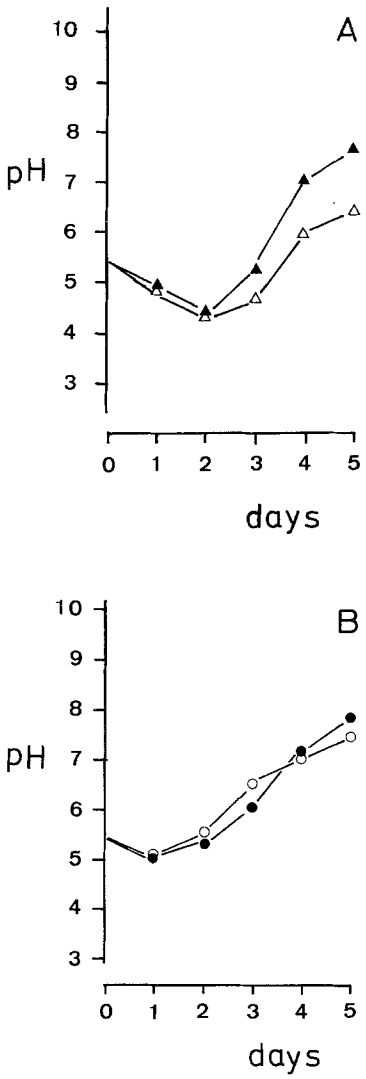

Fig. 3 A and B. pH changes during growth of the mutant and the wild type of *Neurospora crassa* in the presence and absence of additional iron in the medium. Mutant: (**A**) without iron (△) and with the addition of 20 µM $FeCl_3$(▲). Wild type: (**B**) without iron (○) and with the addition of 20 µM $FeCl_3$ (●)

Effect of Hydroxy Acids on Iron Hydroxide Polymer Formation

Organic acids of the citric acid cycle are possible candidates for solubilization of iron. Therefore a simple assay for iron hydroxide polymer solubilization was performed. Siderамine-free mycelia were grown for 1 day under iron deficient conditions and incubated subsequently with 1 mM $FeCl_3$ and 20 mM acid (succinate, malate or citrate). After 3 h the amount of iron hydroxide polymer adsorbed on the mycelia was determined with the aid of desferrioxamine B. As shown in Table 4, prevention of iron hydroxide polymer formation increased in the order succinate, malate, citrate.

Occurrence of Acids in Submerged Cultures

The physiological significance of hydroxy acids during iron supply is dependent on the amount and the time of occurrence in the culture medium. To test whether these acids were excreted during submerged cultivation, the culture filtrate was collected and the amount of succinate, malate and citrate was determined by gas chromatography. Acid production was followed during a growth period of 10 days under iron deficient conditions in the mutant as well as in the wild type. As shown in Fig. 4, the first acid appearing in the culture filtrate was succinate followed by malate and citrate. Whereas succinate decreased after the 3rd day, malate and citrate increased until the 10th day of cultivation. A comparison between the wild type and the sideramine-free mutant revealed that there were only minor differences in the sequence of occurrence and in the proportion of excreted acids. Generally the mutant seemed to be somewhat delayed in the production of malate and citrate, possibly caused by a more pronounced iron deficiency. Considering the absolute amounts of excreted acids, it was observed that the mutant produced remarkably higher amounts of all acids.

Table 4. Effect of acids on iron hydroxide polymer formation[a]

Conc. of iron (mM)	Addition of acid	Recovery of iron (nmol/mg dry weight)	Iron bound %
1	–	685	100
1	20 mM succinate	216	31.5
1	20 mM malate	10	1.5
1	20 mM citrate	–	–

[a] Mycelia grown for 1 day under iron deficient conditions with out siderамines were incubated in the presence of 1 mM $FeCl_3$ and with the addition of 20 mM acid at pH 5.5 for 3 h. The mycelia were filtered, washed and incubated with 1 mM desferrioxamine for 30 min. The amount of iron recovered was determined using the absorption value for ferrioxamine B, $E^{1\%}_{430} = 39$. The value without additional acid is set at 100%

Distribution of Acids Inside and Outside the Cells

A quantitative analysis of the acid content of the mycelia revealed that all acids were present within the cells from the 1st day and increased rapidly between the 2nd and 3rd day (Table 5). The amount of acids occurring in the culture filtrate does not correspond to the actual amount of intracellular acids. These results may reflect pronounced differences in membrane permeability for acids, which seem to decrease in the order of succinate, malate, citrate.

It is noteworthy that citrate is not excreted in detectable amounts during the 1st days of growth, despite the presence of large amounts inside the cells. Comparing the two strains, the intracellular content is very alike at the 3rd day, but the extracellular amount differed by two orders of magnitude. The mutant generally excreted remarkably larger amounts of succinate and malte than the wild type.

Effect of Iron on Acid Production

In order to study the interrelationship between iron supply and acid production during submerged cultivation of *Neurospora crassa*, the intracellular and extracellular amounts of acids were determined after growth in the presence of 20 µM $FeCl_3$.

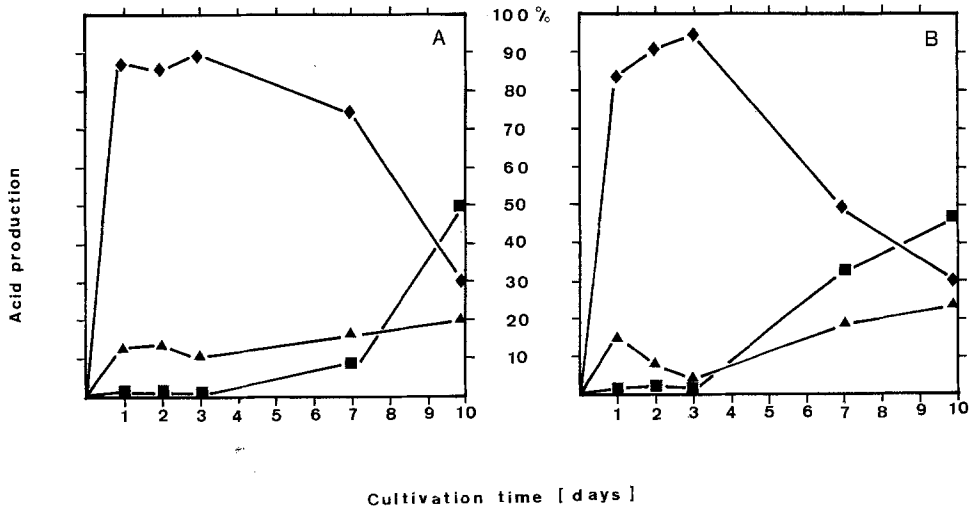

Fig. 4 A and B
Percentage distribution of acids occurring in the culture filtrate of the mutant and the wild type of *Neurospora crassa*. Mutant (**A**), wild type (**B**). Succinate (◆), malate (▲) and citrate (■)

Table 5. Distribution of intracellular and extracellular and extracellular acids during submerged growth of *Neurospora crassa*[a]

Strain	Cultivation time (days)	Acid	Acid content of mycelia (µg/mg dry wt.)	Acid content of culture filtrate (µg/mg dry wt.)
Mutant	1	succinate	0.18	6.16
	1	malate	1.60	4.03
	1	citrate	0.48	–
	2	succinate	0.37	4.32
	2	malate	2.13	3.30
	2	citrate	1.00	–
	3	succinate	11.63	1.80
	3	malate	12.42	0.17
	3	citrate	10.23	–
Wild type	3	succinate	8.47	0.027
	3	malate	12.10	0.004
	3	citrate	12.87	0.022

[a] Culture flasks containing 100 ml minimal medium without additional iron were inoculated with 10^8 conidiospores and incubated at 27° C on a rotary shaker. At the time indicated the acid content of the mycelia and of the culture filtrate was determined by gas chromatography

Table 6. Effect of iron on acid production in the sideramine-free mutant and the wild type of *Neurospora crassa*[a]

Strain	Acid	Mycelia (µg/mg dry wt.)	Culture filtrate (µg/mg dry wt.)
Mutant	succinate	0.005	2.66
	malate	18.76	–
	citrate	23.96	–
Wild type	succinate	0.018	1.22
	malate	6.33	–
	citrate	8.69	–

[a] Iron was added as $FeCl_3$ (20 µM) together with the inoculum. Acids were determined by gas chromatography after 3 days of submerged cultivation at 27° C

As shown in Table 6, the intracellular acid content of the wild type was reduced to 50–70%, whereas the acid content of the mutant was even increased. Another interesting finding is the complete lack of hydroxy acids in the culture filtrate and a general reduction of succinate in the mycelia when iron was present.

Contribution of Acids to Iron Uptake

The contribution of acids during iron uptake was studied by incubating young mycelia of the sideramine-free mutant in the presence of 1 mM succinate, malate or citrate and 10 µM $FeCl_3$ (Fig. 5). Within a period of 90 min, iron was taken up at approximately 0.5 nmol mg^{-1}. Iron uptake in the presence of succinate was relatively high during the first 30 min, whereas malate and citrate showed a slower but continuous increase. These results must be interpreted in the light of the chelating properties of the acids. As succinate is a relatively ineffective iron chelator, the amount of iron taken up during the first incubation period can be regarded as iron hydroxide deposition upon the cell surface. In contrast iron uptake mediated by malate and citrate represents a pure iron chelate uptake, which continually increased with time. Small amounts of all acids, particularly malate, are excreted during the incubation experiments and may influence the obtained results.

Studying the concentration dependent iron uptake in the presence of acids (Fig. 6), a rapid iron uptake could be observed in the presence of succinate, which is

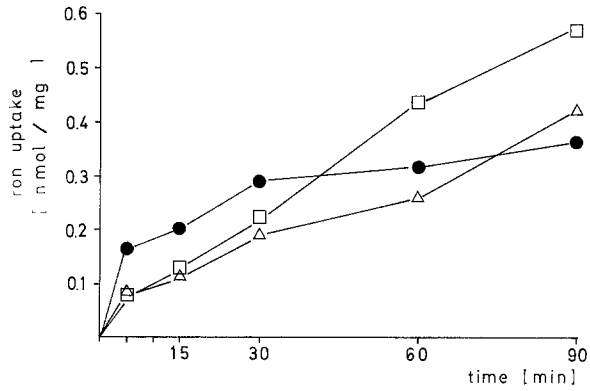

Fig. 5. Uptake of iron by sideramine-free mycelia of *Neurospora crassa* (arg-5 ota aga) in the presence of succinate (●), malate (△), and citrate (□). Mycelia grown for 24 h in submerged culture were incubated at 27° C in the presence of 10 µM $^{55}FeCl_3$ and 1 mM acid. After the times indicated the mycelia were filtered off, washed three times with ice cold 0.9 NaCl solution and counted for radio activity

obviously caused by iron hydroxide deposition on the cells. In the presence of chelating hydroxy acids, such as malate and citrate, considerably lower iron uptake values are observed not exceeding 0.02 nmol mg^{-1}. Whereas citrate iron uptake was saturable at 50 µM, malate iron uptake revealed a more complex uptake curve with a steep increase at higher concentrations indicating iron hydroxide deposition when the ratio of iron to hydroxy acids becomes unfavourable. The kinetic data suggest that malate and citrate reveal different complex stabilities but similar iron transport properties.

Discussion

A generally accepted mode of iron supply among microorganisms is iron transport by iron chelates such as siderochromes. Siderochromes are synthesized and

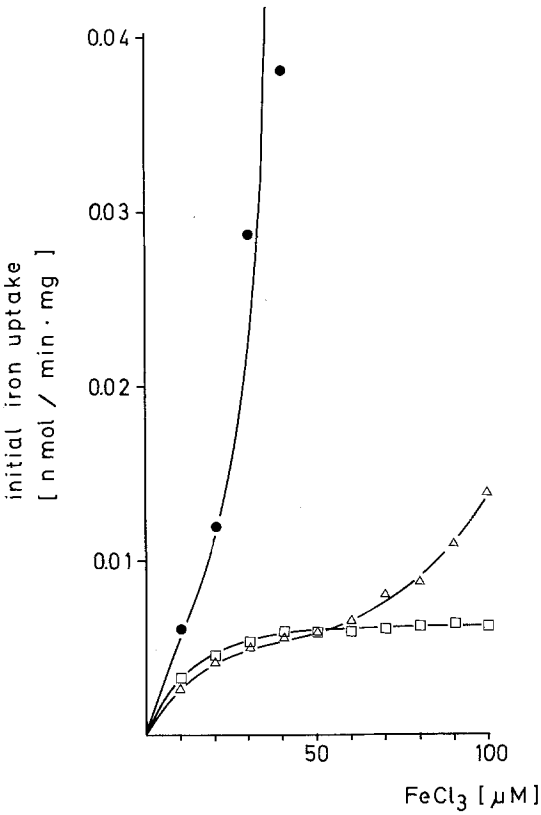

Fig. 6. Concentration dependent uptake of iron by sideramine-free mycelia of *Neurospora crassa* (arg-5 ota aga) in the presence of succinate (●), malate (△) and citrate (□). Mycelia grown for 24 h in submerged culture were incubated at 27° C with increasing concentrations of $^{55}FeCl_3$ in the presence of 1 mM acid. After 10 min the mycelia were filtered off, washed and counted for radioactivity

excreted as desferri-siderochromes when the iron content of the medium decreases. Fungi are known to produce a variety of sideramines which have been shown to be relative specific for the producing strain (Winkelmann, 1974; Wiebe and Winkelmann, 1975). Whether sideramines are also necessary for growth in iron containing media is uncertain. Sideramines have been isolated from mycelia grown in iron containing media, suggesting that they are probably acting in mycelia grown in iron sufficient media.

A variety of fungi, however, are unable to synthesize sideramines. The way in which these organisms take up iron is unresolved. The present investigation describes some features of iron uptake in a sideramine-free mutant of *Neurospora*, which can be regarded as a general model of iron uptake in sideramine-free filamentous fungi.

A basic element of the overall uptake process is the interaction between surface iron hydroxide and excreted hydroxy acids. Iron hydroxide polymer adsorption to the cell surface of chelator-free organisms occurs between a physiological pH range of 4–8. The molecular weight and the size of soluble iron hydroxide polymers may be different depending on the prevailing pH and the presence of acid anions (Spiro et al., 1966). The cell surface seems to promote iron deposition. Iron hydroxide polymer deposition upon the cell surface is common among many bacteria living in fresh water (van Veen et al., 1978). Ensheathed bacteria of the group *Sphaerotilus* have been reported to deposit large amounts of iron hydroxide when incubated in the presence of $FeCl_3$ (Rogers and Anderson, 1976a,b).

It may be assumed that iron deposition upon fungal cells depends on the presence of certain components of the cell wall. Proteins and polysaccharides are possible candidates for adsorption. Iron hydroxide polymer adsorption to the cell surface is, however, not restricted to conditions of excess iron. Even during limited iron supply, such as in minimal medium without additional iron, residual iron may concentrate at the cell surface as iron hydroxide.

The property to concentrate iron at the cell surface allows subsequent utilization after solubilizing by hydroxy acids. The extensive floccoid, wad like structure of the cell surface material enlarges the adsorption area and prevents excape by reducing the solute flow. Formation of mycelial pads additionally supports a subsequent utilization by maintaining high local concentrations of solubilizing acids.

The present results give evidence that both citrate and malate contribute to iron hydroxide solubilization. Their relatively late and sequential occurrence suggests that iron stores within the conidiospores support the early growth period. Only after the cell metabolism has generated excess hydroxy acids, can iron from the cell surface be utilized.

Succinate is a relatively poor iron chelator and therefore does not interfere with iron hydroxide deposition on the cell surface. Malate and citrate completely prevent iron hydroxide deposition. But the ratio of iron to hydroxy acids is of most importance. As long as hydroxy acids are present in lower amounts than iron, iron hydroxide remains attached to the cell surface. There is evidence that hydroxy acid anions integrate into existing iron hydroxide units leading to the formation of smaller molecules (Spiro et al., 1976b). One can imagine that the whole iron uptake process proceeds in a similar way, starting with pure iron hydroxide molecules and ending with 1 : 1 complexes of hydroxy acid iron chelates, which may ultimately be released and taken up into the interior of the cell.

The studies of acid production during submerged growth of *Neurospora crassa* revealed a consecutive appearance of acids in the culture medium in the order succinate, malate and citrate. Succinate and malate accompany the early phase of acidification of the culture medium. Citrate, however, is excreted later,

when the culture filtrate becomes neutral again. The consecutive appearance of acids in the culture filtrate seems to be the result of different membrane permeabilities, as all acids are already present within the mycelia from the 1st day of cultivation. The biological significance of the order of appearance of acids in the culture medium is unresolved. It is generally assumed that acidification of the medium by organic acids supports iron uptake. Production and excretion of acids is initiated directly after germination irrespective of the presence of iron in the medium. It is suggestive that during the 1st days of growth a general overproduction of acids occurs to allow rapid biosynthesis of various cell constituents.

As shown in the present investigation, the addition of iron to the culture medium revealed a significant reduction of the amount of hydroxy acids in the culture filtrate. A simultaneous decrease of intracellular hydroxy acids was observed in the wild type, whereas the mutant continues to synthesize these acids. It is assumed that the decrease of extracellular hydroxy acids is caused by complexing with iron upon the cell surface. Release of hydroxy acids is only observed when the iron content of the cell surface is low.

The very late occurrence of citrate in the culture fluids during iron deficiency is surprising. Citrate was excreted when the pH of the medium again became alkaline, indicating that citrate is obviously not involved in iron transport during the first acidic phase of growth.

It is assumed that malate contributes to iron uptake during the early growth phase after germination, whereas citrate guarantees iron supply during the late alkaline phase of growth. The present model of iron utilization established for sideramine-free fungi seems to be an early evolutionary way to use iron successively from surface iron stores by gradually excreting hydroxy acids, without being injured by toxic concentrations of ionic iron.

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