1 Fungal Siderophores: Structure, Functions and Regulation

Aparajita Das[1], Ram Prasad[1], Abhishek Srivastava[2], Pham Huong Giang[3], Kamya Bhatnagar[1] and Ajit Varma[1]

1.1 Introduction

Fungi are eukaryotic, nonphotosynthetic organisms, and most are multicellular heterotrophs. Classically, the following groups of fungi have been considered:

1) **Slime molds** have a feeding phase of the life cycle (the trophic phase) that are motile and lack cell wall. Foods particles are ingested.
2) **Aquatic fungi** have cell wall and absorb nutrients rather than ingest them. The sex cells and spores of aquatic fungi are motile (zoospores).
3) **Terrestrial fungi** have cell wall and absorb nutrients rather than ingest them. The sex cells and spores are not motile (zoospores). Three major groups of fungi recognized are Zygomycetes (e.g., black bread mold, animal dung fungi), Ascomycetes (e.g., cup fungi, truffles) and Basidiomycetes (e.g., mushrooms or toadstools, puff balls, rusts, smuts). For details see Table 1.1; c.f. Giri et al. (2005)

1.1.1 Significance of Terrestrial Fungi

Fungi play important roles in the environment. Most fungi are either saprophytes or decomposers that break down and feed on decaying organic mate-

[1]Amity Institute of Herbal and Microbial Studies, Amity University, Sector 125, Noida, India, email: ajitvarma@aihmr.amity.edu

[2]International University of Bremen, School of Engineering and Sciences, Campus Ring 6, D-28759, Bremen, Germany

[3]International Centre of Genetic Engineering and Biotechnology (UNO, Triesta, Italy), New Delhi, India

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Table 1.1 Major groups of soil fungi

Group and representative members	Distinguishing characteristics	Asexual reproduction	Sexual reproduction
Zygomycetes *Rhizopus stolonifer* (black bread mold)	Multicellular, coenocytic mycelia	Asexual spores develop in sporangia on the tips of aerial hyphae	Sexual spores known as zygospores can remain dominant in adverse environment
Ascomycetes *Neurospora, Saccharomyces cerevisiae* (baker's yeast)	Unicellular and multicellular with septate hyphae	Common by budding, conidiophores	Involves the formation of an ascus on specialized hyphae
Basidiomycetes *Agaricus campestris* (meadow mushroom), *Cryptococcus neoformans*	Multicellular, uninucleated mycelia, group includes mushrooms, smuts, rusts that affect the food supply	Commonly absent	Produce basidiospores that are born on club shaped structures at the tips of hyphae
Deuteromycetes (Fungi Imperfecti) *Penicillium, Aspergillus*	A number of these are human pathogens	Budding	Absent or unknown

rial or dead organisms. Fungi obtain nutrients to absorb by secreting digestive enzymes onto the food source. The enzymes break down, or digest, the food. The breakdown, or digestion, of organic material can also be called decomposition. Any organism that causes decomposition can be called a decomposer. They are vital links in food webs, primarily as decomposers and pathogens of both plants and animals. They are excellent scavengers, breaking down dead plant and animal tissues, recycling elements back into food webs. Some fungi can establish mutualistic relationships with other organisms in nature. For example, some fungi form mycorrhizae with the roots of plants. The fungus supplies water and minerals and the plant provides carbohydrates and other organic compounds. Mycorrhizal fungi protect the root of plant against attack by parasitic fungi and nematodes. Other fungi grow with algae and cyanobacteria forming lichens. Lichens play an important role in soil formation.

1.1.2 Mycorrhiza

These are fungi which exhibit mutualistic relationships with plant roots; 90% of trees probably have them. Their presence significantly increases the roots' effective absorptive surface area and provides for a direct link between the process of decomposition (which yields raw materials) and the absorption of these materials by plants. Mycorrhizae are beneficial both in nature and agriculture. Plants colonized with them tend to grow better than those without them. Groups of mycorrhizae are given in Fig. 1.1.

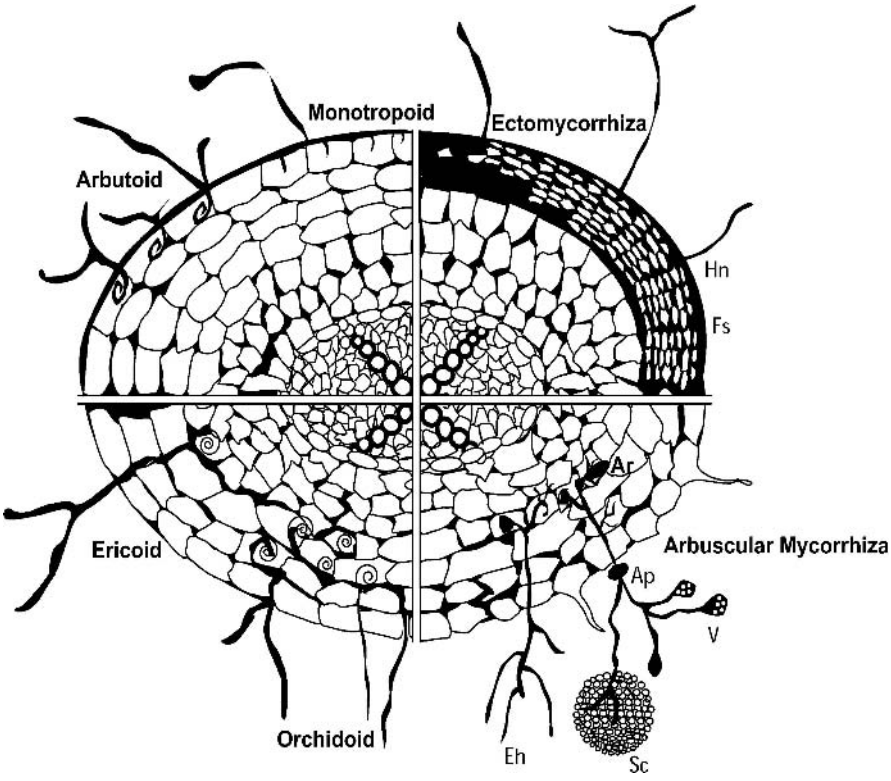


Fig. 1.1. Types of mycorrhizal fungi: Fs-fungal sheath; Eh-extramatrical hyphae; Hn-hartig's; V-vesicle; Sc-sporocarp; Ap-appresorium; Ar-arbuscule; Sc-sporocarp; Ap-appresorium

1.1.2.1 Types of Mycorrhizal Fungi

So far, seven types of mycorrhizae have come into general use over the years on the basis of morphology and anatomy but also of either host plant taxonomy or fungal taxonomy (Srivastava et al. 1996; Smith and Read 1997). These are: ectomycorrhiza, endomycorrhiza or arbuscular mycorrhiza, ericoid mycorrhiza, arbutoid mycorrhiza, monotropoid mycorrhiza, ect-endomycorrhiza and orchidaceous mycorrhiza.

1.1.2.1.1 Ectomycorrhiza (ECM)

Hyphae surround but do not penetrate the root cells. Ectomycorrhizae are commonly found in trees growing in temperate regions. The plant symbionts include

both Gymnosperms and Angiosperms. Some have also been found in the tropics. The willow family (Salicaceae), birch family (Betulaceae), beech family (Fagaceae) and pine family (Pinaceae) have ectomycorrhizal associations. It possibly makes the trees more resistant to cold, dry conditions.

The hyphae grow in between the cortical and epidermal cells of the root forming a network called "Hartig's net". A mantle of hyphae covers the root surface, and mycelium extends from the mantle into the soil. It provides a large surface area for the interchange of nutrients between host and fungi. Most ectomycorrhizal fungi are basidiomycetes, but ascomycetes are also involved.

1.1.2.1.2
Arbuscular Mycorrhiza (AM)

The term refers to the presence of intracellular structures – vesicles and arbusculs – that form in the root during various phases of development. These mycorrhizae are the most commonly recorded group since they occur on a vast taxonomic range of plants, both herbaceous and woody species. The plant symbiont ranges from Bryophytes to Angiosperms. Aseptate hyphae enter the root cortical cells and form characteristic vesicles and arbuscules. The plasmalemma of the host cell invaginates and encloses the arbuscules. Arbuscular Mycorrhizal (AM) fungi belong to nine genera: *Gigaspora*, *Scutellospora*, *Glomus*, *Acaulospora*, *Entrophospora*, *Archaeospora*, *Gerdemannia*, *Paraglomus* and *Geosiphon*, the only known fungal endosymbiosis with cyanobacteria (Fig. 1.2).

1.1.2.1.3
Ericoid Mycorrhiza

In the Ericaceae, heather family, the ectomycorrhizal hyphae form a web surrounding the roots. The ericoid mycorrhizae are endomycorrhizae in the general sense, since the fungal symbiont penetrates and establishes into the cortical cells. Infection of each cortical cell takes place from the outer cortical wall; lateral spread from cell to cell does not occur. Infected cells appear to be fully packed with fungal hyphae. In the ericoid mycorrhizae, the host cell dies as the association disintegrates, thereby restricting the functional life (i.e. nutrient absorption) of these epidermal cells to the period prior to breakdown of the infected cell.

1.1.2.1.4
Arbutoid Mycorrhiza

The arbutoid mycorrhizae have characteristics which are found in both ECM and other endomycorrhizae. Intracellular penetration of cortical cells and formation of a sheath can occur, and a "Hartig's net" is present. A feature distinguishing

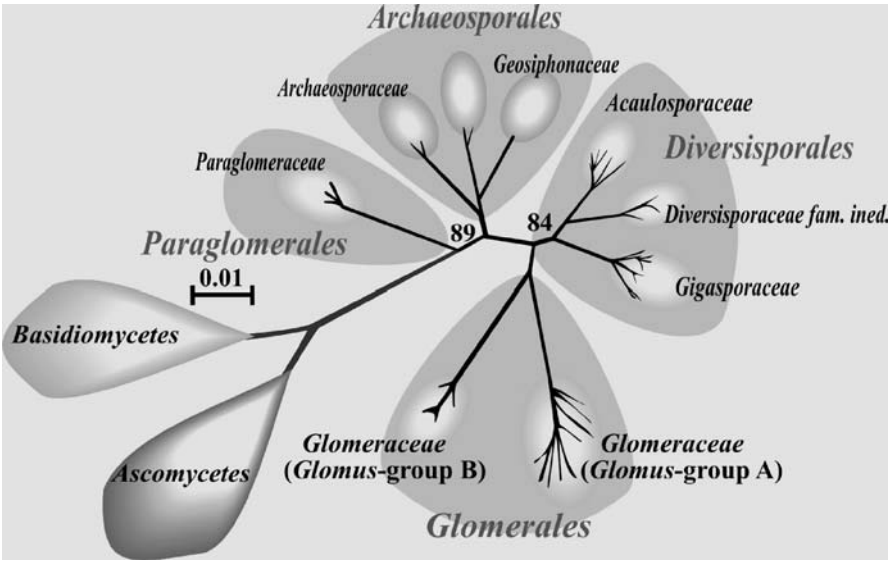


Fig. 1.2. Diagramatic representation of molecular taxonomic position of symbiotic fungi

them from ericoid mycorrhizae is the presence of dolipore septum in internal hyphae. Fungal associate in arbutoid mycorrhizae belong to basidiomycetes.

1.1.2.1.5
Monotropoid Mycorrhiza

This group of mycorrhiza is associated with the achlorophyllous plants in the family Monotropaceae. These mycorrhizae are very similar to the ECM and form a distinct sheath and "Hartig's net". However, they exhibit a distinctive type of intracellular penetration in cortical cells that is unlike other endomycorrhizal types. The fungus forms a peg into the cell wall.

1.1.2.1.6
Ect-endomycorrhiza

They are formed with the members of the Pinaceae. These Mycorrhizae form a "Hartig's net" in the cortex of the root but develop little or no sheath. Intracellular penetration of cortical cells takes place, and thus they are similar to the arbutoid type. Ectendomycorrhizae in Pinaceae seem to be limited to forest nurseries and are formed by a group of fungi called E-strain. These fungi are most likely to be the imperfect stage of ascomycetes; they may cause ect-endomycorrhizae in some tree species and ECM in other tree species.

1.1.2.1.7
Orchidaceous Mycorrhiza

The fungal association is of the endomycorrhizal type, where the fungus penetrates the cell wall and invaginates the plasmalemma and forms hyphal coil within the cell. Once the plant is invaded, spread of the fungus may occur from cell to cell internally. The internal hyphae eventually collapse and or digested by the host cell. Since the symbiosis forms an external network of hyphae, it would seem probable that the fungal hyphae function in nutrient uptake as with other mycorrhizae and that the coarse root system of orchids would be supplemented by the increased absorbing surface area of the hyphae (Smith and Read 1997). A number of basidiomycetes genera have been shown to be involved in the symbiosis, although many reports on isolation of the symbiotic fungus from the roots of orchids have placed the symbionts in the form genus *Rhizoctonia* when the perfect stage was not known or the isolate was not induced to fruit in culture. Orchid seed germinate only in the presence of suitable fungus.

1.1.3
Role of Fungi in Industry

1. Many fungi are valuable food source for humans. Yeast, such as *Saccharomyces*, is an important nutritional supplement because it contains vitamins, minerals, and other nutrients (Table 1.2).
2. Mushrooms are an important food. *Agaricus* (White Button), shiitake, and portabella mushrooms are often found in grocery stores.
3. In other places in the world, people prize the taste of Truffles and Morels, which are Ascocarps found near the roots of trees.
4. Many fungi are plant pathogens that attack grain and fruit. Wheat rust is a Basidiomycete that attacks wheat grains. Other fungi can attack food crops such as corn, beans, onions, squashes, and tomatoes.
5. Fungi are used to produce chemical compounds that are important to the food-processing industry such as citric and gluconic acid. Citric acid is used

Table 1.2. Food products and fungi

Type of Food	Fungus
Cheese: blue, Gorgonzola, Limburger, Roquefort, Camembert	*Penicillium* species
Beer, wine	*Saccharomyces carlsberbensis, Saccharomyces cerevisiae*
Soy products: miso (Japanese), soy sauce, tofu (Japanese)	*Aspergillus oryzae, Rhizopus* species, *Mucor* species
Nutritional yeast	*Saccharomyces species*
Breads	*Saccharomyces cerevisiae*

in soft drinks and candies. Gluconic acid is fed to chickens to enhance the hardness of eggshells.

6. *Ashbya gossypii* is a producer of Vitamin B2, an important nutritional supplement.

1.1.4 A Novel Endophytic Fungus *Piriformospora indica*

Symbiotic but cultivable fungus, *Piriformospora indica*, which is potential candidate to serve as biofertilizer, bioprotector, bioregulator, bioherbicide/weedicide, combats environmental stresses (chemical, thermal and physical) and is an excellent source for the hardening of the tissue culture raised crops/plants. *P. indica* tremendously improves the growth, overall biomass production and the synthesis of secondary metabolites (active ingredients) of diverse medicinal and plants of economic importance. This fungus also protects soil fertility and plant health as well (Fig. 1.3).

The properties of the fungus, *Piriformospora indica*, have been patented (Varma A and Franken P, 1997, European Patent Office, Muenchen, Germany. Patent No. 97121440.8-2105, Nov. 1998). The culture has been deposited at Braunsweich, Germany (DMS No.11827) and National Bureau of Agriculturally Important Microorganisms (NBAIM), Pusa, New Delhi.

Recently this fungus was found to enhance the anti-oxidants in *Baccopa monnieri* and Bacosides (secondary metabolites of *Bacopa monnieri*). A patent entitled '*Piriformospora indica* – a symbiotic fungus promotes biomass of *Bacopa monnieri*, enhances the antioxidant activity and bacoside concentration has

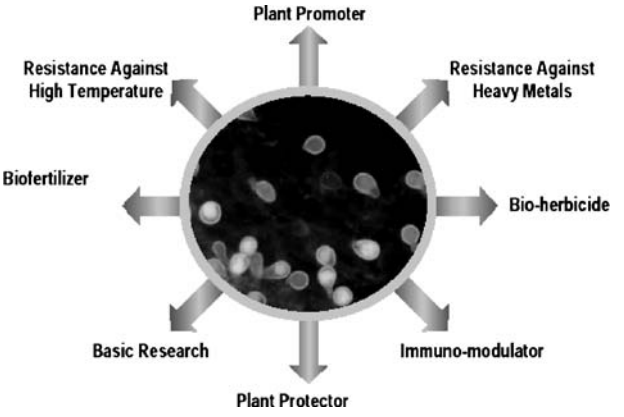


Fig. 1.3. Properties of *Piriformaspora indica*

been filed to seek the protection of this discovery from European and North American Countries. *Bacopa monnieri* is a plant of great economic importance and is known to be used as nerve tonic, adaptogen, antioxidant, antimicrobial, antifungal, sedative, cardio tonic, anticonvulsent, antidepressent and is useful for cancer patients experiencing pain. The fungus *P. indica* is documented to promote plant growth and protects the host against root pathogens and insects. Fungus root colonization promoted the plant growth and enhanced antioxidant activity and the active ingredient bacoside by several times. Symbiotic fungus, *P. indica*, enhances the plant biomass, antioxidant activity and bacoside concentration in *Bacopa monnieri.*

1.2 Siderophores

Bacteria and fungi, in response to low iron availability in the environment, synthesize microbial iron chelates called siderophores.

1.2.1 Bacterial Siderophores

There are a few reports of finding in vivo expression of siderophores by bacterial zoopathogens. For example, siderophores have been detected in sputum samples from the lungs of cystic fibrosis patients with infections due to *Pseudomonas aeruginosa* (Haas B et al. 1991) and enterochelin has been found in peritoneal washings of guinea pigs infected with *Escherichia coli* (Griffiths and Humphreys 1980). Immunoglobulins to siderophores have been detected in some instances. Such a host response is indicative of in vivo synthesis of iron chelators by some pathogens (Reissbrodt et al. 1997).

When we look more deeply into the large group of marine *Vibrios*, we notice that a broad range of structurally different siderophores is produced (Drechsel and Winkelmann 1997). Thus, catecholate siderophores have been detected in *Vibrio cholerae*, *Vibrio vulnificus* and *Vibrio fluvialis*. A mixed-type catecholate-thiazoline-hydroxamate siderophore, named anguibactin, has been isolated from *Vibrio anguillarun* and the citrate-based hydroxamate, aerobactin, has been described in certain marine vibrios. Also the occurrence of ferrioxamine G has been reported in vibrio species and we have recently identified the structurally related dihydroxamate, bisucaberin, in the fish pathogen *Vibrio salmonicida* (Winkelmann et al. 2002). This broad range of structurally different siderophores in the family Vibrionaceae may reflect the existence of a large pool of siderophore biosynthetic genes and may also indicate that the different

genera of the family are more heterogeneous than previously assumed; vibrios are widespread in marine water, but this does not necessarily mean that they are really free-living bacteria. We may assume that most vibrios are somehow associated with particles in marine coastal water. Siderophores have also been isolated from several other marine bacteria, like *Alteromonas*, *Halomonas* and *Marinobacter*, indicating that siderophore production in the marine environment is widespread (Martinez et al. 2000).

1.2.1.1 Ecological Aspects

If we consider siderophore production within different microbial genera, we realize that catecholate siderophores predominate in certain Gram-negative genera, like the Enterobacteria and the genus *Vibrio*, but also in the nitrogen-fixing *Azotobacteria* and the plant-associated Agrobacteria. The reasons that these bacteria use catecholates may be manifold. However, lipophilicity, complex stability, high environmental pH and a weak nitrogen metabolism might favour catecholates. The Gram-positive streptomycetes produce hydroxamate-type ferrioxamines and the ascomycetous and basidiomycetous fungi synthesize ester- and peptide-containing hydroxamate siderophores that are acid-stable and well suited for environmental iron solubilization. Both the *Streptomycetes* and fungi show a versatile nitrogen metabolism with active N-oxygenases.

1.2.2 Fungal Siderophores

Two major responses to iron stress in fungi are a high-affinity ferric iron reductase and siderophore synthesis. Uptake of siderophores is a diverse process, which varies among the different classes of compounds. Three common classes – phenolates, hydroxamates, and polycarboxylates – are observed. Some phytopathogenic fungi produce unique compounds that function as phytotoxins but also chelate iron.

1.2.2.1 Historical Development of Fungal Siderophores

Research in this field began about five decades ago, and interest in it has accrued with the realization that most aerobic and facultative anaerobic microorganisms synthesize at least one siderophore. For details see Table 1.3.

Table 1.3. Development of siderophore research

Title	Authors	References	Year
Hydroxamate recognition during iron transport from hydroxamate-iron chelates	Haydon AH, Davis WB, Arceneaux JEL, Byers BR	J Bacteriol 115:912–918	1973
Siderophores in microbially processed cheese	Ong SA, Neilands JB	J Agric Food Chem 27:990–995	1979
The structure of the fungal siderophore, isotriornicin	Frederick CB, Bentley MD, Shive W	Biochem Biophys Res Commun 105:133–138	1982
Hydroxamate siderophore production by opportunistic and systemic fungal pathogens	Holzberg M, Artis WM	Infect Immun 40:1134–1139	1983
sid1, a gene initiating siderophore biosynthesis in *Ustilago maydis*: Molecular characterization, regulation by iron and role in phytopathogenicity	Mei B, Budde AD, Leong SA	Proc Natl Acad Sci USA 90:903–907	1993
urbs1, a gene regulating siderophore biosynthesis in *Ustilago maydis*, encodes a protein similar to the erythroid transcription factor GATA-1	Voisard C, Wang J, McEvoy JL, Xu P, Leong SA	Mol Cell Biol 13:7091–7100	1993
The role of ligand exchange in the uptake of iron from microbial siderophores by gramineous plants	Yehuda Z, Shenker M, Romheld V, Marschner H, Hadar Y, Chen Y	Plant Physiol 112:1273–1280	1996
Ferric rhizoferrin uptake into *Morganella morganii:* characterization of genes involved in the uptake of a polyhydroxycarboxylate siderophore	Kuhn S, Braun V, Koster W	J Bacteriol 178:496–504	1996
Double mutagenesis of a positive charge cluster in the ligand-binding site of the ferric enterobactin receptor, FepA	Newton SM, Allen JS, Cao Z, Qi Z, Jiang X, Sprencel C, Igo JD, Foster SB, Payne MA, Klebba PE	Proc Natl Acad Sci USA 94:4560–4565	1997
The C-terminal finger domain of Urbs1 is required for iron-mediated regulation of siderophore biosynthesis in *Ustilago maydis*	An Z, Zhao Q, McEvoy J, Yuan W, Markley J, Leon SA	Proc Natl Acad Sci USA 94:5882–5887	1997
The distal GATA sequences of the *sid1* promoter of *Ustilago maydis* mediate iron repression of siderophore production and interact directly with Urbs1, a GATA family transcription factor	An Z, Mei B, Yuan WM, Leong SA	EMBO J 16:1742–1750	1997
Siderophore-mediated iron transport: crystal structure of FhuA with bound lipopolysaccharide	Ferguson AD, Hofmann E, Coulton JW, Diederichs K, Welte W	Science 282:2215–2220	1998
Iron uptake in *Ustilago maydis*: tracking the iron path	Ardon O, Nudelman R, Caris C, Libman J, Shanzer A, Chen Y, Hadar Y	J Bacteriol 180:2021–2026	1998

Table 1.3. *(continued)* Development of siderophore research

Title	Authors	References	Year
Kinetics of iron complexing and metal exchange in solutions by rhizoferrin, a fungal siderophore	Shenker M, Hadar Y, Chen Y	Soil Sci Soc Am J 63:1681–1687	1999
Identification of a fungal triacetylfusarinine C siderophore transport gene (TAF1) in Saccharomyces cerevisiae as a member of the major facilitator superfamily	Heymann P, Ernst JF, Winkelmann G	BioMetals 12:301–306	1999
The *Aspergillus nidulans* GATA factor SREA is involved in regulation of siderophore biosynthesis and control of iron uptake	Haas H, Zadra I, Stoffler G, Angermayr K	J Biol Chem 274:46134619	1999
Siderophore-iron uptake in *Sacharomyces cerevisiae*. Identification of ferrichrome and fusarinine transporters	Yun CW, Tiedeman JS, Moore RE, Philpott CC	J Biol Chem 275:16354–16359	2000
Identification and substrate specificity of a ferrichrome-type siderophore transporter (Arn1p) in *Saccharomyces cerevisiae*	Heymann P, Ernst JF, Winkelmann G	FEMS Microbiol Lett 186:221–227	2000
A gene of the major facilitator superfamily encodes a transporter for enterobactin (Enb1p) in *Saccharomyces cerevisiae*	Heymann P, Ernst JF, Winkelmann G	Biometals 13:65–72	2000
Hydroxamate siderophore synthesis by *Phialocephala fortinii*, a typical dark septate fungal root endophyte	Bartholdy BA, Berreck M, Haselwandter K	Biometals 14:33–42	2001
SREA is involved in regulation of siderophore biosynthesis, utilization and uptake in *Aspergillus nidulans*	Oberegger H, Schoeser M, Zadra I, Abt B, Haas H	Mol Microbiol 41:1077–1089	2001
Siderophore uptake and use by the yeast *Saccharomyces cerevisiae*	Lesuisse E, Blaiseau PL, Dancis A, Camadro JM	Microbiology 147:289–298	2001
The role of the FRE family of plasma membrane reductases in the uptake of siderophore-iron in *Saccharomyces cerevisiae*	Yun CW, Bauler M, Moore RE, Klebba PE, Philpott CC	J Biol Chem 276:10218–10223	2001
Ferricrocin – an ectomycorrhizal siderophore of *Cenococcum geophilum*	**K. Haselwandter, G. Winkelmann**	BioMetals 15:73–77	2002
Identification of members of the *Aspergillus nidulans* SREA regulon: genes involved in siderophore biosynthesis and utilization	Oberegger H, Zadra I, Schoeser M, Abt B, Parson W, Haas H	Biochem Soc Trans 30:781–783	2002
Siderophore uptake by Candida albicans: effect of serum treatment and comparison with *Saccharomyces cerevisiae*	Lesuisse E, Knight SA, Camadro JM, Dancis A	Yeast 19:329–340	2002
Molecular genetics of fungal siderophore biosynthesis and uptake: the role of siderophores in iron uptake and storage	Haas H	Appl Microbiol Biotechnol 62:316–330	2003
The siderophore system is essential for viability of *Aspergillus nidulans*: functional analysis of two genes encoding l-ornithine-*N*-5-monooxygenase (sidA) and a non-ribosomal peptide synthetase (sidC)	Eisendle M, Oberegger H, Zadra I, Haas H	Mol Microbiol 49:359–375	2003

Table 1.3. *(continued)* Development of siderophore research

Title	Authors	References	Year
The siderophore system is essential for viability of *Aspergillus nidulans*: functional analysis of two genes encoding l-ornithine-*N*-5-mono-oxygenase (sidA) and a non-ribosomal peptide synthetase (sidC)	Eisendle M, Oberegger H, Zadra I, Haas H	Mol Microbiol 49:359–375	2003
Characterization of the *Aspergillus nidulans* transporters for the siderophores enterobactin and triacetylfusarinine C	Haas H, Schoeser M, Lesuisse E, Ernst JF, Parson W, Abt B, Winkelmann G, Oberegger H	Biochem J 371:505–513	2003
4'-Phosphopantetheinyl transferase-encoding npgA is essential for siderophore biosynthesis in *Aspergillus nidulans*	Oberegger H, Eisendle M, Schrettl M, Graessle S, Haas H	Curr Genet 44:211–215	2003
Siderophore biosynthesis but not reductive iron assimilation is essential for *Aspergillus fumigatus* virulence	Schrettl M, Bignell E, Kragl C, Joechl C, Rogers T, Arst HN Jr, Haynes K, Haas H	JEM 200:1213–1219	**2004**
Biosynthesis and uptake of siderophores is controlled by the PacC-mediated ambient-pH regulatory system in *Aspergillus nidulans*	Eisendle M, Oberegger H, Buttinger R, Illmer P, Haas H	Eukaryot Cell 3:561–563	2004
A putative high affinity hexose transporter, hxtA, of *Aspergillus nidulans* is induced in vegetative hyphae upon starvation and in ascogenous hyphae during cleistothecium formation	Wei H, Vienken K, Weber R, Bunting S, Requena N, Fischer R	Fungal Genet Biol 41:148–156	2004

1.2.2.2 Ecophysiological Functions of Iron

1.2.2.2.1 Importance of Iron

Iron is required by most living systems. To ensure a supply of the essential metal, pathogenic fungi use a great variety of means of acquisition, avenues of uptake, and methods of storage. Solubilization of insoluble iron polymers is the first step in iron assimilation.

Free iron is a devastating metal. In the Fenton reaction, Fe(II) reacts with H_2O_2 (a normal metabolite in aerobic organisms) to form a hydroxy radical, which binds to critical molecules found in living cells: sugars, amino acids, phospholipids, DNA bases, and organic acids (Byers and Arceneaux 1998; Fleischmann and Lehrer 1985). Microorganisms growing under aerobic conditions need iron for a variety of functions including reduction of oxygen for synthesis of ATP, reduction of ribotide precursors of DNA, for formation of heme, and for other essential

purposes. A level of at least 1 μM iron is needed for optimum growth. Should the supply of Fe(II) dwindle, more is generated by the reduction of Fe(III) by superoxide anions (Byers and Arceneaux 1998; Fleischmann and Lehrer 1985). These environmental restrictions and biological imperatives have required that microorganisms form specific molecules that can compete effectively with hydroxyl ion for the ferric state of iron, a nutrient that is abundant but essentially unavailable.

The two methods most commonly used by microorganisms for solubilization of iron are reduction and chelation. The various means by which fungi acquire iron are listed in Table 1.4. Reduction of ferric iron to ferrous iron by enzymatic

Table 1.4. Mechanisms of iron acquisition by pathogenic fungi (Table adapted from Howard DH (1999) Acquisition, transport, and storage of iron by pathogenic fungi. Clin Microbiol Rev 12:394–404)

Mechanism	Examples	Reference(s)
Reduction of ferric to ferrous iron	*Candida albicans*	Morrissey et al. (1996)
	Cryptococcus neoformans	Jacobson et al. (1998)
	Geotrichum candidum	Mor et al. (1988)
	Saccharomyces cerevisiae http://cmr.asm.org/cgi/content/full/12/3/394/T1 - TF1-b[b]	Lesuisse and Labbe (1994)
Siderophore acquisition of ferric iron		
Hydroxamates (families)		
Rhodotorulic acid	*Epicoccum purpurescens*[a]	Frederick et al. (1981)
	Histoplasma capsulatum[a]	Burt (1982)
	Stemphilium botryosum	Manulis et al. (1987)
Coprogens	*Curvularia lunata*	Van der Helm and Winkelmann (1994)
	Epicoccum purpurescens	Frederick et al. (1981)
	Fusarium dimerum	Van der Helm and Winkelmann (1994)
	Histoplasma capsulatum	Burt (1982)
	Neurospora crassa	Van der Helm and Winkelmann (1994)
	Stemphilium botryosum	Manulis et al. (1987)
Ferrichromes	*Aspergillus* spp.	Charlang et al. (1981)
	Epicoccum purpurescens	Frederick et al. (1981)
	Microsporum spp.	Bentley et al. (1986)
	Neurospora crassa	Van der Helm and Winkelmann (1994)
	Trichophyton spp.	Mor et al. (1992)
	Ustilago maydis	Ardon et al. (1997, 1998)

[a] Examples given are those discussed in the review; both zoopathogens and phytopathogens are included. In some examples, general references are given in which additional reports about the listed fungus are cited

[b] Some strains are reported to be human pathogens (Kwon-Chung and Bennett 1992)

Table 1.4. *(continued)* Mechanisms of iron acquisition by pathogenic fungi

Mechanism	Examples	Reference(s)
Fusarinines	*Aspergillus* spp.	Van der Helm and Winkelmann (1994)
	Epicoccum purpurescens	Frederick et al. (1981)
	Fusarium spp.	Van der Helm and Winkelmann (1994)
	Histoplasma capsulatum	Burt (1982)
	Paecilomyces spp.	Van der Helm and Winkelmann (1994)
Unidentified in report referenced	*Absidia corymbifera*[c]	Holzberg and Artis (1983)
	*Candida albicans*http://cmr.asm.org/cgi/content/full/12/3/394/T1 - TF1-c[c]	Holzberg and Artis (1983); Ismail et al. (1985)
	Madurella mycetomatis http://cmr.asm.org/cgi/content/full/12/3/394/T1 - TF1-c[c]	Mezence and Boiron (1995)
	Pseudallescheria boydii	de Hoog et al. (1994)
	Rhizopus arrhizus http://cmr.asm.org/cgi/content/full/12/3/394/T1 - TF1-c[c]	Holzberg and Artis (1983)
	Rhizopus oryzae http://cmr.asm.org/cgi/content/full/12/3/394/T1 - TF1-c[c]	Holzberg and Artis (1983)
	Scedosporium prolificans http://cmr.asm.org/cgi/content/full/12/3/394/T1 - TF1-d[d]	de Hoog et al. (1994)
	Sporothrix schenckii http://cmr.asm.org/cgi/content/full/12/3/394/T1 - TF1-c[c]	Holzberg and Artis (1983)
Polycarboxylates (rhizoferrin)	Zygomycetes	Van der Helm and Winkelmann (1994)
Phenolates-catecholates (chemical structures not identified)	*Candida albicans* http://cmr.asm.org/cgi/content/full/12/3/394/T1 - TF1-e[e]	Ismail et al. (1985)
	Wood-rotting fungi[f]	
Miscellaneous iron resources		
Hemin	*Candida albicans*	Moors et al. (1992)
	Histoplasma capsulatum	Worsham and Goldman (1988)
β-Keto aldehydes (phytotoxins	*Stemphylium botryosum*	Barash et al. (1982)
Acidification and mobilization	*Neurospora crassa*	Winkelmann (1979)
	Saccharomyces cerevisiae	Lesuisse and Labbe (1994)

[c] Exact chemical structures not provided. Identified as hydroxamates by the Neilands method (Holzberg and Artis 1983)

[d] Assay based on color formation on CAS medium; no further work reported (de Hoog 1994)

[e] Exact chemical structures not provided; phenolates identified by the Arnow assay (Ismail et al. 1985)

[f] Exact chemical structures not given in report; siderophores detected by CAS assay (Fekete et al. 1989) and identified as phenolates by paper chromatography (Fekete et al. 1983)

or nonenzymatic means is a common mechanism among pathogenic yeasts. Under conditions of iron starvation, many fungi synthesize iron chelators known as siderophores. The word siderophore (from the Greek for "iron carriers") is defined as relatively low molecular weight, ferric ion specific chelating agents elaborated by bacteria and fungi growing under low iron stress with a very high affinity for iron. The role of these compounds is to scavenge iron from the environment and to make the mineral, which is almost always essential, available to the microbial cell.

It must be stressed that not all microbes require iron, and siderophores can be dispensed with in these rare cases. Some lactic acid bacteria are not stimulated to greater growth with iron, they have no heme enzymes, and the crucial iron-containing ribotide reductase (Ardon et al. 1998) has been replaced with an enzyme using adenosylcobalamin as the radical generator. Other microbes need iron but grow anaerobically on Fe(II). While nearly all fungi make siderophores, both budding and fission yeast appear to be exceptions (An et al. 1997a, b).

In *S. cerevisiae*, the action of an Fe(III) reductase is followed by that of an Fe(II) oxidase, which generates Fe(III) for uptake. The Fe(III) would tend to polymerize to an insoluble form were it not prevented from so doing (Byers and Arceneaux 1998). Therefore, some method of iron storage must be used to escape the toxic carnage that would result from the occurrence of free iron and to prevent repolymerization of the iron transported into the fungal cell.

Among the alternative means of assimilating iron is surface reduction to the more soluble ferrous species, lowering the pH, utilization of heme, or extraction of protein-complexed metal. Siderophores appear to be confined to microbes and are not products of the metabolism of plants or animals, which have their own pathways for uptake of iron.

Since free iron is toxic, it must be stored for further metabolic use. Polyphosphates, ferritins, and siderophores themselves have been described as storage molecules.

1.2.2.2.2 Ecological Aspects

Siderophores are also involved in mycorrhizal symbiosis, as found in all terrestrial plant communities. One of the major types of mycorrhizae is the ectomycorrhiza, typically formed by almost all tree species in temperate forests. So far, only a few siderophores have been described due to the difficulties with cultivating the mycorrhizal fungi in pure culture under iron limitation. However, siderophores from three ericoid mycorrhizal fungal species, *Hymenoscyphus ericae*, *Oidiodendron griseum* and *Rhodothamnus chamaecistus*, and an ectendomycorrhizal fungus *Wilcoxina* and an ectomycorrhizal fungus *Cenococcusm geophilum*, have been isolated which all produce hydroxamate siderophores of the ferrichrome and fusigen class (Haselwandter and Winkelmann 2002). The ectomycorrhizal fungus *Cenococcum geophilum* was grown in low-iron medium

and the excreted siderophores were extracted, purified and analyzed by HPLC. The principal hydroxamate siderophore produced in *Cenococcum geophilum* was identified as ferricrocin and is confirmed by analytical HPLC, FAB-mass spectrometry and 1H- and 13C-NMR spectra. Although the occurrence of ferricrocin has been shown earlier to occur in the ericoid mycorrhizal fungi, Haselwandter and Winkelmann were the first to report ferricrocin in a true ectomycorrhizal fungus which is taxonomically related to the ascomycetes (Haselwandter and Winkelmann 2002). The siderophore production of various isolates of *Phialocephala fortinii* (a typical dark septate fungal root endophyte) was assessed quantitatively as well as qualitatively in batch assays under pure culture conditions at different pH values and iron(III) concentrations (Bartholdy et al. 2001).

Zygomycetes produce solely aminocarboxylates based on citric acid and amines, which show optimal iron-binding activity at a weakly acidic pH (Drechsel et al. 1992). Although phenolate and catecholate pigments have been detected in higher fungi, defined structures of catecholate-based siderophores have never been reported in fungi. The concomitant production of organic acids by most fungi probably prevents the use of ferric catecholates that are unstable at acidic pH, while ferric hydroxamates are generally stable down to pH 2. Characterization of siderophore classes based on microbial groups, however, is not always possible. The phylogenetic distance between catechol- and hydroxamate-producing genera can be very small and occasionally both siderophore types have been observed in the same genus, and indeed in at least one case in a single siderophore (Carrano et al. 2001). There are reports that both catecholate- and hydroxamate-type siderophores have been isolated from the *Erwinia*/*Enterobacter*/*Hafnia* group, representing closely related genera of the family of Enterobacteriaceae (Deiss et al. 1998; Reissbrodt et al. 1990). Fluorescent pseudomonads and the related non-fluorescent Burkholderia group are well-known producers of linear peptide siderophores (Ongena et al. 2002). These groups of microbes might profit from the generally neutral environment of soil, where acid stability is of minor importance. The alternating d- and l-configuration of peptidic amino acids also makes these siderophores very resistant to microbial proteases.

1.2.2.3
Storage Molecules

The need for iron storage is universal. Soon after uptake, iron is found in the vacuoles of *S. cerevisiae*, where it is perhaps bound to polyphosphates (Lesuisse and Labbe 1994). The vacuoles serve as "major" storage compartments for iron in the yeast (Lesuisse and Labbe 1994). The reduced iron within the compartment is kept in the ferrous form and serves as the substrate for ferrochelatase, which is the enzyme involved in the insertion of iron into heme (Labbe-Bois and Camadro 1994). It is believed that intracellular movement of iron could be effected by intracellular citric and malic acids (Lesuisse and Labbe 1994).

Polyphosphate ferrous iron storage has also been revealed in the low-molecular-mass iron pool of *Escherichia coli* (Böhnke and Matzanke 1995).

Iron-rich proteins have been discovered in animals (ferritin), plants (phytoferritins), and bacteria (bacterioferritins). However, surprisingly few such molecules have been described in fungi. Those that have been are found in members of the Zygomycota (Matzanke 1994a). Three types of ferritins have been described: (i) mycoferritin, which resembles mammalian ferritins; (ii) zygoferritin, a unique form of ferritin found only in the zygomycetes; and (iii) a bacterioferritin found in Absidia spinosa (Carrano et al. 1996).

Ferritin-like molecules have not been discovered among members of the phyla Ascomycota and Basidiomycota. Work on the ascomycetes has focused on *Neurospora crassa* and *Aspergillus ochraceus*. *N. crassa* forms the two predominant hydroxamate siderophores coprogen and ferrichrocin. The major extracellular siderophore formed under conditions of iron depletion is desferricoprogen. Desferriferricrocin is found mostly intracellularly. After uptake of coprogen (the iron-charged desferricoprogen), iron is released and transferred to desferricrocin by ligand exchange (which is not necessarily enzymatically mediated (Matzanke et al. 1987), and the desferricrocin thereby becomes the ferricrocin and serves as the main intracellular iron storage compound (Matzanke et al. 1988). Ferrocrocin also serves as a long-term iron storage siderophore in *A. ochraceus* (Van der Helm and Winkelmann 1994).

The basidiomycetes that have been studied are *Rhodotorula minuta* and *Ustilago sphaerogena*. Biosynthesis of rhodotorulic acid is characteristic of the heterobasidiomycetous yeasts. Other members of the Ustilagnaceae produce ferrichrome type siderophores. Rhodotorulic acid (RA) forms a complex with iron, Fe_2(RA)3 that is not transported across the membrane; rather, the iron is transferred by ligand exchange to an internal pool of rhodotorulic acid that then functions as the iron storage molecule (Matzanke 1994b). It should be noted that energy-dependent ligand exchange occurs at the membrane. It is not a simple exchange between Fe_2(RA)3 and internal desferrirhodotorulic acid; an additional mediator is required (Matzanke 1994b). In *U. sphaerogena*, iron is transported by ferrichrome A, which does not accumulate. The iron storage compound is ferrichrome (Matzanke 1994b). In the ascomycetes and basidiomycetes that have been studied, hydroxamate-type siderophores are the iron storage molecules.

In summary, iron storage ferritin-like compounds have been found only in the zygomycetes. The iron storage function in ascomycetes and basidiomycetes is performed by polyphosphates and hydroxamates.

1.2.2.4 Production of Siderophores

Under conditions of extreme iron stress, fungi produce low-molecular-weight (Mr<1500) ferric iron chelators known collectively as siderophores (Guerinot

1994; Höfte 1993; Riquelme 1996; Telford and Raymond 1996). Most of the fungal siderophores are hydroxamates. However, the zygomycetes form iron-regulated polycarboxylates and there are well-documented reports of phenolate-catecholates in species of the wood-rotting fungi. The compounds were identified by comparison to known phenolic siderophores separated by paper chromatography (Fekete et al. 1983, 1989). There is also an unconfirmed report of phenolates being formed by *Candida albicans*, but this report was based solely on color reactions (Ismail et al. 1985). Siderophores are named on the basis of their iron-charged forms, while the deferrated form (the one that gathers iron) is called deferri-siderophore or desferri-siderophore (Fekete et al. 1983, 1989; Holzberg and Artis 1983; Ismail et al. 1985).

1.2.2.5
Detection of Fungal Siderophores

Detection methods have been covered in detail in several monographs (Fekete 1993; Neilands and Nakamura 1991; Payne 1994a), but since some of the ensuing discussion involves methods employed by investigators, a brief summary is given here. The siderophores are produced in large excess by fungi in iron-starved cultures and turn red upon capture of Fe(III). Thus, a limited-iron medium for the fungus of our choice needs to be designed (Cox 1994); the fungus is grown in the limited-iron medium, periodically iron salts are added to a centrifuged sample, and, when a sample turns red, the culture medium is harvested for further processing.

1.2.2.6
Chemical Types of Siderophores

Two classes of compounds that function in iron gathering are commonly observed: hydroxamates and polycarboxylates.

1.2.2.6.1
Hydroxamates

The hydroxamates all contain an *N*-hydroxyornithine moiety. Descriptions of chemical structures are those given by Höfte (1993). More complete consideration of the chemistry of the hydroxamates has been presented on a number of occasions (Jalal and Van der Helm1991; Matzanke 1991; Telford and Raymond 1996).

There are four recognized families of hydroxamates, as well as other hydroxamates that are reported to be produced by certain fungi.

1. Rhodotorulic acid – the diketopiperazine of *N*-acetyl-l-*N*-hydroxyornithine. It has been found mainly in basidiomycetous yeasts (Van der Helm and Winkelmann 1994). A derivative of rhodotorulic acid is a dihydroxamate named dimerum acid; 3 mol of dimerum acid (DA) is combined with 2 mol of iron (Fe_2(DA)3) to form the iron-bearing ligand. The binding of iron is weaker than that with the other three-hydroxamate families. Dimerum acid is produced by some phytopathogens (e.g., *Stemphylium botryosum* and *Epicoccum purpurescens* (Frederick et al. 1981; Manulis et al. 1987) and by *H. capsulatum* (Burt 1982) and *Blastomyces dermatitidis* among the medically important fungi.
2. Coprogens – these are composed of 3 mol of *N*-acyl-*N*-hydroxy-l-ornithine, 3 mol of anhydromevalonic acid, and 1 mol of acetic acid (Fig. 1.4). Unlike the situation with the rhodotorulic acid family, 1 mol of iron combines with one ligand in the coprogen, ferrichrome, and fusarinine families (Van der Helm and Winkelmann 1994). The coprogens are produced by a number of plant pathogens (Höfte 1993; Manulis et al. 1987), for example *H. capsulatum* (Burt 1982), *B. dermatitidis* and the occasional human pathogens *Fusarium dimerum* and *Curvularia lunata* (Van der Helm and Winkelmann 1994).

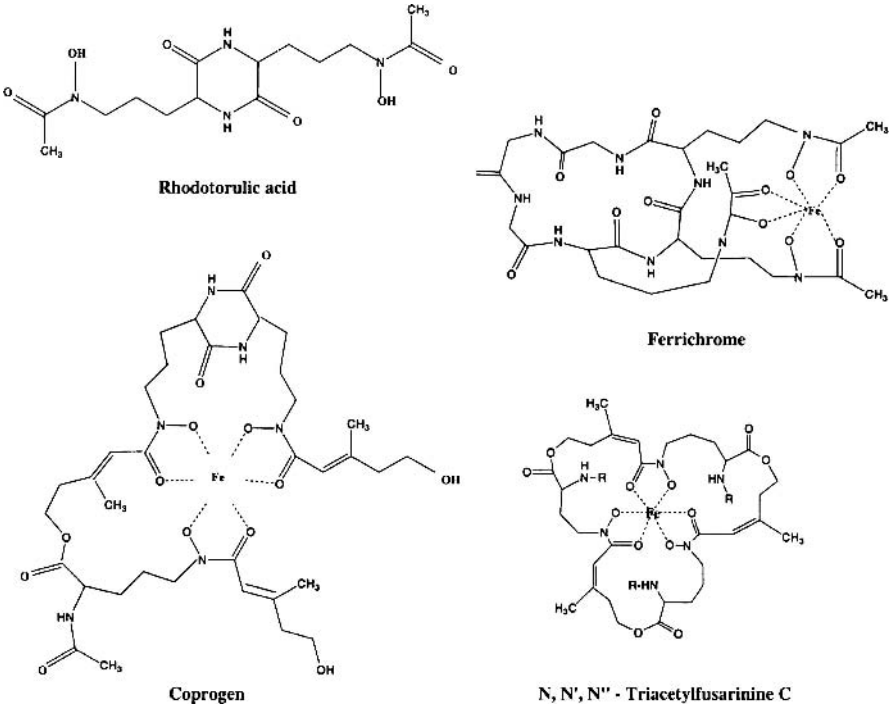


Fig. 1.4. Representative hydroxamate siderophores (adapted from Winkelmann 1993)

The coprogens may be considered trihydroxamate derivatives of rhodotorulic acid with a linear structure, and by such reckoning the number of hydroxamate families would be reduced to three. It is known that the hydrolysis of the ester group of coprogen B results in 1 mol of dimerum acid and 1 mol of *trans*-fusarinine (Winkelmann 1993). In the original report on DA (referred to as "dimerumic acid" in that report) from *H. capsulatum* (Burt 1982), the title implied that it was a degradation product of coprogen B. In fact, hydrolysis of the ester bond of coprogen gives rise to DA and trans-fusarinine (Winkelmann 1993), but DA is the predominant hydroxamate in liquid shake cultures of *H. capsulatum* (Burt 1982).

3. Ferrichromes – cyclic peptides containing a tripeptide of *N*-acyl-*N*-hydroxyornithine and combinations of glycine, serine, or alanine (Fig. 1.4). Among the pathogenic fungi, ferrichromes are produced by some phytopathogenic fungi (Höfte 1993) and by *Microsporum* sp. (Bentley et al. 1986; Mor et al. 1992), *Trichophyton* sp. (Mor et al. 1992), and *Aspergillus* spp. including the important pathogen *A. fumigatus* (Leong and Winkelmann 1998; Van der Helm and Winkelmann 1994). Another function of ferrichromes is the intracellular storage of iron.
4. Fusarinines – also called fusigens, these may be either linear or cyclic hydroxamates. Fusarinine is a compound in which *N*-hydroxyornithine is N-acylated by anhydromevalonic acid. Among zoopathogens, various fusarinines are found in *Fusarium* spp., *Paecilomyces* spp., and *Aspergillus* spp. (Van der Helm and Winkelmann 1994). Compounds identified as *trans*-fusarinine and an unidentified monohydroxamate were observed in culture filtrates of *Histoplasma capsulatum*, but they did not have biological activity (i.e., they did not stimulate growth of the fungus) (Burt 1982). The work on siderophores of *H. capsulatum* was initiated because of the well-known fact that the fungus does not clone in its yeast cell phase of growth on most culture media in vitro (Burt 1982). Burt used hydroxamates isolated from culture filtrates to relieve the growth restriction) (Burt 1982). The same strategy, i.e., growth stimulation, was used by Castaneda et al. (1988) in a study of *Paracoccidioides brasiliensis*. The siderophores coprogen B and DA isolated from *B. dermatitidis* were used to improve the plating efficiency of *P. brasiliensis* (Castaneda et al. 1988).

Unidentified hydroxamates – there are unconfirmed reports of formation of hydroxamates by *C. albicans* (Holzberg and Artis 1983; Ismail et al. 1985; Sweet and Douglas 1991). No exact chemical structures were presented, and it has been suggested by others (Van der Helm and Winkelmann 1994) that long-term cultivation (20 days) of a fungus will result in materials that react positively in tests based on color formation. Cutler and Han (1996) have reported their failure to display siderophores in *C. albicans*. The synthesis of hydroxamate siderophores by *C. albicans* has not been proven by chemical characterization.

Hydroxamates have been reported in other zoopathogens: *Absidia corymbifera*, *Madurella mycetomatis*, *Pseudallescheria boydii*, *Rhizopus arrhizus*, *R. oryzae*, *Scedosporium prolificans*, and *Sporothrix schenckii*. The report of hy-

droxamates from the zygomycetes *A. cormybifera*, *R. arrhizus*, and *R. oryzae* is unconfirmed (Van der Helm and Winkelmann 1994). It appears that an altogether different class of siderophores, the polycarboxylates, is formed by zygomycetes unconfirmed (Van der Helm and Winkelmann 1994). The report on *P. boydii* and *S. prolificans* was based solely on a positive CAS reaction. No studies of siderophore function or chemical characterization were performed (de Hoog et al. 1994). The report on *M. mycetomatis* was based on the CAS reaction (Mezence and Boiron 1995). The report on *S. schenckii* was based on a reaction with iron salts and the use of the Neilands method for hydroxamates (Holzberg and Artis 1983).

1.2.2.6.2
Polycarboxylates

Although there is one incomplete report of hydroxamates among the zygomycetes (Holzberg and Artis 1983), these fungi have not been observed by others to synthesize siderophores of the hydroxamate sort. Instead, a citric acid-containing polycarboxylate called rhizoferrin has been isolated from *Rhizopus microsporus* var. rhizopodiformis (Van der Helm and Winkelmann 1994). The molecule contains two citric acid units linked to diaminobutane (Fig. 1.5). Rhizoferrin is widely distributed among the members of the phylum Zygomycota, having been observed in the order Mucorales (families Mucoraceae, Thamndidiaceae, Choanephoraceae, and Mortierellaceae) and in the order Entomophthorales (Van der Helm and Winkelmann 1994).

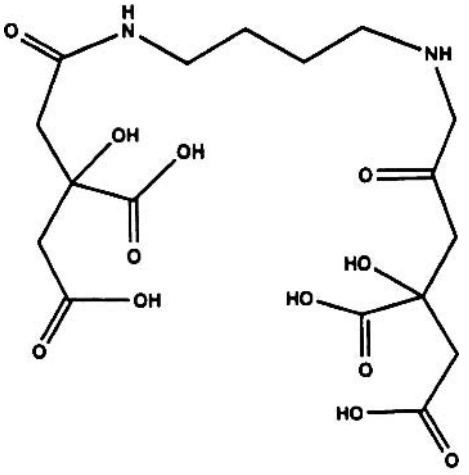


Fig. 1.5. The polycarboxylate rhizoferrin (adapted from **Winkelmann** 1993)

1.2.2.6.3
Phenolates-Catecholates

Most investigators consider that the phenolate-catecholate class of siderophores are not produced by fungi (Höfte 1993; Van der Helm and Winkelmann 1994). Only two exceptions have been reported; the first exception is *C. albicans* (Ismail et al. 1985) and this study was flawed by a lack of chemical structure studies. Phenolate siderophores were not found by two other groups of investigators (de Hoog et al. 1994; Holzberg and Artis 1983) who did, however, report hydroxamates in *C. albicans*. The other exception involves certain wood-rotting fungal species (Fekete et al. 1983, 1989). The siderophores were detected by the CAS dye and identified as phenolates by comparison to standard phenolates (e.g., enterochelin), and both the siderophores and the standard phenolates were separated by paper chromatography and visualized by UV fluorescence (Fekete et al. 1983). Further chemical structure studies were not reported, and the scientist reported that the compounds "...appear to be phenolate in character"(Jellison et al. 1991).

1.2.2.7
Synthetic Pathways of Siderophores

In this part we describe the various synthetic pathway of siderophores, their structural aspects and their mechanism of iron transport.

1.2.2.7.1
Diversity in Siderophore Synthesis

Diversity in siderophore synthesis is observed. Sometimes this diversity is with regard to a number of representatives in a single family, and at other times it is reflected in the synthesis of a number of representatives in several families. Frederick et al. (1981) provided evidence for synthesis of siderophores belonging to all four families of hydroxamates by *Epicoccum purpurescens* and Höfte (1993) reported that *Aspergillus ochraceus* "...can produce up to 10 or more different siderophores...". The reason for diversity is thought to be the ability of organisms to adapt to a wide variety of environmental situations (Höfte 1993; Winkelmann 1993). This idea is especially interesting for pathogens for which a host immune response may be involved (see "Siderophores as Pathogenic Factors" below).

1.2.2.7.2
Biosynthetic and Structural Aspects

The biosynthetic pathways of siderophores are tightly connected to aerobic metabolism involving molecular oxygen activated by mono-, di- and *N*-oxygenases

and the use of acids originating from the final oxidation of the citric acid cycle, such as citrate, succinate and acetate. Moreover, all siderophore peptides are synthesized by non-ribosomal peptide synthetases and in the case of fungal siderophores are mainly built up from ornithine, a non-proteinogenic amino acid. Thus, siderophore synthesis is largely independent from the primary metabolism. Most siderophores contain one or more of the following simple bidentate ligands as building blocks: (1) a dihydroxybenzoic acid (catecholate) coupled to an amino acid, (2) hydroxamate groups containing *N*-5-acyl-*N*-5-hydroxyornithine or *N*-6-acyl-*N*-6-hydroxylysine and (3) hydroxycarboxylates consisting of citric acid or β-hydroxyaspartic acid.

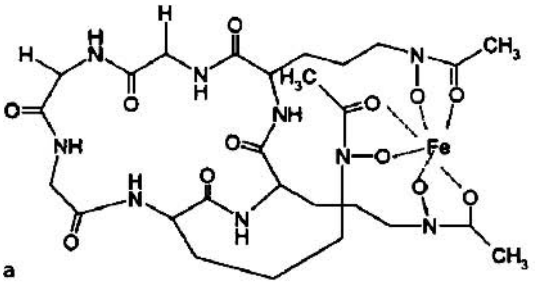
Besides being precursors, most of the monomeric bidentates may also act as functional siderophores after excretion. The iron-binding affinity of bidentate siderophores, however, remains low compared with hexadentate siderophores. A phylogeny of siderophore structures is difficult to delineate. However, starting from simple precursors of each class, one can imagine that extended siderophore structures have been favoured during evolution, resulting in hexadentate siderophores possessing higher stability constants (chelate effect) compared with their monomeric precursors. Thus, higher denticity seems to have a selective advantage in siderophore evolution.

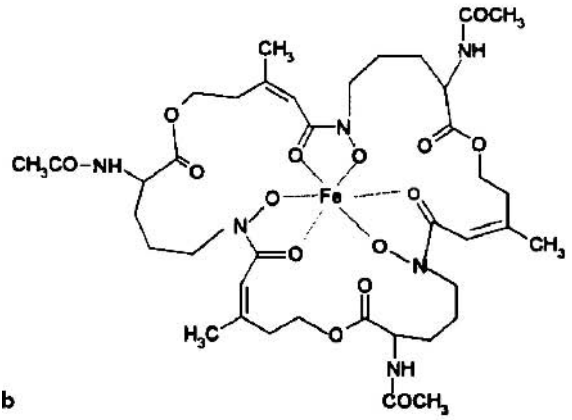
A further aspect of siderophore evolution is the optimization of chelate conformation. Although linear di-, tetra- and hexadentate siderophores have been found in all siderophore classes, there is a tendency for cyclization in the final biosynthetic end products (Fig. 1.6). Examples are enterobactin or corynebactin in the catecholate class, fusigen, triacetylfusarinine and ferrioxamines E and G, as well as the ferrichromes and asperchromes, in the hydroxamate class. Cyclization enhances complex stability, chemical stability and improves resistance to degrading enzymes. Cyclization is regarded as a common feature of secondary metabolism and is found in microbial peptides, polyketides, macrocyclic antibiotics and other bioactive compounds. Cyclization might also be advantageous for diffusion-controlled transport processes across cellular membranes. Moreover, due to a reduction of residual functional groups, the surface of the siderophores becomes non-reactive or inaccessible to modifying enzymes.

1.2.2.8
Transport of Siderophores

1.2.2.8.1
Transport Proteins

In bacterial systems, iron-regulated proteins occur and are expressed under conditions of iron depletion, often in concert with siderophore synthesis (Crosa 1997; Payne 1994b, c). Proteins from the plasma membrane of *N. crassa* that had been grown in iron-replete and iron-depleted media were compared (Van der Helm and Winkelmann 1994). There were no significant differences in the





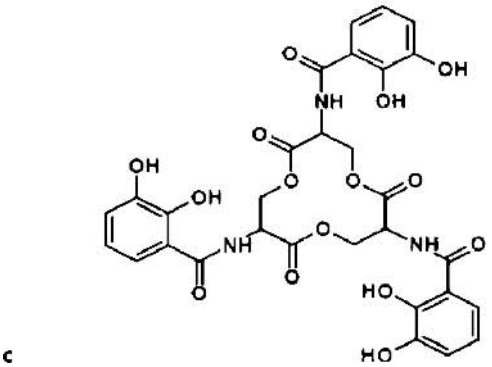


Fig. 1.6. Cyclic siderophores (adapted from Winkelmann 2001)

sodium dodecyl sulfate SDS-gel electrophoretic profiles of the two sources of proteins. There was no observed over-production of membrane proteins like that seen in bacterial systems (Payne 1994b, c). Because at least a fivefold difference in transport rates was observed, the scientists (authors) suggested that the membrane siderophore transport system is constitutively expressed (Van der Helm and Winkelmann 1994). In contrast to the work with *N. crassa*, iron-regulated outer membrane proteins have been found in the mycetoma-causing fungus, *Madurella mycetomatis* (Mezence and Boiron 1995). One unique protein and three amplified ones were observed under iron-limiting conditions (Mezence and Boiron 1995). The situation with other zoopathogens has not been explored.

1.2.2.8.2
Mechanism of Hydroxamate-Iron Transport

There are four described mechanisms of siderophore iron uptake across the cytoplasmic membrane of fungi. The following summary is modified from descriptions given by others (Carrano and Raymond 1978; Chung et al. 1986; Matzanke 1994a; Van der Helm and Winkelmann 1994).

1. Shuttle Mechanism. The intact siderophore-iron complex is taken into the cell. The iron is released by a reductase or by direct ligand exchange in which the recipient siderophore becomes the storage molecule. The gathering ligand is released to capture another iron molecule. This mechanism is the one used for uptake by siderophores of the coprogen and ferrichrome families (Matzanke 1994a; Winkelmann and Zahner 1973).
2. Direct-Transfer Mechanism. Iron is taken up without entrance of the ligand into the cell. The iron transfer is not a membrane-reductive event (Müller et al. 1985) but is a membrane-mediated exchange between the gathering siderophore and an internal chelating agent (Carrano and Raymond 1978). The transfer mechanism may be by ligand exchange (nonenzymatic) to an internal pool of the chelating agent, which then serves as the storage compound (Matzanke 1994a,b). This type of transfer has been reported with the rhodotorulic acid family of siderophores.
3. Esterase-reductase Mechanism. The esterase-reductase mechanism was shown to operate with the ferric triacetylfusarinine C (Winkelmann 1991, 1993). The ester bonds of the iron ligand are split after uptake of the ligand, the fusarinine moieties are excreted, and the ferric iron is reduced and stored (by an unknown mechanism).
4. Reductive Mechanism. Another reductive mechanism appears to be involved in the transport of some ferrichromes, which were shown not to enter cells but, rather, to give up ferric iron by reduction with transport of the ferrous iron (Winkelmann 1991, 1993). The storage mechanisms are not known. Fungi utilizing siderophores that they, themselves, do not synthesize commonly express this sort of transport.

1.2.2.8.3
Mechanism of Carboxylate Transport

The uptake of rhizoferrin has been studied Transport of the entire ligand is observed, and a Km value of 8 mM has been determined (Van der Helm and Winkelmann 1994). It is interesting that both ferrioxamines B and C are taken up at similar rates by *Rhizopus microsporus* var. rhizopodiformis, the species used to study rhizoferrin. This observation probably accounts for the cases of mucormycosis seen in patients treated with Desferal (desferrioxamine mesylate) (Tilbrook and Hider 1998; Boelaert et al. 1993). Phenolate transport has not been studied in either of the instances in which it was reported to occur in fungi (Fekete et al. 1989; Ismail et al. 1985).

1.3 Functions of Siderophores

Although their main function is to acquire iron from insoluble hydroxides or from iron adsorbed onto solid surfaces, siderophores can also extract iron from various other soluble and insoluble iron compounds, such as ferric citrate, ferric phosphate, Fe-transferrin, ferritin or iron bound to sugars, plant flavone pigments and glycosides or even from artificial chelators like EDTA and nitrilotriacetate by Fe(III)/ligand-exchange reactions. Thus, even if siderophores are not directly involved in iron solubilization, they are required as carriers mediating exchange between extracellular iron stores and membrane-located siderophore-transport systems.

The efficiency of siderophores in microbial metabolism is based mainly on three facts. (1) Siderophores contain the most efficient iron-binding ligand types in Nature, consisting of hydroxamate, catecholate or α-hydroxycarboxylate ligands that form hexadentate Fe(III) complexes, satisfying the six co-ordination sites on ferric ions. Moreover, siderophores possessing three bidentates in one molecule (iron-to-ligand ratio=1:1) show increased stability due to the chelate effect. (2) Regulation of siderophore biosynthesis is an economic means of spending metabolic energy, but it also allows for the production of high local concentrations of siderophores in the vicinity of microbial cells during iron limitation. This kind of overproduction may also be operating in host-adapted bacterial and fungal strains, leading to increased virulence. (3) Besides their ability to solubilize iron and to function as external iron carriers, siderophores exhibit structural and conformational specificities to fit into membrane receptors and/or transporters. This has been amply demonstrated by modifying siderophore chemical structure, i.e. using derivatives, enantiomers, metal-replacement studies or by genetic and mutational analysis of receptors and membrane transporters (Stintzi et al. 2000; Huschka et al. 1986; Ecker et al. 1988).

1.4 Siderophores as Pathogenic Factor

1.4.1 Activities

1.4.1.1 Phytopathogens

Many siderophores are produced by both bacterial and fungal plant pathogens, but their role in pathogenesis is largely unknown (Loper and Buyer 1991). The siderophore of the bacterial phytopathogen *Erwinia chrysanthemi*, chrysobactin, has been identified as a virulence factor (Riquelme 1996). However, the fungal phytopathogen *Verticillium dahlia* produces the hydroxamate siderophores coprogen B and DA, but efforts to detect these compounds in plants grown under iron-limited conditions was unsuccessful (Barash et al. 1993).

1.4.1.2 Phytotoxins

Only a single example of phytotoxins is discussed. Stemphylotoxin I and II are produced by *Stemphilium botryosum* f. sp. *lycopersici* (Barash et al. 1982; Manulis et al. 1984) and are ferric iron chelators. Their formation depends on iron concentration but is not as stringently iron regulated, as are the hydroxamates, and their siderophore activity is thus secondary to their primary phytotoxicity (Manulis et al. 1984).

1.4.1.3 Wood-Rotting Fungi

The wood-rotting fungus *Gloeophyllum trabeum* produces iron-binding compounds "that appear to be phenolate in character" (Jellison and Goodell 1988). Partially purified iron-binding compounds were conjugated with bovine serum albumin and injected into rabbits (Fekete 1993). The antisera were then used to immunolocalize siderophore molecules in slices of wood infected with *G. trabeum* (Jellison and Goodell 1988, 1989; Fekete 1993; Jellison et al. 1991). Siderophores are indeed antigenic, and the approach by these workers is appealing in its potential application to mammalian systems.

1.4.1.4
Zoopathogenic Fungi

Studies to reveal the in vivo elaboration of siderophores by zoopathogens have not been performed, but the methods for revealing them directly or indirectly (see the section "Host Response").

1.4.2
Host Response

The siderophores are known to be antigenic (e.g., see "wood-rotting fungi," above). In fact it has been suggested that siderophores may not be effective iron scavengers in vivo because they bind to serum proteins and elicit an immune response (Reissbrodt et al. 1997). Such immunogenicity might compromise the efficiency of siderophores in iron gathering. The immune response is, however, also evidence of in vivo synthesis. For example, enterochelin-specific immunoglobulins are found in normal human serum (Moore et al. 1980). This siderophore is produced by a number of enteric bacteria. Therefore, it is not certain whether the occurrence of anti-enterochelin activity relates to a previous infection (e.g., with *Salmonella*) or is a response to *E. coli* resident in the bowel.

1.4.3
Clinical Applications

As naturally occurring chelating agents for iron, siderophores might be expected to be somewhat less noxious for deferrization of patients suffering from transfusion-induced siderosis. A siderophore from *Streptomyces pilosus*, desferrioxamine B, is marketed as the mesylate salt under the trade name Desferal and is advocated for removal of excess iron resulting from the supportive therapy for thalassemia. The drug must be injected, however, and an oral replacement is needed (Bergeron and Brittenham 1994).

The potency of common antibiotics has been elevated by building into the molecules the iron-binding functional groups of siderophores (Watanabe et al. 1987). The objective here is to take advantage of the high affinity, siderophore-mediated iron uptake system of the bacteria.

1.5 Agricultural Interest

Fluorescent pseudomonads form a line of siderophores comprised of a quinoline moiety, responsible for the fluorescence, and a peptide chain of variable length bearing hydroxamic acid and hydroxy acid functions. Capacity to form these pseudobactin or pyoverdine type siderophores has been associated with improved plant growth either through a direct effect on the plant, through control of noxious organisms in the soil, or via some other route. Nitrogenase can be said to be an iron-intensive enzyme complex and the symbiotic variety, as found in *Rhizobium* spp., may require an intact siderophore system for expression of this exclusively prokaryotic catalyst upon which all life depends.

1.6 Utilization of Siderophores by Nonproducers

In a number of instances, pathogenic fungi have been shown to use siderophores even though they cannot synthesize them. Some examples are given below.

S. cerevisiae. Ferrioxamine B, ferricrocin, and rhodotorulic acid are iron sources for S. cerevisiae. Two mechanisms for utilization of ferrioxamine B have been described: (i) at relatively high concentrations (360 μM), the iron is made available by reductive dissociation, while (ii) at low iron concentrations (7 μM), yeasts transport the entire iron-bearing ligand with iron into the cells and reductively remove the iron internally (Lesuisse and Labbe 1994).

C. neoformans. Growth of *C. neoformans* was stimulated by ferrioxamine B (160 μM) added to an iron-depleted medium (Jacobson and Petro 1987). Although diffusion of the siderophore would dilute the concentration from that applied to the paper disc used, the level of siderophores close to the disc would probably be high enough to indicate a reductive mobilization of Fe^{2+} from the ligand (by analogy to *S. cerevisiae*). Studies on very low concentrations of the siderophore were not conducted, but ligand uptake is also possible.

C. albicans. Ferrichrome and several of its constitutive peptides stimulated the growth of *C. albicans* in an iron-depleted medium (Minnick et al. 1991). There is no information on whether utilization involves reductive mobilization or ligand uptake or concentration-dependent utilization of one or the other mechanisms for iron recruitment.

U. maydis. Studies of the phytopathogen *Ustilago maydis* have provided fascinating evidence for utilization of both synthesized and nonsynthesized siderophores. Under iron-limiting conditions, *U. maydis* produces ferrichrome and ferrichrome B. However, under laboratory conditions, it also uses the bacterial siderophore ferrioxamine B (Ardon et al. 1997, 1998). Ferrichrome was taken up by entire-ligand transport, while the ferrioxamine B was utilized by reductive removal of the Fe(III) and transport of the Fe(II) (Ardon et al. 1998). Nonenzymatic NADH- and flavin mononucleotide-dependent reduction of ferric siderophores has also been recorded (Adjimani and Owusu 1997). Although utilization of an extraneous bacterial siderophore would not affect in vivo recruitment of iron by the phytopathogenic fungus, its saprophytic existence in soil could be influenced. The interest in such an occurrence is heightened by the fact that the concentration of ferrioxamine B in the soil can be as high as 0.1 μM (Lesuisse and Labbe 1994). The same arguments can be raised with regard to zoopathogenic fungi with a saprophytic form of growth that constitutes the infectious phase of the pathogen.

Rhizopus spp. The occurrence of mucormycosis in patients being treated with Desferal (a methanesulfonate salt of desferrioxamine) clearly indicates the utilization of this bacterial siderophore by *Rhizopus* sp. (Boelaert et al. 1993).

P. brasiliensis. The growth of *Paracoccidioides brasiliensis* is stimulated by coprogen B and DA synthesized by *B. dermatitidis* (Castaneda et al. 1988). *P. brasiliensis* may synthesize its own siderophores under conditions of iron stress, but appropriate studies have not been conducted.

Host Iron Proteins. In a mammalian host, iron is bound to transferrin and lactoferrin or stored in ferritin. No fungus has been reported to be able to remove iron from transferrin or lactoferrin, although several bacteria and some animal parasites can do so (Payne 1993; Wilson et al. 1994).

1.7 Acidification and Mobilization

Many fungi can grow anaerobically and will acidify their growth medium. It has been suggested for *Neurospora crassa* that, under acidic conditions, iron could accumulate at the cell surface and be mobilized by excreted hydroxy acids to supply iron to the cell (Winkelmann 1979). This means of iron acquisition has been mentioned for *S. cerevisiae* in its ecological locations in nature (Lesuisse and Labbe 1994). However, no evidence was found for participation of citric acid or other polycarboxylic acids in iron uptake by *C. neoformans* (Jacobson and Petro 1987).

1.8 *Piriformospora indica* and Siderophore

In an independent study, the interaction of *Piriformospora indica* with *Pseudomonas fluorescens* was observed. It was found that *Pseudomonas fluorescens* had completely blocked the growth of the fungus (Fig. 1.7). Towards *Pseudomonas*, the radius never increased more than 0.6 cm.

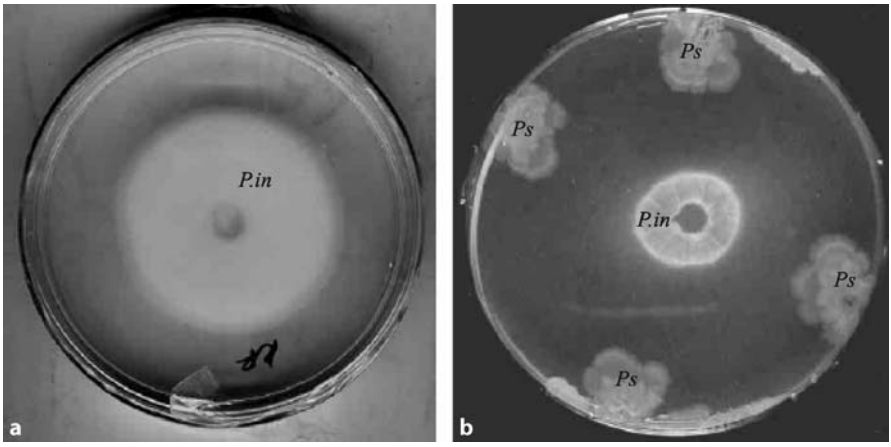


Fig. 1.7. **a** Axenic culture of *P. indica* on Aspergillus agar medium. **b** The interaction of *Piriformospora indica* with *Pseudomonas fluorescens* was observed in which *P. fluorescens* had completely blocked the growth of the fungus *P. indica*

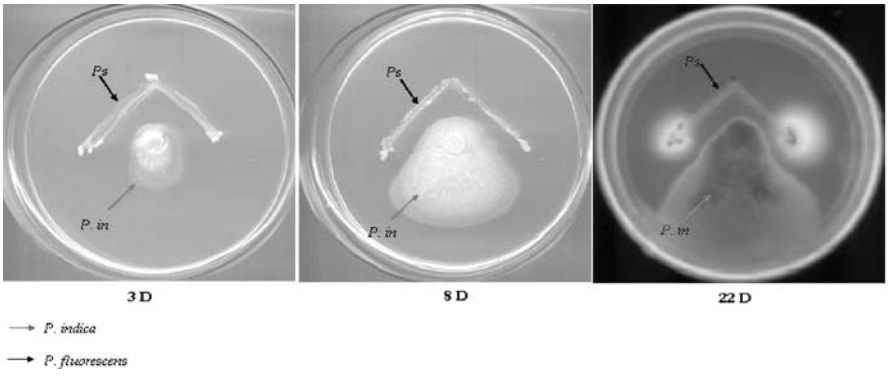


Fig. 1.8. Hyphal growth vs suppression assay showing the inhibitory effect of *Pseudomonas fluorescens* from Day-1 to Day-22 (release of fluorescent compound in the medium is clearly visible in Day-22 Petri plate)

In another set of experiments, *Pseudomonas fluorescens* colonies were raised in an angular manner on the petriplate and the fungus was placed in the centre. The growth of the later was completely blocked. After about three weeks, the synthesis of siderophore pyoverdine (and its derivatives) was detected It seems that the inhibition of fungus is multidirected (Fig. 1.8). In the beginning the inhibition was due to the synthesis of unidentified biomolecules and it was followed by production of siderophore. Identification of siderophore was based on the conventional spectrometry and spectrofluorimetry analysis.

1.9 Methods to Characterize Siderophore

1.9.1 Procedure for Detection of Siderophore

The iron salts procedure commonly used to detect siderophores is the method of Arkin et al. (Neilands and Nakamura 1991; Payne 1994a). The culture supernatants are mixed with a solution containing 5 mM $Fe(ClO_4)_3$ in 0.1 M $HClO_4$. The method is semiquantitative, and the amount of siderophore can be estimated by measurement of the optical density at 510 nm. The test may be used only as a rapid screening method because it lacks both specificity and sensitivity (Neilands and Nakamura 1991). Another colorimetric assay involves the use of the dye Chrome Asurol S (CAS) complexed with hexadecyltrimethylammonium bromide (HDTMA) (Neilands and Nakamura 1991; Payne 1994a; Fekete 1993). The removal of iron from CAS-HDTMA complex by a siderophore turns the dye yellow. The dye may also be included in a nutrient medium to measure the ability of an organism to remove iron from the dye complex (i.e., to make siderophores) by direct cultivation, a use that can be adapted to screening for non-siderophore-producing mutants (Neilands 1994). A caveat is, of course, that the medium must support the growth of the organism sometimes a bit tricky because of the potential toxicity of the HDTMA used to form the dye complex (Neilands and Nakamura 1991; Payne 1994b; Fekete 1993; Neilands 1994). Unfortunately, the CAS assay does have drawbacks. For example, phosphate strips iron off the dye, as also done by cysteine. Since there are several iron-binding substances that are not functional siderophores but nevertheless give positive color reactions with iron salts or CAS (Neilands and Nakamura 1991), the presence of hydroxamates or phenolates must be tested for; carboxylates are a different matter (Van der Helm and Winkelmann 1994). Hydroxamates may be detected by the Csáky assay (Neilands and Nakamura 1991; Payne 1994a), in which the end product detected is nitrite. The test is highly specific for hydroxamates (Neilands and Nakamura 1991). The Neilands assay (Neilands and Nakamura 1991) for hydroxamates identifies a *cis*-nitroso alkali dimer formed by periodic acid oxidation

that is detected at 264 nm. The Neilands assay has a drawback of questionable specificity: the originator has said, "By working so deep in the ultraviolet it is difficult to have confidence that the material being measured is actually the desired dimer" (Neilands and Nakamura 1991). Phenolates-catecholates are identified by the Arnow assay (Neilands and Nakamura 1991; Payne 1994a), in which the centrifugate is treated with nitrous acid, molybdate, and alkali, which yields a pink compound that is detected at 515 nm. All of the color assays are subject to some uncertainties (Neilands and Nakamura 1991; Van der Helm and Winkelmann 1994) and chemical characterization is required to identify the assay reactivity as that of a siderophore (see the next section, on chemical structure).

The biological activity of a hydroxamate may be measured in a bioassay that employs a bacterium, *Aureobacterium* (*Arthrobacter*) *flavescens* JG.9 (ATCC 29091), which requires hydroxamate siderophores for mobilization of iron, as reflected by its growth (Neilands and Nakamura 1991; Fekete 1993). The bacterium uses a wide range of hydroxamates for its growth needs (Neilands and Nakamura 1991). Of course, *A. flavescens* may not have an appropriate receptor for a given compound. Thus, an assay procedure should eventually be developed that uses the siderophore-generating fungus to assess the biologic reactivity of the putative siderophore for the fungus from which it is isolated (Payne 1994b, c). There are bioassays for phenolates. These involve strains of *Salmonella typhimurium* or *Escherichia coli* defective in the biosynthesis of enterochelin (Neilands and Nakamura 1991).

Once it has been determined that a presumed siderophore is a hydroxamate (or phenolate), an excess of iron salts (often $FeCl_3$) is added to the harvested culture medium and the presence of red color is monitored through various procedures designed to isolate, purify, and characterize the compound(s) giving reactivity. This last step, chemical characterization, is essential. There are reports of siderophores for a given fungus that are based on the formation of a red color in the presence of iron salts or a yellow color in the presence of CAS. These initial color reactions for iron chelation may be followed by an assay for hydroxamates or phenolates that are again based on color formation, but no chemical structure studies have been presented It has been pointed out that "a variety of cellular materials from lysed cells may react positively in tests based on color formation" (Van der Helm and Winkelmann 1994). Thus, precise chemical characterization is required for further work with the putative siderophore. Such chemical characterization may be performed by thin-layer chromatography, high-performance liquid chromatography, nuclear magnetic resonance, and mass spectroscopy (Van der Helm and Winkelmann 1994).

1.9.2 Isolation of Siderophore

Since siderophores differ substantially in structure, no uniform procedure is available for their isolation. A preliminary examination by paper electrophoresis

should reveal the charge profile as a function of pH, following which appropriate exchange resins can be applied for retention and elution of the compound(s). Most are water-soluble, and it is thus usually expedient to drive the siderophore into an organic solvent, such as benzyl alcohol or phenol-chloroform, in order to eliminate salt.

The siderophore may be isolated per se or as its iron chelate. The latter has the advantage of visual color, but the iron must be removed before any natural product can be characterized Vigorous hydrolysis in the presence of iron will destroy oxidizable moieties, and direct NMR analysis is ruled out by the paramagnetism of the ferric ion.

Structural characterization is best carried out by a combination of NMR and mass spectroscopy. Both of these techniques are sensitive and capable of providing absolute answers. Less than half of the known siderophores will crystallize; otherwise X-ray diffraction is the method of choice since it affords the configuration of those molecules containing a chiral center (Barash et al. 1993).

1.9.3 Cultivation of *Piriformospora indica*

The fungus is routinely cultivated on Aspergillus medium or Kaefer (Hill and Kaefer 2001). Composition of this medium is given in appendix. Fungal discs were prepared by using bottom of the sterile Pasteur pipette measuring about 4 mm in diameter and one disc was inoculated per petri-plate fortified with Kaefer medium containing 1% agar. These petri-plates were incubated at 28 ± 2 °C in dark. The growth normally commences on 3rd day and after 12 days the fungus completely covers the surface of the agar plate.

The broth culture of the fungus was made in Kaefer medium without agar. Five or more discs were transferred to 250-ml Erlenmeyer conical flasks containing 100 ml medium. The broth culture was incubated on Kühner rotary shaker at constant speed (150 rpm) and temperature (28 ± 2 °C). The maximum growth was obtained after eight days of incubation.

1.10 Conclusions

Siderophores are common products of aerobic and facultative anaerobic bacteria and of fungi. Under conditions of iron starvation, many fungi synthesize low-molecular-weight iron chelators known as siderophores. Three common classes, phenolates, hydroxamates, and polycarboxylates, are examined. Some phytopathogenic fungi produce unique compounds that function as phytotoxins but also chelate iron.

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Appendix

Kaefer medium or Aspergillus Broth or Aspergillus Medium (Hill and Kafer 2001)

Chemicals	**Composition**
Glucose	20 g/L
Peptone	2 g/L
Yeast extract	1 g/L
Casamino acid	1 g/L
Vitamin stock solution	1 ml
Macroelements from stock	50 ml
Microelements from stock	2.5 ml
Agar	10 g
$CaCl_2$ 0.1 M	1 ml
$FeCl_3$ 0.1 M	1 ml
pH	6.5

Macroelements (major elements)	**Stock g/L**
$NaNO_3$	120.0
KCl	10.4
$MgSO_4.7H_2O$	10.4
KH_2PO_4	30.4

Microelements (Trace elements)	**Stock g/L**
Zn $SO_4.7H_2O$	22.0
H_3BO_3	11.0
$MnCl_2.4H_2O$	5.0
$FeSO_4.7H_2O$	5.0
$CoCl_2.6H_2O$	1.6
$CuSO_4.5H_2O$	1.6
$(NH_4)_6$ $Mo_7O_{27}.4H_2O$	1.1
Na_2EDTA	50.0

Vitamins	Percent
Biotin	0.05
Nicotinamide	0.50
Pyridoxal phosphate	0.10
Amino benzoic acid	0.10
Riboflavin	0.25

pH of the medium was adjusted to 6.5 with 1 N HCl
All the stocks were stored at 4 °C except vitamins, which are stored at –20 °C