Fungal Biology

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Fungal Siderophores

From Mineral—Microbe Interactions to Anti-Pathogenicity

Springer

Fungal Biology

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About the Series

Fungal biology has an integral role to play in the development of the biotechnology and biomedical sectors. It has become a subject of increasing importance as new fungi and their associated biomolecules are identified. The interaction between fungi and their environment is central to many natural processes that occur in the biosphere. The hosts and habitats of these eukaryotic microorganisms are very diverse; fungi are present in every ecosystem on Earth. The fungal kingdom is equally diverse, consisting of seven different known phyla. Yet detailed knowledge is limited to relatively few species. The relationship between fungi and humans has been characterized by the juxtaposed viewpoints of fungi as infectious agents of much dread and their exploitation as highly versatile systems for a range of economically important biotechnological applications. Understanding the biology of different fungi in diverse ecosystems as well as their interactions with living and non-living is essential to underpin effective and innovative technological developments. This series will provide a detailed compendium of methods and information used to investigate different aspects of mycology, including fungal biology and biochemistry, genetics, phylogenetics, genomics, proteomics, molecular enzymology, and biotechnological applications in a manner that reflects the many recent developments of relevance to researchers and scientists investigating the Kingdom Fungi. Rapid screening techniques based on screening specific regions in the DNA of fungi have been used in species comparison and identification, and are now being extended across fungal phyla. The majorities of fungi are multicellular eukaryotic systems and therefore may be excellent model systems by which to answer fundamental biological questions. A greater understanding of the cell biology of these versatile eukaryotes will underpin efforts to engineer certain fungal species to provide novel cell factories for production of proteins for pharmaceutical applications. Renewed interest in all aspects of the biology and biotechnology of fungi may also enable the development of "one pot" microbial cell factories to meet consumer energy needs in the 21st century. To realize this potential and to truly understand the diversity and biology of these eukaryotes, continued development of scientific tools and techniques is essential. As a professional reference, this series will be very helpful to all people who work with fungi and should be useful both to academic institutions and research teams, as well as to teachers, and graduate and postgraduate students with its information on the continuous developments in fungal biology with the publication of each volume.

More information about this series at http://www.springer.com/series/11224

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ISSN 2198-7777 ISSN 2198-7785 (electronic)
Fungal Biology
ISBN 978-3-030-53076-1 ISBN 978-3-030-53077-8 (eBook)
https://doi.org/10.1007/978-3-030-53077-8

This Springer imprint is published by the registered company Springer Nature Switzerland AG
The registered company address is: Gewerbestrasse 11, 6330 Cham, Switzerland

Foreword

I am delighted to write the foreword for *Fungal Siderophores: From Mineral—Microbe Interactions to Anti-pathogenicity* edited by an interdisciplinary team of scientists, Dr. Kalyani Dhusia, Dr. Kalpana Raja, and Prof. Pramod W. Ramteke.

The editors have addressed the role of siderophores in various biological domains such as microbiology, pharmacognosy, pharmacology, bioinformatics, and biomedical text mining. While lots of research articles are available on bacterial siderophores, less is known about fungal siderophores. A book specific to fungal siderophores should be beneficial to scientists and researchers. This book covers a wide range of topics and is expected to gain readers from several domains.

In the past few decades, researchers have proven that fungal siderophores epitomize the uptake of iron as well as other essential elements like zinc, magnesium, copper, nickel, and arsenic," in almost every microorganism and plant. Understanding the chemical structures of different fungal siderophores and the membrane receptors involved in the uptake of mineral ions led to new research ideas.

In the current edition, the authors share information on fungal host–pathogen interactions, fungal infections escalating in immunocompromised patients and siderophore therapies involved, virulence control with fungal siderophore of brown rot disease in stone fruits and many more.

Fungal host–pathogen interactions exert a highly priced ranging from the crops cultivation to human health. Severe fungal infections emerge due to the increasing number of immunocompromised patients, aggressive surgical therapy in older patients, comorbid diseases in aged patients, and an increasing number of oncologic diseases, whereas in plants, the mutualistic interactions frequently occur with fungi. Plant-associated fungi are known to exploit tissues of their hosts to retrieve nutrients and shelter. On the other hand, fungal siderophores are investigated to explore the benefits. For instance, in recent years it became clear that the siderophore system constitutes a central element in iron homeostasis of many if not most fungi, affecting growth, oxidative stress resistance, as well as asexual and sexual development. Most fungi produce hydroxamate-type siderophores except for the polycarboxylate rhizoferrin. Fungal requirement for iron could potentially open perspectives toward the development of novel antifungal treatments, for example, iron chelation therapy

or blocking of high-affinity iron acquisition or development of chemical surrogates for siderophores.

Thus, the challenges in pathogenicity and interpretation role of fungal siderophore are both difficult and interesting. Microbiologists are working on them with enthusiasm, tenacity, and dedication to develop new methods of analysis and provide new solutions to keep up with the ever-changing threats. In the current era of global research interconnectivity and interdependence, it is necessary to provide transparent dataset and knowledge to professionals and students. This book provides the latest information on fungal siderophores from a wide range of domains.

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Preface

The microbial-aided survival in agricultural setup is an emerging field in the area of microbiology. Among the microbes, studies on bacterial siderophores are well established and much information is known. Surprisingly, the information on fungal siderophores is minimum. This book presents the current state of knowledge specifically on the mineral–microbe interactions to anti-pathogenicity of fungal siderophores.

In the past few decades, researchers have proven that the fungal siderophores epitomize the uptake of iron and other essential elements like zinc, magnesium, copper, nickel, and arsenic in almost every microorganism and plant. Understanding the chemical structures of different fungal siderophores and the membrane receptors involved in the uptake of mineral ions may lead to new research ideas. To our knowledge, this is the first book on fungal siderophores covering a wide range of information from all possible resources. The book also includes a chapter on applying text mining to process the published biological literature on fungal siderophores. To our knowledge, this is the first attempt to introduce text mining in the field of microbiology.

We focused on the accuracy of the data included in the book, that is, researchers with vast experience and knowledge in microbiology and other related fields such as bioinformatics, biotechnology, biochemistry, and pharmacology have contributed to the chapters presented in this book. The authors have presented all possible information available on fungal siderophores in the chapters they contributed.

New York, NY, USA — Kalyani Dhusia
Madison, WI, USA — Kalpana Raja
Allahabad, India — Pramod W. Ramteke

Contents

Chapter 1
Basics of Fungal Siderophores: Classification, Iron Transport and Storage, Chemistry and Biosynthesis, Application, and More

Shyam Sundar Arputhanantham, Kalpana Raja, Latha Shanmugam, and Vijayakumar Raman

Contents

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K. Dhusia et al. (eds.), *Fungal Siderophores*, Fungal Biology,
https://doi.org/10.1007/978-3-030-53077-8_1

1.1 Introduction

Siderophores are the low molecular weight organic compound produced by most of the microorganisms, especially bacteria and fungi. The primary role of siderophores is to chelate and transport iron across the cell membrane through an energy-dependent process (Renshaw et al. 2002). The ferric form of iron, Fe^{3+}, is insoluble at physiological pH of 7.35–7.40 (Bou-Abdallah 2010). Under such environmental conditions, the microorganisms produce siderophores that exhibit high affinity for Fe^{3+} ion and form siderophore – Fe^{3+} ion complexes (Saha et al. 2012). These complexes are transported across the cell membrane to cystol, where Fe^{3+} ion is reduced to ferrous ion, Fe^{2+} ion for immediate uptake (Fig. 1.1). Apart from iron transportation, siderophores are applied in microbial ecology, agriculture, detoxification of heavy-metal contamination, phyto-pathogenesis, and many more (Renshaw et al. 2002; Saha et al. 2012; Khan et al. 2018).

PubMed database includes 5908 articles on bacterial siderophores and 3582 articles on fungal siderophores (access date 23rd of Sep 2019). Boolean query for searching bacterial siderophores is "bacterial siderophores" OR "bacterial siderophore" OR (bacteria AND siderophore) and for fungal siderophores is "fungal siderophores" OR "fungal siderophore" OR (fungi AND siderophore) OR (fungus AND siderophore). Boolean query restricted to extract articles matching the exact name in the query string retrieved 154 articles for bacterial siderophores and 51 articles for fungal siderophores. We also noticed that only 8 review articles are being published on fungal siderophores when compared to 20 review articles on bacterial siderophores (Winkelmann 2007; Balhara et al. 2016; Ahmed and

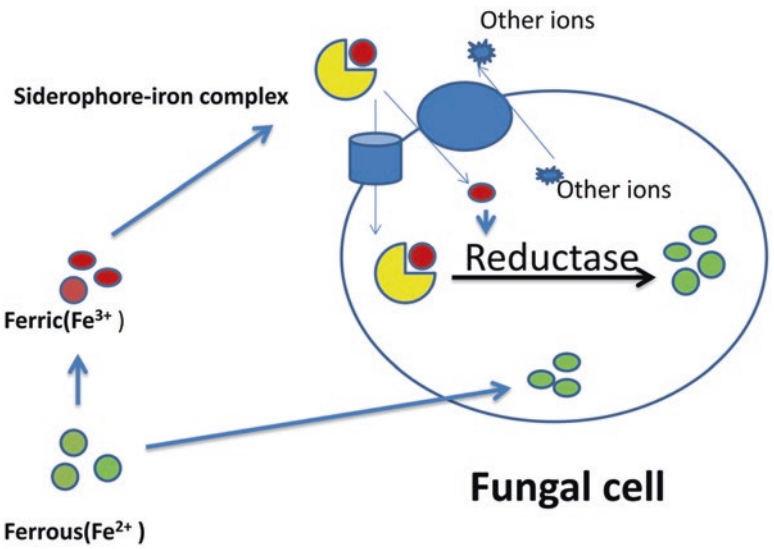

Fig. 1.1 Schematic representation of iron transport through siderophores

Holmstrom 2014; Zheng and Nolan 2012; Haas 2003; Luca and Wood 2000; Riquelme 1996). Thus, it is obvious that much less is known about fungal siderophores when compared to bacterial siderophores. We believe that a brief description on fungal siderophores might be useful to microbiologists working on their biosynthesis, pathogenesis, biomedical application, and other biological aspects.

1.2 Microorganisms and Iron

Microorganisms depend on a variety of metals for their optimal growth and existence. They influence the nature of the metals by modifying their physical properties and chemical states. Biomineralization is an emerging discipline that deals with the process of biogenic mineral formation by microorganisms. It often overlaps with geomicrobiology. Most of the members of Mendeleev's Periodic Table are influenced by microorganisms in the formation of biomass or simple accumulation processes. The microorganisms have their intrinsic vehicle system for carrying the vital metals and minerals through a process called bioprecipitation that consists of three major mechanisms: (i) by generating primary or secondary metabolites, (ii) by changing the microecosystem surrounding the metals, and (iii) by discharging the substances that can precipitate the metals.

The structural components of microorganisms are capable of absorbing metals by interacting with earthen components. The microorganisms as a whole can transform various metals and minerals. The metal-microbe (or metal-microorganism) interactions often result in the oxidation or reduction of various components facilitated by the microorganisms. There are multiple variables such as endogenous chemicals and structural components on which the microbes rely on for their existence. These properties are the primary determinants of the quantity of metals present in the cell. The metal-specific structural components and physiological functions are known in most of the microorganisms. The structural components are essential for the absorption of essential metals such as sodium, potassium, calcium, iron, and many more. The microorganisms have developed a defined and controlled mechanism over the years to acquire metals in the advent of scarcity of the metals. They compete with other living organisms including the host they infect. The general mechanisms through which the metal acquisition takes place comprise of two components: (i) membrane transport system for specific metals and (ii) metal-complexing agents that procure the metals from environment.

The optimal absorption and presence of the essential metals are important because any excess bioavailability of the metals affect the normal growth and development of the microorganisms. Any superfluous amount of metal can lead to both morphological alterations and physiological actions of the microorganisms. The evolutionary capacity of the microorganisms is capable of developing intrinsic mechanisms such as genetically inherited resistance and redox reactions. These intrinsic mechanisms result in lesser toxicity and controlled entry at the cell surface to protect themselves from the metallic overload (Khan et al. 2018). The absorption

of metals by the microorganisms from the natural substrate is achieved by various strategies such as cleavage of chemical bonds, chelation, siderophore-mediated iron acquisition, and redox reactions.

1.3 Classification of Fungal Siderophores

Fungal siderophores are classified into four major categories based on the formation of oxygen ligand for Fe^{3+} ion coordination: hydroxamates, catecholates (or phenolates), carboxylates, and the mixed. The hydroxamate siderophores are further classified into ferrichromes, coprogens, fusarinines, and rhodotorulic acid. Certain fungal species secrete hydroxamate siderophores other than the four general types: ferricrocin, hydroxyl ferricrocin, fusarin C, corogen B, and triacetyl fusarinine C by *Aspergillus* species and *H. capsulatum* (Hass 2014). The production of various types of siderophore is depending on the morphology of the fungi. The hydroxyl ferricrocin is synthesized within the conidial spores, and ferricrocin siderophore is released during the filamentous hyphal growth. These siderophores are responsible for iron storage and distribution in the corresponding parts of the fungi (Blatzer et al. 2011). The catecholate siderophores include enterobactin. The carboxylate siderophores include rhizoferrin (Fig. 1.2).

The hydroxamate siderophores are the most common ones produced by both bacteria and fungi (Hofte 1992). They consist of C (=O) N-(OH) R functional group, with R being an amino acid or its derivative. The two oxygen atoms present in the functional group form a bidentate ligand with the Fe^{3+} ion. The binding between the siderophores and Fe^{3+} ion is strong and protects the complex against hydrolysis and enzymatic degradation (Winkelmann 2007). The hydroxamate siderophores are detected by Neilands spectrophotometric assay, the electrospray ionization-mass spectrometry, the modified overlaid chrome azurol S, and Csaky's assay (Neilands 1981; Perez- Miranda et al. 2007; Karuna et al. 2010). The catecholate siderophores and carboxylate siderophores are commonly produced by bacteria (Dave et al. 2006). Apart from bacterial and fungal siderophores, two marine organisms, *dinoflagellate Prorocentrum minimum* and blue-green algae, are known to produce

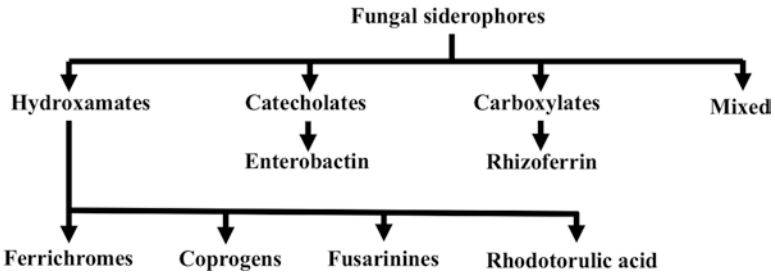

Fig. 1.2 Classification of fungal siderophores

siderophores (Armstrong and Baalen 1979). Siderophores with functional groups other than C (=O) N-(OH) R, lysine derivative (e.g., mycobactin), ornithine derivative (e.g., pyoverdine), and histamine derivative (e.g., anguibactin) are also recognized.

1.4 Fungal Siderophores and Iron Transport

The siderophores are closely associated with metals, especially iron. Their biosynthesis is regulated by the internal concentration of iron (Renshaw et al. 2002). A wide range of living organisms including the plants compete for iron through siderophores (Ahmed and Holmstrom 2014). The primary mechanism is through the suppression of the plant diseases induced by the biological control agents via the production of antimicrobial secondary metabolites such as siderophores, antibiotics, volatile substances, and many more. The living organisms utilize more than one mechanism for the optimal iron transport. Among them, two mechanisms are widely observed: (i) transportation through siderophores that are specific to each organism and (ii) adherence to iron in the form of ferric oxide. The second mechanism is simple and favors a much faster iron transport.

The iron-chelated siderophores are transported inside the cell by siderophore iron transporters. The iron acquisition of the organism depends on specific siderophore iron transporters. There are ten siderophore iron transporters in *Aspergillus* species. MirA and MirB are the siderophore iron transporters found in *A. nidulans*. MirB transports triacetyl fusarinine C in *A. nidulans* and *A. fumigatus*. The siderophores with the transporters are internalized and the iron from triacetyl fusarinine C is released by esterase B. The iron from the iron-siderophore complex is also released by reductase (Howard 1999). In fungi, the iron transportation is mainly through siderophores and most of the fungi secrete more than one type of siderophores. The iron transportation is achieved through a mechanism, the siderophore-mediated transport system (SMTS) that requires energy. The transported iron is utilized in enhancing the fungal growth. SMTS helps the fungi to compete effectively with the growth of other microorganisms by limiting the availability of iron. Though the system is believed to be useful in acquiring other metals, iron is the only documented essential element acquired by fungi. SMTS consist of four major transportation mechanisms (Zheng and Nolan 2012; Haas 2003; Luca and Wood 2000):

(a) Vehicle transport is facilitated by the penetration of iron-siderophore complex into the fungal cell and subsequent intracellular breakup by fungal reductase.
(b) The siderophores mediated taxicab transport that results in ligand exchange and subsequent iron transfer.
(c) Hydraulic acquirement is facilitated by the influx of the iron-mediated siderophores and consequent breakup by fungal intracellular mechanisms.
(d) The iron-siderophore complex triggers the reductive procurement that results in reduction of iron adjacent to fungal membrane and subsequent uptake of Fe^{2+}.

1.5 Fungal Siderophores and Iron Storage

Apart from contributing to iron uptake in restricted microecosystem, the siderophores play a pivotal role in storing iron in the cytoplasm of the fungi. Fungi principally utilize two distinct approaches for iron storage: (i) deposition of iron in the vessels and (ii) iron storage facilitated by siderophores. *A. fumigatus* uses both strategies for iron storage. Unlike bacteria, the fungi infecting the plants and animals do not have the ferritin-mediated iron storage. The iron storage happens even in the fungal spore forms. The fungi capable of generating the siderophores have the potential to extract iron from the proteins present in the host. The siderophores triacetyl fusarinine C and fusarinine secreted by *A. fumigatus* are more potent and capable of extracting iron bound to transferrin, a protein present in the host. For *Aspergillus* spp., the primary method of acquirement of iron is through siderophore production because of their inability to get iron from either heme or ferritin or transferrin. In *A. fumigatus*, iron permease was superfluous and is not essential for pathogenicity, but deletion of siderophore-producing genes results in increased pathogenicity as evident from increased virulence and formation of conida. In addition, *A. nidulans* needs siderophores for its existence, and deleting siderophore-producing genes resulted in abnormal growth and development. After binding with ferric iron, siderophore is moved to the cytoplasm of *A. fumigatus* through siderophore iron transporters, and iron is stored in the cytoplasm of vacuoles.

The specific iron storage exists in certain fungal species. The fungi grouped under *Mucorales* order have no mechanism to generate siderophores with hydroxamate class. Rhizoferrin, a siderophore belonging to carboxylate class produced by mucormycetes, has poor affinity to obtain iron from the proteins of the hosts, and hence rhizoferrin is a poor infecting agent in humans. As mucormycetes cannot use its own siderophore, they use the iron vehicles that act as exogenous siderophores. The patients with the risk of iron intoxication are prescribed with defuroximine, a potent chelating agent. The patients are reported with the episodes of siderophore-mediated mucormycosis.

1.6 Chemistry and Biosynthesis

Most of the fungal siderophores are hydroxamates with few exceptions. The biosynthesis of hydroxamate siderophores is regulated by an iron-dependent mechanism in most of the fungal species including *Epichloë festucae*, *Ustilago maydis*, and *Saccharomyces cerevisiae* (Holinsworth and Martin 2009; Forester et al. 2018; An et al. 1997a, b). Genes such as Sid1, Sid2, SidN, and urbs1 are known to be involved in the biosynthesis pathway (An et al. 1997a, b). The hydroxamates establish a stable ferric iron-binding bidentate. N^5-Hydroxy-l-ornithine is a nonprotein synthetic amino acid that contributes to the formation of hydroxamates through acylation. N^5-Hydroxy-l-ornithine is generated by the hydroxylation of l-ornithine that has a variety of functional groups such as acetyl, hydromevalonyl, and many more

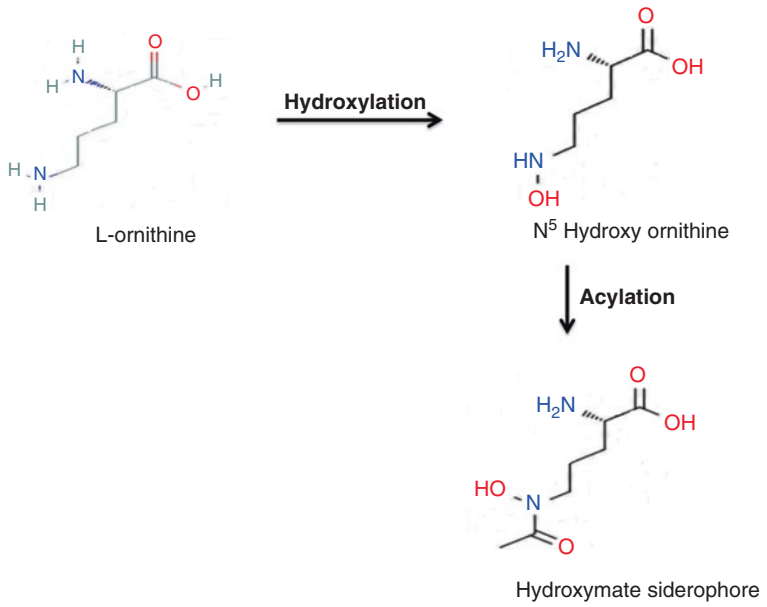

Fig. 1.3 Biosynthesis of hydroxamate siderophore

(Fig. 1.3). The moieties are connected through a peptide or ester bond to form hexadentate structures with improved affinity for ferric form of iron. An iron-chelating hydroxamate, hexadehydroastechrome, from *A. fumigatus* is less known and needs to be tested further for its utility (Renshaw et al. 2002; Armstrong and Baalen 1979).

Even though siderophores are synthesized from different structural families, most of the siderophores produced by a single organism is limited to a specific structural family. *Mucorales* synthesizes rhizoferrin and *Penicillium bilaii* synthesizes catecholate pistillarin which are polycarboxylates. The chemical stability is enhanced with the cyclization of siderophore, which is evident with a few fusarinines and most of ferrichromes. Similar to *Rhodotorula* spp., *basidomycetous* yeasts produce rhodotorulic acid that contains two N-acetyl-N-hydroxy-1-ornithine connected together as a diketopiperazine nucleus. The fusarinines are formed from the three *N*-cis-anhydromevalonyl-N hydroxy-l-ornithine units that are connected by the ester bonds. The coprogens containing three units of N-*trans*-anhydromevalonyl-N-hydroxy-l-ornithine are formed from trans-fusarine units. Among these, two units are linked together to form diketopiperazine ring, and the third unit forms ester with the OH group of diketopiperazine ring at the C-terminal. The organic reactions such as N-acetylation and N-methylation are responsible for the varieties within the coprogens family (Renshaw et al. 2002; An et al. 1997a, b). *Epichloë festucae*, an endophyte, produces a siderophore of ferrichrome variety that has a novel structure. The epichloënin A siderophore structure contains tetra-glycines, single glutamine, and three *N-trans* anhydromevalonyl-N-hydroxy-l-ornithine groups (Renshaw et al. 2002; Armstrong and Baalen 1979; An et al. 1997a, b).

1.7 Role of Iron in Fungal Physiology

Iron is the fourth abundant element in our planet next to oxygen, silicon, and aluminum. It is an essential element in almost all living beings except the *Lactobacillus* genus. It is an indispensable component for microbial growth and development. Various biological processes such as the synthesis of DNA are contributing to endogenous enzyme activity (Hofte 1992; Winkelmann 2007). The environmental iron exists in both reduced (Fe^{2+}) and oxidized forms (Fe^{3+}). The ability to donate or accept electrons between the reduced and oxidized forms enables iron to play an integral role in the biological reactions (Neilands 1981). The iron required by the fungi is not readily available in nature. Several fungal species have established various mechanisms to acquire iron. The release of siderophores and the organic acid surge are the two main strategies employed by fungi for ensuring the availability of iron for their physiological processes (Perez- Miranda et al. 2007; Karuna et al. 2010). The dependency of fungi on iron to execute their normal physiological functions makes iron as an important limiting factor for their growth.

The fungi depend on iron for various metabolic activities including redox-active reactions, absorption and active uptake of iron, translocation, solubilization/weathering, and biogenic mineral production (Dave et al. 2006). During the redox-active reactions, insoluble Fe^{3+} ion is converted to soluble Fe^{2+} ion. Multiple mechanisms are proposed for the procurement of iron absorption and active uptake by fungi. The most common ones are (i) the facilitated uptake by iron-chelating agents like siderophores, (ii) reductive iron assimilation, (iii) acquiring ferrous iron with less pull, and (iv) acquiring iron from heme and its subsequent breakdown. *Candida* spp. that are pathogenic, *Cryptococcus neoformans* that causes opportunistic infections, and *Saccharomyces cerevisiae* that is extensively beneficial in food production do not produce siderophores. Instead, these microorganisms utilize the iron chelated by other siderophore-producing organisms through various mechanisms such as reductive iron assimilation, heme uptake, and low-affinity iron uptake to acquire iron. The morphology of most of the microorganisms that do not produce siderophore resembles the morphology of yeast. It is interesting to note that certain yeast species like *Schizosaccharomyces pombe o*r *Aureobasidium pullulans* produce siderophores. The reductive iron assimilation is absent in species like *Aspergillus nidulans*. Iron toxicity and deficiency are controlled by cells through a defined iron regulatory mechanism.

1.8 Iron Sensing and Transcriptional Regulation

S. cerevisiae, a poor prototype of many fungal species, is extensively studied for its iron homeostasis function. *C. glabrata* has the iron-regulating mechanism similar to *S. cerevisiae*. Other fungal species matching the iron-regulating mechanism of *S. cerevisiae* are unknown. In *S. cerevisiae* and *C. glabrata*, an Aft transcription activator upregulates the genes involved in iron uptake under the iron-deprived environment. The process is achieved by the binding of Fe-S clusters present in the

mitochondria to the glutaredoxins (Arstol and Hohmann-Marriot 2019; Gerwien et al. 2016). In iron-excess environment, the Fe-S clusters in mitochondria activate the iron-responsive regulator yap 5 that targets two genes, CCC1 that codes for the vacuolar iron importer and CUP1 that codes for the copper-binding metallothionein (Ueta et al. 2012; Li et al. 2008, 2012; Pimentel et al. 2012).

1.9 Siderophores and Pathogenicity

In some species, production of siderophores serves as virulence component which controls pathogenicity. The variant of *Microbotryum violaceum*, a plant pathogen, produces little quantity of siderophores and it is almost devoid of pathogenic ability. The growth of fungi that are pathogenic to plants may be controlled by the utilization of atypical siderophores produced by nonpathogenic fungi. Such fungi are deemed to be useful for plants. The published literature confirms that the plants are capable of acquisition of iron through siderophores to a minimum level (Renshaw et al. 2002; Saha et al. 2012, 2015).

1.10 Applications

Fungal siderophores can either be beneficial or detrimental to our environment based on their use in bioremediation process. The aforementioned examples clearly highlight the utility of siderophores in the treatment of nuclear wastes and in healthcare (Fig. 1.4). Mostly, siderophores are controlled by an endogenous mechanism that recognizes iron. The energy-dependent transportation of siderophores into the cell utilizes receptor proteins to which they bind. The receptor proteins identify the

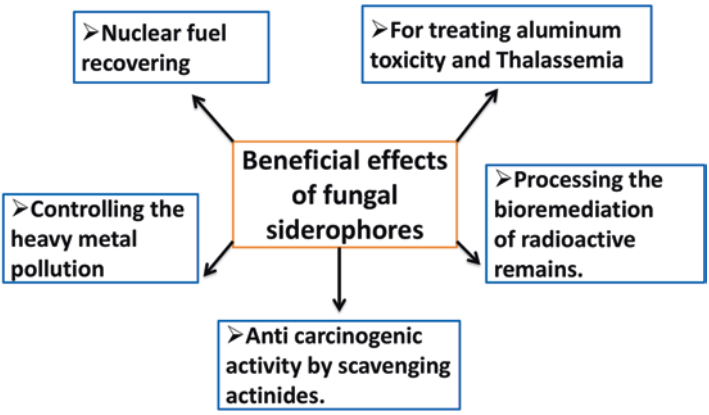

Fig. 1.4 Beneficial effects of fungal siderophores

siderophore through N-acyl moiety encircling the metal and the coordinating dimensions of ferric iron. In addition to iron, siderophores can bind to other metals such as actinides, lead, chromium, and aluminum ions. There is an increasing interest within the research community to optimize siderophores in terms of biological remediation of toxic substances and ecological scavenging.

Fungal siderophores are found to be useful in the field of medicine and biotechnology (Renshaw et al. 2002; Ahmed and Holmstrom 2014):

(i) Fungal siderophores have significant role in nuclear fuel recovering, biochemical remediation of fields contaminated with metals, and retreatment of industrial wastes.
(ii) Clinically, siderophores are useful in treating aluminum toxicity and thalassemia, a type of hereditary hemolytic disease. Ferrioxamine B is the most extensively tested siderophore for its potential medical use. DFO siderophore is useful in treating acute lymphoblastic lymphoma. A variety of siderophores have been documented for their antineoplastic activity.
(iii) The intoxication with aluminum can precipitate neurodegenerative diseases such as Alzheimer's disease. The ability of siderophores to uptake the elements other than iron is being investigated. However, promising evidences have not yet been achieved till date.
(iv) The fungal siderophores and their analogues are found to be useful to scavenge actinides. Actinides are the elements ranging from actinium to lawrencium in the periodic table. The actinides are found to be carcinogenic in nature.
(v) The environmental pollution from the industries, motor vehicles, sewage, and sludge lead to heavy-metal pollution. The most common are from seven metals, mercury, cadmium, arsenic, copper, lead, nickel, and chromium. The weapons, nuclear power stations, and their testing protocols lead to significant hazards. Fungal siderophores might be useful in controlling the heavy-metal pollution. Future studies on the ability of the fungal siderophores to absorb the hazardous metals might be an interesting research topic to explore.
(vi) The chelating nature of fungal siderophores is useful in processing the bioremediation of radioactive remains.

1.11 Potential Research Directions

The influence of the chemical structures of siderophores on various medical and biotechnological applications can be investigated using computational approaches and bioinformatics. The ecological housekeeping activity and the environmental applications of fungal siderophores are less known. These are the potential research areas for microbiologists. The effectiveness of structurally different variants of siderophores in iron-scarce environments such as saline water and certain type of soils are yet to be explored. An in-depth structural analysis and genomic studies may enhance the existing healthcare applications. The association between genes and fungal siderophores may open up new knowledge on the diseases and related pathways.

1.12 Conclusion

Siderophores are secreted both in bacteria and fungi especially for iron uptake. While bacterial siderophores are widely studied, less information is known about fungal siderophores. This motivated us to write a brief introduction on fungal siderophores, their classification, biosynthesis, iron uptake and storage, application in medicine and biotechnology, and many more. In addition, we included the potential research directions on fungal siderophores that might be useful for researchers from diverse disciplines including microbiology, computational biology, and bioinformatics.

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Chapter 2
Inhibition of Siderophores in Blocking Fungal Infection

Sonam Bhatia and Shaminder Singh

Contents

2.1 Introduction

A perilous increase in the number of microbial infections has led to develop throngs of antibiotics for increasing the quality and expectancy of life. However, the gains are just transient as the pathogens resist the antibiotic attack and generate the resistant forms called "persisters" which become drug tolerant. These pathogens are found to utilize a number of ways to evade antibiotic attack and thus acquire MDR (multidrug resistance) forms which are resistant to multiple antimicrobials event in combined therapeutic regimes (Sunenshine et al. 2007; Beck-Sagué et al. 1992). Thus, the chance of developing resistance increases among pathogens that can withstand antibiotic stroke and thereby spread of the infection. The resistance can be acquired either by plasmid/chromosome encoded or can be efflux pump medi-

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K. Dhusia et al. (eds.), *Fungal Siderophores*, Fungal Biology,
https://doi.org/10.1007/978-3-030-53077-8_2

ated, etc. (Gootz 2010). These pathogens also have the dexterity to communicate with each other by the process of "quorum sensing" where the process of cell-to-cell signaling is under the control of "autoinducers" which are classified as signaling molecules. Quorum sensing regulates the expression of virulence gene and the traits that are involved in biofilm formation, thus making the pathogen more resistant toward antibiotic attack. The severity of antimicrobial resistance catches the attention of the World Health Organization (WHO) which is drafting the global action plan on the problem of antimicrobial resistance and the ways to combat this crisis (WHO 2014). Various national and international agencies are striving to promote awareness on the problem, rational use of antibiotics, measures for controlling outbreaks, and design of new antibiotics or adjuvant therapeutics which can increase the sensitivity of known antibiotics/antimicrobials (White House Executive Order 2014; Chaudhary 2016).

One feasible strategy to overcome antimicrobial resistance is to target the physiological pathways responsible for acquisition of essential microbial nutrients (Clatworthy et al. 2007). Iron is a vital micronutrient for virtually all forms of life where its main role is to act as cofactor of key enzymes which plays a role in energy generation, DNA replication, RNA synthesis and other cellular processes, etc. and ultimately responsible for the survival of microorganism (Skaar 2010). Thus, acquiring iron is crucial for the sustainability of pathogen inside the host. It is well documented that pathogen depends on host machinery for the requirement of iron and during inflammation and febrile conditions, the sequestration of iron and other micronutrients from the pathogen provides "nutritional immunity" to the host (Vasil and Ochsner 1999). Limiting the iron requirement to pathogens interferes with its metabolic processes and thus inhibits their growth. In contrast, microbial pathogens, viz., bacteria and fungi, have evolved with the complex system to uptake iron from host, including hemoproteins, other iron-binding proteins (transferrin), and iron chelators called "siderophores" (Cornelis and Matthijs 2002). These are low molecular weight (< 1 kDa) compounds which have strong affinity for iron; their complexes with iron serve as a means of transport of iron across the cell membranes. These diverse compounds have great structural and functional properties (Renshaw et al. 2002).

These "iron carriers" are produced and secreted by bacteria, fungi, and monocotyledons plants under the condition of low iron stress and, thus, serve as carrier for uptaking iron for pathogenic microbes from the host environment. Figure 2.1 depicts the pathway of uptake of iron (III) by hydroxamate-based fungal siderophores of *Aspergillus fumigates* (Moore 2013). The fungal cell wall is made of chitin and glucan and allows limited permeability of nutrients to periplasmic space and plasma membrane (Farkas 1985). However, this permeability keeps on altering during different phases of growth. During iron-deficient conditions, the siderophores are released into the external environment and form complex with iron (III) form. This complex is well recognized by membrane proteins which facilitate the transport of bulky iron-siderophore complex across the cell

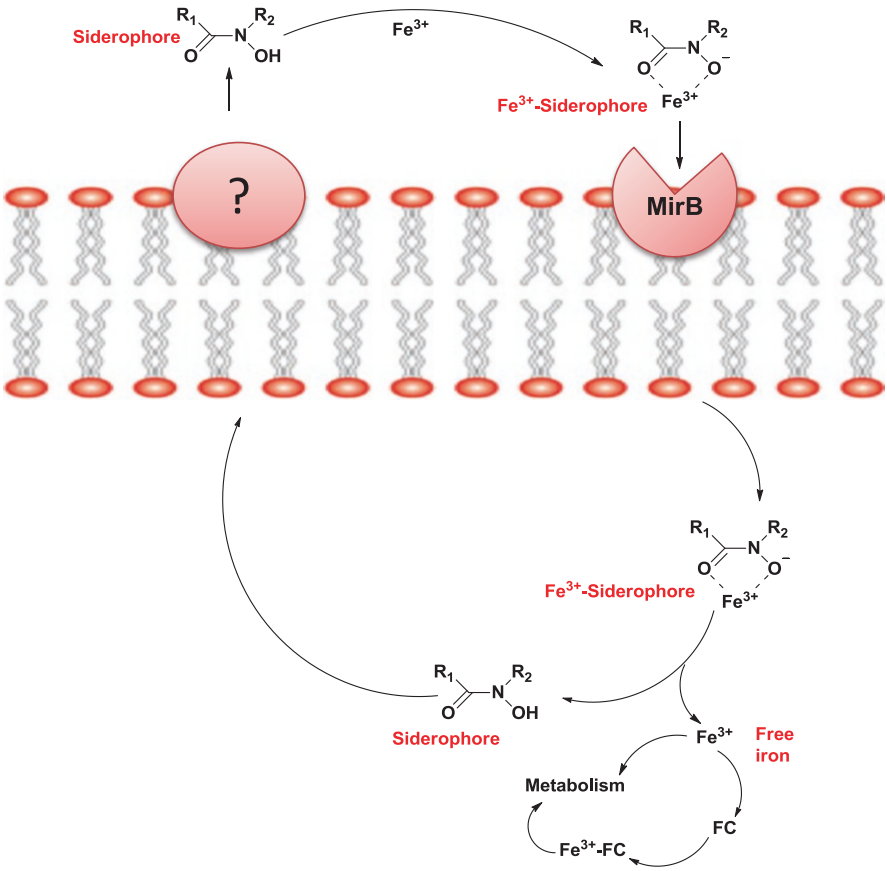


Fig. 2.1 Pathway depicting the uptake of ferric iron through hydroxamate-based siderophore in *Aspergillus fumigatus*

wall (Raymond-Bouchard et al. 2012). The research in this field have gained attention five decades ago, and interest in exploring the chemical and biological perspective of siderophore reached the momentum when it was realized that most aerobic and facultative anaerobic microorganisms synthesize at least one siderophore for their sustainability (Neilands 1995; Haas 2003). The coordination property of siderophores is well studied by Abdallah et al. where it was proven that these compounds form complexes with only higher oxidation state of iron (Albrecht-Gary et al. 1994). Apart from forming iron complexes, these siderophores also play a crucial role in process of quorum sensing. Recently, the investigation suggests that there exists a well-defined link between process of quorum sensing and siderophores and iron uptake mechanism (McRose et al. 2018). Kamino et al. have demonstrated that microbial population in marine environment respond to artificial addition of siderophores and homoserine lac-

tone (autoinducers) and, thus, chances of their possible identification and isolation increase to many folds by adopting such strategy (Kanoh and Kamino 2001).

The most life-threatening human fungal pathogens like *Aspergillus*, *Candida*, and *Cryptococcus* utilize the siderophore-mediated iron uptake mechanism either for their survival or virulence (Steinbach 2010; Park et al. 2009). Moreover, the risk of infection increases too many folds in immunocompromised patients which easily gained opportunistic infections. Thus, interfering with the iron acquisition mechanism and developing nutritional deficiency by blocking siderophore production leads to the development of new class of antifungals which are safer for mammalian host.

2.2 Nature of Siderophores and Their Production

Blocking of siderophore production in fungal pathogens is considered as a novel approach for developing antifungals based on fungal-specific target. There are numerous enzymes found in a microorganism which synthesizes siderophores. These enzymes can be targeted to achieve bacteriostatic or bactericidal action by depleting their iron requirement. Siderophores are typically divided into two classes: (a) hydroxamate and (b) α-carboxylates (only exception rhizoferrin) classes, respectively (Renshaw et al. 2002). This classification is developed depending on the nature of functional groups which are coordinating with the ferric iron. The hydroxamate-containing siderophores consist of N^5-acetyl-N^5-hydroxyornithine residue as the iron-binding ligand, which can be further subdivided into four structural classes, viz., (i) rhodotorulic acid, (ii) ferrichromes, (iii) fusarinines, and (iv) coprogens, depending on their substitution (Van der Helm and Winkelmann 1994) with different acyl groups as shown in Fig. 2.2. In

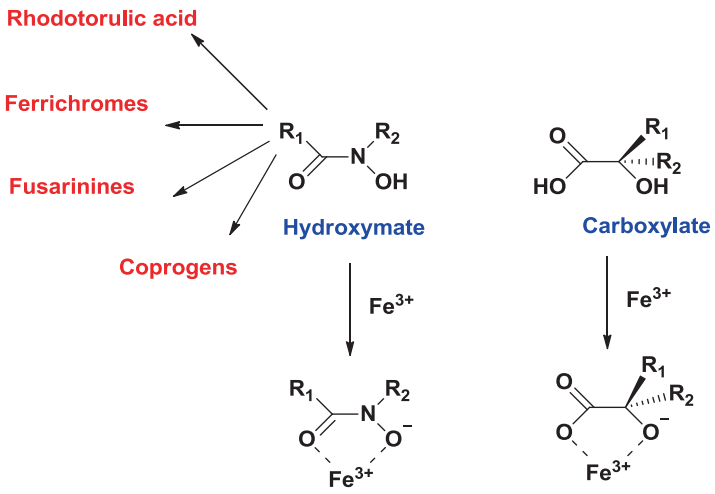


Fig. 2.2 Classification of diverse category of fungal siderophores

contrast to this, α-carboxylate-based siderophores are produced by the fungal group *Rhizopus microsporus* and *Zygomycetes* which synthesized rhizoferrin (Baakza et al. 2004). However, rhizoferrin represents a fairly simple molecule consisting of citric acid linked to diaminobutane but possesses potential metal-binding properties.

The requirement of iron uptake and virulence of one of the major opportunistic fungi *A. fumigatus* is also dependent on the production of siderophore molecules (Schrettl et al. 2004). It produces varied siderophores for acquisition of iron from extracellular and intracellular environment. *A. fumigatus* synthesize fusarinine type of siderophores for procurement of iron from extracellular location, viz., fusarinine C and its acetylated derivative triacetylfusarinine C (TAFC) (Miethke and Marahie 2007; Hissen et al. 2005). However, ferricrocin [a ferrichrome (FC) representative] fulfils the intracellular iron requirement of *A. fumigatus* (hyphae) for carrying out distribution and storage of iron inside the cell. The major enzymes involved in the biosynthesis of hydroxamate-type siderophores are produced by non-ribosomal peptide synthetase (NRPS) multienzymes (Mootz et al. 2002; Eisendle et al. 2003). NRPS is a large multifunctional enzyme which is capable of synthesizing peptides in absence of mRNA and ribosome by linking various amino acids (proteogenic and non-proteogenic) through peptide or thioester bond. Mostly, fungal siderophores are comprised of hydroxamate motifs which are synthesized using non-proteogenic amino acids (e.g., L-ornithine) which are further processed by undergoing several reactions on the multiple domains of NRPS enzyme. Some siderophores like TAFC and hydroxyferricrocin need post-synthetic modifications after their release from NRPS domains (Eisendle et al. 2003).

The first step involved in the biosynthesis of hydroxamate-based fungal siderophores is hydroxylation at N5 position of L-ornithine (provided from mitochondrial pool) to form N5-hydroxylated ornithine which is catalyzed by ornithine-N5-monooxygenase (NMO) as represented by a flow diagram in Fig. 2.3. This oxygenation process utilizes the O_2, NADPH, and FAD as cofactor. The enzyme NMO is encoded by *SidA* gene of *A. fumigatus* which plays a crucial role in biosynthesis of siderophore. This gene is also involved in spreading the virulence of pathogen as confirmed through gene deletion studies in mice (Hissen et al. 2005). The next step involves acylation at N5 position by a transfer of acyl group (acetyl or anhydromevalonyl) from acyl coenzyme A and its derivatives. The enzyme involved in acylation is anhydromevalonyl or acetyl coenzyme A-N5 -transacylase. These enzymes are encoded by *SidF* (upregulated by iron starvation) and *SidL* gene (which is constitutively expressed) and lead to synthesis of TAFC and FC type of siderophores, respectively (Eisendle et al. 2003; Blatzer et al. 2011). The mevalonate biosynthetic pathway is linked to siderophore biosynthetic pathway via anhydromevalonyl-CoA. The genes responsible for the synthesis of anhydromevalonyl-CoA are *SidI* (encodes acyl-CoA synthase) and *SidH* (encodes enoyl-CoA hydratase). The next step involves the NRPS enzymes, which link the different hydroxamate units generated in previous steps through ester or peptide bonds and, thus, synthesize macrocyclic peptides which are capable of chelating iron for nutritional requirement. These NRPS enzymes are co-

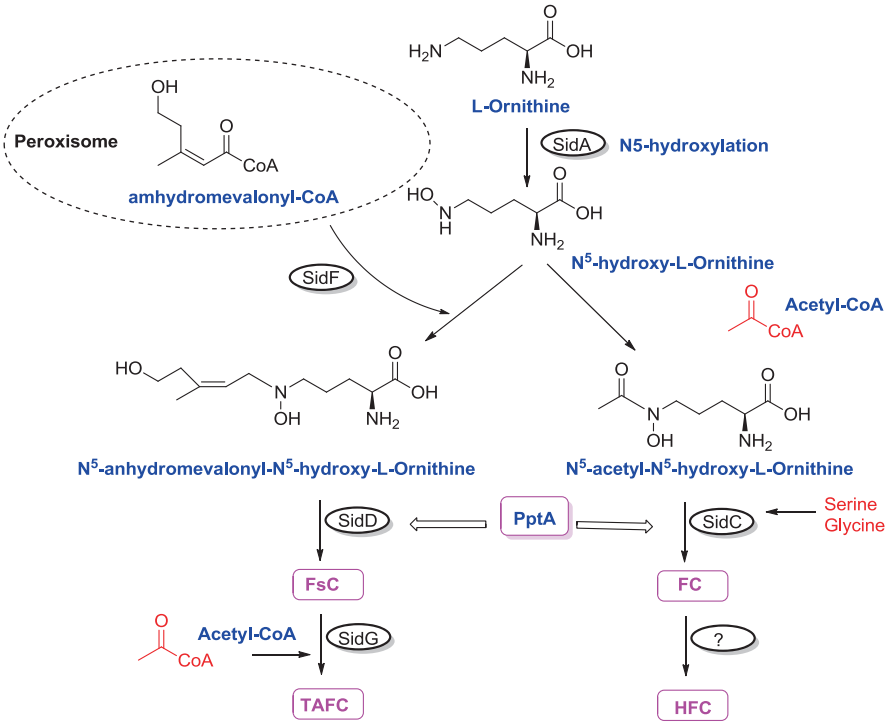


Fig. 2.3 Representation of siderophore biosynthetic pathway

expressed and co-regulated in condition of iron stress. The chief NRPS enzymes involved in biosynthesis of siderophores in *A. fumigatus* is encoded by *SidD* (FC and TAFC synthesis) and *SidC* (FC synthesis) (Yasmin et al. 2012). TAFC is produced by acetylation of FC by the enzyme transacetylase encoded by *SidG* gene. The activation of NRPS enzyme is led by PptA (phosphopantetheine transferase enzyme) which converts apo-NRPS into its activated (holoenzyme) form (von Döhren 2004).

2.3 Screening of Compounds as Inhibitors of Siderophore Biosynthesis

The general protocol followed for screening the inhibitors of siderophore biosynthesis is to grow a bacterial culture in iron-limiting conditions. Those compounds which prevent the growth of bacteria under such conditions are considered as potential candidate. This protocol is more effective in pathogens which have single mechanism for iron uptake, while those having more than one system for iron acquisition were found

to be less susceptible toward drugs targeting the single system. However, the relation between siderophore biosynthesis and virulence is complex, and thus, this architecture networking needs to be explored more in terms of its understanding at cellular level.

2.3.1 Screening Through Biomonitor Organism

A generic screen was identified for finding biosynthetic inhibitors of siderophores which proves to be beneficial in terms of cost and effectiveness. The opportunistic fungal pathogen *A. fumigatus* is widely used as biomonitor organism (generic screen) for screening purpose. This protocol is divided into two steps wherein at first step, *A. fumigatus* is grown in either iron-deficient or iron-rich medium. Compounds which show sluggish growth of pathogen in iron-limiting medium but allow growth in iron-rich medium are taken forward for further evaluation. In the second step, the fungi are grown again in iron-limiting medium, and those compounds which have shown limited production of siderophore which is confirmed and quantified by colorimetric assay were considered as potential candidates. In presence of ferric ions, the Fe(III)-siderophore complex displays red color which shows reduction in intensity in presence of blockers of siderophore biosynthetic pathway (Pinto and Moore 2009).

2.3.2 Screening of Compounds by Using Structural Similarity Approach

This is yet another approach based on identification of siderophore inhibitors which resemble siderophores more closely in terms of their structural features. This strategy as of now is more utilized in targeting bacterial pathogenesis of *Mycobacterium tuberculosis* and *Yersinia pestis*. Mycobactin and yersiniabactin are the corresponding siderophores produced by these organisms (Stirrett et al. 2008). The pharmacophoric features of the compounds were identified which cause inhibition of siderophore production, and thus, the pharmacophore can be used for screening the library of compounds which can act as structural mimics of siderophore. Based on EC_{50} values and interference in bacterial growth, the screened compounds were categorized as bacteriostatic and bactericidal. Ravichandiran et al. conducted docking-based studies to screen plant-based inhibitors of *SdiA*. The top five hits were assessed for their binding affinity, and in vitro assay was performed where compound 7-(1-bromoethyl)-3,3-dimethyl-bicyclo [4.1.0] heptan-2 was found to interact with PHE 63, TYR 67, TRP 71, and VAL 86 amino acid residues in the active site of *SidA* where it competes with the natural ligand (Ravichandiran et al. 2012).

2.4 Blocking Siderophore as an Alternative Approach Toward Antifungal Therapy

The physiological conditions of host cell are not favorable for the growth of pathogen under scarcity of free iron concentration due to the presence of iron-binding proteins, e.g., transferrin and ferritin. Further, the condition is more aggravated by the release of iron chelators and iron sequestering agents which compete with fungal siderophores for the acquisition of iron. However, the immune function of host is misbalanced in condition of severe fungal infections where the recruitment of iron deficiency itself in the pathogen can be a way to bring fungicidal effect. Such condition can be generated by blocking the synthesis of siderophore where the following discussed approaches were proven to be highly beneficial in mitigating the fungal pathogenesis and its virulence.

2.4.1 Enzymatic Inhibitors Blocking the Production of Siderophore

The highly impactful approach to inhibit iron-dependent fungal infection is to target iron requirement itself which will act as a fungicidal condition, preventing the growth of fungus. One of the best ways to achieve this condition is to block the biosynthesis of siderophore. The generalized protocol adopted for identifying compounds which can inhibit siderophore production was to grow the pathogen (*A. fumigatus,* biomonitor organism) in iron-limiting and iron-rich medium as described earlier in Sect. 2.3.1 (Pinto and Moore 2009). Catalytic site inhibitors of important enzymes which are involved in biosynthetic pathway of siderophore production are method of choice followed by many research groups who are active in this area. The initial step of N5 hydroxylation of L-ornithine in siderophore biosynthetic pathway is a crucial step and catalyzed by NMO encoded by *SidA* gene which can be a target of choice in *A. fumigatus* due to absence of homologue of *SidA* in humans. The crystal structure of this protein with and without ligand is present in Protein Data Bank (PDB) which can be utilized for designing site-directed inhibitors of NMO. By using fluorescence polarization (FP) binding assay, screening of library of putative inhibitors of NMO has been carried out which results in identification of sanguinarine sulfate as a competitive inhibitor in *A. fumigates* (Olucha and Lamb 2011). The developed assay exhibits good tolerance to temperature, incubation time, and concentration of dimethylsulfoxide (Qi et al. 2012; Robinson et al. 2015). Subrado and coworkers utilized the homologous approach and screened 2320 compounds using FP-based high-throughput screening assay system for identifying inhibitors of NMO. The study suggested that celastrol (a natural quinone analogue) was found to inhibit NMO in a noncompetitive fashion by reported MIC of 2 μM (Martín del Campo et al. 2016). In biological evaluation, the compound shows no growth of pathogen (*A. fumigatus*) on fungal growth medium. Absence of growth clearly reflects that the celastrol binding leads to *SidA* inhibition and, thus, blocks

the biosynthesis of siderophore. Similar approach was also applied for targeting kynurenine monooxygenase (KMO) for reducing the pathogenesis of *Trypanosoma brucei* among African population suffering from neurodegenerative disorders. The high-affinity KMO inhibitor (Ro-61-8048) was recognized which was found to be effective in manipulating the kynurenine pathway in a highly reproducible mouse model of human African trypanosomiasis (Rodgers et al. 2009; Zwilling et al. 2011). Hence, such site-directed inhibitors can be explored using high-throughput screening which can be a virtuous approach for targeting antifungal therapy. In contrast to this, another approach involves the blocking of multifunctional NRPS enzymes which synthesize macrocyclic secondary metabolites including siderophores. The secondary metabolism and virulence is closely linked in fungal pathogens invading plants and humans (Keller et al. 2005). *A. fumigatus* and *C. neoformans* are the examples of human-invading fungus which produces melanin as a product of secondary metabolism which contributes in their pathogenicity by quenching reactive oxygen species and protecting hyphae by invasion from human monocyte. This NRPS synthesis is activated in the biological system by phosphopantetheine transferase (PptA) which catalyzed the processes of phosphopantetheinylation that proves to be a novel target for antifungal therapy in *A. fumigatus* through gene-specific mutant studies (Horbach et al. 2009; Allen et al. 2011). Through FP-based assay, the screening of potential inhibitors against PptA was carried out where the activity of targeted enzyme can be monitored by quantifying the polarization signals. This protocol can be extended further for the determination of toxicity of potential inhibitors for evaluating the therapeutic index of lead compounds. Another advantage for targeting PptA was to design pathogen-specific inhibitors as the PptA enzyme was found to differ considerably in its sequence between host (humans, plants, animals) and pathogen (bacteria and fungi). Hence, this target is newly identified and can be explored in a logical manner for identifying pathogen-specific antifungal drugs. The diverse approaches adopted against siderophore-linked iron uptake process in fungal pathogens are enlisted in Table 2.1.

Table 2.1 Different targets used against siderophore-linked iron uptake process in fungal pathogens for the development of antifungal drugs

Enzymatic inhibitors			Trojan horse approach		
			Siderophore-drug conjugates (SDC)		
Siderophore biosynthetic pathway	Isoprenoid biosynthetic pathway	Antifungal proteins	Natural SDC	Synthetic SDC	Metal-siderophore complex
N^5-Hydroxy-monooxygenase inhibitors (NMO)	Statins alone and in combination	Anti-*Aspergillus* protein	Albomycin	Isocyanurate-based siderophore	Ga-siderophore complex
Phosphopantetheine transferase inhibitors (PptA)		Anti-*Saprolegnia* protein	Ferrimycin	Compound 9924129	Co-siderophore complex
NRPS adenylation domain inhibitors			Salmycin	Siderophore-beta lactam complexes	–

NRPS enzymes have various domains each with specific functions like adenylation, thiolation, condensation, etc. These domains are either arranged in linear or nonlinear (as present in fungal NRPS) fashion. Researchers are also exploring the inhibitors of adenylating enzymes by employing rational drug design strategy to inhibit stand-alone adenylating domain. So far, this approach is utilized for inhibiting the bacterial species like *Mycobacterium tuberculosis*, *Pseudomonas aeruginosa*, and *E. Coli* (Finking et al. 2003; Ferreras et al. 2005). On similar lines, the inhibitors of salicylate adenylation enzymes (salicyl-AMS) were also examined in the hope that there mechanism of blocking the substitution of acylphosphate group in adenylating enzymes will prove to be a novel way to curb siderophore biosynthesis. However, this strategy is having limitation in terms of toxicity as this enzyme is having close homology with aminoacyl-tRNA synthetase of host which is an important regulating enzyme in ribosomal protein synthesis. This problem can be overcome by targeting selectively NRPS-adenylating domains of pathogen by employing ligands carrying macrocyclic structural framework, and thus, these ligands inhibit the enzymes in different conformational fashion as compared to aminoacyl-tRNA synthetases, and thus such selective inhibition approaches open new routes to develop novel antibiotics (Cisar et al. 2007).

In case of fungi, the several approaches adopted to inhibit adenylate domain specifically are still in the emerging phase (Stack et al. 2007). Lee et al. have reported the crystal structure of the third domain of siderophore-synthesizing NRPS enzyme of *Neotyphodium lolii* fungal pathogen (Lee et al. 2010a, b). The structural insights revealed the large binding pocket of adenylating enzymes for binding bulky peptidal substrate. The active site was found to have 17 amino acid residues with the divergence in signature sequences which were found in comparison to prokaryotes. In such scenario, the rational use of homology modeling is quite effective for comparing the structure and sequence of unknown domains with sequence of known specificity. Thus, the major focus was to develop precise methods for identifying substrate specificity toward domains of unknown specificity in NRPS enzymes. In spite of available literature on structural features of eukaryotic NRPS and its mechanism of regulation, more such studies are needed to understand thoroughly the nature of this complex process, in order to design domain-specific inhibitors against fungal NRPS (Lee et al. 2010a, b).

2.4.2 Statins

Drugs belonging to statin class are often prescribed for lowering blood cholesterol levels. HMG-CoA reductase is the rate-controlling enzyme for mevalonate pathway which leads to biosynthesis of cholesterol. Thus, statins block the synthesis of mevalonate by inhibiting HMG-CoA reductase through competitive inhibition as shown in Fig. 2.4. During biosynthesis of siderophores, mevalonate motif acts as an important structural template in structural framework of extracellular siderophores. Hence, mevalonate

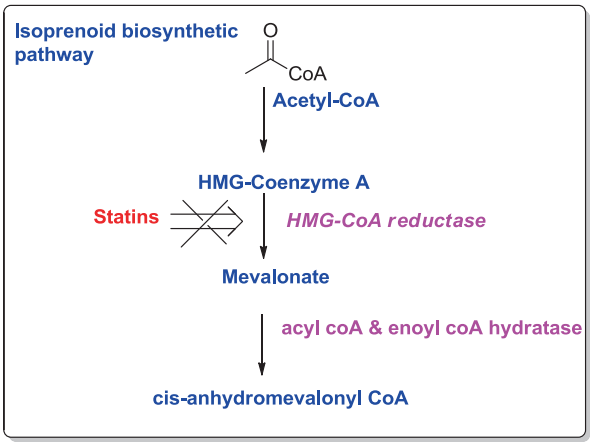


Fig. 2.4 Sketch of isoprenoid biosynthetic pathway inside peroxisome which leads to synthesis of mevalonate

pathway can be targeted by inhibiting HMG-CoA reductase by statins class of drugs like simvastatin and lovastatin which can interfere in the biosynthesis of siderophores. Further, isoprenoid biosynthetic pathway and siderophore biosynthetic pathway are interconnected by cis anhydromevalonyl-CoA which is transported across peroxisome to cytoplasm for entering into siderophore biosynthetic pathway (Leal et al. 2013).

The potential of statins in the field of antifungal therapy has been investigated, and it was found that these drugs have promising application, either alone or in combination with other antifungal drugs to treat infections (Galgóczy et al. 2009; Macreadie et al. 2006). There was also a report that antifungal activity of statins is observed only in higher concentration. So, in order to reduce its dose, the combined formulation of statins and azoles was tried, and these combinations show synergistic interaction. On this track, the combined dose form of lovastatin and voriconazole was studied, and these formulations were found to be effective against various pathogenic fungi (especially *Zygomycetes*) (Chamilos et al. 2006). Further, more such combinations are under development phase for curbing the opportunistic infections caused by fungal pathogens.

2.4.3 Peptide-Based Inhibitors

Recently, research group of Balhara and coworkers identified an antifungal protein which is called AAP (anti-*Aspergillus* protein) isolated from *E. coli* DH5α that can be found to show antimycotic effect against pathogenic isolates of *Aspergillus* spp. with a MIC in range of 5.62–31.25 μg ml^{-1}. The protein was purified using anion exchange chromatography and found to be falling in the range of 28–66 kDa after staining with silver stain. Further, from toxicological point of view, the isolated AAP was found to be safe for human erythrocytes at a dose 1000 μg ml^{-1} (Balhara

et al. 2014). The connection of AAP protein in interfering with siderophore biosynthetic pathway was established using mass spectrometry analysis which discloses the structural similarity of trypsin-digested fragments of AAP with siderophore biosynthetic protein in *A. fumigatus*.

Similarly, Zhang et al. reported the antifungal peptide for saprolegniasis (fungal fish disease) isolated from *Pseudomonas protegens*. The compound was found to be active against *Saprolegnia* spore germination and hyphal growth. MIC value of the purified protein against *Saprolegnia* spores was identified to be 0.0625 mg ml^{-1}. The stability of investigated peptide falls in the temperature range of 30 °C and 60 °C with narrow working pH range of 5.0–9.0. These investigations demonstrate the potential of *P. protegens* as measure for saprolegniasis in aquaculture industry.

Hence, this approach can be exploited to search for more such organisms producing antifungal proteins that can interfere with iron uptake machinery of pathogenic microbes by interrupting the biosynthetic pathways of siderophore production.

2.4.4 Trojan Horse Approach

One effective method to circumvent the permeability-mediated drug resistance was to utilize "Trojan horse" strategy. This concept is based on targeting the siderophores as antimicrobial weapon and killing the bacteria by either limiting the iron uptake mechanism of pathogen or by complexing the drug with siderophore as carrier and killing the pathogen by entering into it. This approach of siderophore-drug conjugation is found to be effective against bacterial infections (Fig. 2.5) (Górska et al. 2014). Figure 2.5 is a general sketch which represents how effectively the drug can be bound to siderophores and this strategic combination can be used effectively in many ailments. The recent studies on the mechanism of iron transport systems in yeast will help in investigating the potential of "Trojan horse" approach for delivering the drug inside the fungal cells. The presence of microbial membrane receptors for uptaking the iron from iron-siderophore complex is explored, and the mechanistic details of transporter proteins involved in the process help in designing novel drug delivery systems for antifungals. The drug is found to be covalently attached to iron (III)-siderophore complex by a linker. The function of linker is to attach the antibiotic to the siderophore and, thus, monitor its release inside the fungal cells. Trojan horse approach (THA) can also be employed for transporting heavy metal ions like Ga^{3+} into bacterial cell and thus could interfere in cellular iron uptake (Haas et al. 2015).

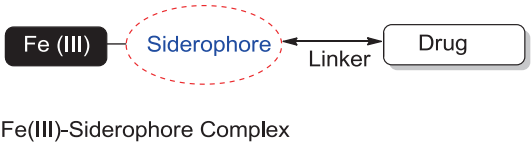


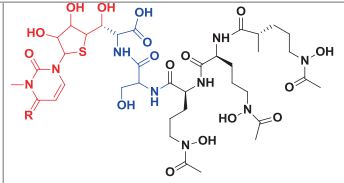
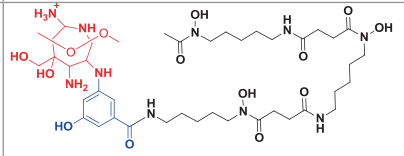
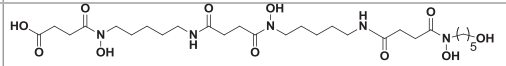
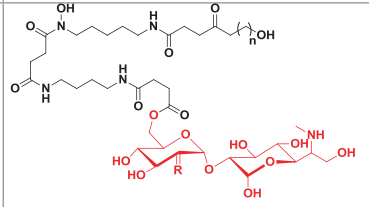
Fig. 2.5 Sketch of SDC employed in THA

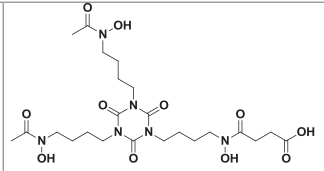
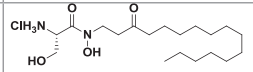
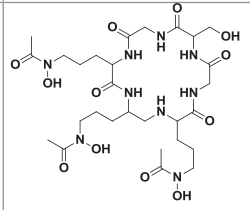
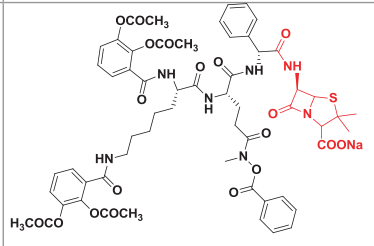
There are also few reports of natural siderophore-antibiotic complexes, viz., albomycins, ferrimycins, danomycins, and salmycins which are produced by *Streptomyces* or *Actinomyces*. The albomycin complex is made of tris(N^5-acetyl-N^5-hydroxyornithine) peptidal moiety which resembles functionalities of fungal siderophore (Table 2.2) (Pramanik et al. 2007). This moiety is having affinity for FhuA receptor and FhuD binding protein, from where it is transported to cytoplasm by ABC transporter which is located in the cell membrane. After entering into the cell by a facilitated transport, the toxic component of albomycin displays the antimicrobial activity by preventing the protein synthesis through inhibition of aminoacyl-tRNA synthetase (Schauer et al. 2008).

Ferrimycin (Table 2.2) is found to be effective against Gram-positive bacteria, particularly *Staphylococcus aureus* and *Bacillus* sp. Similarly, danomycins and salmycins also have antimicrobial action. Apart from this many research groups have synthesized artificial siderophores or conjugated antibiotics with well-established siderophores in order to modulate the pharmacokinetics of known drugs (Braun et al. 2009). In this category, Malouin and coworkers investigated the potential of isocyanurate- and hydroxamate-based SDC against *Candida* spp. The combined drug was 13C-desketoneoenactin (DE) (Table 2.2); the addition of siderophore and its concentration has positive effect on inhibitory action of drug (Bernier et al. 2005). This inhibitory activity is further increased to 16-fold when the conjugate was tested on organism growing in iron-depleting medium. Further, the studies on *Candida albicans* strain devoid of CaSit1/CaArn1 siderophore transporter protein have shown no uptake of ferrichrome or the synthetic siderophore by the organism for its growth promotion. It was also observed that the organism was less responsive toward SDC as compared to its wild strain. Hence, the design of new synthetic siderophores which can be selectively transported through CaSit1/CaArn1 transporter protein can be identified to improve/restore the activity of known drugs. In this category, need for the exploration of novel drug-siderophore linkers also emerges which can modulate the release of drug in desired concentration (Bernier et al. 2005).

Recently, Co(II) and Co(III) complexes of hydroxamate-based siderophores, desferricrocin (DFR) and triacetylfusarinine (TAF) (Table 2.2), were synthesized and investigated for their antifungal effect against solid stress agar cultures of *Penicillium brevicompactum* and *A. fumigates* (Farkas et al. 2018). The complexes of cobalt were found to be more stable in comparison to iron (III) complexes. Most of the antifungal drugs develop resistance due to altered permeability across the cell membrane, cell wall, and efflux of drugs by specialized pumps or reduce transport by active transport proteins, etc. These resistance mechanisms can be bypassed if mixed SDC will be synthesized and transported into the cell through different uptake pathways. The advantage of this THA has led to the development of new SDC which includes complexes of ciprofloxacin, ampicillin, amoxicillin, etc. (Ji et al. 2012). Mollmann and coworkers have synthesized siderophore moieties based on catechol framework. The metal-binding capacity of these moieties is analyzed by performing chrome azurol S (CAS) assay. Several complexes of synthetic siderophore and aminopenicillin conjugates were synthesized and found to be effective against *P. aeruginosa* infection. Compound 9924129 is one such conjugate of

Table 2.2 Examples of different types of siderophore with their chemical structures and uses

Name	Type	Chemical structures	Use
Natural occurring			
Albomycin	Sideromycins (antibiotic containing siderophore moiety)		Antimicrobial
Ferrimycin	Sideromycins (antibiotic containing siderophore moiety)		Antimicrobial
Danoxamine	Siderophore		Antimicrobial
Salmycin	Sideromycins (antibiotic containing siderophore moiety)		Antimicrobial

Synthetic			
Isocyanurate-based siderophore	Siderophore-antibiotic conjugate		Antifungal
13C-Desketoneoenactin (DE)	Antibiotic		Antifungal
Cobalt complexes of hydroxamate-based siderophore	Metal-siderophore complex		Antifungal
Compound 9924129	Siderophore-antibiotic conjugate		Antimicrobial

siderophore-ampicillin and found to be effective against both the wild and mutant strain of bacteria (Table 2.2) (Möllmann et al. 2009). Large number of drugs can be conjugated where their transport can be made effective by membrane receptors and pumping channels. Thus, THA can be exploited as novel approach to combat the problem of resistance in fungal infections with less toxic implications.

2.5 Conclusion

The various strategies that can be exploited for the development of antifungal drugs by targeting siderophore-mediated iron uptake system are enlisted in Table 2.1. Enzymes employed in biosynthetic siderophore production are majorly focused, and several studies pertaining to their characterization and inhibition are still in preliminary phase, but further understanding would open the ventures for pathogen-specific and nontoxic antifungal formulations. The considerable difference in the sequences of intermediary enzymes between host and pathogen helps in designing pathogen-specific inhibitors (da Silva et al. 2002). The major enzyme involved in siderophore synthesis is NMO which is found to be crucial for virulence. Analysis of active site of enzymes using X-ray crystallography and in silico methods helps in developing site-specific inhibitors. No compound is yet established to have credentials to be used at clinical level, but since the importance of targets in establishing virulence of pathogen is well proven, so there is some hope to develop drugs targeting siderophore-mediated iron transport among fungal pathogens (Balhara et al. 2016). Further, metal complexes of siderophore also have potential in diagnostics as radiotracer. Trojan horse approach (THA) was also found to have promising results in the fungal infections caused by resistant strains. Hence, by exploiting these approaches, more effective drugs in the near future will likely to flourish which can solve the riddle of antimicrobial resistance in the world of medicine.

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Chapter 3
Association of Fungal Siderophores in Human Diseases: Roles and Treatments

Saranya Jayapalan, Archana Prabahar, and Shankar Arumugam

Contents

Abbreviations

ABPA	Allergic bronchopulmonary aspergillosis
AFS	Allergic fungal sinusitis
IA	Invasive aspergillosis
PET	Positron emission tomography
TAFC	Triacetylfusarinine C

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https://doi.org/10.1007/978-3-030-53077-8_3

3.1 Introduction

3.1.1 Human Fungal Diseases

A number of diseases that impact human are caused by fungal infections, affecting over one billion people worldwide (Brown et al. 2012; Guinea et al. 2010; Marr et al. 2002). Like other microbiota, fungi also classified as helpful and harmful to human. In the case of harmful fungi, they invade the human body and adopt to the environment; they can be challenging to kill since the grow inside the body and reinfect the host again. Fungal diseases affect seriously the lifetime of human, but they are curable with antifungal drugs and different treatments invented in recent times (Denning 2006; Nascimento et al. 2003; Arikan et al. 1999). Most of the fungal infections are harmless; in some cases fungal infections lead to life-threatening problems that attack the blood, lungs, and other major human organs. Fungal pathogens are known to exist in the host system mainly based on the immune efficiency of the host. Importantly patients with immunosuppression such as those with impaired immune system (due to HIV, cancer, or undergoing treatments like chemotherapy) are at an increased risk of fungal infections (Denning 1998; Denning and Stevens 1990; Washburn et al. 1988). Invasive aspergillosis is a severe and aggressive fungal disease that arises in severely immunocompromised hosts (Denning 1998). *Aspergillus* (Denning 2017), *Candida* (Morrissey et al. 1996), *Pneumocystis jirovecii* (Carmona and Limper 2011), *Cryptococcus* species (Nyhus et al. 1997), *Histoplasma capsulatum* (Timmerman and woods 1999), and mucormycetes (Jamed William et al. 2006) are the most significant fungal pathogens responsible for the majority of fungal diseases. *Candida albicans* is responsible for mucosal disease (Moyes and Naglik 2011), *Aspergillus fumigatus* for the majority of the allergic fungal diseases (Latge 2001), and *Trichophyton* spp., particularly *T. rubrum*, for skin infections (Achterman and White 2013; White et al. 2014). Dermatophytosis is a type of fungal disease caused by dermatophytes, wherein keratinophilic fungi affect keratinized tissues of the body like the hair, nails, and skin (Carrillo-Munoz et al. 2007; Walsh and Groll 1996; Ghannoum 2009).

3.1.2 Aspergillosis

Aspergillosis is a universal and invasive infection affecting the human respiratory system which is caused by inhaling spores of the mold *Aspergillus*, existing in the environment (Vonberg and Gastmeier 2006). Diseases caused by *Aspergillus* are commonly called aspergillosis. In 1842 the very first case of human *Aspergillus* infection was reported, and it was aspergilloma (Cawley 1947). Two different non-invasive types of *Aspergillus* disease exists, viz., aspergilloma and allergic

bronchopulmonary aspergillosis (ABPA) (Latge 1999). Aspergilloma is a condition typically seen in patients with previously grown cavities in the lungs, while ABPA is generally found in patients with atopy and cystic fibrosis (Knutsen and Slavin 2011; Eaton et al. 1999; Denning et al. 2012). The failed immune system which is not able to prevent the entry of *Aspergillus* spores to the bloodstream via the lungs, which leads to the acute invasive aspergillosis stage.

Several groups of *Aspergillus* organisms, for example, *A. flavus*, *A. fumigatus*, *A. nidulans*, *A. niger*, and *A. terreus*, are known to cause fungal infections (Perfect et al. 2001; Baddley et al. 2001; Enoch et al. 2006). Among these fungi, *A.fumigatus* is known to be accountable for more than 90% of infections, followed in occurrence by *A. flavus* and *A. niger* (Lass-Florl et al. 2005; Balajee et al. 2009).

Aspergillosis is not transmissible from one person to another (Pegues et al. 2002). Symptoms of invasive pulmonary aspergillosis classically include fever, shortness of breath, chest pain, cough, or hemoptysis, although also include asthma, pneumonia, sinusitis, or rapidly progressing systemic illness.

3.1.2.1 Types of Aspergillosis

A wide range of diseases were caused by *Aspergillus* with respect to the health conditions and immune system of the individual, invasive aspergillosis to chronic forms of aspergillosis. *Aspergillus* spare known to infect the open spaces found in the human body which includes pulmonary cavities affected by previous lung disorders (Bronchiectasis, Lung tumor and Tuberculosis), the sinuses and the infection of external auditory canals.

Allergic bronchopulmonary aspergillosis (ABPA): ABPA is a form of lung disease that occurs due to *Aspergillus* infection, which is a hypersensitive reaction to *A. fumigatus*. This allergic reaction causes lung inflammation and weakens the immune system and leads to pulmonary fibrosis and finally lost lung function (Denning 2001). ABPA is a complex syndrome to diagnose, it is mostly affects the individuals who is having asthmatic patients and cyctic fibrosis patients (Latgé 1999).

Allergic fungal sinusitis (AFS): AFS is a common type of sinus fungal infection where *Aspergillus* causes inflammation of the sinuses. Various symptoms caused by this sinus infection include drainage, stuffiness, and headache. These patients are known to develop nasal polyps and asthma, and also the sense of smell of the patient is affected at chronic condition. When left untreated, this may lead to the loss of vision in affected patients (McClay et al. 2002).

Aspergilloma: Aspergilloma is also referred to as “fungal ball” which is a rounded mass of fungal hyphae ball that grows mostly in the lungs or sinuses. In most of the cases, the fungal balls in the lungs are due to the inhalation of small spores of

A. fumigatus with rare cases due to non-fumigatus. Patients suffering with underlying cavitary lung disease such as tuberculosis, sarcoidosis, bronchiectasis, systemic immunodeficiency, and cystic fibrosis are mainly affected by aspergilloma. The fungal development occurs within the cavity and is noninvasive and usually asymptomatic (Latgé 1999). The allergens and toxins secreted from the fungi inside the host organism may induce the symptomatic changes like cough, weight loss, and hemoptysis during massive and fatal stages of the infection (Chen et al. 1997).

Chronic pulmonary aspergillosis: Chronic pulmonary aspergillosis mainly affects the individuals with tuberculosis. In this condition one or more fungal balls may form in the lungs, and this likely to becomes a long-term (3 months or more) condition (Denning et al. 2011).

Invasive aspergillosis (IA): IA is a complex infection in human that is known to affect patients with weakened immune systems, such as those who are immunosuppressed due to organ transplant or a stem cell transplant. IA usually affects the lungs of the patients, but sometimes spreads to other parts of the body (Kramer et al. 1991; Kemper et al. 1993).

Cutaneous (skin) aspergillosis: In cutaneous infections of *Aspergillus*, the fungus enters the body through the pores in the skin and causes infection in patients with immune suppression (Hogan et al. 1996). Human immunodeficiency virus (HIV)-infected patients infrequently develop cutaneous aspergillosis; this invasive aspergillosis spreads to the skin from some other parts of the body, mainly the lungs. Several investigations stated the various conditions of cutaneous aspergillosis in HIV patients at different age groups (Jo-Anne H. van Burik et al.). Some of the non-HIV-infected immunocompromised patients with this infection include burn victims, cancer patients, infants, and bone marrow and solid organ transplant patients.

3.2 Role of Siderophores in Fungal Diseases

The need of new efficient therapeutics is in demand to overcome the resistance of fungal pathogens against the available antifungal agents and drugs. Iron acquisition mechanisms could be an efficient approach for fungal pathogens. Fungal siderophores are the high-affinity iron-chelating compounds secreted by fungi, serving as iron transporters to membrane (Neilands 1995). *Aspergillus* spp. have the ability to synthesize their own siderophores, in which iron is crucial for the growth and development of microorganisms. *Aspergillus* is one of the important opportunistic human fungal pathogens which secrete their own siderophores under iron-limiting conditions which is crucial for growth and development of fungal organisms and plays a significant role in virulence and propagating infection (Diekmann and krezdorn 1975).

Table 3.1 List of fungus and their respective siderophores

Aspergillus species	Siderophores
Aspergillus flavus	Aspergillic acid Ferrichrysin
Aspergillus FSY-01	Neoaspergillic acid
Aspergillus FSY-02	Neoaspergillic acid
A. fumigatus	Ferricrocin Fusarinine C N,N′,N″-triacetylfusarinin C
A. nidulans	Coprogen Ferrichrome Fusarinine C N,N′,N″-triacetylfusarinin C
A. niger	Ferrichrome
A. sclerotiorum	Aspergillic acid
A. terreus	Coprogen Dimerumic acid Ferrichrysin

Iron uptake mediated by siderophores are studied more deeply in the *Aspergillus* genus such as *A. fumigatus*, *A. flavus*, *A. nidulans*, etc. Unlike *Candida* species, *Aspergillus* species produce different hydroxamate-type siderophores such as ferricrocin (FC), hydroxyferricrocin, fusarinine C (FsC), coprogen B, and triacetylfusarinine C (TAFC) (Diekmann and Krezdorn 1975; Adjimani and Emery 1987; Schrettl et al. 2004; Hissen et al. 2005; Howard et al. 2000).

The list of siderophores produced by *Aspergillus* species is given in Table 3.1. Putative siderophore transporters determined in *Aspergillus* species are also shown.

From Table 3.1 it is observed that *A. nidulans* produce coprogen, ferrichrome, FsC, and TAFC. *A. fumigatus* synthesized its own siderophores such as FC, FsC, and TAFC. The two significant siderophores FsC and TAFC are commonly found in both *A. fumigatus* and *A. nidulans* (Park et al. 2016; Raymond-Bouchard et al. 2012; Haas et al. 2003).

3.3 Association of Siderophoric System in *Aspergillus* Species and Infections

Aspergillus species are commonly found in the environment and are known to play a vital role in carbon recycling of decaying organic debris. Though we inhale numerous amounts of conidia, several spores are removed by the lungs and hence prevent normal persons from developing *Aspergillus* infection, which may otherwise cause a serious threat to human health (Trick and Jarvis 1998). IA is mainly observed in immunocompromised patients which is mainly caused by *Aspergillus fumigatus*,

while *A. nidulans* very rarely causes IA. Due to delay in phagocytosis, there is difference in immune response of *A. fumigatus* against *A. nidulans* and also the recognition characteristics of *A. Nidulans* involves in slower cell migration and phagocytosis and also increases cytokine production. The biosynthetic pathways of *A. fumigatus* and *A. nidulans* are essential to understand the association of siderophoric systems involved in aspergillosis disease and their associated genes.

3.3.1 Aspergillus fumigatus

Aspergillus fumigatus produces more asexual spores that are widespread in the environment. These spores spread in the environmental air constantly inhaled by human and are usually removed by a functional immune system (Brakhage and Langfelder 2002; Latge 2001). However, patients with weakened immune system due to organ or bone marrow transplantation and cancer chemotherapy or those with renal failure have a higher risk of these fungal infections, termed as aspergillosis. *A. fumigatus* is commonly referred to as the most common airborne fungal pathogen in human.

3.3.2 Aspergillus nidulans

A. nidulans is an important aerobe which survives in anaerobic environment. Occasionally, *A. nidulans* causes human disease and belongs to filamentous model fungus. *A. nidulans* is a well-studied fungus which is closely related to several *Aspergillus* species, such as *A. fumigatus* (Brookman and Denning 2000). *Aspergillus nidulans* produces two main siderophores such as triacetylfusarinine C (TAFC) and ferricrocin (FC) (Charlang et al. 1981; Oberegger et al. 2001). The filamentous fungus *A. nidulans* synthesizes three major siderophores, namely, fusigen, triacetylfusarinine C, and ferricrocin. Putative genes of *A. nidulans* such as metalloreductase-encoding gene are also extracted (Oberegger et al. 2002).

3.3.3 Siderophore Biosynthetic Pathway in A. fumigatus *and* A. nidulans

Siderophores are known to play an essential part in the iron metabolism and fungal virulence. *A. fumigatus* and *A. nidulans* expel the siderophore triacetylfusarinine C (TAFC) which is involved in iron acquisition. An extensive literature survey was performed to identify the genes associated with siderophoric pathway, and input data were taken from publicly available databases for the analysis. Table 3.2 gives the *Aspergillus* spp. with their associated genes and siderophoric activity. About 20 genes are known to be responsible for iron homeostasis maintenance in *A.*

Table 3.2 *Aspergillus* spp. with their associated genes and siderophoric activity

Aspergillus spp.	Associated genes	Siderophore activity
A. nidulans	AN6238	Siderophore iron transporter, putative
	sidC	Ferricrocin synthetase (nonribosomal peptide siderophore synthase)
	sidD	Putative siderophore synthetase
	AN7801	Putative siderophore-degrading esterase
	sidF	Siderophore biosynthesis acetylase AceI, putative
	AN6239	Siderophore biosynthesis lipase/esterase, putative
	sidL	Siderophor Biosynthesis protein, putative
	mirB	Siderophore iron transporter MirB
	mirA	Siderophore iron transporter mirA
	mirC	Siderophore iron transporter mirC
A. fumigatus	sidD	Fusarinine C is involved in siderophore production and is known as extracellular nonribosomal peptide synthetase (NRPS)
	sreA	GATA transcription factor that represses genes in siderophore
	sidL	Siderophore biosynthetic pathway. GNAT-type acetyltransferase
	sidF	Extracellular hydroxyornithine transacylase involved in siderophore biosynthesis
	sidA	L-Ornithine N5-oxygenase helps in biosynthesis of siderophore and iron starvation
	zrfB	Zinc transporter that helps in heme biosynthesis and in the production of triacetylfusarinine C siderophore
	Afu8g01310	Helps in siderophore transport
	sidJ	Siderophore biosynthesis lipase/esterase
	sidC	Putative NRPS which is required for ferricrocin siderophore biosynthesis
	acuM	Putative genes for extracellular siderophore production
	mirB	Putative siderophore SrbA-regulated during hypoxia involved in iron transport
	mirC	Putative siderophore transporter
	Afu7g04730, sit1	Putative siderophore transporter
	mirD	Putative siderophore transporter
	hapX	bZIP transcription factor involved in transcriptional activation of the siderophore system

(continued)

Table 3.2 (continued)

Aspergillus spp.	Associated genes	Siderophore activity
A. niger	An03g01550	Siderophore biosynthetic process
	An05g01780, An04g05570	Domain with siderophore transmembrane transporter activity
	An16g01150	Ferric-chelate reductase activity involved in siderophore transport
	An01g00720	Helps in transport of siderophores
	An03g03530, An16g04320	Involved in hydrolase activity and siderophore catabolic process
	sidF	Siderophore biosynthetic pathway
	An02g14190	Involved in siderophore transmembrane transport
	An01g02370	Involved in iron transport and siderophore biosynthetic pathway
	An12g05510	Siderophore iron transporter
A. oryzae	AO090701000976	Plays an important role in siderophore biosynthetic process
	dffA	L-Ornithine N5-oxygenase involved in siderophore biosynthesis
	AO090701000932	Siderophore transportation and plasma membrane localization
	AO090124000035	Helps in siderophore transport and plasma membrane localization
	AO090005000471	Helps in ferrichrome transport and transmembrane transport
	AO090103000170, AO090701000114	Essential for hydrolase activity, acting on ester bonds and in siderophore catabolic process
	sidF	Siderophore biosynthetic process and peroxisome localization
	AO090003000906	Siderophore biosynthetic process and cytoplasm localization
	AO090001000692	Siderophore transmembrane transport
	sidC	Putative NRPS; ortholog of the siderophore biosynthetic enzyme of *A. fumigatus*

fumigatus, whereas *A. nidulans* is less pathogenic and is also involved in iron metabolism of filamentous fungi (Haas 2012). From the available information, a network of *A. nidulans* and *A. fumigatus* was constructed using Cytoscape 3.7.1 with genes and protein targets involved in biosynthetic pathway which are shown in Figs. 3.1 and 3.2, respectively.

Common genes between *A. fumigatus* and *A. nidulans* network are shown in Fig. 3.3. Genes such as mirB, mirC, sidD, sidL, and sidF are commonly occurring in both the species and have common protein targets. Low-affinity iron acquisition and siderophore-aided high-affinity iron uptake are acquired by both *A. fumigatus* and *A. nidulans* (Schrettl et al. 2004).

Triacetylfusarinine C is composed of three N2-acetyl-N5-anhydromevalonyl-N5-hydroxyornithine residues which are further cyclically connected by ester bonds

Fig. 3.1 *A. nidulans* network with genes and protein targets involved in biosynthetic pathway

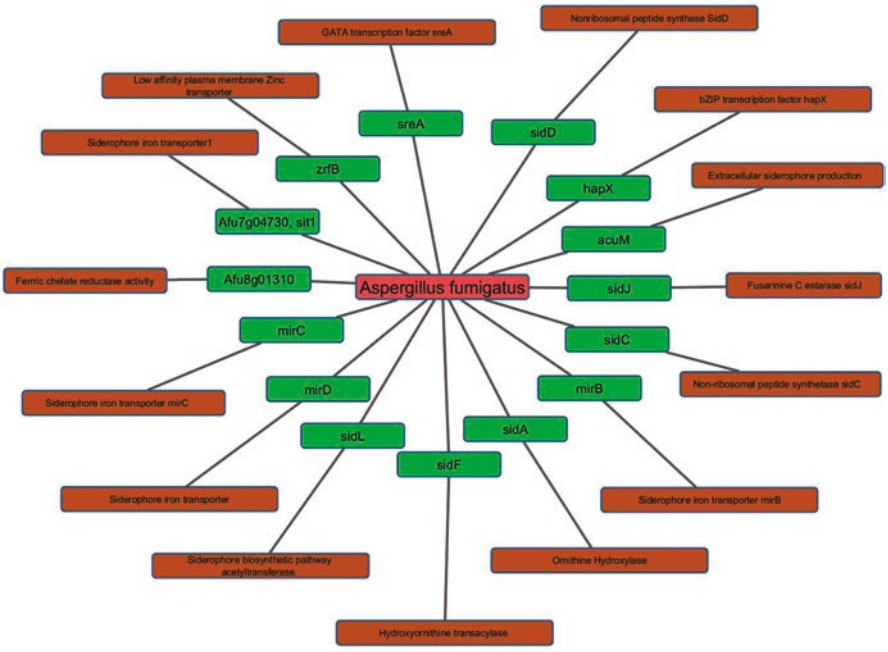

Fig. 3.2 *A. fumigatus* network with genes and protein targets involved in biosynthetic pathway

Fig. 3.3 Common genes and protein targets in *A. fumigatus* and *A. nidulans*

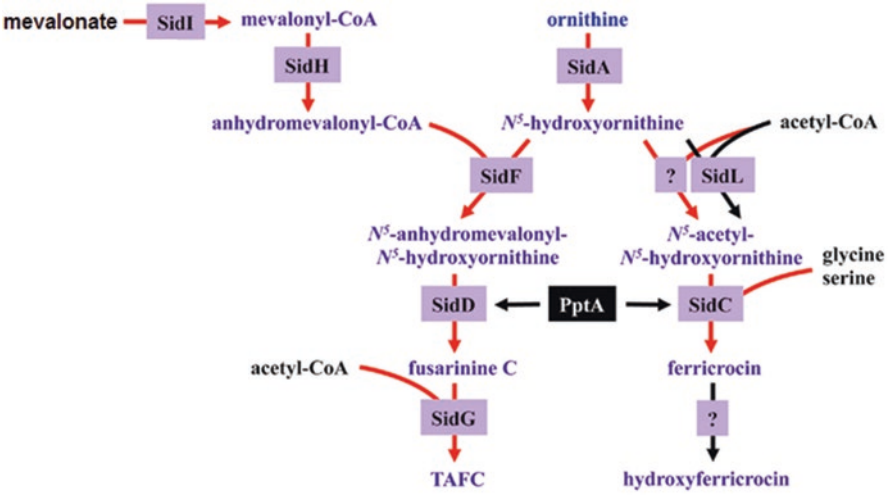

Fig. 3.4 The siderophore biosynthetic pathway of *A. fumigatus* and *A. nidulans*. (Adapted from Haas 2012)

which further convert to fusarinine C is a cyclic hexapeptide with the structure Gly-Ser-Gly-(N5-acetyl-N5-hydroxyornithine)3 (Haas et al. 2008). The siderophore biosynthetic pathway is shown in Fig. 3.4. The primary step in the siderophore biosynthesis involves the hydroxylation of ornithine catalyzed by the ornithine

monooxygenase SidA (Olucha et al. 2011). Second step involves the splitting of extracellular and intracellular siderophores. Extracellular siderophores are produced by transferring the anhydromevalonyl to N5-hydroxyornithine using transacylase SidF (Schrettl et al. 2007). This is produced by CoA ligation and dehydration catalyzed by SidI and SidH, respectively (Yasmin et al. 2011). FC biosynthesis involves two transacetylases such as SidL and an unknown enzyme that is upregulated by the starvation of iron. The assembly of FsC and FC is catalyzed by two NRPS such as SidD and SidC. Later, SidG catalyzes N2-acetylation of FsC for forming TAFC.

The extracellular and non-extracellular siderophores are vital for growth during iron limitation (ΔsidA mutant) in *A. fumigatus* and *A. nidulans* (Eisendle et al. 2003; Schrettl et al. 2004). Hence, the removal of siderophore biosynthesis leads to complete a virulence of *A. fumigatus* in murine model of aspergillosis. The lack in either extracellular (ΔsidI, ΔsidH, ΔsidF, or ΔsidD mutants) or intracellular siderophores (ΔsidC mutants) leads to partial attenuation of virulence (Schrettl et al. 2007; Yasmin et al. 2011). Biosynthetic pathways of fungal siderophores thus provide the potential targets for novel and selective therapy.

3.4 Diagnosis of Aspergillosis

Diagnosis of Aspergilloma is quite and difficult process, as *Aspergillus* species are commonly found in environment it is difficult to differentiate from other molds under microscopic examination (Ruhnke et al. 2003). Mycological investigation and diagnosis of invasive aspergillosis is difficult with the low diagnostic yield and sensitivity of the cultures particularly from lower respiratory secretions (Tarrand et al. 2003; Mennink-Kersten et al. 2004). Blood test may be preferred to diagnose people under early stage of invasive aspergillosis with weakened immune systems. Diagnosis is mainly by clinical experiments and aided with imaging techniques – computerized tomography (CT) scan, respiratory secretion (sputum) test, tissue and blood test, and biopsy (Nalesnik et al. 1980; Patterson et al. 1986; Kuhlman et al. 1987). Recently highly specific antibody-guided imaging technologies have been described for the in vivo diagnosis of fungal diseases in animal models, with high quality for translation to human disease diagnosis (Rolle et al. 2016).

Clinical diagnosis of invasive pulmonary aspergillosis (IPA) remains tremendously difficult, due to the fact that patients remain with nonspecific symptoms, and also does not have responsive biomarker activity during diagnostic procedures. Radiographic imaging of the lungs of the affected individual is the commonly used diagnostic method in these invasive fungal infections. Their regularity determined in chest CT report may reflect the presence of IPA, but it is not sufficient for complete diagnosis of this fungal disease (Nucci et al. 2010).

In case of *Aspergillus* sinusitis, fungal cultures are required to diagnose the infection accurately. Particularly fungi do not give fine stain with route stains, so the specially synthesized silver-impregnated fungal stains (Gomori methenamine

silver) with fungal cultures are essential for the deep diagnosis of sinusitis and its subclassification (Kim et al. 2012). Polymerase chain reaction (PCR) detects the genus/species of fungus in >70% of chronic rhinosinusitis (Kim et al. 2005), and cultures from tissue samples provide positive reports in 30–70% of cases of chronic fungal rhinosinusitis (Chakrabarti 2009; Challa 2010).

3.5 Treatment of Aspergillosis

With overall 200,000 known fungal species, only a few percentage have been described to be associated with human illness and diseases (Denning 1996). The mortality rate of invasive aspergillosis is compensated with the use of antifungal treatments; attainment of complete cure from the treatment is highly dependent on time of diagnosis, the type of invasive aspergillosis, and the immune response of the particular patient (Latge 2001). Since 1990, itraconazole and amphotericin B are the two important antifungal drugs used commonly for invasive aspergillosis (Denning 2006; Nascimento et al. 2003). Initially itraconazole was given to the patients whose body cannot tolerate amphotericin B. The kind of azole drugs, including itraconazole, inhibits the cytochrome p-450 which is involving in the ergosterol biosynthesis. Ergosterol is sterol which aids the formation of a significant element of the fungal cellular membrane (Nascimento et al. 2003).

Several allergic aspergilloses such as ABPA or allergic *Aspergillus* sinusitis, the commonly suggested antifungal drug is itraconazole; corticosteroids are also optionally used (Ng et al. 1994). In recent days, the newer and most effective voriconazole (Vfend) is used as standard antifungal drug for invasive aspergillosis (Tkacz and DiDomenico 2001). Amphotericin B also used in some cases as an optional medication.

IA in patients is treated with antifungal drugs such as voriconazole. After the approval of voriconazole in the United States in 2002 for clinical use, it has been used as an antifungal agent. Voriconazole is more effective against itraconazole-resistant strains of *A. fumigatus* (Arikan et al. 1994). The antifungal medications such as lipid amphotericin formulations, isavuconazole, posaconazole, itraconazole, micafungin, and caspofungin are employed to treat aspergillosis. People who are severely affected with aspergillosis may be treated by surgery. In the case of immunocompromised adults with invasive aspergillosis, caspofungin drug is given optionally to the patients who did not respond to tolerate other antifungal drugs. The new treatments and techniques against aspergillosis lead to the improved drug therapy and antifungal research, but many of the drug targets are yet to be studied. The development of new therapeutics needs to reduce the mortality and improve the survival rate of the patients with invasive aspergillosis.

3.6 Applications of Fungal Siderophores in Molecular Imaging

The low molecular weight chelators produced by fungi are referred to as siderophores and are key essential components to forage essential iron (Neilands 1995). These attractive iron-chelating properties of siderophores make them an exclusive system for fungal virulence and aid in diagnosis of fungal infections (Franceschini et al. 2012; Leal et al. 2013).

Positron emission tomography (PET) is a kind of nuclear medicine imaging method which is also used in the diagnosis of fungal aspergillosis to observe the changes in various metabolic processes in the body of patients (Denning 1998).

To overcome the limitations and disadvantages of CT scan when diagnosing the various fungal infections, fungal siderophores are widely used as imaging agents in disease diagnosis and improved therapy. *A. fumigatus* produces a siderophore, namely, triacetylfusarinine C (TAFC), for iron acquisition due to its application toward the disease diagnosis. TAFC is considered to be a specific marker for invasive aspergillosis. TAFC is a main siderophore of the two important fungi such as *A. fumigatus* and *A. nidulans*, while *A. terreus* and *A. niger* produce other siderophore types.

Recent researches showed that PET imaging can be done with the aid of siderophores for the diseases caused particularly by *A. fumigatus*. Aspergillosis in animal models was detected by PET with the use of gallium-68 chelated by TAFC or bacterial siderophore ferrioxamine for selective accumulation in fungal cells. Thus, TAFC acted as potential PET tracer with great sensitivity when it is labeled with the radionuclide gallium-68 (68Ga). The two siderophores such as triacetylfusarinine (TAFC) and ferrioxamine E (FOXE) were radiolabeled with 68Ga and used in PET imaging of illness in rats caused by *A. fumigatus* (Petrik et al. 2010, 2012, 2014).

In a research study, an attempt has been made by coupling CT and PET with [18F]-fluorodeoxyglucose ([18F]-FDG), an important marker of metabolic activity. Though new diagnostic approach is developed, it showed limited advantages in the invasive fungal disease diagnostics (Christopher 2018).

3.7 Conclusion

Aspergillus infections are emerging as life-threatening yet underappreciated and underdeveloped as compared to other microbial infections. New strategies for defense against *Aspergillus* infections are desperately needed to improve the survival rates of the patients with different forms of disease, and they are necessary to develop new therapeutics and treatments. In this chapter, we discussed various infections caused by *Aspergillus* species and their virulence in different stages of the disease. The two major *Aspergillus* pathogens *A. fumigatus* and *A. nidulans* were taken for the study, and their siderophoric system involved in biosynthetic pathway

has been discussed. We pinpoint the genes and targets involved in the biosynthetic pathways of *A. fumigatus* and *A. nidulans*. Applications of siderophores in the field of molecular imaging provide the contribution of TAFC in PET particularly in disease diagnosis. Hence these data suggest that siderophores as iron chelators are playing a crucial role in pathogenesis of *Aspergillus* infections and also contribute in antifungal therapy.

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Chapter 4
Siderophores Mediated Iron Acquisition and Virulence of Brown Rot Disease in Stone Fruits Caused by *Monilinia fructicola* in Jammu and Kashmir

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K. Dhusia et al. (eds.), *Fungal Siderophores*, Fungal Biology,
https://doi.org/10.1007/978-3-030-53077-8_4

4.1 Background

Fresh fruits and vegetables are considered essential for health as they provide all essential dietary supplements. Post-harvest damage prevention of these commodities is a matter of concern as it has lot of economic implications, both at producer and consumer level. It also affects the availability of raw material and products based on these items, especially in developing countries. The statistics has revealed that post-harvest fruit and vegetable damage can reach to very high values, representing over 25% of total production in industrialized countries and over 50% in developing countries. A variety of biotic agents are responsible for this damage to fruit and vegetable items. One major damaging group of organisms is fungus. Many strategies are being followed world over to control these organisms. The control of fungal phytopathogens is carried out through the application of synthetic fungicides. However, their continuous and consistent use has led to environment problems and are becoming health hazards for humans and animals. As a consequence, the worldwide trend of consumers to buy agricultural products that come from systems without application of synthetic products has started growing rapidly.

Therefore, global attempts are under way to search for alternatives to control post-harvest phytopathogenic fungi. Several species of fungi cause post-harvest diseases, including species belonging to *Alternaria, Aspergillus, Botrytis, Fusarium, Geotrichum, Gloeosporium, Mucor*, *Monilinia, Penicillium, Rhizopus*, and other genera. The control of these phytopathogens is commonly carried out through the use of synthetic fungicides, but their use is limited due to environmental and toxicological risks. Furthermore, the repeated and continuous use of fungicides has led to the development of fungus-resistant strains, making the fungicides ineffective against these strains.

One of the most critical physiological processes during in vivo pathogenesis is the maintenance of iron homeostasis. The most quarantined fungal pathogen of stone fruits is *Monilinia* spp. It has been identified as the brown rot disease of stone fruits and has developed strains resistant to several of the chemical fungicides. Therefore, the recent emergence of resistance, the toxicity paradigm, and the limited efficacy of conventional fungicides require the identification of de novo targets in the metabolism of phytopathogenic fungi.

One alternative can be seen in siderophores. They interfere with the action of pathogens because they can inhibit enzymes important for their establishment in post-harvest fruit wounds for survival, virulence, propagation, or resistance to oxidative stress predicted in vivo during infection. Therefore, the biosynthetic pathways of the fungal siderophores along with their recruitment and utilization mechanisms are an ideal target for specific pathogens' resistance-friendly strategy that would block the proliferation of pathogens without causing any damage to the host.

Iron is the essential component for various life processes (photosynthesis, enzyme cofactor, redox reagent, respiration, nucleoside, and amino acid synthesis) of the plant. The symptoms of iron deficiency include loss of photosynthesis, chlorosis, and various harmful processes at the cellular and molecular level.

To avoid iron deficiency, many plants rely on excretion mechanisms of phytosiderophores (i.e., chelated compounds, common in iron-sequestering herbs) from the roots and the secretion of siderophores by a group of microbes to facilitate absorption of the Fe complex under iron deficiency, for maintaining iron homeostasis by controlling the absorption. Use and storage of iron depends on its environmental availability (Neilands 1995). Although iron is one of the most abundant elements on earth, bioavailability is lower in aerobic conditions (in the presence of oxygen and neutral pH), mainly because ferric iron (concomitant oxidation of Fe^{2+} to Fe^{3+}) reacts with oxygen to form insoluble ferric hydro-oxide.

Pathogenic fungi of the genus *Monilinia spp*. includes numerous aggressive and economically important pathogens of the *Rosaceae*, *Ericaceae*, and *Empetraceae*. They are distributed all over the world and are one of the most limiting factors for the production stone and pome fruits, and also berries all over the world. These pathogens are often referred to as "brown rot" agents. The host range of *Monilinia spp*. includes apple, pear, quince, cherry, apricot, plum, peach, nectarine, almond, etc.

Four species of *Monilinia spp*. are considered economically significant: *M. fructicola*, *M. laxa*, *M. fructigena*, and *M. polystroma* are known pathogens that influence rosaceous fruit production worldwide. Other species such as *M. oxycocci*, *M. baccarum*, *M. urnula*, and *M. vaccinii-corymbosi* inhabit the Vaccinium host especially in North America. *M. fructicola* is identified in America, Australia, and New Zealand. It has been reported for the first time in French peach orchards and then in Spain (Garcia-Benitez et al. 2016), Switzerland (Bosshard et al. 2006), Hungary (Petróczy and Palkovics 2009), and Italy (Pellegrino et al. 2009) on peaches.

Monilinia spp. are economically important pathogens, very difficult to control. In this paper we have highlighted the metabolic pathways; specifically siderophore biosynthesis, uptake and utilization, triggered in the *Monilinia spp.* in iron starving conditions and further importance of siderophores can interfere with the action of the *Monilinia spp*. because they may inhibit enzymes important for their installation in the wounds post-harvest infection of brown rot of stone fruit.

4.2 Siderophore and Fungal Pathogenesis

Siderophores are specific chelators of ferric iron of low affinity and low molecular weight (<10 kDa). Siderophores are secreted by all prokaryotic and eukaryotic microorganisms, except *Saccharomyces cerevisiae*, *Candida albicans*, and *Cryptococcus neoformans*. Those who cannot produce siderophores can use siderophores produced by other microorganisms, called *xenosideróforos*. The microorganisms secrete one or more siderophores through specific receptors on the surface of the cells depending on the type and severity of the deficiency. Its biosynthesis is controlled by the availability of iron and produced by bacteria, fungi, and plants in limited iron conditions to stimulate plant growth to extract iron from the environment and make the mineral available for the cell near the root (Ahmed and Holmström 2014). In fungi, there are extracel-

lular and intracellular siderophores. They can participate in the transport or storage of ferrous iron (Winkelmann 2007). Siderophores are either excreted to mobilize extracellular or intracellular iron produced mainly for iron storage. They are specific growth inhibitors of several phytopathogenic fungi, including *Phytophthora parasitica*, *Pythium ultimum*, *Fusarium oxysporum vera dianth*i, and *Sclerotinia sclerotiorum*.

Siderophores act as plant growth regulators (Verma et al. 2011; Yadav et al. 2011), biological control agents (Verma et al. 2011), and bioremediation agents.

Iron is the essential component for various vital processes (photosynthesis, enzyme cofactor, redox reagent, respiration, synthesis of nucleosides and amino acids) of the plant. For avoiding deficiency of iron, various plants seem to rely on the excretion of phytosiderophores (i.e., chelate compounds, common in grasses that sequester iron) by the roots and secretion of siderophores by a group of microbes to facilitate the Fe complex uptake under iron deficiency conditions, which binds with high affinity for iron (Mino et al. 1983; Neilands 1995; Takagi 1976). Although iron is one of the most abundant elements on earth, bioavailability is less in aerobic conditions (in the presence of oxygen and at neutral pH), primarily because ferric iron (concomitant oxidization of Fe^{2+} to Fe^{3+}) reacts with oxygen to form insoluble ferric hydroxides. Conversely, excess iron or incorrect storage of iron is deleterious as "free iron" (reduced ferrous) catalyzes the production of cell-damaging reactive oxygen species via the Fenton reaction. To maintain iron homeostasis, the delicate balance of sufficient iron supply while preventing iron-induced toxicity and subsequent cell-damage regulated strategies for the careful control of iron uptake, utilization, and storage have evolved in a diversity of organisms.

To date, siderophore secretion systems have been described in only a few microorganisms. Siderophores are exported from the microbial cell by efflux pumps. As for fungi, little was known until recently about the role of iron in the interactions between fungi and hosts, and about the general regulatory mechanisms that govern iron homeostasis. In fungi, siderophore uptake is mediated by high-affinity transporters from the major facilitator family, which have strong specificity for their substrates (Haas 2014). However, a successful microbe must be able to acquire iron from its host's iron-limiting environment through the expression of high-affinity iron absorption systems. Two main systems compromised by fungi are the assimilation of reductive iron and the absorption of iron assisted by siderophores, in plants. Furthermore, its importance for the acquisition of iron for interactions between fungi and hosts and the regulatory mechanisms that influence this aspect will be examined. Recent reports have shown that some fungal siderophores act as determinants of virulence (Eisendle et al. 2006; Oide et al. 2006) and play a role in maintaining symbiotic fungal plant interactions (Johnson 2008). Conversely, in other fungi, the components of the reductive iron assimilation pathway are necessary for virulence (Ramanan 2000).

Siderophores have been biochemically characterized from various fungi and their structures elucidated. The majority of fungal siderophores identified to date belong to *Zygomycotina*, *Ascomycotina*, and *Deuteromycotina.* Generally, fungi produce a hydroxamate type of siderophore. Exceptions are the carboxylate-type siderophore rhizzoferrin produced by several Mucorales and the catecholate pistillarin produced by the marine species *Penicillium bilaii*. Most widely studied fungi

Table 4.1 Types of fungal siderophore secretion in plants

Fungal phytopathogen	Siderophore	Plants	References
Fusarium graminaerum	Triacetyl-fusarinine C	Cereals	Oide et al. (2006)
C. heterostrophus	Ferricrocin	Maize	Oide et al. (2006)
Cochliobolus heterostrophus	Coprogen; neocoprogens	–	Oide et al. (2006)
Fusarium graminaerum	Ferricrocin	–	Schwecke et al. (2006) and Oide et al. (2006)
Ustilago maydis	Ferrichrome	Maize	Yuan et al. (2001)
Epichloe¨ festucae	Fusarinine	Ryegrass	Johnson et al. (2007)
Epichloe¨ festucae	Ferricrocin	Ryegrass	Johnson et al. (2007)
Magnaporthe grisea	Ferricrocin	Rice	Hof et al. (2007)
Alternaria alternate	Dimethyl coprogen	Citrus	Chen et al. (2013)
Colletotrichum Graminicola	Coprogen, coprogen B, and methyl-coprogen B	Maize	Albarouki et al. (2014)
Zymoseptoria tritici	Ferricrocin, fusarin C	Wheat	Derbyshire et al. (2018)

for siderophore production are *Aspergillus fumigatus* and *Aspergillus nidulans* having 55 similar types of siderophore. The types of siderophores produced in plants are listed in Table 4.1.

Fungal hydroxamate siderophores can be divided into four structural families: fusarinines, coprogens, ferrichromes, and rhodotorulic acid. Hydroxamate siderophores form strong iron(III)-binding hexadentate, tetradentate, and bidentate ligands. The hydroxamate group is formed by acylation of the non-proteinogenic amino acid N5-hydroxy-L-ornithine, which is obtained by hydroxylation of L-ornithine, with acetyl or more complex groups such as anhydromevalonyl.

To avoid the periods of iron-starvation as well as to overcome iron toxicity, all cells require means to store iron. In fungi, two strategies for iron storage are most accepted: (i) vacuolar iron deposition and (ii) siderophore-mediated iron storage. In contrast to bacteria, plants, and animals, most fungi appear to lack ferritin-mediated iron storage and detoxification.

To increase affinity for Fe(III), most fungal siderophores include three of these moieties linked by ester or peptide bonds to form hexadentate structures. Cyclization of the siderophores, as found in ferrichromes and some fusarinines, improves the chemical stability and prevents degradation by enzymes. On the basis of the functional group present, the structure of siderophores varies in fungi, N^5-acyl-N^5-hydroxyornithine is the basic structural unit derived from L-ornithine except neurosporin siderophore (Renshaw et al. 2002).

In this study, we will highlight the importance of fungal siderophore; specifically siderophore biosynthesis, uptake, and utilization, triggered in the fungi in iron starving conditions and the various putative targets viable in these pathways to be recruited as novel disease resistance target and further role in the development of fungal-specific reliable disease management strategies. Siderophores are the weapons released by pathogenic fungi to conquer the battle for iron acquisition. Thus, the

purpose of this study is to present the siderophore biosynthesis pathways in *Monilinia spp.* causing brown rot in stone fruits focusing on the siderophore production along with their uptake and utilization mechanisms and represent an ideal target for pathogen-specific, host friendly therapeutic strategy, which would block the proliferation of fungus without causing any harm to the host. This study comprehensively compiles the information currently available to better understand siderophore biosynthesis pathways in *Monilinia spp.* related to BR resistance.

4.3 Pathways of Fungal Siderophores Biosynthesis

The progress in the knowledge of the novel targets of the siderophores biosynthesis path has undergone great progress in recent years; there is a substantial contradiction between the information provided by the considerable number of well-characterized biosynthetic pathways of siderophores. The mechanisms are potentially attractive objectives with respect to the control of pathogens.

The siderophore biosynthesis starts from precursors such as citrate, amino acids, dihydroxybenzoate, and N^5-acyl-N^5-hydroxioritine. Some genes have been described for the secretion of siderophores in bacteria and fungi that also have homologous genes in other microbial species. The siderophores are produced and assembled from non-ribosomal cytoplasm synthase. In general, the biosynthesis pathways of siderophore can be differentiated as being either dependent on or independent of an enzyme NRPSs (non-ribosomal peptide synthetases). In this section, the biosynthesis of siderophores will be discussed as aspects of secretion, absorption, and release of iron siderophores.

There are two main pathways for siderophores biosynthesis:

4.3.1 Non-Ribosomal Peptide Synthetases (NRPSs) Multi Enzymes Dependent

Nonribosomal peptide synthetase (NRPS) enzyme complexes synthesize nonribosomal peptides are called modular multidomain enzymes. Nonmodular NRPS enzymes are found in siderophores biosynthetic pathway like EntE and VibH in enterobactin, and VibE in vibriobactin.

4.3.2 NRPS-Independent

Non- ribosomal peptides (NRPs) are synthesized by a large multimodular enzymes NRPSs in which each module are associated for the incorporation of an amino acid into the peptide chain. Generally, the number and order of the modules determine the number and order of the amino acids in the peptide product (Crosa and Walsh 2002). Characteristic for NRPSs are the core domains for adenylation, thiolation, and condensation. The activation domain (A) of each module of the NRPS recognizes and

activates a specific amino acid such as its acyl adenylate by reaction with ATP. This activated ester is then covalently connected as its thioester in the thiolation domain (T). The condensation domain (C) catalyzes the direct transfer to another intermediate of acyl amino acid in the adjacent posterior form to form a peptide bond. The NRPS synthesizes both the chromophores (Mossialos et al. 2002) and the peptide chains of pyoverdine (Crosa and Walsh 2002). The generated NRPSs are very different: they are mostly part of proteogenic and non-proteogenic amino acids and can be linear or branched cyclically with a variable length. They show extensive secondary modification after its synthesis outside from the ribosome. Many fungal NRPSs have a high economic and/or ecological value such as β-lactam antibiotics, the immunosuppressant Cyclosporin A, but also mycotoxins such as glyoxin.

4.3.2.1 NRPS-Dependent Biosynthesis

ATP pyrophosphate is the main enzyme involved in this process, which is widely used in exchange assays and to determine the substrate specificity of the adenylation domains within the multienzyme synthetase. The formation of hydroxy acid by capturing the activated carboxyl group with hydroxylamine is an alternative method of analysis of the enzymes involved in the formation of acyl adenylate. Hydroxamic acid can be converted into its ferric complex and can be detected spectrophotometrically.

The NRPS is a set of peptides of broad structural diversity and biological activity. For the recognition, activation, and modification of each incorporated amino, they have a different unitary structure and each unit is responsible for its specific function to finally form a peptide product (Lautru 2004). The number and order of units in the PRPS are responsible for determing their size and sequence.

NRPS is an important mechanism for the biosynthesis of extra- and intracellular iron chelating siderophores that help the growth of fungi (Eisendle et al. 2006). In *A. fumigatus*, the studies identified the expression of three NRP synthetase genes (sidC, D, and E), which are, at various levels, susceptible to regulation by the level of free iron present in the culture medium. It has been shown that the expression of sidD is significantly upregulated in culture medium that limit iron (iron free or 20 mM free iron), concomitant with the production of siderophores and that the related sidD protein is present, as determined using the combined approach of 2D-PAGE/MALDI-TOF and TOF/TOF mass spectrometry. Previous studies on the expression of microarray in *S. cerevisiae* have shown that the disabled Open Reading Frame (ORF) can be expressed at the level of transcription (Harrison et al. 2002).

Therefore, functional identification of NRP synthetases, at the protein level, has been important since then pseudogenes may undergo transcription due to the presence of functional promoters (Lee et al. 2005). Furthermore, the identification of NRP synthase gene expression can be problematic (Cramer et al. 2006). Significantly, one gene (NPS6) has been identified in the pathogenic fungus of the *Cochliobolus heterostrophus* plant that contributes both to the virulence of the fungi in maize and resistance to oxidative stress (Lee et al. 2005). The elimination of NPS6 in the plant pathogenic ascomycetes *Alternaria brassicicola*, *Cochliobolus miyabeanus*, *C. heterostrophus*, and *F. graminearum* produced a reduced virulence and a greater sensitivity to H_2O_2 (Oide et al. 2006).

The NRPS chain extension unit contains three chains:

1. *Adenylation domain (A):* Adenylation domain specifically identifies the substrate and catalyzes the adenylation of its carboxyl group.
2. *Thiolation domain (T):* Thiolation domain is also called as the peptidyl carrier protein (PCP) domain. It utilizes terminal thiol of a post-translationally installed phosphopantetheine arm to bind the activated carboxyl group of the adenylate.
3. *Condensation domain (C):* It catalyses acylation of the resulting thioester with activated acyl group attached to the T domains in upstream unit. In some NRPS unit, C domain is replaced by a heterocyclization (CY) domain that catalyzes the heterocycle formation by a reaction of β-amino thiol group in the substrate attached to the T domain of the upstream unit.

The chain initiation unit contains only A and T domains. The growing chain is covalently bound to the T domain in successive unit throughout the assembly process.

Thioesterase domain (TE): This domain is usually present in the final unit. Hydrolysis or cyclization result in the release of assembled chain from the NRPS. Some TE domains catalyze NADH-dependent cleavage of the acyl thioester attached to the T domain.

4.3.2.2 NRPS-Independent Biosynthesis

Compared with NRPS-dependent pathway, enzymology of non-ribosomal-peptide-synthetases-independent siderophore (NSI) biosynthesis has been neglected for nearly three decades. Neilands and colleagues reported the first genetic characterization of the NIS biosynthetic pathway of aerobactin 1980 (Challis 2005; de Lorenzo et al. 1986). Aerobactin siderophore is a common metabolic product of different bacteria such as Vibrio, Yersinia, Salmonella, and E. (Challis 2005). The enzymes involved in aerobactin biosynthesis are LucD, a mono-oxygenase-dependent FADH2 that converts L-lysine L-N6-huydroyl-lysine using molecular oxygen as a co-substrate (Challis 2005). The enzymes involved have specific properties of the substrate according to biochemical characterization. Several NIS synthetases were reported, for instance, DesD, a cluster of gene des ABCD, pubC, and put AB (Barona-Gomez et al. 2006; Challis 2005).

In contrast with most other genes encoding fungal NRPSs, NPS6 is widely conserved among filamentous ascomycetes (Lee et al. 2005). The authors also demonstrated that the application of exogenous Fe3+ restored the mutant NPS6 virulence in exposed plants and that the gene encodes the extracellular hydroxamate siderophore. NPS6 appears to be an orthologue of sidD, indicating that sidD (or peptide sidD product) can partially mediate resistance to oxidative stress in fumigatus and contribute to the virulence of the fungus. Furthermore, the removal of sidC in *A. nidulans* led to the inability to produce ferricrocin siderophore and upregulation of some antioxidant enzymes (e.g., catalase and superoxide dismutase) (Eisendle et al. 2006). More

recently, it has been reported that NPS2/SidC is responsible for the biosynthesis of ferricrocin in *F. graminearum*. Based on the analysis of gene expression, protein identification, and siderophore detection, we hypothesized that NRP synthesizes sidC, D, and E, potentially biosynthesis the siderophores responsible for iron acquisition in *Monilinia spp.* although direct proof of this remains outstanding. Thus, although advances in NRPS are emerging, further work is urgently required to fully elucidate the nature of this important process, in particular, adenylation domain specificity and NRP product identification and function.

4.4 Siderophores and Plant Immune Responses

Plants are exposed to various biotic stresses; therefore they have developed a wide range of endogenous defense mechanisms against potential plant pathogens. In addition to preformed physical and chemical barriers, plants can detect attacks of pathogens and activate various complex signaling cascades, which lead to induced resistance against pathogens. Induced innate immune responses include phosphorylation processes, accumulation of reactive oxygen species (ROS), cell wall rigidification and degradation of biomolecules, deposition of the callose, defense hormonal signaling, and expression of pathogenesis-related (PR) gene encoding pathogenesis (Nurnberger et al. 2004).

To activate these defenses against potential microbial pathogens, the plants are equipped with a complex sentinel system comprising proteins dedicated to the recognition of inducers received from pathogens. Recognition of preserved motifs in microbial molecules called microbe-associated molecular patterns (MAMP) requires membrane-anchored pattern recognition receptors (PRRs). At the time of infection, components such as oligo-galacturonide or the peptides are released from the plant cells and are called damage-associated molecular patterns (DAMPs). It can be recognized by specific PRRs and activated defense mechanisms. Plants also contribute a complex recognition system associated with resistance proteins that directly or indirectly allow recognition of proteins secreted by pathogens, known as effectors, with reference to the fact that these proteins are attributed to the promotion of infections (Nurnberger et al. 2004).

More recently, Albarouki et al. (2014) demonstrated that secretion of coprogens from the hemibiotrophic fungus *C. graminicola* on maize activate defense responses. Interestingly, genes associated with coprogen biosynthesis are suppressed during the first biotrophic phase of invasion and are upregulated during the necrotrophic phase. Thus, the fungus tightly controls the production of siderophores at the early stages of infection probably to avoid the plant's immune system.

To understand the molecular mechanisms associated with immunity mediated by siderophores in leaves better, the plant immune system triggered by siderophores in *A. thaliana* has been extinsevely studied.

The deposition of callose in the leaves of *Arabidospsis* throughout the leaf vascular system suggested due to secretion of siderophores resulting the cell wall rigidity. The deposition callose differs from the well-described pattern induced by pathogens and MAMP, which appear in the inter vein zone. One of the most widely accepted hypothesis is that the siderophores mediated transient changes in iron status in the vascular system, causing ROS generation, followed by accumulation of callose along the veins.

4.5 Mechanisms of Siderophores Action on Plant Immune Response

As highlighted in this paper, siderophores primarily possess two opposing potential roles during infection processes: pathogenesis effectors involved in host invasion and defense elicitors. This dual property is also found in some protein effectors secreted by fungal pathogens (Bent and Mackey 2007).

4.5.1 Siderophore Regulation via Their Iron Scavenging Effect

Iron scavenging by siderophores may alter the stoichiometric metal balance and this may result in siderophore-mediated activation of plant immune responses. For example, if any metalloproteins bind with a particular metal, it can lead to disturbance in maetal balance (e.g., Zn^{2+} replaces Fe^{2+}) thereby resulting in a danger signal. This alarming signal could be detected through pathogenic effectors that are injected into plant cells to promote infection (Block et al. 2008). Surprisingly, these effectors strongly activate immune responses and the hypersensitive response, leading to cell death. In the case of siderophores, no cell death has ever been reported. Aznar and his coworkers (2014) reported that deferrioxamine (DFO) triggers the defense at a concentration that coincides with the concentration of iron in the leaf.

The immune responses are not activated below this level suggesting that the plant can tolerate the presence of strong iron scavengers at a certain level, corresponding to their own iron content. Above this level of level, the “danger signal” is inactivated. Choi et al. (2007) demonstrated that the activation of immunity by the elimination of iron is effective in mammal cells as well as in plants. As discussed above, in Arabidopsis, immunity mediated by siderophores requires transporter IRT1 indicating that, siderophores mediated defense caused not only due to the strong iron deficiency but also due to the uptake of metals. Thus, exceptionally in this case, the effect of iron starvation caused by the scavenging only triggers immunity. In contrast, the disturbance in metal homeostasis due to iron scavengers is probably the signal that activates defensive responses in both animals and plants (Aznar and Dellagi 2015).

4.5.2 Siderophores Regulation by Cell Receptors

Animals can neutralize iron scavengers from pathogens through siderocalins, by binding with certain iron–siderophores, that limiting microorganism iron nutrition. However, microorganisms can secrete more than one siderophore, but some siderocalins, such as Lcn1, can recognize and bind siderophores with diverse structures. In plants, no such recognition mechanism has been reported so far. Although a phylogenetic study of the plant lipocalin family identifies proteins that show some similarity with animal lipocalins, siderocalins are not reported in this study (Frenette Charron 2005). Aznar and Dellagi (2015) performed a BLAST search to identify proteins similar to siderocalins, using the protein coding for Neutrophil gelatinase-associated lipocalin (NGAL) and Lcn1 and found no plant orthologues, and they suggested if siderophores recognition exists in plants, then it is probably based on another type of protein. Information is still lacking, whether siderophores can be identified by specific receptors in plants, similar to MAMPs and how they are identified by PRRs (pattern recognition receptors). Albarouki et al. (2014) reported in maize that the accumulation of ROS and upregulation of the PR gene occur in response to both siderophores coprogen and Fe-coprogen, indicating that a receptor could detect siderophores in maize. The transcriptomic analysis of siderophores DFO is very similar to the transcriptome of plants infected by plant pathogens (Aznar and Dellagi 2015).

This similarity may determine that the ratio of the signaling cascade activated after detection of siderophores is similar to that of a MAMP or an attack of pathogens. Dellagi et al. (2009) suggested that the SA upregulation via DFO depends on proteins NPR1, EDS5, and SID2, which are considered to be main regulators of the plant's response to pathogens (Quartin et al. 2001). It is believed that Response Regulator Protein (RRP) is dedicated to the recognition of microbial siderophores. Alternatively, siderophores can determine the activity of one or more proteins, in the case of pathogenic effectors, which is associated with the modification of a metalloprotein. The immune response will be activated if this target protein is protected by resistance proteins, such as those in nucleotide-binding leucine-rich repeat NB-LRR family. In Maize, for example iron scavenging effect does ssem to involved in the process of activation of defense by the coprogen (Albarouki et al. 2014).

4.6 Siderophore-Mediated Plant Immunity Activation

Recently, molecular approaches suggested that various plant species are able to activate immune programs in response to siderophore treatment. For example, in maize, the effect of the coprogen seems more reminiscent of "priming" (Albarouki et al. 2014), while in *Arabidopsis* the coprogenic effect resembles direct stress (Aznar and Dellagi 2015). In the studies described below, oxidative stress has been reported in several cases. ROS production is associated with the combination of pyochelin and pyocyanin in tomato cells, with the siderophores Psb374 in tobacco cell cul-

tures (van Loon et al. 2008), and with siderophores in *A. thaliana*, in which differential expression of genes related to the metabolism of ROS is also observed. Changes in the cellular redox balance can trigger a diffusible signal that causes the systemic diffusion of defense responses. Siderophore-mediated defense mechanism leads to the production of ROS, formation of antifungal compounds, and activation of plant pathogenesis–related PR pathways against pathogen (Fig. 4.1). ROS production can be derived from a Fenton effect generated by a strong and rapid modification of the distribution of iron at the cellular level and at the plant level. The role of ROS may be different in treated tissues or systemic tissues, for example, in the study by van Loon et al. (2008) in tobacco plant cells, there were only direct cellular effects of siderophores, although the effect of systemic metal signaling. Mobilization in entire plants has not been monitored. Immunity activated by siderophores seems to depend on several hormones according to the biological system considered. In rice, De Vleesschauwer et al. (2008) determined that ET and JA are necessary for systemic immunity induction, while in *Arabidopsis*, SA is required for protection against *P. syringae* pv *tomato* DC3000 (Aznar and Dellagi 2015).

This difference in hormonal signaling may be due to specific mechanisms of the perception of the siderophores in each organ (leaf or root), that depend on the affinity of the siderophores for iron, or for differences in signaling response between plant species. A comparison of the underlying mechanisms of immunity activation through the roots or leaves needs more future investigation. Another promising approach concerns the investigation of immune signaling activated in fungi-host-pathogen because they have different iron signaling networks.

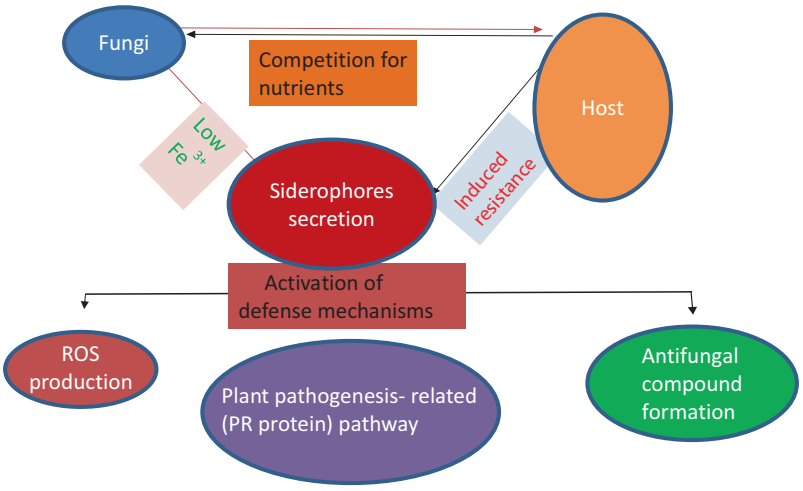

Fig. 4.1 Siderophore-mediated defense mechanism in host plant

4.6.1 Role of Siderophores in Fungal Pathogenicity

In plant fungal pathogenesis, the roles of siderophores vary between the different pathosystems, and the importance of the siderophores for fungal pathogenesis was first reported in the pathogen of maize *C. heterostroph* (Lee et al. 2005; Oide et al. 2006). Later it was reported that iron metabolism mediated by siderophores is necessary for a complete virulence in the pathogen of rice *C. miyabeanus*, the wheat pathogen *F. graminearum*, and the *Brassicaceae* pathogen *A. brassicicola* (Table 4.1). Hof et al. (2007) revealed that the inhibition of the synthesis of ferricrocin siderophore in *Magnaporthe grisea* influences its pathogenicity of rice. However, siderophore apparently has no role in the virulence of basidiomycete maize pathogen *Ustilago maydis*. Siderophores are also an important factor of virulence of the human pathogen *Aspergillus fumigatus* (Hissen et al. 2005; Schrettl et al. 2007).

4.6.2 Role of Siderophores, in (ROS) Generation, and Fungal Sexual Development

Siderophores, iron-scavengers, have a crucial role in oxidative defense. Papanikolaou and Pantopoulos suggested that excess of intracellular iron can cause oxidative stress through the Fenton reaction while on the other hand, heam is essential for the function of many peroxidases, an important family of enzymes in the detoxification of hydrogen peroxide. Thus, it is important in fungi to maintain the iron concentration carefully. Eisendle et al. (2006) showed that oxidative stress causes upregulation of intracellular ferricrocin siderophore in *A. nidulans*. Similarly, this increase is also shown when exposed to excessive iron conditions, although it is not clear whether this is due to the iron itself or due to the iron-mediated oxidative stress resulting in the cell.

Deletion of siderophore synthetase sidC inhibits the production of ferricrocin, resulting in different phenotypes, including inefficient use of iron, delayed germination in iron replete conditions, and inhibiting the formation of cleistothecia in homothallic conditions. Additionally these phenotypes, the conidia of the sidC mutant, show sensitivity to H_2O_2. In *A. fumigatus*, a similar relationship of siderophores has been reported in oxidative defense mechanism. In the null mutant siderophore, ΔsidA observed severe sensitivity to O_2, while the absence of intracellular or extracellular siderophore observed low sensitivity separately. This suggested that both intra- and extracellular siderophores play a pivotal role in mediating oxidative damage and showed redundancy in the system. The cross-talk between ROS detoxification and siderophores is not limited to *Aspergillus* species. In *Alternaria alternata*, the elimination of NPS6, NRPS essential for the biosynthesis of extracellular siderophores, increases the sensitivity to H_2O_2.

In addition, the expression of NPS6 in *A. alternata* is regulated by NOX, YAP1, and HOG1, genes involved in oxidative defense mechanisms (Chen et al. 2014). Deletion of NPS6 in *Cochliobolus miyabeanus*, *Fusarium graminearum*, and *Alternaria brassicicola* also resulted in enhanced sensitivity to oxidative stress (Oide et al. 2006).

4.7 Role of Siderophores in Fungal Virulence

A strong competition for iron between the host and fungi may establish during fungal infection, consequently, fungi have developed specialized systems to scavenge iron from the host by taking up hemes or glycoproteins associated in iron transport, such as transferrin and lactoferrin. In addition, siderophores are able to compete for iron directly due to their high affinity for this element (Caza and Kronstad 2013). For instance, in fungus *Aspergillus fumigatus*, both intracellular and extracellular siderophores are responsible for virulence.

Siderophores of phytopatogenic fungi are able to multiply in the host and promote infection (Haas et al. 2008). Oide et al. (2006) reported that, in four ascomycete species, *Cochliobolus miyabeanus*, *C. heterostrophus*, *Fusarium graminearum*, and *Alternaria brassicicola*, siderophores are essential for resistance to hydrogen peroxide and for full pathogenicity in their respective hosts, maize, rice, wheat, and *Arabidopsis thaliana* (Oide et al. 2006).

More recently, the secretion of siderophores by pathogenic hemibiotrophic fungus *Colletotrichum graminicola* in maize was reported to be required for its virulence and resistance to oxidative stress (Albarouki et al. 2014). Virulence of the pathogenic fungus *A. alternata* on citrus is compromised in a siderophore-deficient fungal mutant (Chen et al. 2013). Similarly, in apple, fire blight–causing agent *Erwinia amylovora* takes advantage of its siderophore DFO to infect apple seedlings and flowers and for its resistance to hydrogen peroxide.

The biosynthesis of siderophores is regulated by well-characterized transcription factors in fungi and bacteria (Haas et al. 2008; Troxell and Hassan 2013). These transcription factors detect the iron status in the environment and regulate the expression of their target genes by binding with cis-regulatory sequences. Taken together, these studies reported that siderophores play an important role in the virulence of different types of plant fungal pathogens. This data revealed that the pathogenic fungus can assist the siderophore production and virulence factor during the disease process based on the iron status of the host. However, recent advances have raised several interesting questions about the mechanisms associated with plant siderophores.

4.8 Future Prospects

Brown rot diseases caused by *Monilinia* spp. causes severe losses of stone and pome fruits every year. Due to their low pH, higher moisture content and nutrient composition fruits are susceptible to attack by pathogenic fungi, which cause decaying or rotting and make them unfit for consumption by producing mycotoxins. The storage period and market life of stone fruits is limited due to post-harvest brown rot diseases. In India, this crop is mostly cultivated in the Jammu and Kashmir region. Therefore, the present study was carried out with the main objective to undertake for the management of the brown rot pathogen that causes decaying in stone and pome fruits under storage conditions in the

Kashmir Valley. The crucial role of the siderophore system for fungal virulence and the differences to the plant iron acquisition mechanisms might help to properly manage fungal infections. Specifically, the fungal siderophores biosynthetic pathway represents a promising target for selective therapeutic intervention. Phylogenetic and structutral analysis suggest that NPS6 is conserved among diverse species of filamentous ascomycetes, in contrast with the previously identified fungal NPS genes involved in virulence, which appear to be restricted to one or a few species or races in particular *Monilinia spp.* including NPS genes is still presents many challenges in terms of understanding, exploitation and even nomenclature. In the environment, plants can be exposed to a variety of microorganisms that can be beneficial or pathogenic, all of which are likely to produce siderophores. The relative stability and iron affinity of these siderophores may play an important role in plant health. Siderophores clearly cause important physiological modifications in plants. A better understanding of the mechanisms through which fungal siderophores affect plant immunity may hold promise for designing new crop protection. The convergence of established technologies such as genome sequencing, in silico annotation and metabolite profiling, in addition to "secondary metabolite cluster signatures" combined with the application of emerging and novel genomic (e.g., fungal gene deletion and silencing) and proteomic strategies (i.e., recombinant adenylation-thiolation domain activation and activity analysis) sets the ideal scene for rapid and unambiguous advances in dissecting the global importance of NRPS in filamentous fungi. Thus, the detailed understanding of fungal iron uptake strategies may help to develop novel strategies to combat pathogenic fungi and to improve plant protection. To date, conjugates of siderophores with a variety of commercially available antibiotics with different cellular targets have been developed. It is tempting to speculate whether corresponding conjugates of fungal siderophores with different fungicides, for example, azoles, dicarboximides, or strobilurins, could be used in a corresponding Trojan Horse strategy to combat plant pathogenic fungi.

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Chapter 5
Application of Siderophore in Crop Productivity and Remediation of Heavy Metal-Contaminated Soil

Anuj Saxena

Contents

5.1 Decreasing Soil Fertility Impact on Agriculture

With population boon and rapid urbanization and industrialization, there is a need for enhancing agriculture productivity on shrinking agricultural land (Balasubramanian and Choi 2010; Chen et al. 2011; Agarwal et al. 2015). Healthy soil is the foremost and crucial element for sustainable agriculture and crop productivity which rely on efficient nutrient and water cycling. Soil health is dependent on physical (soil texture, water-holding capacity, moisture content, soil temperature, etc.), chemical (pH, redox potential, minerals, organic matter, cation exchange capacity, etc.), and biological (physicochemical properties of soil, organic matter, and rhizosphere biota) characteristics (Kibblewhite et al. 2008; Frac et al. 2018; Meena et al. 2019; Yu et al. 2019). But contrary to it, injudicious and heavy application of agrochemicals in the past few decades adversely affected soil structure, soil chemistry, and most importantly soil biota (Amundson et al. 2015; Datta et al. 2016; Gouda et al. 2018). Soil health not only plays a crucial role in crop productivity but also maintains soil biota, reduces carbon footprint through carbon sequestration,

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K. Dhusia et al. (eds.), *Fungal Siderophores*, Fungal Biology,
https://doi.org/10.1007/978-3-030-53077-8_5

and helps in controlling plant diseases in an eco-friendly biological way (Saxena et al. 2013; Benbi 2018; Sarfraz et al. 2019; van Bruggen et al. 2019).

Before independence, India was like a ship-to-mouth country as it was a massive importer of food. With the efforts of agricultural scientists during the Green Revolution, India was able to transform itself from a begging bowl to a bread basket (Yadugiri 2011). This wide gap filled by high-yielding and more nutrient-demanding cultivars of staple and other crops has gradually transformed the soil into nutrient deficient. Application of agrochemicals has depleted the beneficial soil microflora also. Recognizing this fact, nowadays, stress is being laid on biological inoculants to replenish the soil health.

5.2 Metal Contamination

Due to anthropogenic activities, natural resources are depleted at a faster rate. A large number of metals are continuously being added to the environment causing water, air, and soil pollution. These metals find their way in the food chain through biomagnifications and exert toxic effects at successive trophic levels in plants, animals, and human beings (Jagtap and Bapat 2015). Globally, more than 20 million hectares of land mass is adulterated by different heavy metals (Liu et al. 2018).

5.3 Biological Inoculants

New successful, reliable, eco-friendly, cost-effective technologies are necessary to recover the contaminated and mineral-deficient soils caused due to intensive farming and natural or anthropogenic sources. There is an urgent need to put a restriction on excessive use of agrochemicals in agriculture system without compromising the agricultural productivity. Many workers have reported the application of different biological inoculants for growth promotion and disease management in crops (Saxena et al. 2013; Munir et al. 2019; Raklami et al. 2019). But biological inoculants have not been widely accepted by farming community so far. The enhancement in growth and productivity of crop plants by biological inoculants depends on many biotic and abiotic factors, e.g., soil type and characteristics (texture, pH, moisture content, temperature, porosity, etc.) climatic conditions, crop type, genotype and nature of biological inoculants, inoculation technology (like root-dip inoculation, seed treatment with or without surfactant, challenge inoculation), addition of supplementary or co-inoculants or chemical additives, nutrient mobilization, interaction of crop plants with biological inoculants, and agricultural practices (Zhang et al. 2000; Bashan et al. 2014; Pii et al. 2016; Sarfraz et al. 2019). There are several reports indicating application of biological inoculants for phytoremediation of contaminated and degraded soil (Sipahutar et al. 2018; Wani and Khan 2013; Khanna et al. 2019). The biological inoculants regulate hormonal and nutritional balance, induce pathogen resistance, and solubilize nutrients for easy uptake by plants.

Biological inoculants interact with rhizospheric microorganisms synergistically and antagonistically to promote plant growth (Ali and Vidhale 2013; Vejan et al. 2016).

5.4 Siderophore

Siderophores are iron-chelating secondary metabolites of low molecular weight synthesized by microbes (Sinha et al. 2019), fungi (Renshaw et al. 2002; Manzoor et al. 2019), graminaceous plants (Garnica et al. 2018), phytoplankton (Reid et al. 1993), cyanobacteria (Arstøl and Hohmann-Marriott 2019), etc. under low-available iron conditions. Siderophores have binding ability not only with iron but also with a variety of other metals making them available or excluded from the rhizosphere offering vigorous growth and enhanced crop productivity and their possible application in phytoremediation studies. Nowadays, siderophores have gained worldwide importance due to their role as biocontrol, biosensor, bioremediation, and chelating agent.

Siderophore-mediated responses strongly depend on pH and available iron (Jin et al. 2010). Different siderophores differ in their chemical structures, properties, and stability constant with iron. Identification of microbial strain on the basis of siderophore is known as siderotyping. Siderotyping is being used by many workers for taxonomical identification and characterization of microbes (Bosne and Levy Frebault 1992; Meyer et al. 2002; Mulet et al. 2008). About 500 different siderophores have been reported so far (Paul and Buttinger 2005). Siderophores are broadly divided into the catecholate, hydroxamates and carboxylate (Ali and Vidhale 2013). Mostly bacteria and fungi produce hydroxymate siderophore (Balagurunathan and Radhakrishnan 2007; Ali and Vidhale 2013).

5.5 Benefits of Siderophore

The source of environmental iron is lithosphere in its ore form. Iron is quite copious on earth. It is ubiquitous and redox active, one of the most essential and crucial element for existence of plant, animal, or microbial life. Iron acts as an essential component in many enzyme-mediated metabolic processes like respiration, photosynthesis, anabolism of nucleic acids, porphyrin, vitamins, siderophores, and aromatic compounds (Aguado-Santacruz et al. 2012; Rout 2015). Siderophore determines the density and structure of rhizospheric microbial biota and enhances the possibility of obtaining culture of several unculturable microorganisms by making the iron available to them (Saha et al. 2016). In agriculture, siderophore promotes the growth and productivity of crop plants by promoting the iron availability and uptake, especially in iron-deficient soils. It has been noticed that apart from iron, siderophores can enhance the availability of other mineral nutrients by

complexing with them (Renshaw et al. 2002; Duckworth et al. 2009; Harrington et al. 2015).

Considering the adverse effects of agrochemicals on soil health, the environment, and the biotic community, including humans, the siderophore can ideally be used in the agriculture system as they not only enhance mineral availability but also biologically control the pests and pathogens of crop plant. Several workers have reported the role of siderophore as a biocontrol agent to suppress phytopathogens (Tariq et al. 2010; Sasirekha and Srividya 2016). Pandey et al. (2005) demonstrated siderophore-mediated growth enhancement in Indian mustard. There are reports on siderophore-mediated phytoextraction and bioremediation of metal-contaminated environment (Hernlem et al. 1999; Rajkumar et al. 2010; Gaonkar and Bhosle 2013). Siderophore also helps in soil mineral weathering (Ahmed and Holmstrom 2014).

5.6 Mechanism of Action

Plant cell decreases hydrophobicity under low-available iron environment generating iron deficiency signals. Siderophores are low-molecular-weight proteins that chelate the iron more efficiently and make them available to the plants or microbes. In the cellular environment, the ferric iron is converted into ferrous iron, which remains available for utilization by microbes and plants. Many of the siderophore-producing bacteria may stimulate plant growth by ensuring iron availability to plants and by producing phytohormones and organic acids that solubilize phosphate and other lithospheric nutrients present in soil in complex state. Siderophores can play a role in the prevention of pathogenic microbes from plants by depriving iron and elicit induced resistance. Liu et al. (2007) have shown that pathogen attack depletes the intracellular iron leading to transcription of pathogenesis-related genes for oxidative burst regulating the plant defense. Saxena (2003) reported transcript accumulation for peroxidase and phenylalanine lyase during induction of systemic resistance in pear millet against downy mildew disease as expressed by inoculation with plant growth-promoting rhizobacteria (PGPR) (Figs. 5.1 and 5.2).

Many siderophore-producing bacterial and fungal *strains* promote plant growth by enhancing iron solubility. Thus, siderophore chelates the iron and makes it available to the plants. Han et al. (2018) identified the role of *sip* gene in iron absorption in *Vibrio anguillarum* 775. Siderophore-interacting proteins (SIPs) by utilizing ferric siderophore complex make the ferrous iron available to microbial system. Defense response genes are induced in both incompatible and compatible plant–pathogen interaction. However, mRNA accumulation for many plant defense genes is more rapid during interaction involving plant expressing resistance (R) gene corresponding to virulence gene of the pathogen (Christensen et al. 2002; Davis et al. 2002). There are studies related with differential transcript accumulation in multicell hypersensitive response interaction (Burrow et al. 2000; Fossdal et al. 2001; Christensen et al. 2002).

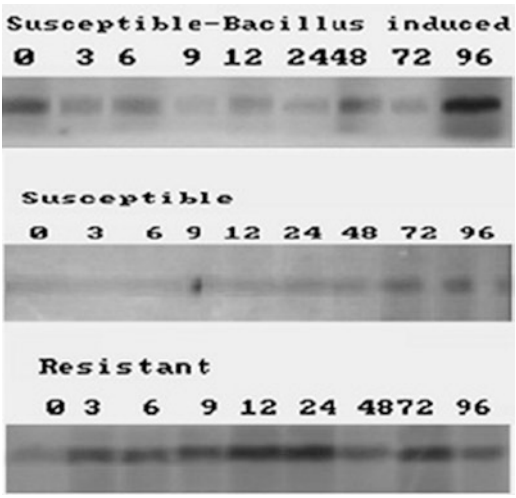

Fig. 5.1 Pattern of accumulation of peroxidase transcripts in downy mildew susceptible, resistant, and susceptible induced with ISR 5 for resistance (equal loading with respect to time). (After Saxena 2003)

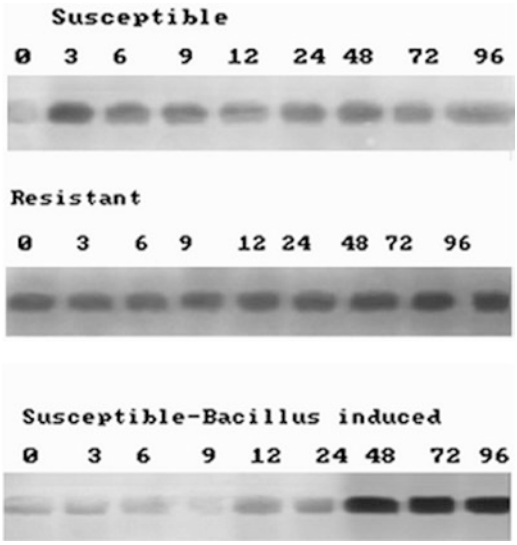

Fig. 5.2 Pattern of accumulation of phenylalanine lyase transcripts in downy mildew susceptible, resistant, and susceptible induced for resistance. (After, Saxena 2003)

5.7 Future Prospects

Considering the potential importance of siderophore in environmental, agricultural, and medical field, there is a need to explore the molecular mechanisms of siderophore pathway (Trindade et al. 2019). Siderophore can be used in combination with other biofertilizers to increase crop productivity. Much of the knowledge on siderophore is based on observations in vitro. However, the field conditions are marked different from control conditions (Bossier et al. 1988). Application of biological inoculants for siderophore-mediated responses may pose potential biohazards

(Ferreira et al. 2019). Therefore, its biosafety issue must be resolve before large-scale field application. There are some initial reports indicating the synthetic siderophores. Williams et al. 2019 have reported coelichelin and several derivatives as ferric iron delivery vehicles for *Pseudomonas aeruginosa*. Sridhar et al. (2018) have synthesized gobichelin B siderophore which is found in *Streptomyces* sp. Sockwell and Wetzler (2019) have reported polyhydroxamic acid siderophores, which showed a higher preference for Fe^{III}. Pros and cons of biological inoculants and natural or synthetic siderophores at field level are yet to be established.

Acknowledgment Visiting fellowship from Indian Academy of Sciences is deeply acknowledged. Author is thankful to Dr. Kalyani Dhusia for her support and going through the manuscript.

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Chapter 6
Interventions to Ameliorate Heavy Metal Contaminated Soils Employing Fungal Siderophores

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K. Dhusia et al. (eds.), *Fungal Siderophores*, Fungal Biology,
https://doi.org/10.1007/978-3-030-53077-8_6

6.1 Introduction

Inordinate metal contamination is one of the notable results of industrialization, seen in mining, oil refining, vehicles, paints, and many other activities. The related anthropogenic effects have frequently brought about natural contamination. Contamination of the earth continues to expand at a disturbing rate because of human excesses, such as urbanization, innovative progression, dangerous agrarian practices, and rapid industrialization, all of which corrupt nature. Overwhelming amounts of industrial metals are discharged into the Earth, injurious because of their poisonous qualities that cause extreme danger to life forms exposed to elevated levels of such contaminants. Metals are among the natural fundamental elements of plants, but at increased levels, these metals interfere with the metabolic responses of life forms. Poisonous metals, for example, copper, zinc, lead, arsenic, mercury, and cadmium, are the most common heavy metals that are not useful to plants, decreasing plant development because of diminished photosynthetic functions, decreased plant mineral sustenance, and diminished movement of basic catalysts. Significant metals are cytotoxic at low concentrations, will cause harmful development in humans, and furthermore affect other properties of living creatures. Fungal siderophores are involved in bioremediation by cleaning up toxic heavy metal contamination in the soil environment to reduce the impact on the ecosystem.

Bioremediation is the cleanup of toxins and pollutants from the soil to reduce their impact on the ecosystems, including copper, zinc, and cadmium as the most common heavy metals. Two bioremediation techniques are remediation in situ by bioventing and remediation ex situ by slurry-phase and solid-phase methods. Soil treatment by solid-phase bioremediation includes composting, land farming, and bio-piles.

6.1.1 Remediation In Situ

Remediation of contaminated soil in situ is useful for treating areas containing low levels of heavy metal contaminants. Adding a sufficient amount of nutrients to the soil enhances the degradation of contaminants by the activity of indigenous soil microflora, the naturally occurring microbes. This process of in situ bioremediation is known as bioventing. In bioventing, oxygen as air is sent through the injector into the contaminated areas of soil. Wells are inserted into the soil so that air can be sucked or blown through it. Similarly, many inorganic fertilizers and nutrients such as phosphorus and nitrogen are also pumped through these injection wells, increasing the rate of growth of microorganisms in the soil. Such

remediation is aerobic and is most effective in cleaning larger areas. Microbes acts as catalysts and minimize toxicity to humans by reducing the levels of toxic contaminants in the soil.

6.1.2 Remediation Ex Situ

The soil excavated or pumped from the site is treated to remove contaminants by ex situ bioremediation. Because this technique removes the soil before treatment, faster decontamination of soils and the ability to treat a wide range of toxins in soils with easier manageability are its main advantages.

6.1.3 Slurry-Phase Technique

As the name implies, a slurry is prepared by using a bioreactor to mix the contaminated soil with water and various reagents. Harmful substances present in the soil will contact the microorganisms by homogenization. Thereafter, oxygen and supplements are introduced to the slurry, blended to create an ideal environment for the microorganisms to break down the contaminants. The soil is tested when separation of water is complete, and thereafter supplanted in that location with naturally existing microflora. A faster rate of bioremediation is its advantage.

6.1.4 Solid-Phase Technique

Bioremediation by the solid-phase process treats a contaminated soil by digestion in aboveground reactors. Anaerobic conditions inside the reactor, which acts as a fermenter, are maintained for a considerable period of time to ensure that optimum treatment of soil and water can occur. Anaerobic microbes thus grow and degrade the waste. Diatomaceous earth and granulated charcoal are mixed with the slurry to increase microbial growth. As soon as degradation is complete, the slurry is transferred to another large concrete tank and treated with fresh microbial inocula. Now, aerobic conditions are well maintained so soil wastes can be mineralized completely by the activity of these aerobic microbes. The decontaminated or treated soil is then returned to the original field from which it was excavated. Maintenance is much easier in this type of soil treatment of soil, but its requirements for larger space and longer time for decontamination are disadvantages when compared to the slurry-phase bioremediation method.

6.1.5 Land Farming

Land farming is a simple process that helps in the mixing and maintenance of soil on flat land. Degrading wastes by the existing microbes in wet mud or sludge is known as land farming. Light loamy soil is uniformly spread over a pad to the depth of 0.5 foot. Residual liquid or oil sludge is collected from the soil and piled over the loamy soil to a height of 1 foot. Phosphates, limestones, and nitrates are uniformly spread over the mixture. Thereafter, the wet mud is watered to provide more than 30% water saturation in the pad. The mixture is turned over regularly to allow air to mix with the excavated soil so that the microorganisms present in the soil will break down. Microbial growth is maximum when the pH is between 7 and 8 and temperature is between 20 °C and 30 °C. In this method, the indigenous microbes degrade 60–70% of the soil wastes in the wet mud within 4 months. After complete degradation of wastes, the sludge material is excavated and replaced on the original field from where it was excavated.

6.1.6 Composting

Decayed organic matter that is rich in microorganisms can degrade the wastes of explosives which cannot be treated easily by any other methods. This process of degrading explosive wastes is known as composting. Explosives such as trinitrotoluene (TNT), octahydro tetra nitro-tetra zocine (HNT), and hexahydro trinitrotriazine (RDX) are treated by composting. In a compost pit, liquid that contains the explosives is added to a pile of decaying organic matter. Microbes present in the organic matter will degrade the explosives and reduce their concentration in the compost. At 50–60 °C, about 80–90% of wastes can be degraded within 70 days in compost piles. By 150–170 days, the concentration of explosives in the soil will be less than 1%. Thereafter, the compost pile is excavated from the pit and used to fertilize the soil. This method of bioremediation is much cheaper when compared to incineration of explosives. Composting allows maximizing the water levels and air levels in which the microorganisms have more access. Composting is of three types: static pile, in-vessel, and windrow composting methods.

6.1.7 Soil Bio-piles

In this procedure, heaps of soil are placed over the top of a vacuum siphon. The vacuum siphon pulls air through the soil to enable oxygen to pass through the dirt to the microorganisms. Contaminants that may transform into gas are effectively gathered as they are basically sucked with the airstream through the dirt.

6.2 Nature of Heavy Metals

The closeness of these overwhelming metal concentrations in the Earth has become a subject of serious blowing stress because of their high lethality and nonbiodegradable toxic nature. These substantial metals consequently can be changed only through assimilation, methylation, complexation and changes in valency state. These changes influence the versatility and bioavailability of metals. At low fixations, metals can fill in as a vital segment in life forms, regularly serving vital capacities as co-factors of catalysts for better protein action. Moreover, such metals at certain limits concentrate, which may harm the natural framework. Introduction of substantial metal mixes into nature for the most part instigates morphological, cytological, and physiological changes in the microbial networks, applying a particular burden on the microorganisms (Verma and Jaiswal 2016), by and large the destination.

Metal-safe microorganisms can debase substantial metals (Gadd 1993). Fortunately, microorganisms can influence the reactivity and portability of metals and therefore can be utilized to detoxify some metals, counteracting further metal defilement. Bioremediation is slowly being acknowledged as the safe method in restoring soils with overwhelming concentrations of heavy metals because of its environmentally friendly nature, as contrasted with the more usual compound and physical strategies, which are usually costly and also ineffectual when metal concentrations are low. Such methods also deliver significant amounts of poisonous slop (Ekperusi and Aigbodion 2015; Ayangbenro and Babalola 2017).

The cost viability of remediation was determined by Blaylock et al. (1999), who statead more than half the cost could be avoided when natural remediation was used for the cleaning of one section of land of Pb-fouled soil as contrasted with ordinary techniques such as landfill and exhuming. The efficacy of microorganisms to lower contaminant levels depends appropriately on the usual environmental conditions for their progress and processing based on the physical factors that support their growth (Verma and Jaiswal 2016).

6.3 Principles Involved in Bioremediation

Bioremediation is a system to expel ecological soil contaminants in the environment that utilizes the organic components innate in microorganisms and also in plants to overcome dangerous toxins and reestablish the biological system to its unique condition. Microorganisms are mostly utilized in bioremediation to remove substantial metals (components with 5 g/cm^3 density and greater) in the contaminated condition (Banik et al. 2014). Notwithstanding the normal existence of substantial metals (Cobbina et al. 2015), they are widely utilized in industry, horticulture, and military activities. These procedures have caused overwhelming concentrations of persistent metals in the Earth, which cause hazards to our general well-being and all biological

systems. The high concentrations of substantial amounts of metals in the Earth are also credited for some perilous maladies, including malignant growths and cardiovascular diseases. The disposal of these substantial metals requires their fixation and regulation as they cannot be corrupted by any organic, physical, and compound procedures (Naz et al. 2016). Along these lines, utilizing microorganisms for overwhelming metal disposal and ecological cleaning is a powerful methodology because of their capacity to collaborate with metals in the site. For example, microorganisms can change excess substantial metals from one condition of oxidation to another (Xiong et al. 2015).

Fundamentally, microorganism-combined remediation depends on the opposition of the organisms used to the substantial metal that is actuated freely or through metal ionic bonding. The essential standards of bioremediation include diminishing the solvency of the contaminants by evolving chemical changes from the polluted condition. Various reports have included redesigning the adsorption of pentachlorophenol by modifying the hydrogen ion concentration in fluid configurations. Removal of pentachlorophenol, which is pH dependent, from watery areas alternatively uses the absorption potential of *Aspergillus niger* and *Mycobacterium chlorophenolicum* methods. Microorganism surface assimilation becomes entirely irreversible at pH 5.4, whereas complete activity is regained at pH 7.0. However, at pH 6–8, greater results are found on the surface assimilation of lead against pentachlorophenol by microorganism biomass for liquid methods (Jianlong et al. 2000). The outcomes acquired by different workers feature the significance of utilizing the appropriate pH for ideal execution of microorganisms utilized in bioremediation.

Bioremediation advances rely on central chemical reactions that form dynamically and on water-based biological science by infusing reagents into contaminated water where structural debasement occurs, with furthur removal of varied toxins and impurities by mixtures that decrease the responses. Redox responses include artificially changing unsafe contaminants into harmless or less poisonous discharges that are progressively steady, less portable, or latent. This reaction assumes an essential function in the change of lethal metals, particularly arsenic, chromium, mercury, and selenium (Nematian and Kazemeini 2013; Kabata-Pendias 2010), making the residue harmless (Gadd 1986; Rajapaksha et al. 2013). Redox responses in debased soil dregs are frequently influenced by the physical and chemical properties of the medium, however, which can be controlled by expansion of natural revisions, for example, manures and biochar (Bolan et al. 2013; Beiyuan et al. 2017). The usage of traditional rectifications, for instance, of metal wastes in the soil, may destroy the soil and cause isolates within many Earth microorganisms by changing pH scale, reducing the solubility of some metals, and creating allochthonous microorganism biomass and open enhancements (Alburquerque et al. 2011; Chen et al. 2015).

Biochar is an after-effect of the pyrolysis of harvested plant remains, animal excreta, and municipal waste that can be used to reinforce microorganisms for degradation by making nature progressively exceptional (Ok et al. 2015). Some broad reviews have portrayed the potential estimation of biochar as a convincing administrator in the immobilization of metals and normal toxins (Mohan et al. 2014; Ahmed et al. 2016; Rizwan et al. 2016; Yuan et al. 2017).

Biochar can give, acknowledge, or exchange electrons inside the surroundings abiotically or through natural pathways (Klupfel et al. 2014; Saquing et al. 2016). Many scientists recommend that biochar will likewise encourage transporting forms of microbe electrons and show comparative utilitarian qualities to soil redox dynamic issues (Graber et al. 2014). Biochar acts by expanding the pH of tainted soils along these lines, influencing the availability of excessive metals for the uptake of plants.

The versatility and harmful nature of arsenic, copper, and lead depends on their oxidation states and redox responses (Violante et al. 2010; Tandon et al. 2013). Another oxidative course for the change of As(III) to As(V) detailed utilizing dirt bolstered with zero-valent nanoparticles of iron by blending ferric nitrate with alcohol of momentarily accessible tea. Removal of arsenic (III) up to 99% in sullied water was accomplished. The viability of remediation relies upon such factors as the nature of the living beings used, the predominant ecological components at the debased site, and the level of the toxins in that condition (Azubuike et al. 2016). Remediation can similarly be harnessed through the use of microbes and by floras that bond, extricate, and reclaim toxins from the earth, that is, plant-based remediation. The available metal is absorbed by plants, which forms a part of nutrient translocation, and thereby the level of the contaminant is diluted (Tak et al. 2013).

6.4 Bioremediation Involving Microorganisms

The remediation of metals by microorganisms is outlined in Table 6.1.

Microorganisms are set for substantial decrease and discharge of mercury-polluted water (Horn et al. 1992). Microorganisms complement the normal cycle of mercury in the earth. A few microbes can change mercury into an innocuous structure that appears in positive relationship between the existence of the sheltered organisms and the spread of mercury blends in dirtied sediment. Detoxification of mercury by methylation leads to remediation (Robinson and Touvinen 1984). Fungi are omnipresent, and some prevail in metal-contaminated living spaces, where they can take up and fix lethal metals and radionuclides to an organic product assemblage that can be utilized by parasites and fungal organisms (Gadd 1986; Brown and Hall 1990).

Table 6.1 Heavy metal pollution reclamation by microorganisms (Bernard et al. 2018)

Microbial group	Responsible organism	Metallic minerals	Metal in ionic level (mg/l)	Sorption efficiency (%)
Bacteria	*Acinetobacter* sp.	Cr	16	87
	Sporosarcina saromensis (M52)		50	82.5
	Bacillus cereus		1500	81
	Bacillus cereus (immobilized)		1500	96
	Bacillus circulans MN1		1110	71.4
	Bacillus cereus in addition to 0.5 glucose		1	78
	Bacillus cereus		1	72
	Bacillus sp. SFC		25	80
			50	43
	Bacillus subtilis		0.57	99.6
	Desulfovibrio desulfuricans (KCTC 5768) (immobilize on zeolite)		100	99.8
			200	56.1
			50	99.6
Bacteria	*Cellulosimicrobium* sp. (KX710177)	Pb	50	99.33
			100	96.98
			200	84.62
			300	62.28
	Methylobacterium organophilum		–	18
	Gemella sp.		0.3	55.16 ± 0.06
	Micrococcus sp.			36.55 ± 0.01
	Bacillus firmus		–	98.3
	Pseudomonas sp.		1	87.9
	Staphylococcus sp.		0.183	82.6
	Streptomyces sp.		0.286	32.5
	Bacillus iodinium		100/1.8	87
Bacteria	*Desulfovibrio desulfuricans* (KCTC 5768) (immobilize on zeolite)	Cu	50	97.4
			100	98.2
			200	78.7
	Staphylococcus sp.		1.536	42
	Streptomyces sp.		1.129	18
	Enterobacter cloacae		100	20
	Desulfovibrio desulfuricans (immobilize on zeolite)		100	98.2
	Bacillus firmus		–	74.9
	Flavobacterium sp.		1.194	20.3
	Methylobacterium organophilum		–	21
	Arthrobacter strain D9		0.05	22
	Enterobacter cloaceae		100	65

(continued)

Table 6.1 (continued)

Microbial group	Responsible organism	Metallic minerals	Metal in ionic level (mg/l)	Sorption efficiency (%)
	Micrococcus sp.		0.3	38.64 ± 0.06
	Gemella sp.			50.99 ± 0.01
	Micrococcus sp.		0.3	38.64 ± 0.06
	Pseudomonas sp.		1	41
	Flavobacterium sp.		0.161	25
	Alcaligenes faecalis (GP06)		100/19.2	70
	Pseudomonas aeruginosa (CH07)		100/17.4	75
Bacteria	*Desulfovibrio desulfuricans* (immobilize on zeolite)	Ni	50	90.3
			100	90.1
			200	90.1
	Micrococcus sp.		50	55
	Pseudomonas sp.		1	53
	Acinetobacter sp. B9		51	68.94
Bacteria	*Vibrio parahaemolyticus* (PG02)	Hg	5	90
			10	80
	Bacillus licheniformis		0.1	73
	Vibrio fluvialis		0.25	60
Bacteria	*Bacillus firmus*	Zn	–	61.8
	Pseudomonas sp.		1	49.8
Consortium organisms	*Acinetobacter* sp. and *Arthrobacter* sp.	Cr	16	78
	P. aeruginosa and *B. subtilis* (P + B)		570/2	99.6
	S. cerevisiae and *B. subtilis* (Y + B)		570/16	97.2
	S. cerevisiae and *P. aeruginosa* (Y + P)		570/4	99.3
Fungi	*Aspergillus versicolor*	Cr	50	99.89
	Immobilized *Saccharomyces cerevisiae* (Y bead)		570/0	100
	Gloeophyllum sepiarium		–	94
	Saccharomyces cerevisiae (Y)		570/25	95
	Aspergillus niger (FIST1)		–	64.7
	Mutant *S. cerevisiae*		–	98.7
	Sphaerotilus natans		200	60
	Saccharomyces cerevisiae		–	99
	Sphaerotilus natans		200	82
	Phanerochaete chrysosporium (immobilized on loofa wipe)		100	98
Fungi	*Aspergillus versicolor*	Cu	50	29.06
	Sphaerotilus natans		200	58

(continued)

Table 6.1 (continued)

Microbial group	Responsible organism	Metallic minerals	Metal in ionic level (mg/l)	Sorption efficiency (%)
	Aspergillus lentulus		100	99.7
	Aspergillus niger		–	50
Fungi	*Aspergillus versicolor*	Ni	50	30.05
	Aspergillus sp.		50	90
	Aspergillus niger		–	58
Algae	*Spirogyra* sp.	Cr	5	98.23
	Spirulina sp.		5	98.3
Algae	*Chlorella vulgaris*	Pb	50 mg dm^{-3}	99.4
	Chlorella vulgaris		51.79	99.4
	Nostoc sp.		1	99.6
Algae	*Chlorella vulgaris*	Cu	50 mg dm^{-3}	97.7
	Spirogyra sp.		5	89.6
	Spirulina sp.		5	81.2

6.5 Fungal Bioremediation

Continuous resistance toward excessive metals exhibits the fungal potential to degrade metals. Although the components of this resistance are not completely comprehended, the technique utilizes a specific procedure of concealment on collective processes of a plant breeding framework toward mercury by the fungus *Trichoderma harzianum*, which displays a potential role for hydrophobin, which can separate nonpolar particles in liquid medium. Therefore, resistance was communicated through a comparative development on control culture rate (Puglisi et al. 2012). For the most part, two components have been proposed for substantial metal resilience in parasites. The first component contains an extracellular (chelation and cell wall-binding) sequestration, and the second has a direct sequestration of metal in which the proteins are completely different than those binding to the ligands to defend against harming the target-sensitive metal cell. In this manner, extracellular components are basically suggested in the evasion of metal passage, whereas intracellular frameworks decrease the metal weight in the cytosol. In the primary system, distinctive natural atoms that have no place with the network of the cell divider are discharged by the parasitic cell to chelate metal particles. The nearness of different anionic structures, for example, glucan and chitin, gives a contrary charge to the cell surface of microorganisms, which enables microorganisms to attach to cations of the metals. In the living instrument, the transport of metal proteins could be fashioned with flexibility, either by removing particles of metal on the cytoplasm or via permitting sequestration onto the vacuolar section (Jain and Arnepalli 2016).

6.5.1 Standards Involved in Fungal Siderophore Bioremediation

Fungi generally have four phases in iron transportation. (1) Within the automotive framework, the Fe^{3+} is moved toward the cell, where Fe^{3+} is completely free at the time; the proteins are decreased by attack, and the unattached siderophores are furthur reused. The framework associated with the example is mainly used to transport the ferrichrome by some compelling species. (2) For instance, in *Ustilago maydis* within the taxi element, the Fe^{3+} obtained from extracellular cells is affected by an intracellular film. Species of *Rhodotorula* most commonly use these elements for transportation (Winkelmann and Huschka 1987). (3) In the framework of interaction with liquid, all Fe^{3+} is completely removed to some subtractive point and caused to degrade. The Fe^{3+} is then converted to Fe^{2+} within the cellular level. Further, the component is employed within the intake of Fe^{3+}-triacetylfusarinine structures by means of *Mycelia sterilia* (Adjimani and Emery 1988). (4) The most subtractive tool used does not help in the transportation of iron over the cellular layer. Therefore, the conversion of Fe^{3+} to Fe^{2+} happens at the cell level, and this section is carried out by the intake of ferrous iron from ferrichrome by a *Sphaerogena* fungus. The general system of iron procurement is given in Fig. 6.1.

Mercury is a harmful ingredient to plants, so that after such procedures it may (1) alter the oxidizing framework (Israr and Sahi 2006), (2) change the photosynthesis structure, (3) suppress plant improvement and yield age through upgraded takeup and maintaining physiological processes (Patra and Sharma 2000), and (4) begin damage by chemical agents that are produced within a cell, causing mutations. Mercury can adhere to DNA and disrupt the genetic information (Chenkci et al. 2009). The impact of mercury on all social functions of the living species and natural framework shapes, including microbial and very large scale, interferes with the soil process. The acquired structure of mercury

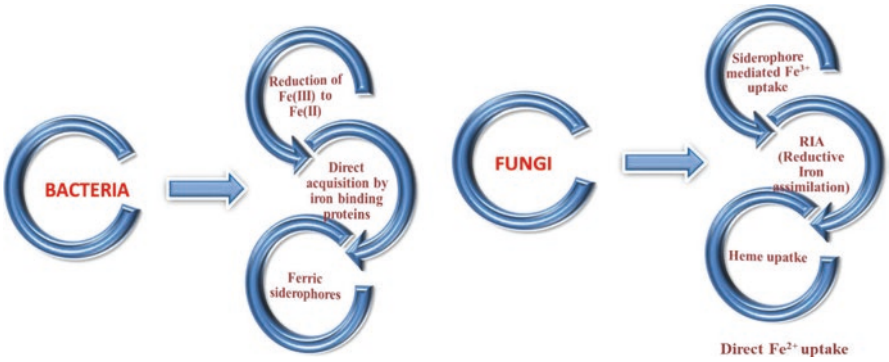

Fig. 6.1 Mechanism of iron acquisition

affects orchestrated grouping of microorganisms that are sensitive; in any case, the fabricated mercury is an impediment to living beings, for example, mercury-resistant minuscule life forms (HgR) (Ranjard et al. 1997).

Fungi are recognized for their greater ability to harvest a wide diversity of cell proteins needed for growth, such as organic acids and enzymes. Their waste biomass might be utilized as a viable biosorbent for the evacuation, decrease, and detoxification of modern effluents. Be that as it may, these effluents contain high groupings of substantial metals that may enter into human and nonhuman populations through the natural order, causing numerous metabolic issues in the influenced individual. Thus, it is important to expel the overburdening metals from soil and wastewater through easy innovation, for example, turn-around assimilation, dissolvable extraction, lime coagulation, or particle trade. Concoction precipitations for evacuation of substantial metals are wasteful or over-the-top expensive, particularly when the grouping of the overwhelming metal particles is low, in the range of 1 to 100 mg/l (Iram 2012).

As indicated by the *Soils in the Environment Encyclopedia* (2004), omnipresent fungi rule in soil with high natural issue. Developments have very important characteristics and monetary basics for the Earth. The organisms have a place in Eukaryota and its phyla, for example, the species of Ascomycota, the Zygomycota, the Basidiomycota, and the Chytridiomycota, in addition to a casual gathering of mitosporic parasites (once the growth Imperfecti or Deuteromycota). These organisms are disseminated worldwide and have the ability to develop in a wide variety of living spaces such as in extraordinary situations, for example, deserts or remote ocean silt. Most parasites develop in the earth; however, a few animal types live to some extent or exclusively in the amphibian condition.

Seagoing parasites include those living in liquid regions of the ocean, animate entities perpetually documented in term of the wide microorganism system within the Earth. Within the usual setting, development can interface with varied life forms, for instance, new growth patterns or blue-green algae as lichens. This affiliation assumes employment in soil starting by that suggested state of enduring. Contagious relationships with plants return as interdependence (mycorrhizas) and interdependency (plant-infective growths). In relationship with the soil, the fauna has an exceedingly similar structure as its connected plants. The key is the development structure, which includes a sort of string filament that develops by top growth and occasional spreading to form a plant part that saturates the niche, within which a parasite is developing. The space across hyphae shifts with age, species, and food intake; where they are heterotrophs in nutrition, for instance, they use mounted (natural) C sources as substrate.

Respiration will be either oxygen greedy or anaerobic, and depends on the facultative nature. The dietary intake might begin from other animal sources either as parasitic or mutualistic relationships, whereas others are saprotrophs. The infectious part of a plant, such as spores all around, shows up as an organic phenomenon or sexual reproductive structure, the sclerotia (thick, energetically pigmented hyphal collection), or hyphal components. The natural ability and cruciality of fungi might remain in latent resting stages known as rhizomorphs, conidia, and spores. These

structures allow parasites to engage and induce growth in very negative conditions, for example, in different seasons, or if the density of the host population has been lost.

Moreover, a twofold division is assumed, either to arise as a new form of their limits. If temperature, water, and supplements are adequate, reinstating reproductive structure growth is possible. Developments increase enhancement, usually in natural structures in which their elementary occupation is as a decomposer. Parasites connected with C, N, or P are the foremost elementary employments on soil sequences. Thus, the rule-of-thumb employment of parasites is counteracted by polysaccharides incorporated to the enzyme polyphenol oxidases. Parasitic-mediated corruption on compounds with a face can create a grouping of blends, for example, traditional acids (siderophores) that immobilize crucial metals. Thus, immobilization occurs after sequestration on mycelia that are discharged following death and lysis on pathogens. Eucarpic life is a form that requires a basic occupation for transport onto the soil surface.

6.5.2 Contextual Investigations in Fungal Bioremediation

The components of impacts on soil structure segments include (1) catching soil molecules together, utilizing its strands, and (2) separating typical issues tied to the Earth with its degradative supermolecules. Soil-dwelling organisms excrete immense amounts of a holddown feature of hydrophobic proteins (hydrophobin) that serve to confirm hyphal dividers.

All things considered, these blends apart from coating soil particles may reliably affect the capacity to absorb water. As hyphae are linked throughout the world, this is one of the main mechanisms causing physical fixing of the soil. The genus *Aspergillus* is viewed as an operating master in ruined plots as clarification for human and creature illnesses and as creating a director passing on basic metabolic operations. As in the categorical *Aspergillus* species, *Aspergillus flavus* follows a general course. It develops better with water temperatures between 0.86 °C and 0.96 °C; the ideal temperature is 37 °C. However, parasitic movement will be seen at temperatures from 12 °C to 48 °C, and furthur rise in temperature adds to human pathogenicity (Hedayati et al. 2005).

Aspergillus flavus uses much of its time on Earth living as an organism within the dirt. It takes into account action as an associated improvement recycler, proceeding via the plant (Scheidegger and Payne 2003). So, progression will create disruptive conditions, thus showing its strength with another life structure for the substrate within the earth or a plant. Development is manifestly capable to overwinter in a similar way in a plant part, which tends to sprout to create an extra hypha or turn out conidia (abiogenetic spores) that are more dispersed within the earth and air. *A. flavus* as such actually differs little from *Aspergillus oryzae*. The genetic science may be particularly surprising as *A. flavus* could be a customary microorganism whereas the sequenced species of *A. oryzae* are being used in soy, creating issue by living for

innumerable years (Hedayati et al. 2005). The genus *Aspergillus* has been established on a basic dimension on allotments in structural and social features (Raper and Fennel 1965) that are accumulated in nine species and two hybrids. Species such as *A. flavus* seeking affirmation within the genus *Aspergillus* are rendered questionable by their morphological and organic chemistry properties.

The conidiophores are variably long, sharp, and spiked; they are characterized as uni- or biseriate. By unfolding to the total cyst, phialides are indicated in all points. Conidia are global to subglobose, noticeably spores, dynamically 3.5–4.5 mm wide.

Bioremediation of mercury mining utilizing movements of fungi is noted to survive and detoxify metals by one or two structures together with valence modification, intra- and living precipitation, and dynamic uptake. The large surface volume capacity for detoxifying metals is a potential choice instead of assembled steps for bioremediation of incapacitated techniques of metals and robust waste (Li et al. 2009).

Parasitic metal changes are visible in the conservative and stationary stages. The irresistible convenience of metals happens through heterotrophic (chemoorganotrophic) analytics, for instance, solubilizing the lead material by *A. niger*, green lead ore ($Pb_5(PO_4)3Cl$), and methylation of metals to yield temperamental subordinates (selenium) that might offer one way for cleaning. The metal immobilization technique connects biosorption or metal knowledgeable within the cell (Gadd et al. 2014). Further utilizing *A. niger*-pretreated biomass to eliminate inorganic (Hg^{2+}) and alkyl group mercury (CH_3Hg^+) by using a fluid strategy (Kapoor et al. 1999) is comparable to *A. versicolor* (Spry and Wiener 1991). The actual *A. fumigatus* and *A. flavus* species are stated to have a high assurance from metal, for instance, zinc contamination on material waste products (Moneke et al. 2010), and likewise as atomic number 82, Zn, Cu, and atomic number 28 from Egyptian paper rush gushing (Tamer and Tunali 2006). Metals are difficult to collect by means of parasitic biomass (Zafar et al. 2007). Huge amounts of mercury offer group action information regarding bioavailability and reactivity (Issaro 2009). In this manner the evolution strategy remains very large; as an example, soil with low bioavailable mercury focus has to be compelled to be initially increased up to its metal bioavailability before being remediated utilizing bioremediation strategy. On the off-chance that it is low proficiency, then other remedies have to be expedited. Some remedial forms of progress for mercury-polluted soil are created. With everything considered, the essential rationalization behind mercury concentration within the dirt for the usage of remedy advancements is 260 mg/kg (Wang et al. 2012). Strategies for extraction are mainly needed to remove mercury at more than 260 mg/kg, whereas modification frameworks are accessible to modify and reduce the values of mercury.

Mycoremediation could be a type of bioremediation in which enhancement area units are applied to clean the location. The period of time of mycoremediation unambiguously recommended is the employment of parasitic mycelia in bioremediation. One in each of the basic livelihoods of parasites within the Earth is weakening, which is completed by corruption of natural objects. The plant part secretes extracellular proteins and acids that are numerous polymers and polyoses, the two noteworthy structure bases of plant fiber. These area unit seasoning mixes are composed of long chains of carbon and chemical elements, primarily resembling varied

natural pollution. Mycoremediation allows investigating the privileged contagious species that specialize in a specific infection. Mycofiltration could be a comparable methodology, using parasitic mycelia to channel dangerous waste and microorganisms from water and also from the soil.

Considered that *Aspergillus flavus* is superbly useful for the remediation of nickel from watery media. As wished, bio-clearing means that this parasite was compelled to fill in for treating leachate, emanating moreover conjointly dirtying the water areas destined for risky metallic scrap. Thus, advantageous composed efforts with AM have been planned joining the contraptions of liberal metallic plant coverings (Hildebrandt et al. 2007) containing water (Auge 2004; Ruiz-Lozano and Azcon 1996).

Sullied soils areas are the foremost component delineated by negative soil structure, low water restriction, everyday problems, and supplements. In such regard, revisions to the dirt before the inoculation of AM growths has been prescribed. The mycorrhizal flowers benefited by elements solubilized from rock phosphate via *Aspergillus niger* through an atomic ^{32}P debilitative structure; also, the creators exhibited an impressive association between the excellence through *A. niger*-handled dry olive cake (DOC) and *Glomus mosseae* for plant development (Alguacil et al. 2008). Similarly, Caravaca (2005, 2006) laid down unified prescriptions, as amendment of the integrity of mycorrhizal protection against *Glomus intraradices* helps conjointly in the enlargement of advanced DOC. They broadened their advance in *Juniperus oxycedrus*, which is far better than each treatment associated self-sufficiently. Valls (2000) have used the notably artificial *Ralstonia eutropha*, associated indicator of soil, to sequester metals from soils. McGrath (1995) tested unfavorable impacts of the metals on the action and assembled a mixture of soil microorganism masses. Soil debasement for the foremost half makes changes within the mixture and causes AM to not be able to resist human impacts (Koomen et al. 1990).

Tinker (1981) noted that excessive extents of overwhelming metals can delay, scale back, or possibly totally exclude AMF plant organ germination and AM organization at fixations such that phytotoxic effects are not visible. Similarly, noted an unpleasant affiliation among Zn fixation in soil treated with city-mechanical slop and AM organization in grain. Higher damage from zinc, copper, lead, and other metallic elements of native upgrades from slop-contaminated regions, but with regard to removal from pure soils, has been addressed by Gildon and Tinker (1983) and Daniel (2006), who separated the introduction of *Armillaria* rhizomorphs into functions in degrading the metals in soils and also to cause mensuration against excessive metals. Eliminating the available toxic metals that generally ensured discrimation during helpful circumstances was considered the first-ranked goal. Niu (1993) stated that at low hydrogen ion concentrations these will work as biogeochemical directors for bioaccumulation of dissoluble varieties. Iqbal (2005) showed that *A. niger* and *Penicillium* sp. have contributed to promote bioadsorption of the most distant elements; chromium and nickel have diverse multimetallic paths that constituted abuse of the thready parasites of metallic-debased areas. Parameswari (2010) showed that advances derived against liberal metals having metallic biosorption ability can be used for expulsion with the watery metallic approach; moreover,

what is seen more is no temporary entreaty among the area of metal removal and biosorption unallowed. Such organisms are a straightforward unit of the soil sustenance web, giving sustenance to aggregations that live in the dirt. They will be associated as a very important component in the deterioration of waste issue (Rhodes 2013).

The significance of mycoremediation lies in utilizing parasites in bioremediation, and the long strings of hyphae assist with soil particles and roots, framing a filamentous body known to endure overpowering metals (Baldrian 2003). These organisms can change themselves to develop under different unusual states of pH, temperature, supplement availability, and high metal fixation (Anand et al. 2006). Zafar (2007) showed promising biosorption for cadmium and chromium by two parasitic genera, *Aspergillus* and *Rhizopus*, isolated from contaminated rural soil. Species of Ascomycetes and Basidiomycetes are the parasites most usually detailed for extreme metal elimination from polluted soils (Narendrula-Kotha and Nkongolo 2017). The oxalate stones formed by microorganisms additionally can remove toxicity from substantial amounts of metal (Michael et al. 2014). The filamentous structure of the hypha profoundly infiltrates through deeper soil, so substantial amounts of metals are adsorbed. Root exudates additionally assume a critical function in changing metal bioavailability, as the arrival of certain natural mixes assembles metals by shaping metal buildings as well as giving supplement and vitality to microbial networks, thus bolstering plant development and survival.

Microorganisms have been employed in a significant application procedure to remove metals, showing improvements known to be step-by-step tolerance toward metals, which has favored organisms whose surface-to-volume sum is superior to an autochtonus population. Soils and mineral substrates are not merely a part of the growth of biota (Volesky 2007).

6.6 Conclusion

A number of significant subatomic apparatuses have been developed for the hereditary and metabolic buildup of microorganisms that can contain natural contaminants and allow ecological cleanup. Certainly, these new systems will provide much simpler development of new or improved strains than previously. When taking up hereditary alterations, the procedure should be fully comprehended, and any exploration should dependably consider the genuine dangers and advantages included while expanding these innovations for society. Fungal organisms have the biochemical and biological capacity to degrade ecologically natural synthetic compounds and to diminish the dangers related to metals, metalloids, and radionuclides, either by blend modification or by affecting substance bioavailability. As opposed to minuscule life forms, fungal development does not require endless water stages for unlimited dispersal. Their hyphae move transversely over air–water interfaces, associate with air-filled soil pores, and form into soil pores. Parasitic mycelia similarly aid the advancement of extrahyphal microorganisms, transport supplements

among spatially confined source and sink areas, and transport hydrophobic contaminants. Organisms co-utilize numerous natural synthetic mixtures and thus do not rely upon the use of mixed carbon and energy sources. Toxin-debasing parasitic chemicals incorporate a few extracellular oxidoreductases basically proposed to disintegrate lignocellulose, as cell-bound catalysts, enabling growth to follow on a wide scope of toxic substances with biochemical and natural capacity to avoid ecological natural synthetics and to lessen the risk related to metals, metalloids, and radionuclides, either by concoction revision or by affecting bioavailability. Parasites also do not require unlimited water stages for dispersal. Their hyphae cross air–water interfaces and connect with air-filled soil pores. Contiguous mycelia also aid development of additional hyphal microscopic organisms, transport supplements among spatially isolated source and sink areas, and transport hydrophobic natural contaminants. Growths coprocess numerous natural synthetic compounds and hence do not rely upon the utilization of mixed carbon and energy sources. Toxin-corrupting parasitic proteins incorporate a few extracellular oxidoreductases basically expected to disintegrate lignocellulose, just as do cell-bound compounds, enabling organisms to effectively manage a wide scope of contamination.

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Chapter 7
Contrasting Role of Fungal Siderophore in Metal Ion Complex Formation

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K. Dhusia et al. (eds.), *Fungal Siderophores*, Fungal Biology,
https://doi.org/10.1007/978-3-030-53077-8_7

7.1 Introduction

Plants and microbes have vast significance in our day-to-day life. Iron is considered to be an abundant element in the soil (earth's crust) and essential for all life processes such as respiration, DNA synthesis, tricarboxylic acid cycle and production of various small molecules like amino acids, lipids, and sterols. Being an essential element in earth's crust, the bioavailability of iron is limited in the habitat (soil and sea) owing to its low solubility. This property of iron results in its poor uptake by plants, which eventually makes iron an essential nutrient for plant growth. In aqueous and oxygenated conditions, the iron is found in its supreme state, which accumulates in the form of minerals such as iron oxides and iron hydroxides and is not ready to be utilized as such by organisms. In order to overcome this restricted process, the microbial flora of soil such as *Pseudomonas* spp., *Enterobacter* genus, *Bacillus* spp. produces special iron-binding tiny carriers, called 'siderophore', that help to scavenge iron from these mineral phases (oxides and hydroxides) by forming soluble iron (Fe^{3+}) complexes which are readily taken up by the environment through active transport mechanism (Philpott 2006).

Siderophore came from Greek words sidero that means 'iron' and phore that means 'carriers', and in combination, it is termed as 'iron carrier'. Siderophores are small, low-molecular-weight (<10 kDa) iron-chelating compounds, secreted by plants and microorganisms (bacteria and fungi) to maintain their iron requirement. These are also produced by rhizospheric bacteria in iron-limiting conditions in order to increase the plant growth by scavenging iron from the environment and make it available to the cell near the plant roots (Sah and Singh 2015; Li et al. 2016).

7.2 Siderophore-Mediated Iron Transport

Iron transport in siderophore is an energy-dependent mechanism. Type and stereoselectivity of siderophore are specific factors in recognition and transport of iron-siderophore complexes in microbes. The complexation also depends on metal ion coordination geometry as well as N-acyl residues present at the periphery of central metal ion. For instance, the coordination of metal centre and configuration of ligand affect the stability of complex. In case of *Rhodotorula pilmanae*, configuration of macrocyclic rings of siderophore is favoured, whereas in contrast to this, in *Penicillium parvum*, *Neurospora crassa* and *Aspergillus quandricinctus*, L-*cis*-ferrichrome is found to be a stable configuration. Further, the geometrical stability of complex also depends on the types and number of N-acyl residues surrounding the iron coordination centre (Huschka et al. 1986).

In spite of having specific transport mechanism of siderophore, many microbes may utilize multiple transport system as well as more than one type of siderophore at a time for efficient transport of metal ion. For example, microorganism like *Agaricus bisporus* has variable transport systems for fusarinines and ferrichromes,

whereas *Neurospora crassa* represents different recognition sites for coprogen- and ferrichrome-type siderophore system (Howard 1999).

In fungal species, majority of literature suggested that *Saccharomyces cerevisiae* has two high-affinity iron transport mechanism. In first reductive mechanism, ferric iron (Fe^{3+}) is identified to be reduced by a number of inducible membrane-bound reductase, namely, Fre1p–4p, usually present at cell surface. Across plasma membrane, the reduced iron (Fe^{2+}) is then transported by involvement of permease-oxidase, namely, Ftr1p and Fet3p. This mechanism is utilized by variety of Fe^{3+}-siderophore complexes such as ferrichrome, triacetyl fusarinine C and rhodotorulic acid for their transportation after being reduced by cell-surface-bound reductase. On the other hand, the second mechanism involves the uptake of iron (Fe^{3+})-siderophore complex as an intact form into the cell. All the proteins that are involved in the transport of so far identified complexes by this type of mechanism belong to major facilitator superfamily, namely, Sit1p (also known as Arn3p which helps in transporting ferrioxamine B, ferrichrome and ferrichrome A), Arn1p (helps in transporting ferrirubin, ferrirhodin and ferrichrome A), Taf1 (also known as Arn2p, which helps in transporting triacetyl fusarinine C) and Enb1p (helps in transporting enterobactin). The uptake specificity may vary among receptors as well as among strains (Renshaw et al. 2002). For instance, Arn1p specifically transports ferrichrome-type siderophores around the iron centre which have branched-chain ornithine-N5-acyl residues but does not support the short-chain acetyl hydroxamic residues present in siderophore such as ferrirubin and ferrichrome. Likewise, Arn2p is found to specifically transport triacetyl fusarinine C, whereas Sit1p has been found to be less specific. Arn1p and Sit1p transport the complex via cell surface and are then rapidly internalized as both are localized in intracellular vesicle layers (Seneviratne and Vithanage 2015). Table 7.1 represents the list of siderophore transport supported by different receptors.

Table 7.1 Various receptors involved in siderophore transport in fungi (Bairwa et al. 2017; Raymond 1994)

Fungal species	Protein/receptors	Siderophore transported
S. cerevisiae	Arn1	Ferrichrome and ferrichrome A transport
	Arn2/Taf1	Triacetyl fusarinine C transport
	Arn3/Sit1	Ferrichrome and ferrichrome A transport
	Arn4/Enb1	Enterobactin transport
Candida albicans	Arn1/Sit1	Ferrichrome-type xenosiderophore transport
C. glabrata	Sit1	Ferrichrome transport
Cryptococcus neoformans	Sit1	Ferrioxamine transport
A. fumigatus	Sit1	Ferrichrome and ferrioxamine B transport
	Sit2	Ferrichrome transport
	MirB	Triacetyl fusarinine C transport
Histoplasma capsulatum	Mfs1, Abc1	Putative siderophore transporter
Rhizopus oryzae	Fob1, Fob2	Ferrioxamine binding at cell surface

7.3 Fungal Iron Regulation

In fungi, regulation of iron uptake is necessary to maintain iron homeostatic processes. For this, four different mechanisms of iron uptake have been suggested in fungi at molecular level by different mechanistic-based studies (Haas 2014). These four mechanisms involve (a) ferric iron (Fe^{3+}) uptake through siderophores, (b) reductive iron assimilation, (c) heme uptake and d) direct iron uptake. These four mechanisms are explained as follows.

7.3.1 Iron (Fe^{3+}) Uptake Through Siderophore

Each and every fungal species exhibit siderophore-iron transporter (SIT)-mediated extracellular iron uptake mechanism. SIT constitute majorly facilitator protein family, which acts as a proton-coupled symporters potentiated by plasma membrane. In addition, high solubility and high energy factors render iron-chelated siderophore to combat during microbial growth. On the other hand, triacetyl fusarinine (TAFC) and fusarinine C (FsC) facilitate intercellular release of iron by partial hydrolysis by esterase (Estb) enzyme (Howard 1999).

7.3.2 Reductive Iron Assimilation (RIA)

In fungi, iron acquisition with high affinity is usually achieved either by secreted siderophores (iron chelators) or by reductive iron assimilation (RIA) mechanisms (Fatima et al. 2017). In order to start iron uptake, iron is first reduced from ferric (Fe^{3+}) to more soluble ferrous (Fe^{2+}) form by localized ferrireductases present in the plasma membrane of fungal species. Soon after this, the ferrous iron is re-ionized which is further imported by protein complex consisting of iron permease (FtrA) and ferrioxidase (Fetc) genes. The protein such as permease also transports metals other than iron such as copper and zinc.

7.3.3 Heme Uptake

In contrast to bacteria, binding and uptake of iron, in fungi, are done only with heme component. For instance, *Candida albicans* heme uptake mechanism involves the glycosylphosphatidylinositol (GPI)-anchored cell surface mannoprotein, namely, Rbt5P, but the details regarding its transport mechanism are still unknown. In order to utilize heme-iron complex, the uptake requires intercellular degradation of heme with heme oxygenase (Hmx1p, localized in endoplasmic reticulum).

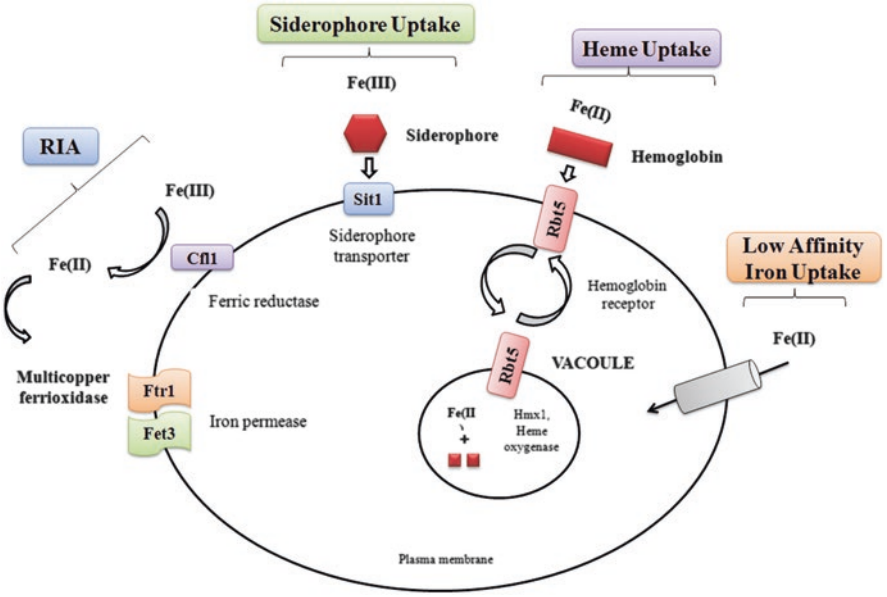


Fig. 7.1 Different approaches in fungal iron acquisition

7.3.4 Low-Affinity Iron Uptake

The iron in the form of ferrous (Fe^{2+}) is taken up by permease Fet4p. The system is non-specific for Fe^{2+} form of iron as it also transports other metals such as zinc and copper. In *Saccharomyces cerevisiae*, the iron supply is associated with mobilization of iron from vacuole which is facilitated by fluid-phase endocytosis and Smf1p protein belonging to natural resistance-associated macrophage protein (NRAMP) family (Kosman 2003).

All the four iron regulation mechanisms are pictorially represented in Fig. 7.1.

7.4 Types of Siderophore

Chemically based on the interaction sites, siderophore has been categorized into two main groups, namely, first 'enterobactin', the strongest iron chelator which shows interaction between iron and catecholate hydroxy groups, and second 'hydroxamate' that acquires N-hydroxylated amide bonds (e.g. ferrichromes in fungi). Atoms like nitrogen (N), oxygen (O) and sulphur (S) can also participate in the coordination of iron in carboxylate groups (Drechsel et al. 1995; Butler and Theisen 2010). Mixed type of siderophores involves those donating groups that do not belong to hydroxamates or aromatic hydroxy group category. The description for various types of siderophores (shown in Fig. 7.2) is summarized as follows.

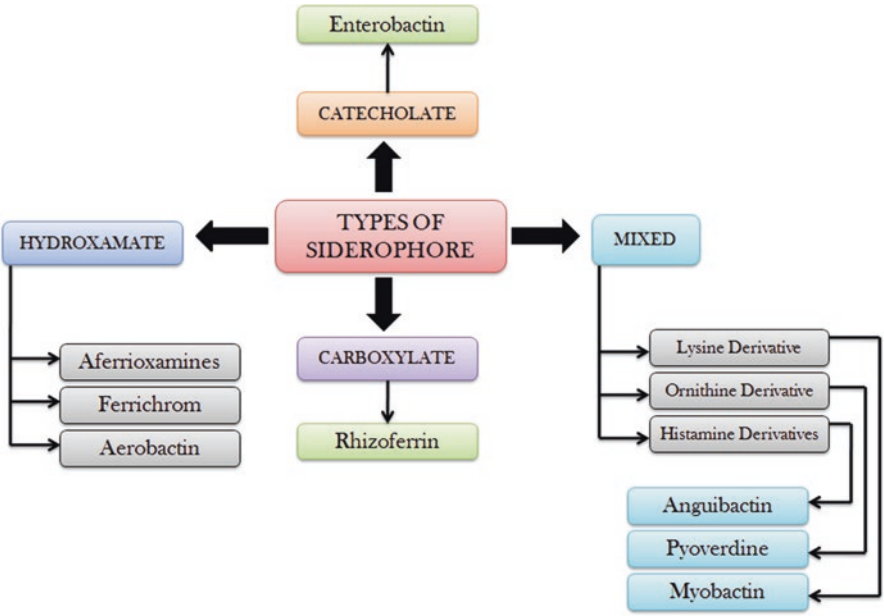


Fig. 7.2 Schematic representation of different types of siderophores

7.4.1 Hydroxamate Siderophores

Chemically, these are specifically tri-hydroxamate type of siderophores reportedly found in fungi and majorly produced by fungal species that belongs to Zygomycotina (Mucorales), Ascomycotina (*Aspergillus* spp., *N. crassa*) and Deuteromycotina (*Fusarium dimerum*) subdivision of fungi. Hydroxamate siderophores have strong correlation among their hydroxamate groups and bound ligands as they known to form hexadentate, tetradentate and bidentate complexes. In hydroxamate class mainly, ferrichromes are of main concern with respect to ecological importance due to their potential to chelate iron from soil and supply to plant species, whereas other naturally occurring siderophores are not as effective because of their affinity towards other metal ions too. From the literature reports, ferrichrome derivative such as ferrioxamines also exhibits antibiotic activity (named as ferrimycines). In addition, rhodotorulic acid, dimerium acid, alkaligens and putrebactin also belong to hydroxamate-type siderophore family (Garnerin et al. 2017).

7.4.2 Carboxylate Siderophores

A new class of siderophore was identified by Winkelmann from fungi *Rhizopus microspores* belonging to zygomycetes, which contain 'hydroxyl' and 'carboxyl' moieties as donor groups to iron (Fe^{3+}) which are solely responsible for metal bind-

ing. This type of siderophore is termed as 'carboxylate siderophore' well known as 'rhizoferrin' which is isolated from *Rhizopus* spp. using ion-exchange column chromatography. Structurally, rhizoferrin and its analogues contain 1,4-diaminobutane symmetrically acylated to the terminal carboxylate of citric acid through amide bonds (Drechsel et al. 1995).

7.4.3 Catecholate Siderophore

This class of siderophores is known to have phenolate or 2,3-dihydroxybenzoate (DHB) as a binding moieties. Catechol (also called as pyrocatechol), naturally occurring organic, colourless compound, is the ortho-isomer of the three isomeric benzenediols and found in trace amounts. *Azotobacter vinelandii*, in iron-deficient medium, forms various types of catecholate-based siderophores such as monocatecholate aminochelin, dicatecholate azotochelin and tri-catecholate protochelin (Baakza et al. 2004). Basically, all these types of naturally occurring siderophores contain negatively charged oxygen donors as hard Lewis base which binds with Fe^{3+} which act as hard Lewis acid as per its chemical nature.

7.4.4 Mixed Ligand Siderophores

This class of siderophores, also called heterobactins, contains combined donor groups of hydroxamate and catecholate together. Siderophore of this type includes mixed ligand of lysine, ornithine and histamine derivatives. For instance, mycobactins (*Mycobacterium* spp.) contain hydroxamate and phenolate donor groups as chelating ligands (Mohammad et al. 2011).

7.5 Mechanism of Binding of Iron in Cell

In fungi, siderophore-mediated iron (Fe^{3+}) uptake can be regulated by four mechanisms, namely, shuttle, hydrolytic, taxicab and reductive. Three of them, i.e. shuttle, taxicab and hydrolytic mechanisms, depend upon the specific recognition of several siderophore. (a) In shuttle mechanism, the iron (Fe^{3+})-siderophore complex initially enters the cell membrane, and soon after that, it releases the metal from ligand (e.g. ferrichrome in fungal species, viz. *Ustilago sphaerogena* and *Ustilago maydis*) which ultimately leads to excretion of free siderophore (Sah and Singh 2015). This mechanism is depicted in Fig. 7.3.

The hydrolytic mechanism involves the transportation of iron (Fe^{3+})-siderophore complex in its intact form into the cell (e.g. uptake of ferric triacetyl fusarinine C in *mycelia sterilia*). Simultaneously, both reductive and degradative steps occur inside

the cell that lead to the reduction of iron from ferric (Fe^{3+}) to ferrous (Fe^{2+}) form and also the cleavage of ester bonds in siderophore (triacetyl fusarinine C) in the presence of specific esterase resulting in monomeric fusarinines which are further excreted as shown in Fig. 7.3. This mechanism is helpful for removing toxic metals such as aluminium (Al^{3+}), gallium (Ga^{3+}) and chromium (Cr^{3+}) from the cell as these metal-siderophore chelates have similarity to the prototype iron (Fe^{3+}) complex and their complexes are taken up inside the cell where these metals remain bounded to the monomeric fusarinine, so further reduction of these metals is restricted resulting in their excretion (Sanz-Ferramola et al. 2013), whereas, (c) in taxicab mechanism, iron in ferric form is transferred from extracellular siderophore to intracellular ligands (e.g. ferric rhodotorulate in *Rhodotorula pilimanae*) across the cell membrane as the extracellular siderophore does not enter the cell membrane (Gerwien et al. 2018). However, (d) in reductive mechanism, the reduction of iron (Fe^{3+})-siderophore complex occurs at the membranes instead of transporting that complex inside the cell, and the reduced ferrous form is taken up by the cell membrane. This type of mechanism is used for transporting ferrichrome siderophores in some fungal species such as *Ustilago maydis* (Trivedi et al. 2016).

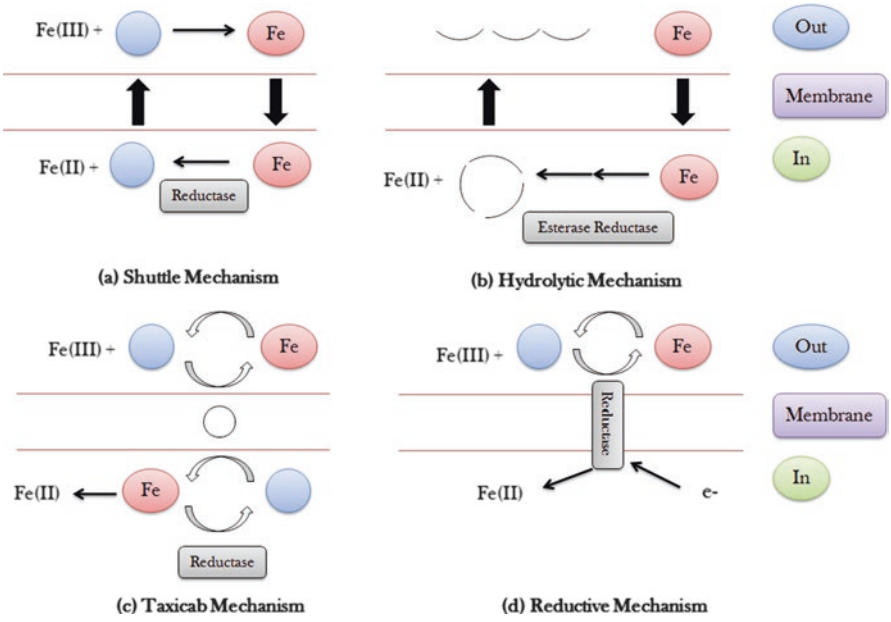
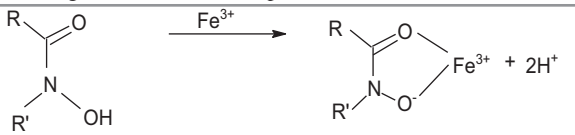
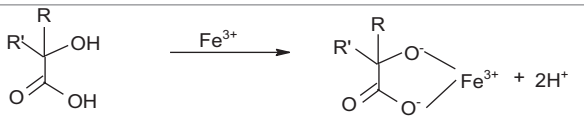
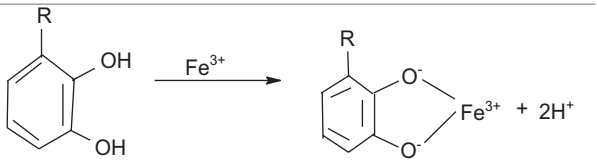
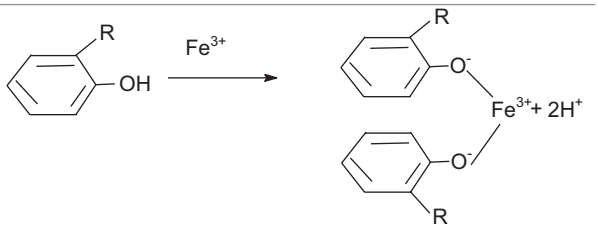


Fig. 7.3 Depiction of various types of iron uptake mechanism via siderophore across cytoplasmic membrane in fungi

7.6 Binding Sites in Siderophores

The stability of iron-siderophore complex strongly depends on the nature of binding sites present in varied siderophore structure with different functional motifs. All the different types of siderophores reported till date form extremely stable, highly specific complex containing high-spin ferric iron. The different moieties that explain the anchoring in most siderophore-iron complex belong to hydroxamates, catecholate, carboxylate and mixed-type categories as shown in Table 7.2. The first three types show some similarities in respect to their iron-binding affinity. These three classes hire two sites of the iron centre that form stable five-membered ring structure complexing metal iron to form siderophore-iron complex. Oxygen shows high affinity towards iron (Fe^{3+}), and also classified under hard donor ligand, further, a lone pair of oxygen atom always coordinates with iron cation in order to increase the strength of coordination complex. Using this donor-acceptor interaction, the selectivity of siderophore ligand for iron improves many folds, and this helps in transportation of iron across the cell membrane. Mixed type of siderophore consists of different binding sites. For example, rhizoferrin, a fungal siderophore, contains two molecules of citric acid usually linked with an additional chain. Further, it requires two hydroxycarboxylic acid and two carboxylic acid groups for iron coor-

Table 7.2 Binding site of iron in different types of siderophore (Wittenwiler 2007)

Sl. no	Class of siderophore	Binding site of iron in siderophore
1.	Hydroxamate	
2.	Carboxylate	
3.	Catecholate	
4.	Mixed ligand (phenolate type)	

dination (Neilands 1995). Table 7.2 illustrates the structural representation of iron-siderophore complex among different classes of siderophore.

The physico-chemical parameter, i.e. pKa values of binding groups, has a great influence on the siderophore's complex stability as the oxygen atom in these complexes is interacting in a non-protonated state. From the available literature, hydroxamic acid has been shown to have pKa values in the range of 8–10; however, acetohydroxamic acid (main compound for monohydroxamate ligand) has shown to have 9.29 pKa value. The pKa values of carboxylates lie in the range of 3.5–5 which contributes to efficient iron imbibitions by carboxylate siderophores under low-pH conditions. The microbes living in acidic medium (such as fungi) use carboxylate siderophores for iron immobilization. In spite of this fact, the carboxylate siderophores couldn't compete with stronger siderophores such as hydroxamates and catecholates as they are fully protonated at physiological pH. In catecholate, a model monomer has been synthesized in such a way that once nitro group is added on para position, then the pKa values of resulting alcohol groups were found to be 6.69 and 10.83. On the other hand, after substituting it with hydrogen atom, the resulting pKa values were 9.26 and 13.3. The pKa values of binding groups have an impact on the affectivity of siderophores as the oxygen atoms only bind with the groups in non-protonated state. These values are not easily accessible and effected by side chain modifications (Neilands 1995). The pKa values for hydroxamate, catecholate and carboxamate groups exhibit the partly deprotonated states in the presence of iron at neutral pH. Proton-independent pKa values do not demonstrate the actual iron-binding efficiency of siderophores at physiological pH due to incomplete deprotonation. For better analysis, pH analogous to pFe values is considered to be a better approach for comparing the true relative abilities of iron binding with different siderophores by giving negative decadic logarithm of free iron concentration. As per standards, the total ferric concentration and total ligand concentration are considered to be 10^{-6} M and 10^{-5}, respectively. The chelation efficiency is strongly influenced by pH of medium; thus, pFe value is a pH-dependent value. For instance, at serum pH (i.e. 7.4), in the presence of enterobactin and aerobactin, the concentration of free iron is observed to have different pFe values which are 35.5 and 23.4, respectively (Wilson et al. 2016; Miethke and Marahiel 2007).

Phytosiderophores are hexadentate ligands that coordinate with Fe^{3+} from all six coordination sites which explore a variety of combinations at the three binding sites to form a potential iron-siderophore complex. In the contrary, many siderophores use only one type of binding site to form the stable complexes with iron such as trihydroxamate siderophore (ferrioxamine) and tri-catecholate siderophores (enterobactin) (Renshaw et al. 2002).

7.7 Metal Ion Complex Formation with Siderophore

Several fungal species have been investigated with complex regulatory arrangements during intake of secondary metabolites, namely, mycotoxins (produced by mycotoxigenic fungi) and its detoxification process of converting these metabolites

from more toxic to less toxic form. The metals such as iron, zinc, copper, chromium, gallium and manganese interact with the siderophore produced by different fungi to make stable siderophore-metal complexes resulting in homeostasis regulation. These metal ions may interact with variety of siderophore. Table 7.3 highlights some of the examples of metal-ion-siderophore complexes along with their functions.

Table 7.3 Different metal ion complexes with siderophores

Sl. no.	Metal ion	Fungal source	Siderophore and derivatives	Functions	References
1.	Iron (Fe)	*Saccharomyces cerevisiae*	Catecholate, hydroxamate, ferrioxamine, ferricrocin	Facilitates two types of high-affinity iron transport systems, namely, reductive and non-reductive transport	Senthilnithy (2008)
2.	Iron (Fe)	*Paracoccidioides yeast*	Hydroxamates	Target for antifungal chemotherapy	Lesuisse et al. (1998)
3.	Iron (Fe)	*Trichoderma* species	Hydroxamates, carboxylates	Crop enrichment, improvement of medical interest and management of fungal disease on crops	Baila et al. (2014)
4.	Iron (Fe)	*Aspergillus* species, *Penicillium oxalicum*, *Aureobasidium pullulans*, *Phanerochaete chrysosporium*	Hydroxamates	Iron mobility and transport through soil	Ghosh et al. (2017)
5.	Iron (Fe-III)	*Rhizopus microspores*	Carboxylates (rhizoferrin)	Relieve the iron-restricted growth by iron transport into the cells of the indicator strains	Drechsel et al. (1995)
6.	Iron (^{55}Fe) with 13C-desketoneoenactin	*Candida* species	Ferrichrome, hydroxamates	Drug targeting and promotes growth in an iron-restricted medium	Baakza et al. (2005)
7.	Cobalt (Co-II/III)	*Penicillium brevicompactum*, *Aspergillus fumigates*	Hydroxamates (desferricrocin, desferrioxamine, desferricoprogen, tri-acetyl fusarinine)	Antifungal effect	Bernier et al. (2005)
8.	Gallium-68 (^{68}Ga)	*Aspergillus fumigates*	Hydroxamates	Infection imaging	Farkas et al. (2018)

(continued)

Table 7.3 (continued)

Sl. no.	Metal ion	Fungal source	Siderophore and derivatives	Functions	References
9.	Gallium-68 (^{68}Ga), zirconium-89 (^{89}Zr)	*Aspergillus fumigates*	Ferrichrome, ferrichrome A, tri-acetyl fusarinine C, desferrioxamine, desferrioxamine E, coprogen, fusarinine C, ferricrocin	Infection imaging	Petrik et al. (2012a, b), Haas et al. (2015) and Petrik et al. (2010, 2014, 2015)
10.	Gallium-67 (^{67}Ga)	*Ustilago sphaerogena*, *Aspergillus fumigates*	Ferrichrome, ferrichrome A, rhodotorulic acid, tri-acetyl fusarinine C, malonichrome, desferrioxamine	Significant iron transport in microbes	Petrik et al. (2016), Emery and Hoffer (1980), Conti and Eriksson (2016) and Velikyan (2014)
11.	Chromium (Cr)	*Ustilago sphaerogena*	Desferrioxamine	Analogous to iron-siderophores that competitively inhibit utilization of iron-siderophore	Stintzi et al. (2000)
12.	Gallium-67 (^{67}Ga), indium-111 (^{111}In)	*Escherichia coli*, *Salmonella typhimurium*	Enterobactin (tricatecholamide analogue)	Ligand for radiopharmaceuticals	Crowley et al. (1988) and Petrik et al. (2017)
13.	Cobalt (Co)	*Fusarium solani*	Ferricrocin	Synthesized intercellular siderophore for iron storage, and highlighted the importance of iron tolerance against cobalt	Moerlein et al. (1981)
14.	Zinc (Zn)	*Candida albicans*, *Aspergillus fumigates*	Zincophores	Significant for identification of novel virulence factors in microbes	Rasha (2017)
15.	Zinc (Zn) and copper (Cu)	*Fusarium* species	Ferricrocin	Imposed toxic effect to the fungi present in soil	Wilson et al. (2012)
16.	Zinc (Zn), copper (Cu) and iron (Fe)	*Rhizophagus irregularis*	Enterobactin, coprogen	Significant for plant colonization	Islam and Datta (2015)
17.	Copper (Cu II), manganese (Mn-II), zinc (Zn-II), magnesium (Mg-II)	Mucorales, *aspergilli*, *penicillia*, *Neurospora crassa*, *Fusarium dimerum*	Hydroxamates, carboxylates	Improved binding affinity of siderophore	Tamayo et al. (2014)

7.8 Biological Functions of Siderophore

Siderophore affects the plant and microbes in several significant ways. These are discussed in detail as follows.

7.8.1 An Iron-Scavenging Compound

Siderophores have the ability to support the growth factor in all fungi and auxotrophic organism despite the fact that they are producing or non-producing species. Several auxotrophic organisms found to be in underdeveloped stage in the absence of these compounds require siderophores for their growth. For instance, *Pilobolus kleinii* utilizes coprogen as an essential growth factor (Dave and Dube 2000). The siderophore-mediated transport system facilitates and enhances the efficient competency among microbes for the consumption of available iron to make survival of one type of microbe over the other. This is advantageous in case of non-pathogenic species producing more siderophores as it competes more efficiently for iron and limiting the growth of pathogenic species (less competent) of the same organism at the same time (e.g. *Fusarium species*, *Aspergillus ochraceus*) (Aznar et al. 2014).

7.8.2 Virulence Factor

The siderophore-mediated transport system also plays an important role in microbial pathogenicity. Host pathogenic microorganism acquires iron to survive, and restricting its availability to others is considered to be an effective defence mechanism of host microbes that suggest that siderophores can act as virulence factors. For example, *Microbotryum violaceum* siderophore (mutant of plant pathogen) accumulates less rhodotorulic acid as compared to its wild type that exhibits minimal pathogenicity. On the other hand, mutant of *Ustilago maydis* (plant pathogen) showed defectiveness in siderophore production and is found to be as virulent as its wild-type strains. In addition, phototoxic and immunosuppressive effects also contribute to virulence in pathogenic organism in terms of chelating activity in siderophores. For example, *Alternaria cassiae* produces siderophore such as coprogen and ferricrocin which act as phytotoxins and several other siderophores such as desferrichrome which exhibits immunosuppressive effect in mouse model (Ecker et al. 2018).

7.8.3 *Intracellular Iron Storage*

Apart from solubilizing and transporting iron, siderophore also facilitates intracellular iron acquisition in mycelia and spores that effect germination. In case of *Neurospora crassa*, several intercellular siderophores produced by the species such as excretory coprogen and intracellular ferricrocin found in both hyphae and conidia are essential for its germination and iron storage. It is also well established that iron-siderophore complexes maintained in hyphae for the incorporation of spores help in sporulation and also transport iron to mycelial mitochondria. Several fungal species have both types of inter- and intracellular siderophores such examples *Penicillium chrysogenum*, *Aspergillus nidulans*, *Rhodotorula minuta* and *Ustilago sphaerogena* (Renshaw et al. 2002).

7.9 Applications of Siderophore

Siderophore and its different derivatives have broad scope in medical sciences and offer several applications in the field of biotechnology, microbial ecology and biomedical science. Various studies demonstrate the effective role of some siderophore in the management or treatment of human diseases and infections (Popat et al. 2017; Nagoba and Vedpathak 2011; Ahmed and Holmstrom 2014; Saha et al. 2016; De-Serrano 2017; Dimkpa 2016; Fine 2000; Banner and Woolf 2004). These are as listed in Table 7.4 which are as follows.

Table 7.4 Enlisting of biotechnological, ecological and biomedical applications of siderophores

Sl. no.	Area	Description
Biotechnological and ecological applications		
1.	Microbial ecology and taxonomy	The method of identification and characterization of microorganisms based on siderophore type they produce is known as 'siderotyping' which is further divided into analytical (HPLC analysis) and biological (specific DNA recognition) approaches
2.	Biocontrol of fish pathogens	Siderophores limit the iron availability in pathogenic fish (by making siderophore-iron complexes) which is necessary for the interaction in microbial virulence
3.	Optical biosensor	Pyoverdine has been investigated as an element of the sensor for molecular recognition in the identification of iron bioavailability in ocean water and soils

(continued)

Table 7.4 (continued)

Sl. no.	Area	Description
4.	Nuclear fuel reprocessing	Desferrioxamine B (hydroxamate and catecholate functionalities) is supposed to form strong complexes and have high-stability constants due to hard oxygen anions which help in separation of contaminants (acetanilide) during Purex process (fuel reprocessing)
5.	Bioremediation of environmental pollutant	Due to high affinity of siderophore towards metal ions such as Cd, Zn, Cu, Ni, and Pb, siderophores are extremely effective in solubilizing and increasing mobility of these metals and possess strong affinity to form stable complexes, thus making it useful in bioremediation
6.	Bio-bleaching of pulps	Siderophore (isolated from *Gloeophyllum trabeum*) effectively reduces the proportion of chemical used in bleaching process and can be an environment-friendly approach
7.	Siderophore promotes plant growth	Siderophores provide nutrition in form of iron to support their growth when the bioavailability of iron is low
8.	Soil mineral weathering	Siderophores help in protecting microbes against environmental stress by adhering to mineral surfaces
9.	Biogeochemical cycling of iron in the ocean	Siderophores make the iron available to phytoplankton in marine waters which plays an important role in their growth
Biomedical applications		
10.	Selective drug delivery-Trojan Horse strategy	Development of siderophore-antibiotic conjugates called 'sideromycins'
11.	Treatment of disease condition with iron overloading	Effective in conditions like haemochromatosis, sickle cell disease and thalassemia (major)
12.	Management of Malaria	Siderophore (such as desferrioxamine B) produced by *Streptomyces pilosus* has activity against *P. falciparum* (in vitro and in vivo)
13.	Removal of overload of transuranic elements like aluminium and vanadium	Siderophore named Desferal interacts with aluminium and vanadium ions and forms complexes which are readily excreted from the body via urine and faeces
14.	Cancer therapy	Several siderophores such as dexrazoxane, O-trensox, desferriexochelins and desferrithiocin are found to be effective in cancer therapy
15.	Antidote for iron poisoning	Deferoxamine B is used as antidote in acute iron poisoning and high iron loading conditions such as thalassaemia major and aluminium poisoning in chronic renal dialysis. Deferoxamine B is considered to have a high binding affinity for ferric form of iron that helps in removing excess iron from tissues and circulatory system

7.10 Conclusion

From recent investigations, it can be concluded that siderophores are the key components in iron transport in phototrophs and microorganisms. Structural variation and ligand specificity in siderophore as well as membrane receptors regulate the iron uptake process; hence, this field has immense potential for further exploration in the field of biomolecular science. The siderophore plays significant role in environmental applications and is also investigated as potential strategy in the field of biotechnology (agriculture, bioremediation and biosensor) and medicines (diagnosis and treatment). In a search of advance-level revelations, the metagenomic approach with detailed chemical examinations may be employed to improve the current environmental applications that also give new realm of investigation for siderophores. With the metal-chelating ability, siderophores are known to have potential applications in the field of medicine and biotechnology. Apart from iron binding (Fe^{3+}), the variety of siderophore is also investigated for binding with other metals including Pb^{2+}, Cr^{3+}, Al^{3+} and actinide ions. The study of metal-microbe conjugation highlights the significance of microbes which provides suitable environment for growth and reproduction of various forms of life. From the established literature, it is clear that siderophores represent the vital organic compounds for iron uptake among microbial and plant species. Siderophore variability in terms of their structural and functional characteristics and membrane receptors involving metal coordination in relation to microbial communities should be thoroughly investigated to establish the role of siderophore at profound level in the field of advance-level therapy in medical science.

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Chapter 8
Chemistry and Biomedical Applications of Fungal Siderophores

Mohamed Saleem Abdul Shukkoor and Shaik Ibrahim Khalivulla

Contents

8.1 Introduction

Siderophores are synthesized and released by various fungi to bind iron from the environment. Most of the fungi synthesize siderophores except *Saccharomyces* species (Neilands 1993; Lesuisse 1994). Fungal siderophores have diverse biomedical applications ranging from diagnostics to treatment of microbial resistance. Various fungi synthesize siderophores (Renshaw et al. 2002; Varma et al. 2007). Chemically, hydroxamate moiety is present in most of the fungal siderophores and subsequently they belong to hydroxamate type (Leong 1986). The major pharmaceutical applica-

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K. Dhusia et al. (eds.), *Fungal Siderophores*, Fungal Biology,
https://doi.org/10.1007/978-3-030-53077-8_8

tion of siderophores is Trojan-horse strategy whereby the siderophore conjugates are introduced into the microbial cells to kill them (Mislin and Schalk 2014).

Siderophore-iron complexes are transported into the fungal cell through "siderophore-iron transporters" (SITs), a group of transporter proteins belonging to the major facilitator protein superfamily. These receptors are conserved in all fungal species including the species such as *Saccharomyces cerevisiae*, *Candida* spp. and *Cryptococcus neoformans* which are not producing siderophores. Interestingly, many of the fungal species are capable of utilizing the siderophores synthesized by other species through these receptors (Philpott and Protchenko 2008; Petrik et al. 2017).

8.2 Chemistry of Siderophores

Numerous fungal siderophores have been isolated and their physicochemical and spectral properties have been characterized very well. Most of them are of hydroxamate-type, except the polycarboxylate rhizoferrin (Thieken and Winkelmann 1992). They are biosynthetically derived from L-ornithine. Fungal siderophores consist of bidentate hydroxamate ion ligands. Hydroxamate ions are derived from hydroxamic acid by consecutive deprotonation of hydroxyl and replacing the hydrogen with alkyl attached on nitrogen (Fig. 8.1).

The presence of mesomeric effect (Fig. 8.2) due to more electronegative oxygen atoms and pulling nature of π-electrons, the hydroxamate and α-hydroxycarboxylate ion shows two resonating forms by the delocalisation of the π-electrons and negative charge on oxygen.

N^5-hydroxy-N^5-acylornithine is the basic unit for the fungal hydroxamate siderophores. The hydroxamate functionality originates through subsequent hydroxylation and acylation of the δ-amino group of L-ornithine (Fig. 8.3). The ornithine is a non-proteinogenic amino acid.

The high electron density on two oxygen atoms on hydroxamate ions (bidentate ligands) forms effective conjugation with iron (III) (Ferric, Fe^{3+}) in fungal siderophores. Each siderophore contains three hydroxamate ions in their structure, except rhodotorulic acid (2) and dimerum acid (3), which contains only two hydroxamate ions (Fig. 8.8). Three bidentate ligands of hydroxamate ions forming six coordinate covalent (dative) bonds with central iron (III) gives the octahedral complex structure. The hybridisation involved in the iron (III) is explained by the valence bond theory.

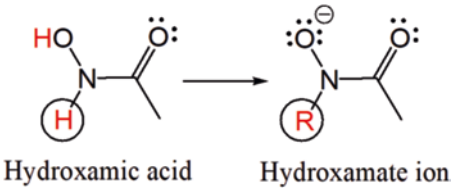


Fig. 8.1 Conversion of hydroxamic acid to hydroxamate ion

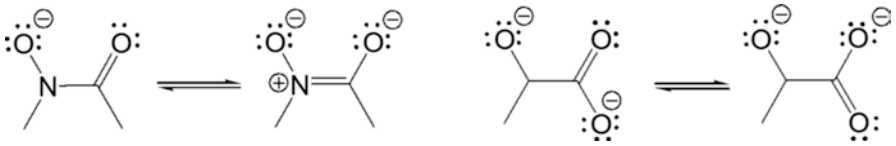


Fig. 8.2 Hydroxamate (left) and α-hydroxycarboxylate (right) bidentate ion ligands resonating forms

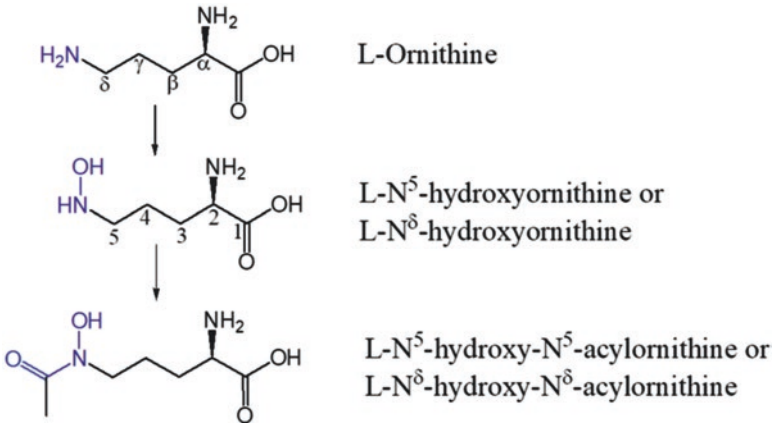
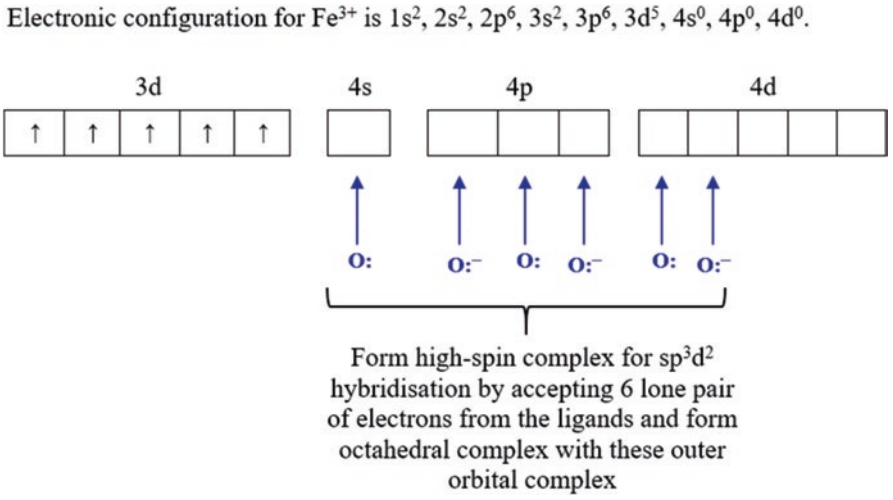


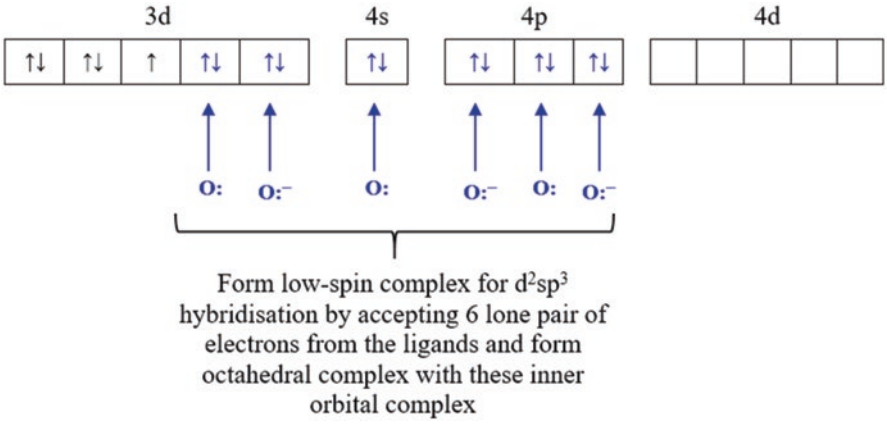
Fig. 8.3 Origin of the basic unit, N^5-hydroxy-N^5-acylornithine of fungal siderophores

Atomic iron (Fe) electronic configuration is $1s^2, 2s^2, 2p^6, 3s^2, 3p^6, 3d^6, 4s^2, 4p^0, 4d^0$.

The loss of two 4s electrons and one 3d electron forms the Fe^{3+} ionic state. In this form, due to five unpaired electrons in 3d orbital the spin of the ion is high, called high spin ion which is an unstable form. If this ion forms a complex with three bidentate ligands, then six an empty orbitals are required on the metal to receive the six coordinated lone pairs of electrons. To form this complex, the valence (outer) orbitals one 4s, three 4p (4_{px}, 4_{py}, 4_{pz}) and two 4d ($4d_{x^2-y^2}$, $4d_{z^2}$) orbitals get hybridised to give a set of six equivalent sp^3d^2 hybrid orbitals. A ligand orbital of oxygen (hexadentate hydroxamate ions) each with a lone pair of electrons makes a coordinate (dative bond) with these empty hybridised orbitals of Fe^{3+} metal ion and forms an octahedral structure of the complex.



Due to the involvement of valence (outer) orbitals, the energy of these orbitals is quite high and the complex is reactive and labile. An octahedral complex with low energy involvement is also possible by the use of inner d-orbitals. In this case, the unpaired electrons in d-orbitals are forced to pair up, then spin of unpaired ions will reduce and becomes low spin complex. In this case also, the hybridisation is d^2sp^3, hence the structure of the complex is octahedral.



With an exception of α-hydroxycarboxylate type of chelation in rhizoferrin (**1**) (Fig. 8.4), isolated from *Rhizopus microsporus* var. *rhizopodiformis*, which is composed of citric acid and diaminobutane (Drechsel et al. 1991), siderophores reported from the fungus are all hydroxamate type of chelation.

Structurally, the fungal hydroxamate siderophores are classified into three families, namely, coprogens, fusarinines and ferrichromes.

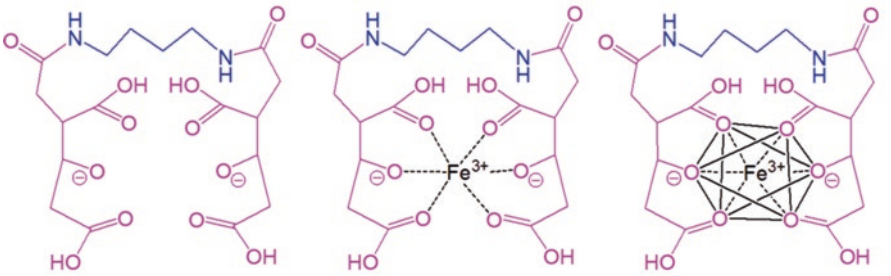


Fig. 8.4 Example for d^2sp^3 hybridised Fe^{3+} ion and the structure of **1** and its Fe^{3+} octahedral complex

8.2.1 Coprogens

Coprogens (Table 8.1) are connected by a head-to-head dipeptide bond of two N^5-hydroxy-N^5-acyl-L-ornithine monomers to form a cyclic dipeptide or diketopiperazine unit in the middle and hydroxamate members towards the end with different substituents. Dihydroxamate members (**2**, **3**) (Fig. 8.5) of the coprogen family (also called Rhodotorulic acid family) are rhodotorulic acid and dimerum acid. In the trihydroxamate members of the coprogen family, the additional monomer of substituted N^5-hydroxy-N^5-acyl-L-ornithine is linked either side by an ester bond (**4–15**) (Figs. 8.6 and 8.7).

8.2.1.1 Dihydroxamate Coprogens

Rhodotorulic acid (**2**), containing two acetyl groups, was first isolated from the yeast *Rhodotorula pilimanae* in 1967 by Atkin et al. (Atkin and Neilands 1968), and from fungus *Ustilago inflorescentiae* by Muller et al. (Muller et al. 1985). The dimerum acid (**3**) formed by a head-to-head peptide bond of *trans*-fusarinine moiety was first isolated from the fungus *Fusarium dimerum* (Diekmann 1970) and connected with two *trans*-anhydromevalonoyl residues.

8.2.1.2 Trihydroxamate Coprogens

In the trihydroxamate coprogen members, a N^5-hydroxy-N^5-acyl-L-ornithine (or *trans*-fusarinine) and *trans*-fusarinine form a head-to-head dipeptide bond, and an additional substituted N^5-hydroxy-N^5-acyl-L-ornithine (or substituted *trans*-fusarinine) moieties laterally attaches to the *trans*-fusarinine side chain to form an ester bond. In these compounds, the common chemical groups are ornithyl residues and the middle N^{δ}-*trans-anhydromevalonoyl* groups. In coprogen (**4**), the third *trans*-fusarinine, the free amine (N^2) group hydrogen is replaced with acyl group.

Table 8.1 Fungal hydroxamate coprogen siderophores

	Name of the siderophore	*Fungal species*	References
(i) Dihydroxamate members	Rhodotorulic acid (**2**)	*Ustilago inflorescentiae*	Atkin and Neilands (1968) and Muller et al. (1985)
	Dimerum acid (**3**)	*Fusarium dimerum*	Diekmann (1970)
(ii) Trihydroxamate members	Coprogen (**4**)	*Penicillium* sp.	Pidacks et al. (1953)
	Coprogen B (**5**)	*Fusarium dimerum*	Diekmann (1970)
	Hydroxycoprogen (**6**)	*Alternaria longipes*	Jalal and Helm (1989)
	Hydroxyneocoprogen I (**7**)	*A. longipes*	Jalal and Helm (1989)
	Hydroxyisoneocoprogen I (**8**)	*A. longipes*	Jalal and Helm (1989)
	Isoneocoprogen I or Triornicin (**9**)	*Epicoccum purpurascens* (Syn. *E. nigrum*) (triornicin)	Frederick et al. 1981)
	Isotriornicin or Neocoprogen I (**10**)	*Epicoccus purpurascens* (Isotriornicin) and *Curuularia lunata* (Neocoprogen I)	Frederick et al. (1982) and Hossain et al. (1987)
	Neocoprogen II (**11**)	*Curuularia lunata*	Hossain et al. (1987)
	N^{α}-dimethyl coprogen (**12**)	*A. longipes*	Jalal et al. (1988)
	N^{α}-dimethyl neocoprogen I (**13**)	*A. longipes*	Jalal et al. (1988)
	N^{α}-dimethyl isoneocoprogen I (**14**)	*A. longipes*	Jalal et al. (1988)
	Palmitoylcoprogen (**15**)	*Trichoderma strain*	Anke et al. (1991)

This compound was first isolated in 1953 from *Penicillium* strains (Pidacks et al. 1953). Figure 8.6 shows the presence of three bidentate hydroxamate ligands in coprogen (**4**) that help to hold the ferric ion (Fe^{3+}) firmly and can expect other trihydroxamate copragens (Fig. 8.7) form similar Fe^{3+} complexes. In most of the trihydroxamate coprogen members, the N^2 substitution is acyl group.

The species *Epicoccum purpurascens* (Syn. *E. nigrum*) belongs to the family Leptosphaeriaceae which is a rich source of siderophores with four major constituents of 87% of the total weight, ferricrocin, coprogen, triornicin, and isotriornicin, and among the compound neocoprogen I (triornicin) (**9**) is having slight antitumor activity. The compounds **9** and isotriornicin (**10**) are positional isomers with the exchange of acetyl and (*E*)-5-hydroxy-3-methyl-2-pentenoyl hydroxamate groups (Frederick et al. 1982). Compound **10** is the first example of trihydroxamate siderophore in the solid state with Δ-*trans* geometry of iron coordination (Hossain et al. 1987).

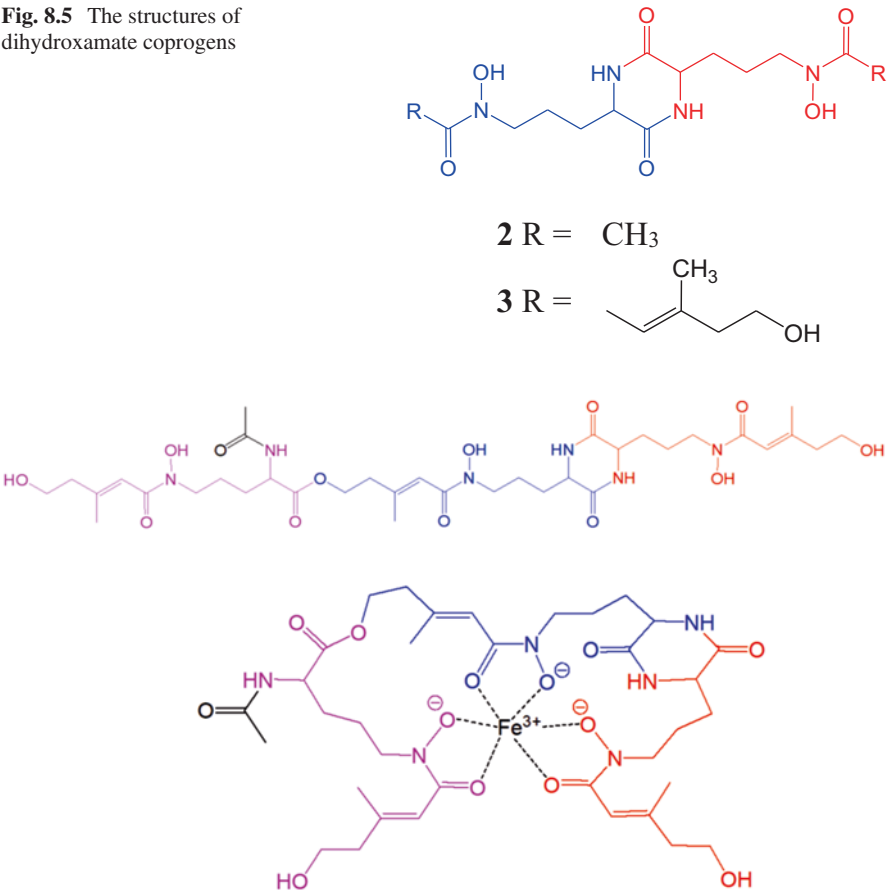


Fig. 8.5 The structures of dihydroxamate coprogens

Fig. 8.6 Coprogen (**4**) and its Fe^{3+} ion complex

Structurally, the compounds N^{α}-Dimethyl neocoprogen I (**13**) and N^{α}-Dimethyl isoneocoprogen I (**14**) are similar to isoneocoprogen I (ferric triornicin) (**9**) and neocoprogen I (**10**), respectively, except that they possess N^{α}-dimethyl groups instead of the N^{α}-acetyl group. These compounds contain an N^{δ}-anhydromevalonoyl group at one end of the molecule and an N^{δ}-acetyl group at the other end. In N^{α}-dimethyl neocoprogen I (**13**), the N^{δ}-acetyl group is at the diketopiperazine end, while in N^{α}-dimethyl isoneocoprogen I (**14**), this group is in the ester-linked ornithyl residue (Jalal et al. 1988).

Structures of three hydroxycoprogens **6–8** are hydroxyl analogues of **4**, **9** and **10**, respectively, in which one of the terminal *trans*-anhydromevalonic acid residues is replaced by *trans*-4,5-dihydroxy-3-methyl-2-pentenoic acid residues (Jalal and Helm 1989).

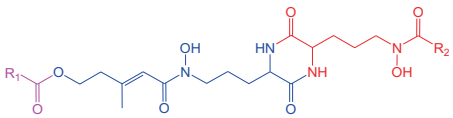
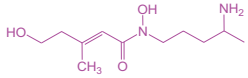
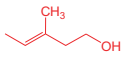
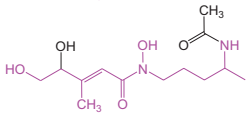
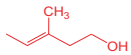
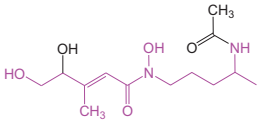
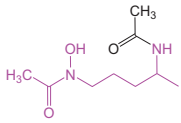
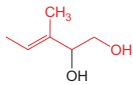


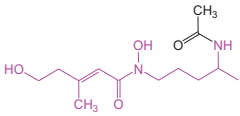
Fig. 8.7 Structures of trihydroxamate coprogens

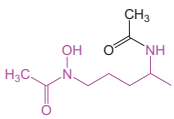
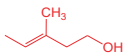
5 R_1 =  R_2 = 

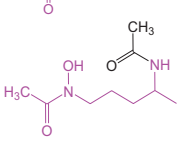
6 R_1 =  R_2 = 

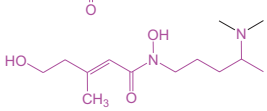
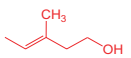
7 R_1 =  R_2 = CH_3

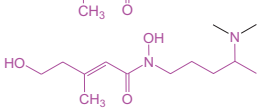
8 R_1 =  R_2 = 

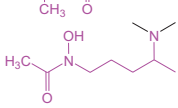
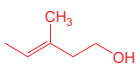
9 R_1 =  R_2 = CH_3

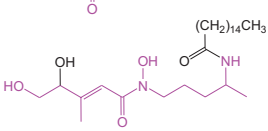
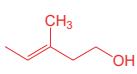
10 R_1 =  R_2 = 

11 R_1 =  R_2 = CH_3

12 R_1 =  R_2 = 

13 R_1 =  R_2 = CH_3

14 R_1 =  R_2 = 

15 R_1 =  R_2 = 

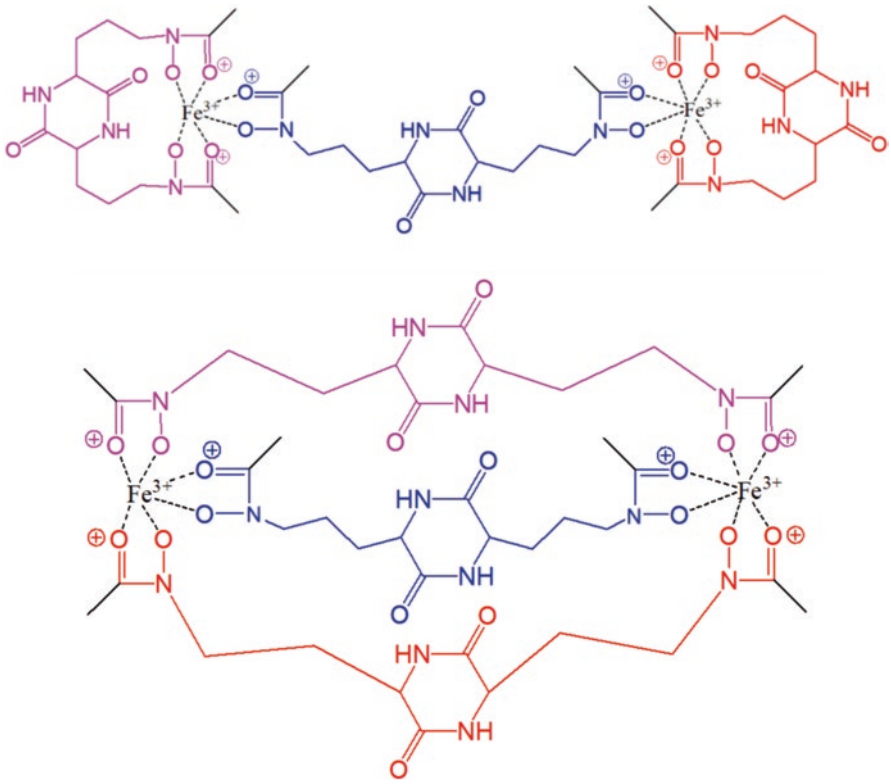


Fig. 8.8 $Fe(III)_2Ligand_3$ type of complexes of **2**

A lipophilic coprogen derivative, palmitoylcoprogen (**15**) is only found inside the cells and not excreted into the culture broth (Anke et al. 1991).

Bidentate ligand copragens generally capture the Fe^{3+} by forming the $Fe^{3+}{}_2Ligand_3$ type of structural complexes (Scarrow and Raymond 1985). For example, the three molecules of rhodotorulic acid makes two ferric ions complex as shown in Fig. 8.8.

Mixed complex of siderophores are also observed with the combination of **3** and *Cis*-fusarinine (**16**) showed in Fig. 8.9 (Jalal et al. 1986).

8.2.2 Ferrichromes

All of these ferrichrome siderophores (Tables 8.2 and 8.3) have a common hexapeptide ring composed of three identical *δ-N*-acyl-*δ-N*-hydroxyornithine groups in its ornithine side chains, but the structure of the acylating acid and their peptide ring constitution may differ in different ferrichromes. For example, ferrichrysine (**17**) is a consecutive cyclic hexapeptide of three amino acids (2 molecules of serine and 1 molecule of glycine) and three molecules of *δ-N*-acetyl-*δ-N*-hydroxyornithine

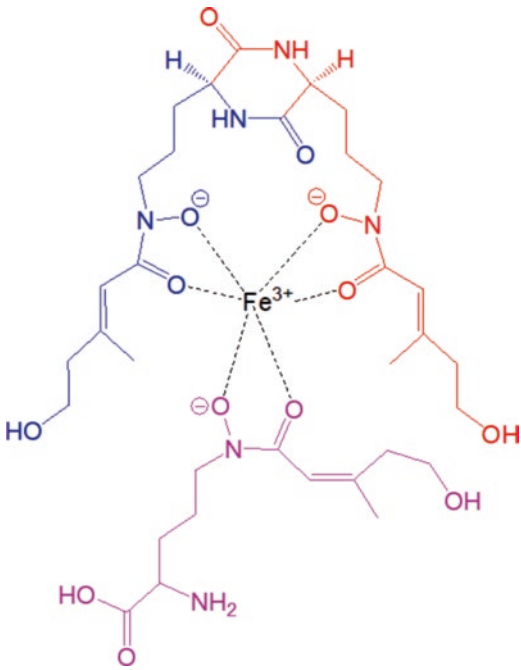


Fig. 8.9 Mixed siderophore complex of **3** and **16**

(Ser_1-Ser_2-Gly-Orn_1-Orn_2-Orn_3) forming a stable Fe^{3+} octahedral complex with three dissimilar ornithyl *δ-N*-acyl groups (Tables 8.2 and 8.3).

Asperchrome Bl, B2 and B3 (**19–21**) (Fig. 8.12, Tables 8.3 and 8.4) are functional isomers with two long ornithyl *N*-acyl groups and a short *N*-acetyl group with difference in the position of *N*-acyl group on the three ornithines present on the hexapeptide ring.

Structurally, the substitution of the acyl group in ferrichrome C (**32**) by malonic acid results in malonichrome (**36**) (Fig. 8.12, Tables 8.3 and 8.4) found in *Fusurium roseum* (Emery 1980).

The efficiency of iron transport by the siderophore in fungal systems increase when the 3-methyl-5-hydroxy-2-pentenyl residues in the ornithines are gradually replaced by the acetyl residues (**17 > 23–25 > 19 > 35**) (Fig. 8.12, Tables 8.3 and 8.4). The efficiency also depends on (i) the position of the odd acyl group when the compounds are isomeric (**19 > 20**) (Fig. 8.12, Tables 8.3 and 8.4) and (ii) the hydrophobicity of the acyl groups (asperchromes **>35**) and of the peptide ring (**18 > 35**) (Fig. 8.12, Tables 8.3 and 8.4). Thus, the efficiency of the ferrichromes depends on minor structural variations, the acyl part, and also the peptide ring of the siderophore which is involved in hydrophobic interactions with the siderophore-binding protein and possibly with the outer lipid membrane (Jalal et al. 1984).

Deml et al. isolated tetraglycyl ferrichrome (**38**) (Fig. 8.10), the first heptapeptide ferrichrome, from *Neovossia indica*. Although it contained increased ring size and conformational flexibility when compared with its hexapeptide analogues, there

Table 8.2 Fungal hydroxamate ferrichrome siderophores

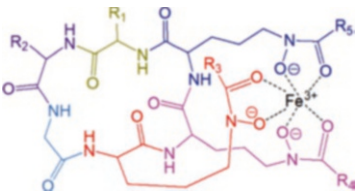
Ferrichromes	The amino acid substitutions	Acylating acid in ornithyl δ-N-acyl groups
Asperchrome A (**18**)	Ser-ala-Gly	*Trans*-β-methylglutaconlylic acid
Asperchrome B1 (**19**)	Ser_1-Ser_2-Gly	Two groups derived from *trans*-5-hydroxy-3-methyl-*Z*-pentenoic acid and one group from acetic acid
Asperchrome B2 (**20**)	Ser_1-Ser_2-Gly	*Two groups derived from trans*-5-hydroxy-3-methyl-*Z*-pentenoic acid and one group from acetic acid
Asperchrome B3 (**21**)	Ser_1-Ser_2-Gly	[a]
Asperchrome C (**22**)	Ser_1-Ser_2-Gly	[a]
Asperchrome D1 (**23**)	Ser_1-Ser_2-Gly	Two groups derived from acetic acid and one group from *trans*-5-hydroxy-3-methyl-*Z*-pentenoic acid
Asperchrome D2 (**24**)	Ser_1-Ser_2-Gly	[a]
Asperchrome D3 (**25**)	Ser_1-Ser_2-Gly	[a]
Asperchrome E (**26**)	Ser_1-Ser_2-Gly	[a]
Asperchrome F1 (**27**)	Ser_1-Ser_2-Gly	[a]
Asperchrome F2 (**28**)	Ser_1-Ser_2-Gly	[a]
Asperchrome F3 (**29**)	Ser_1-Ser_2-Gly	[a]
Ferrichrome (**30**)	Gly_1-Gly_2-Gly_3	Acetic acid
Ferrichrome A (**31**)	Ser_1-Ser_2-Gly	*trans*-β-methylglutaconic acid
Ferrichrome C (**32**)	Gly-ala-Gly	Acetic acid
Ferrichrysin (**17**)	Ser_1-Ser_2-Gly	Acetic acid
Ferricrocin (**33**)	Gly_1-Ser-Gly_2	Acetic acid
Ferrirhodin (**34**)	Ser_1-Ser_2-Gly	*cis*-5-hydroxy-3-methyl-*Z*-pentenoic acid Or *cis*-anhydromevalonoylic acid
Ferrirubin (**35**)	Ser_1-Ser_2-Gly	*trans*-5-hydroxy-3-methyl-*Z*-pentenoic acid Or *trans*-anhydromevalonoylic acid
Malonichrome (**36**)	Ala-Gly_1-Gly_2	Malonic acid
Sake colorant A (**37**)	Ser-ala-Gly	[a]
Tetraglycyl ferrichrome (**38**)	Gly-Gly-Gly-Gly	*cis*-anhydromevalonic acid
Des(diserylglycyl) ferrirhodin (**39**)		Acetic acid

[a]Not confirmed

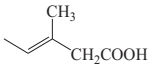
was no significant change observed in the rate of uptake of this molecule into the fungal cell. Furthermore, the authors suggested that the iron coordination centre and its residues might play a significant role in siderophore transport in fungal cells. The structure of the compound was confirmed on the basis of spectral analysis but the complete structure of the compound has not been given (Deml et al. 1984).

All fungal hydroxamate ferrichrome siderophores contains cyclic hexapeptide moieties with six L-amino acids, among three of peptides having attachment of trishydroxamate residues. The attachment of hydroxamate with three peptides are

Table 8.3 Structures of fungal hydroxamate ferrichrome siderophores



A =



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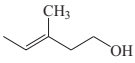
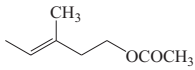
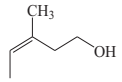


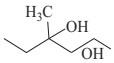
C =



D =



E =



Siderophore	R_1	R_2	R_3	R_4	R_5	Fungal species	Reference
18	CH_2OH	CH_3	A	A	A	*Aspergillus ochraceous*	Jalal et al. (1984)
19	CH_2OH	CH_2OH	CH_3	B	B	*A. ochraceous*	Jalal et al. (1984)
20	CH_2OH	CH_2OH	B	CH_3	B	*A. ochraceous*	Jalal et al. (1984)
21	CH_2OH	CH_2OH	B	B	CH_3	*A. ochraceous*	M.A.F et al. (1984)
22	CH_2OH	CH_2OH	C	B	B	*A. ochraceous*	Jalal et al. (1984)
23	CH_2OH	CH_2OH	B	CH_3	CH_3	*A. ochraceous*	Jalal et al. (1984)
24	CH_2OH	CH_2OH	CH_3	B	CH_3	*A. ochraceous*	Jalal et al. (1984)
25	CH_2OH	CH_2OH	CH_3	CH_3	B	*A. ochraceous*	Jalal et al. (1984)
26	CH_2OH	CH_2OH	D	B	B	*A. ochraceous*	M.A.F et al. (1984)
27	CH_2OH	CH_2OH	E	B	B	*Aspergillus* sp.	Jalal and Helm (1991)
28	CH_2OH	CH_2OH	B	E	B	*A.* sp.	Jalal and Helm (1991)
29	CH_2OH	CH_2OH	B	B	E	*A.* sp.	Jalal and Helm (1991)
30	H	H	CH_3	CH_3	CH_3	*Ustilago sphaerogena*	Neilands (1952)
31	CH_2OH	CH_2OH	A	A	A	*U. sphaerogena*	Garibaldi and Neilands (1955)
32	CH_3	H	CH_3	CH_3	CH_3	*Aspergillus, Cryptococcus, Neurospora sp*	Llinás and Neilands (1976)
17	CH_2OH	CH_2OH	CH_3	CH_3	CH_3	*A. melleus and A. terreus.*	Walter and Deér (1963)

(continued)

Table 8.3 (continued)

33	CH_2OH	H	CH_3	CH_3	CH_3	*A.* sp.	Walter and Deér (1963)
34	CH_2OH	CH_2OH	D	D	D	*A.* sp.	Walter (1963)
35	CH_2OH	CH_2OH	B	B	B	*Penicillium* sp	Walter (1963)
36	CH_3	H	CH_2COOH	CH_2COOH	CH_2COOH	*Fusarium roseum*	Emery (1980)
37	CH_3	CH_2OH	CH_3	CH_3	CH_3	*A. ochraceous*	Llinás and Neilands (1976)
38*	H	H	CH_3	CH_3	CH_3	*Neovossia indica* (Mitra)	Deml et al. (1984)

[a]An additional glycyl residue present in the peptide ring

Table 8.4 Fungal fusarinine siderophores

Name of the siderophore	*Fungal species*	References
Cis-fusarinine (**16**)	*Fusarium roseum*	Emery (1965)
Trans-fusarinine (**42**)	*Fusarium dimerum*	Diekmann (1970)
Fusarinine A (**43**)	*Gliocladium virens*	Jalal et al. (1986)
Fusarinine B (**40**)	*Gliocladium virens*	Jalal et al. (1986)
Fusarinine C (Fusigen) (**41**)	*Rhodothamnus chamaecistus*	Haselwandter et al. (1992)
N,N′,N″-triacetylfusarinine C (**44**)	*Penicillium* sp.	Moore and Emery (1976)
Neurosporin (**45**)	*Neurospora crassa*	Eng-Wilmot et al. (1984)

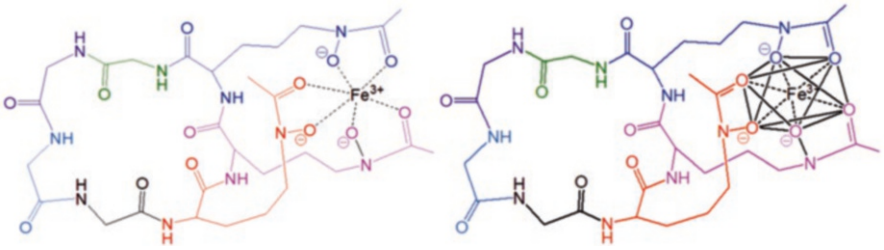


Fig. 8.10 The structure of tetraglycyl ferrichrome (**38**) and its Fe^{3+} octahedral complex

first and second position, either glycine, alanine or serine, and third position is glycine. The exceptions include **38** and **39**, **38** is a cyclic heptapeptide with four amino acids in ring made up of four glycine residues (Deml et al. 1984) and, an acyclic linear tripeptide of diserylglycyl amino acid substitutions. Des(diserylglycyl)ferrirhodin (**39**) (Fig. 8.11) unlike to a typical ferrichrome back bone. (Jalal et al. 1985). In this compound, the absence of characteristic ferrichrome family feature of cyclic hexapeptide ring and the presence of terminal N^5-acylated hydroxy ornithine groups in **39**, identical with those found in fusarinine B (**40**), fusigen (**41**) and ferrirhodin (**34**), can be observed (Jalal et al. 1985).

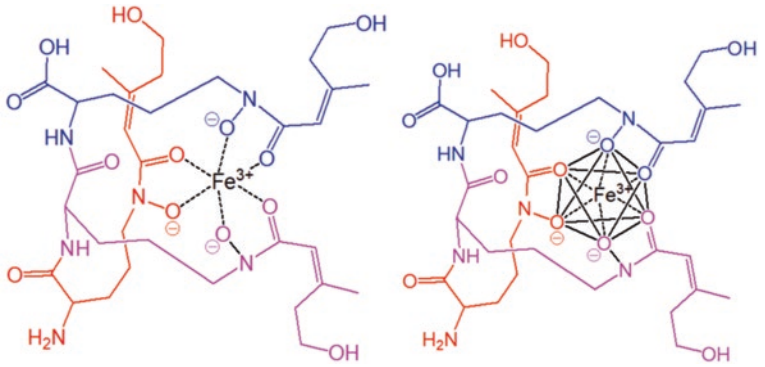


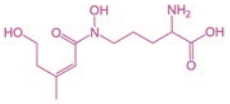
Fig. 8.11 The structure of des(diserylglycyl)ferrirhodin (**39**) and its Fe^{3+} octahedral complex

8.2.3 Fusarinines

Cis-fusarinine [*δ-N*-(5-hydroxy-3-methyl-*Z*-2-pentenoyl)-*δ-N*-hydroxy-L-ornithine] (**16**) is the simplest compound (Fig. 8.12) composed by the substitution of acyl group of N^5-hydroxy-N^5-acylornithine with *cis*-anhydromevalonic acid (5-hydroxy-3-methyl-*Z*-2-pentenoic acid) (Renshaw et al. 2002). It is the first ornithine hydroxamate with the absence of peptide linkage. The other fusarinines are either *cis*- or *trans*- of linear dimers, trimers or cyclic trimers. These monomers are connected by a labile aminoacyl-ester bond. The *cis*- dimer and trimer of fusarinine are named as fusarinine A (**43**) and B (**44**), respectively (Jalal et al. 1986), and the cyclic trimer of it is named as fusarinine C or fusigen (**41**) (Haselwandter et al. 1992). The *trans*-trimer **44** is the major product present in most of the *Aspergilli* (Moore and Emery 1976), which shows a highest binding and the highest chemical stability. Neurosporin (**45**) was isolated as a minor constituent from the fungal species *Neurospora crassa.* All the fungal siderophores contain L-isomer of N^5-hydroxy-N^5-acylornithine, except (**45**) (Eng-Wilmot et al. 1984).

8.3 Biomedical Applications

Several studies have demonstrated the efficacy of siderophores in the management of acute and chronic iron overload, infections, sickle cell anaemia, acute intoxications, malaria and malignancies (Kalinowski and Richardson 2005; Hatcher et al. 2009; Yu et al. 2012). Currently, various studies are ongoing on the applications of siderophores in the biomedical field.



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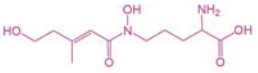
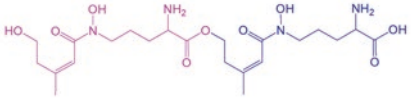
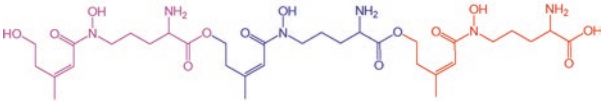


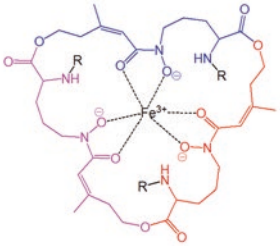
42



43

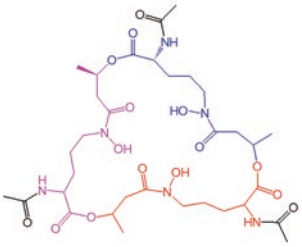


40



41 R = H

44 R = CH_3CO



45

Fig. 8.12 Structures of dihydroxamate fusarinines

8.3.1 Iron Overload

Iron overload mainly occurs in the children. The usual antidote is desferrioxamines, a group of siderophores produced from bacterial species such as *Streptomyces* and *Nocardia*. Desferrioxamines are trihydroxamate-type siderophores. In these bacteria, lysine and ornithine are used as precursors for the biosynthesis of it. Desferrioxamine B is the major siderophore produced by *Streptomyces pilosus* (Gunter et al. 1993), a bacteria. Siderophores have been used for the management of iron overload conditions such as β-thalassaemia and aluminium overload (Crichton et al. 2002; Origa et al. 2005). Not much work has been carried out on fungal siderophores.

8.3.2 Biosensors and Nanosensors

Recently, siderophores have been employed in biosensors and nanosensor platforms to detect iron and other metal ions in the environment and detection of microorganisms (Nosrati et al. 2018). Some siderophores such as pyoverdines are naturally fluorescent allowing them to be exploited in the detection of many metals (Palanché et al. 1999). Not much work has been carried out specifically on fungal siderophores.

8.3.3 Antimicrobial Activity

Banin et al. have evaluated the desferrioxamine-Ga (non-radioactive) conjugate for its antimicrobial activity against *Pseudomonas aeruginosa*. The desferrioxamine-Ga conjugate showed significant antimicrobial effect by push-pull mechanism whereby the siderophore released Ga inside the bacterial cell in exchange of iron and the toxicity of gallium interfered with all metabolic processes (Banin et al. 2008).

Sideromycins are conjugates of an antibiotic and siderophore. Their minimum inhibitory concentrations are usually 100 times lower than their parent molecule. The conventional antibiotics are conjugated with suitable siderophore and used. The microorganism takes up the siderophore in order to get the iron while the antibiotic also gets delivered inside the cell. This is called as Trojan horse strategy in antimicrobial chemotherapy (Möllmann et al. 2009). This strategy effectively circumvents the antibiotic resistance developed by the microorganism. There are naturally occurring sideromycins such as albomycins, salmycins and ferrimycins (Górska et al. 2014). The Trojan horse strategy may also utilize gallium ion in a siderophore conjugate to release gallium into the bacterial cell. The gallium may prevent biofilm formation and interfere with the bacterial metabolism (Juárez-Hernández et al. 2012). The successful application of Trojan horse strategy led to the development of

trihydroxamate-ciprofloxacin conjugates and biscatecholate-monohydroxamate mixed ligand siderophore-carbacephalosporin conjugates (Wencewicz and Miller 2013; Wencewicz et al. 2013). Several studies have shown that this kind of Trojan horse strategy could overcome the antibiotic resistance in microorganisms (Möllmann et al. 2009; Górska et al. 2014; Mislin and Schalk 2014). However, resistance development may occur to the transport of siderophores into the microbial cell as it requires the availability of specific outer membrane receptors (TonB receptors) and loss of these receptors may cause resistance to the entry of siderophore-antibiotic conjugates (Ferguson et al. 2000; Górska et al. 2014). However, more understanding of those receptors and siderophores is progressing and it is anticipated that the emergence of resistance to siderophore conjugates may be minimized. One of those approaches involved the usage of mixed siderophore conjugates to target multiple receptors of iron transport (Wencewicz and Miller 2013; De Serrano 2017; Schalk 2018).

Fungal cells are mostly similar to mammalian cells with respect to the metabolic processes concerned. Due to this property, the availability of selective and unique antifungal therapeutic targets are limited. Recent evidence indicates that invasive fungal infections are resistant to current pharmacotherapy and cause significant mortality. Hence, the development of newer antifungal agents are required (Szebesczyk et al. 2016; Wilson et al. 2016). Brumbagh et al. developed a vaccine with siderophore receptor, yersiniabactin receptor (FYuA), in a murine model against pyelonephritis (Brumbaugh et al. 2013). Recently, Smith et al. developed a vaccine against urinary pathogenic bacteria *Escherichia coli* by using protein conjugates of yersiniabactin and aerobactin in a murine model (Smith et al. 2016).

8.3.4 Molecular Imaging

It is important to know the exact location of infection inside the human body for appropriate treatment. The radioimaging with siderophores helps in this aspect by giving the location of the microbial mass. Radionuclides such as gallium-68, zirconium-89, copper-64 and scandium-44 are used in positron emission tomography (PET) in clinical diagnostics. Attaching radiometals to the biomolecules requires a chelator to be attached with stability and without compromising the binding affinity to the target (Petrik et al. 2017). These molecular imaging molecules can be synthesized via the exchange of iron and the introduction of suitable radiometal to the natural (iron-)siderophore complex. Alternatively, a suitable chromophore can be used to modify the natural siderophore for better optical imaging (Nudelman et al. 1998; Ouchetto et al. 2005; Szebesczyk et al. 2016). Triacetylfusarinine C (TAFC) was successfully radiolabelled with ^{68}Ga using few micrograms of siderophores and the complex exhibited high chemical stability (Helbok et al. 2010). Several 68Ga-labelled siderophores exhibited excellent imaging properties. However, only ^{68}Ga-TAFC and ^{68}Ga-ferrioxamine E were found to be suitable for uptake by fungal cells in a culture with better pharmacokinetic and metabolic prop-

erties (Petrik et al. 2012). TAFC also exhibited high specificity towards *Aspergillus* species when compared to other bacterial and fungal species (Petrik et al. 2014). ^{89}Zr also has been found to be useful for the imaging purposes. However, it has long half-life and less positron-emitting property. Overall, radiolabelling of siderophores has a promising potential to be exploited in clinical diagnostics due to its selectivity towards particular microbial species (Petrik et al. 2017). Apart from radiolabelling, siderophores have been conjugated with fluorescent probes such as fluorescein, rhodamine, 7-nitrobenz-2-oxa-1,3-diazole and anthracene are employed for optical imaging and monitoring of siderophore transport inside and the microorganisms (Nudelman et al. 1998; Larcher et al. 2013).

8.3.5 Metal-Binding Property

Other than iron, siderophores also can bind with other metals such as aluminium (Al^{3+}), zinc (Zn^{2+}), gallium (Ga^{3+}), chromium (Cr^{3+}) and plutonium (Pu^{3+} and Pu^{4+}) (Neu 2000; Miethke and Marahiel 2007). This property of siderophores is exploited in bioremediation exercises when environmental cleaning is required due to contamination by harmful heavy metals such as plutonium (De Serrano 2017). Neubauer et al. demonstrated that hydroxamate-type siderophore, desferrioxamine B, in high pH conditions, was able to bind to Co(III) better than Fe(III) (Neubauer et al. 2000). The siderophores produced by *Fusarium solani* were found to be effective in solubilizing Cu and Zn in vitro (Hong et al. 2010).

8.4 Conclusion

It is obvious that fungal siderophores mostly belong to hydroxamate type and play an important role in several biomedical applications. However, a lot of work has been done on bacterial siderophores and plant siderophores when compared with fungal siderophores. The Trojan horse strategy is very promising against several resistant infections such as invasive fungal infections. Hence, more research work is required to be done on fungal siderophores especially on their antifungal therapeutic application against invasive fungal infections.

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Chapter 9
Fungal Siderophores: Prospects and Applications

Sharanya Manoharan, Oviya Iyappan Ramalakshmi, and Sathishkumar Ramasamy

Contents

9.1 Introduction

Iron is an essential element for all living kinds, trivial for the synthesis of amino acids, DNA, and sterols as well as involvement in enzymatic processes and oxidation-reduction reactions. Iron is abundant in the Earth's crust; however its bioavailability is low due to the oxidation by atmospheric oxygen into ferric hydroxides. Though the rivalry in acquiring environmental iron exists, excess of iron utter its toxicity via oxidative stress and damaged ROS (Reactive Oxygen Species). Therefore, iron homeostasis has to be tightly regulated. Pathogenic and

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K. Dhusia et al. (eds.), *Fungal Siderophores*, Fungal Biology,
https://doi.org/10.1007/978-3-030-53077-8_9

nonpathogenic organisms employ different strategies to ensure iron supply as well as in avoiding iron toxicity (Haas 2014).

9.1.1 Iron Acquisition in Fungi

Fungi employ different strategies to access iron; they are (1) a reductive iron acquisition (RIA) pathway, (2) siderophore transporters for endogenous or exogenous siderophores, (3) secreted cell surface reductants, and (4) endocytic pathway for haem internalization. However, all the four pathways are not involved in the iron acquisition of individual fungal species; instead, any one of the strategy would be utilized (Potrykus et al. 2014). For example, *Aspergillus fumigatus* utilize endogenous siderophores, RIA system, as well as low-affinity iron uptake (Hissen et al. 2005). The different strategies of sequestering iron employed by different fungal types are reported for *Candida albicans*, *Cryptococcus neoformans*, and *Histoplasma capsulatum* (Potrykus et al. 2014). Fungi store the iron in the form of vacuolar iron deposition and siderophore-mediated iron storage (Haas et al. 2008).

9.2 Fungal Siderophores

Siderophores are low molecular weight polyketide/polypeptide compounds and non-ribosomally synthesized, secreted molecules in a wide variety of organisms which help the organism to survive in both iron-deficit and iron-excess conditions. It works by sequestering the environmental iron as well as iron chelators (Condon et al. 2014). Siderophores are broadly classified into three groups as hydroxamate, hydroxycarboxylate, and catecholate (Fig. 9.1); among these the substructure variations were observed (Sorensen et al. 2014).

The major type of fungal siderophores is hydroxamate; however, exceptions are identified in *Mucorales* and *Penicillium bilaii* which produce carboxylate-type rhizoferrin and catecholate pistillarin, respectively (Capon et al. 2007). The hydroxamates are classified into five groups based on its functional groups in the side chain (Winkelmann 2007), for example, fusarinines, coprogens, ferrichromes, and rhodotorulic acid (Haas 2014). Recent studies identified mixed-type siderophores with

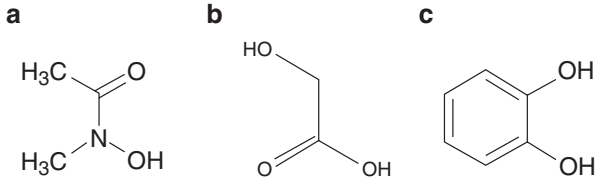

Fig. 9.1 Siderophores (a) hydroxamate, (b) hydroxycarboxylate, (c) catecholate

the ability to bind different iron moieties, and the siderophores vary based on their affinity toward iron (Aznar and Dellagi 2015). Among the fungal organisms, *Saccharomyces cerevisiae*, *Cryptococcus neoformans*, and *Candida albicans* lack the production of siderophores and instead utilize the siderophores produced by other organisms; those are called xenosiderophores. Moreover, these organisms undertake RIA, heme-bound iron, and low-affinity iron uptake mechanism (Kaplan and Kaplan 2009).

The presence of ferrichromes, the coprogens, and fusigen siderophores was abundant among fungal species, as well. Ahmed and Holmstrom (2014) reported that the polished mineral surfaces highly influence the production of siderophores at higher concentration than the minerals surrounding bulk soil. Recently, a novel metachelin A (O-glycosylated and N-oxidized coprogen) and metachelin B (O-glycosylated) and a coprogen-type siderophore were identified from the insect-pathogenic fungus *Metarhizium robertsii* (Krasnoff et al. 2014).

9.3 Fungal Siderophore Synthesis and Function

Synthesis of siderophores both in fungi and bacteria is similar (non-ribosomal peptide synthetase, NRPS), but differs with a unique fungal siderophore transporter (SIT) uptake mechanism (Sorensen et al. 2014). After uptake, iron-free siderophores are hydrolyzed and recycled by the cellular vesicles (Sorensen et al. 2014). The presence of siderophore transporters (SIT) exclusively in fungi enhances the uptake of different types of small chelators and secures their own needs of iron requirement from the bacterial uptake (Saha et al. 2013).

Boyce and Andrianopoulos (2015) have reviewed the genes responsible for the biosynthesis of siderophores and their ability to acquire iron from the host critically for intracellular growth. So far, genes responsible for siderophore biosynthesis are identified from *Ustilago maydis* (*sid1*) (Mei et al. 1993), *Aspergillus nidulans* (*sidA*) (Eisendle et al. 2003), *A. oryzae* (*dffA*) (Yamada et al. 2003), *A. fumigatus* (*sidA*) (Schrettl et al. 2004), *Fusarium graminearum* (*SID1*) (Greenshields et al. 2007), *Cochliobolus heterostrophus* (*SIDA*) (Turgeon et al. 2008), and *Rhizophagus irregularis* (Tamayo et al. 2014). Moreover, the genes regulating the homeostasis in the organisms, viz., *C. albicans*, *Aspergillus* sp., and *Cr. neoformans*, are explained by Chen et al. (2014).

Different types of siderophores are produced by the fungus with each performing its respective functions like extracellular iron acquisition, intracellular iron storage, conidial iron acquisition, as well as storage during infections. These different siderophores are encoded by different gene sequences, where the study on silencing the siderophore-producing genes of *A. fumigatus* prevented the infections in murine models of pulmonary aspergillosis (Bills et al. 2014). The intracellular and extracellular siderophores are mostly produced through the genes of non-ribosomal peptide synthetases (NPS), where Oide and his colleagues (2015) pointed the importance and function of the genes NPS1, NPS2, and NPS6 in cereal pathogen *F. graminearum*,

while NPS2 is responsible for the intracellular siderophore production and on gene deletion impairing the sexual spore production. The deletion of NPS6 gene rendered sensitivity to iron starvation, oxidative stress, and reduced virulence. Both NPS1 and NPS6 are responsible for extracellular siderophore production among which NPS1 accounts for the synthesis of malonichrome (a ferrichrome-type siderophore compound) in *F. graminearum*. The triple mutant (*nps*1*nps*2*nps*6) strain of *F. graminearum* lacks all three siderophores and demonstrated damage in all the attributes such as spore production, iron sequestration, ROS, and virulence factor (Oide et al. 2015).

In *A. fumigatus* hydroxamates fusarinine C and triacetylfusarinine C serve in capturing extracellular iron, ferricrocin for distribution of iron within the cell and hyphal storage, hydroxyferricrocin for iron conidial storage, germination, and oxidative stress resistance (Khan et al. 2017). The biosynthesis pathway for triacetylfusarinine, ferricrocin, and hydroxyferricrocin of *A. fumigatus* was schematically represented by Khan et al. (2017) and also includes the catecholate siderophore mapping from the KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway database.

The impact of fungal siderophores on the fungal sexual development was observed when the fungus lacks the siderophore ferricrocin storage resulting in the formation of deprived ascospore development in strains of *Cochliobolus heterostrophus* (Oide et al. 2007) and affecting asexual sporulation in *Aspergillus fumigatus* and *Magnaporthe grisea* (Hof et al. 2009; Schrettl et al. 2007). This postulates that siderophores might be involved in delivering iron at the appropriate time (Oide et al. 2007).

Intriguingly, Forester et al. (2017) studied the functions of the genes SidN, SidC, and SidA in the symbiotic, endophytic fungi *Epichloe festucae* with host ryegrass (*Lolium perenne*), indicated SidA gene encodes the precursor, ornithine N^5- monooxygenase is responsible for the production of epichloenin and ferricrocin, which are encoded by SidN and SidC, respectively. Also studies on genes and gene mutation clearly depicted the contrasting functions of intracellular and extracellular siderophores in maintaining the iron homeostasis. Furthermore, the cellular energy uptake for the siderophore biosynthesis is remarkably high in microbes; hence to sustain the situation, a tight regulation might be followed under significant iron shortage (Oberegger et al. 2001).

The vision on fungal siderophores has been increased in the recent decades; therefore this chapter comprises the applications and advancements in siderophore studies in fungal species (Fig. 9.2).

9.4 Applications of Fungal Siderophores

Fungal siderophores have vast application in the field of agriculture, environment, and health care.

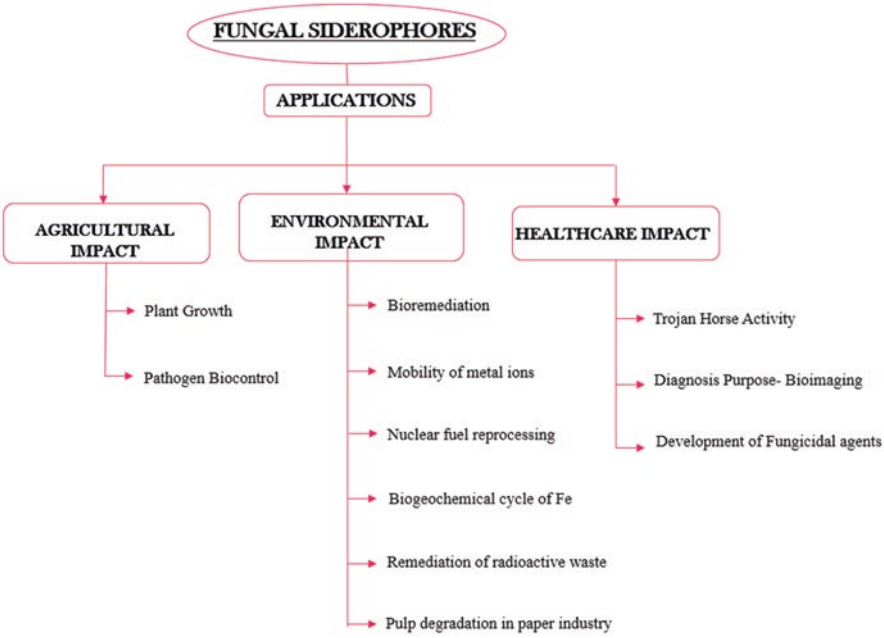

Fig. 9.2 Overview of applications of fungal siderophores

9.4.1 Agriculture Impacts

In general, microbial siderophore has significant impact on plant growth and pathogen biocontrol, where Ahmed and Holmstrom (2014) described the mechanisms a plant could exhibit in fetching iron (Fe) from the environment. First is the reduced form of ferrous oxide (Fe(II)) are donated by microbes to the transport system of plants, i.e., apoplast of the plant roots through high redox potentiality, which is further reduced to ferric form (Fe(III)) by plants for its utilization (Mengel 1995). Second is the existence of ligand-exchange process which exists between the microbial siderophores and phytosiderophores (Masalha et al. 2000). Siderophores are apparently connected with rhizosphere microflora and render greater benefits for both plants and microbial niche. Besides, microbial siderophores possess added advantage by acting as an alternative for hazardous pesticides (Sulochana et al. 2014); in specific, the mycorrhizal sorghum plants took Fe in higher concentration than the nonmycrorrhizal plants, and this might be due to the biofertilizer property of fungal siderophore (Caris et al. 1998). The ectomycorrhizal fungi are associated with plant nutrition (Van Scholl et al. 2008), and Yadav et al. (2011), through his investigation, observed that siderophores of *A. niger*, *Penicillium citrinum*, and *Trichoderma harzianum* significantly promoted the lengthening of chickpeas' shoot and root.

Extracellular siderophores are crucial for maintaining mutualistic relationship with plants; for instance, interaction of fungi, *Epichloe festucae*, with ryegrass showed symbiotic relationship, as well as to improve the sensitivity of plant's oxidative stress (Johnson et al. 2013). More such fungal extracellular siderophores like coprogens from *Cochliobolus heterostrophus* and *Magnaporthe grisea* and triacetylfusarinine C from *Aspergillus nidulans* and *A. fumigatus* were identified (Lee et al. 2005; Oide et al. 2006).

Further, siderophores gained more importance since pathogenic fungus with impaired siderophore synthesis has less lethality and reduced virulence (Haas et al. 2008) as in human pathogen *A. fumigatus* was shown to be affected with the survival rate (Schrettl and Haas 2011). Therefore targeting siderophore biosynthesis renders a greater opportunity to combat fungal infection in both human and plants. The AmcA deficiency (a putative mitochondrial transporter protein AmcA) in *A. fumigatus* reduced the siderophore production where cellular polyamine content reduction was observed and the precursor ornithine usage was prioritized at cellular level instead of taking part in siderophore biosynthesis. This increases the organism's susceptibility toward the drug eflornithine to affect polyamine biosynthesis (Schafferer et al. 2015). Mutating the genes of fungal siderophore synthesis increased sensitivity of fungal strains toward the ROS toxicity produced by plants as a response of fungal colonization. Chen et al. (2014) observed that fungal proteins (NADPH oxidase, the redox-responsive transcription factor, mitogen-activated protein kinase) involved in host ROS resistance are interlinked with the expression of genes responsible for biosynthesis of siderophores. However, the knowledge about underlying mechanisms related to oxidative stress, iron homeostasis, and fungal pathogenesis are unrevealed.

The siderophore-associated mechanisms of immunity manipulation in plants show the involvement of siderophore groups like carboxylate (achromobactin, citrate), hydroxamate (desferrioxamine E, ferrichrome), catecholate (enterobactin, chrysobactin), phenolate (pyochelin, yersiniabactin), and mixed type (aerobactin and pyoverdin) (Aznar and Dellagi (2015). The phytopathogenic ascomycetes *Cochliobolus miyabeanus*, *C. heterostrophus*, *F. graminearum*, and *Alternaria brassicicola* required the siderophores to resist the activity of hydrogen peroxide in their respective host's maize, rice, wheat, and *Arabidopsis thaliana* (Oide et al. 2006). The same has been observed in the hemibiotrophic fungus *Colletotrichum graminicola* (Albarouki et al. 2014) and *A. alternata* (the causative agent of infection in citrus) (Chen et al. 2013) ,and the siderophore desferrioxamine renders support in the fire blight-causing agent *Erwinia amylovora* by facilitating the infection on apple seedlings and flowers (Dellagi et al. 1998).

The defense strategies in the case of plants which included phosphorylation events, reactive oxygen species (ROS) accumulation, cell wall rigidification, increased deposition of callose, upregulated genes of pathogenesis-related proteins, and, even more, the induced systemic resistance (ISR) on any disturbance for its stoichiometric balance of metal homeostasis are reported (Aznar and Dellagi 2015). The mechanism of immunity induction by siderophores varies among the plant

species; therefore, more scientific researches are required to understand the mechanisms undertaken by the plant defense system.

The bacterial siderophores are observed with antagonistic activity for the fungal pyoverdin siderophore of *Pseudomonas putida* which effectively controlled the *Fusarium* wilt in radish through the mechanism called induced systemic resistance (ISR) in plants (De Boer et al. 2003). Bacterial siderophores have the capacity to reduce the ferric ions of rhizosphere which indirectly affect the growth of fungus (Sulochana et al. 2014). Siderophores treated spores of *Aspergillus* sp. lacks the germination and mycelial growth (Sulochana et al. 2014). Siderophores being utilized for antagonizing plant pathogens might also possess multiple activities toward host plant and the other microbial niches (Thomashow and Bakker 2015). In wide variety of plants, these siderophores might result in priming which is said to be plant defense regulator (Conrath et al. 2002). The iron competition in the soil portrayed the mechanism in biological control of soil-borne diseases (Thomashow and Bakker 2015).

Plants utilize the bacterial siderophores in controlling pathogenesis of disease-causing fungi. The siderophore pyoverdine from *Pseudomonas* controls the wilt diseases of potato caused by *Fusarium oxysporum* (Schippers et al. 1987), fungus *Gaeumannomyces graminis* which is the basis for growth deficiency of wheat and barley (Voisard et al. 1989), phytopathogens of peanuts, maize (Pal et al. 2001), etc. Recent study by Maindad et al. (2014) reported the capability of acinetobactin-like siderophore produced by *Acinetobacter calcoaceticus* (common niche of wheat rhizosphere) in inhibiting the plant pathogen *F. oxysporum*.

9.4.2 Environment Impacts

Siderophore applications in the benefit of agriculture sector are even considered as worthy under the environmental impacts. The contribution of siderophores for the environmental welfare is quite enormous. Extracellular siderophores on the way of sequestering iron make the mobility of other metal ions (divalent, trivalent, and actinides) (Renshaw et al. 2002; Dahlheimer et al. 2007) in addition to its role of plant and microbial nutrition. Siderophores apart from binding iron have the ability to complex with essential elements like molybdenum, manganese, cobalt, and nickel facilitating their availability for microbial cells. Siderophores based on their ligand functionalities are able to solubilize and increase the mobility of wide range of metals (Cd, Cu, Ni, Pb, Zn and actinides such as Th(IV), U(IV), and Pu(IV)), which could be useful as an environmentally friendly, cost-effective tool in bioremediation (Rajkumar et al. 2010). This might be able to solve the metal pollution caused by manufacturing industry, sludge applications, nuclear power stations, and mining (Wasi et al. 2013). The siderophore molecule desferrioxamine B chelates Co(III) with greater affinity than Fe(III) especially under high pH conditions (Neubauer et al. 2000). For example, siderophore produced by *Fusarium solani* contributes in mobilizing Cu and Zn (Hong et al. 2010). Siderophore-mediated iron

dissolution enhances the soil mineral weathering due to their stable Fe(III) complex-forming capability (Matzanke 1991). This activity was higher when siderophores function synergistically with low molecular mass organic acids (LMMOAs) (Matzanke 1991). The siderophores also dissolve elements such as rare-earth elements (REE), lanthanum (La), cerium (Ce), praseodymium (Pr), neodymium (Nd), promethium (Pm), samarium (Sm), europium (Eu), gadolinium (Gd), terbium (Tb), dysprosium (Dy), holmium (Ho), erbium (Er), thulium (Tm), ytterbium (Yb), and lutetium (Lu) along with yttrium (Y) which plays an important strategy, i.e., mobilizes the immobile trace elements with the existence of desferrioxamine B in the igneous rock region and eventually supports the natural bioleaching progress (Kraemer et al. 2016).

Siderophore production in marine environment enhances the photochemical cycling of Fe for phytoplankton and in the process of biogeochemical cycle of Fe (Amin et al. 2012). With the higher Fe stability constants, siderophores inhibit the growth of several fish pathogens by competing the enzyme transferrin produced by fish pathogens which is one of the mechanisms to infect the host by bacteria (Yano 1996). This augmented the use of siderophore production as probiotics in fish farming (Dimitroglou et al. 2011). Moreover, high affinity to Fe(III) and strong stability constant properties of siderophores claim its usage as optical biosensor in sensing the Fe bioavailability in the ocean (Chung Chun Lam et al. 2006). Oil spills and spread of petroleum hydrocarbons pose a serious threat in the marine ecosystem (Das and Chandran 2011). The siderophore contributes in the biodegradation of petroleum hydrocarbons through indirect mechanisms of Fe acquisition (Barbeau et al. 2002). In the field of biosensing Fe(II) and Fe(III) availability in ocean, the siderophore molecules could be encapsulated in thin-film format over a quartz substance using sol-gel method (Sharma and Gohil 2010) or could be immobilized on a porous sol-gel glass (Yoder and Kisaalita 2011).

Siderophores have been proposed for the remediation of radioactive waste and nuclear fuel reprocessing. The functional groups anionic hydroxamate and catecholate exhibit strong oxodonor ability which attracts the Lewis acids to form a stable complex; this ability has triggered the application of siderophore in reprocessing irradiated nuclear fuel. In Purex process, solvent extraction techniques are used to recover the uranium (U) and plutonium (Pu) from the fission products like titanium (Ti) and neptunium (Np). Np being an actinide has strong affinity toward the oxygen anions of siderophores, which could be applied in the selective removal of Np from the solvent phase (Taylor et al. 1998).

Fungal siderophores are utilized in the degradation process of the pulp materials produced in the paper industry, where the waste materials produced here have a greater impact on environmental pollution such as global warming, human toxicity, ecotoxicity, photochemical oxidation, acidification, nitrification, and solid wastes (Bajpai 2010). Siderophores are considered as an active environmentally friendly alternative in treating the pulp since it reduces 70% of chemicals required to bleach kraft pulp (Bajpai 2004). Brown-rot fungi especially have higher influence due to their wood-decaying capacity through the production of catecholate and hydroxamate siderophore. For example, a brown-rot fungus *Gloeophyllum trabeum* mediates

redox cycling processes to form reduced Fe which on further reaction with hydrogen peroxide generates more number of oxygen radicals for cellulose, hemicellulose, and lignocellulose depolymerization (Xu and Goodell 2001; Arantes and Milagres 2007). It was reported that bio-bleaching of pulps by fungus is facilitated by acting on the viscosity (Milagres et al. 2002), where different percentage of viscosity loss were observed for *G. trabeum* (10.8%), *Perenniporia medulla-panis* (13.6%), and *Tinea versicolor* (14.4%), and also by altering the lignin structure (Wang et al. 2008).

The saprophytic fungi *A. fumigatus* and *A. nidulans* produce 55 similar types of siderophores which are reported mainly in the recycling purpose of environmental carbon and nitrogen. The iron capturing by siderophore in the soil environment notably reduces the toxicity emitted by asbestos fiber and chrysotile, which naturally has the ability to release high concentration of iron (Mohanty et al. 2017). And the activity was relatively highly observed by the fungal siderophores than bacteria.

9.4.3 Health Care

In the present era, fungal infections among immunocompromised patients and resistant clinical strains are highly threatening mankind and even provoke the nonpathogenic to a virulent strain. Perhaps the iron uptake and transport mechanism of fungi are found to be unique; the fungal siderophore biosynthetic pathway might serve as a selective therapeutic intervention. The genomics study revealed the specific genes responsible for siderophore biosynthesis which on further extension with proteomics analysis would render the elucidation of structural and functional knowledge on enzymes. This might facilitate the design and development of selective fungicidal agents.

In fungi, as mentioned earlier, the importance of siderophores is revealed for germ tube formation, sporulation, and stress resistance, and even the mutant studies indicated the attenuation of fungal virulence (Scharf et al. 2014). The mutant studies on the genes responsible for extracellular and intracellular siderophore under the iron-limitation as well as iron-sufficient condition clearly depicted its functionality in the fungi *A. fumigatus*. Mutating the genes sidI, sidH, sidF, and sidD in *A. fumigatus* which are responsible for extracellular siderophores, there was decreased growth, conidiation, and oxidative stress resistance. And eliminating the genes involved in the intracellular siderophore production (sidC and sidL) blocked the sexual development as well as reduced conidia formation. Another approach of inactivating the entire siderophore system resulted in the high sensitivity due to iron starvation and is directly related to the virulent factors of the pathogen (Haas 2014). *C. albicans* being a commensal in the gut region of mammals exhibits SFU1 expression to shut down the Fe uptake to overcome intracellular toxicity due to Fe abundance (Chen et al. 2011). Several other importance of siderophores for fungi specify their predominant essentiality in infecting animal or plant hosts. Siderophore biosynthesis being unique in fungi, drug development would be highly appreciated to

combat fungal infections in human beings with reduced toxicity and side effects. Sulochana et al. (2014) also suggested that siderophores could be benefitted as drug in health-care issues. Carroll and Moore (2018) illustrated that the enzymes which take part in the non-ribosomal peptide synthetase (NRPS)-independent siderophore biosynthetic pathway are to be considered as potent anti-microbial agents. A few reports persevered the relativity which exists between melanin biosynthesis and Fe acquisition, as the genes involved in melanin formation (LAC1, ATX1, and CCC2) regulate the Fe uptake (Walton et al. 2005).

Despite targeting the siderophore biosynthesis, the siderophores produced by the fungus could be utilized as "Trojan horse" toxins by uptaking the antibiotics along with iron inside the cells which itself affects the pathogen's development (Ahmed and Holmstrom 2014). The "Trojan horse" mechanisms in the treatment of iron overload disease, thalassemia mobilizes and decreases the iron storage in the body (Kontoghiorghe and Kontoghiorghes 2016).

Broadly, animal and plant hosts circumvent the fungal invasion using iron as a defense component, and it is said to be "nutritional immunity" (Weinberg 2009). Mammals encompass a strategy called hypoferremia which triggers the immunogenic signals such as IL8 production to dodge the chemical structures of strong iron chelator produced by bacteria and fungus. With the worse condition existing in the treatement of fungal infections, Lin et al. (2014) serendipitously discovered human transferrin as a novel antimicrobial agent by sequestering iron and disrupting membrane potential of fungi *Candida albicans* (Lin et al. 2014). Few mammalian proteins called siderocalins (e.g., neutrophil gelatinase-associated lipocalin, NGAL, and tear lipocalin, TL) interact with siderophores and lead to endocytosis and degradation (Aznar and Dellagi 2015).

The siderophore-mediated iron acquisition also assists the microbes in their survival rate within the macrophages (Nevitt and Thiele 2011). The dimorphic ascomycete *Histoplasma capsulatum*, the basidiomycete *Cr. neoformans*, the yeast-like ascomycete *C. glabrata*, and the dimorphic ascomycetes *Paracoccidioides brasiliensis* and *Blastomyces dermatitidis* are capable to survive the phagocytic pathway, despite the defense mechanism exhibited by host's immune system (Gerwien et al. 2018). The fungus *H. capsulatum* maintains the intraphagosomal pH at 6.5 which apparently inhibits phagosome function as well as iron accessibility (Hilty et al. 2008).

Furthermore, siderophores are widely used to reduce the growth of cancerous cells, and the relationship that exists between iron overload and cancer progression has been reviewed thoroughly by Huang (2003). The hydroxamate desferrioxamine reduces the tumor succession in neuroblastoma as well as induces apoptosis in malignant melanoma cells (Schrettl et al. 2010).

Diagnosis is a crucial technique where a lot has to be improved to determine the accurate localization, characterization, and risky stages at the earliest of a diseased condition which would influence the success rate of the therapy. Molecular imaging techniques such as radiological, optical diagnosis in the clinical practice are capable of identifying the infections, but however have some limitations over specificity. Dessferioxamine in complex with nonradioactive gallium was designed as an

antioxidant agent and employs "push and pull" mechanism delivering gallium and fetching iron which in turn renders the antimicrobial activity (Banin et al. 2008). Complexing zirconium-89 with dessferioxamine and other bifunctional chelators would perhaps bring related advancement in the field of bioimaging applications of siderophores, since radionuclide-based technologies are highly specific in tracing the molecular targets that enhance the understanding on pathophysiology in a diseased condition (Petrick et al. 2017).

9.5 Experimental Techniques

Experimental techniques in the field of studying siderophores have to be improved a lot where Meyer et al. (2002) suggested that the techniques could be more advantageous with quick and unambiguous nature. Siderotyping is a novel characterizing technique evolved in determining the strains according to the siderophore types produced by them. The two different methods are analytical and biological, where high-performance liquid chromatography (HPLC) coupled with mass spectrometry is used mostly for analytical siderotyping and biological methods include direct measurement of siderophore-mediated Fe in the microbial cells as well as molecular biology methods to recognize the specific DNA sequences for siderophores (Bach et al. 2000). Fluorescence microscopy ambient ionization mass spectrometry was used to detect siderophores (desferrichrome, triacetylfusarinine C) in the White-nose syndrome (WNS), suggesting their functional role in the infection and/or tissue invasion (Mascuch et al. 2015).

RP-HPLC technique has been employed for siderophore identification in *A. fumigatus which causes aspergillosis* (WinkelStroker et al. 2015). *However due to lack of active uptake by the pathogen leading to signal intensification at the infection site, radiotracers were used with high specificity and sensitivity for fungal infections* (Haas et al. 2015). Molecular imaging technique has been implemented for siderophore typing where the target was to "steal" the host iron which is tightly sequestered by host proteins such as hemoglobin, transferrin, etc. (Haas et al. 2015). Radiophore tagging was also employed to identify xenosiderophores by nonproducers like *Saccharomyces* sp. and *Candida* by using radiophore tagged xenosiderophore like Ga-TAFC (triacetylfusarinine C) and Ga-FOXE (ferrioxamine E) (Haas et al. 2015). However, molecular imaging of the siderophores comes with its own challenges where siderophore system is greatly affected in patients undergoing antifungal treatment: either the reduced iron uptake mechanisms affect the sensitivity toward the radiotracer uptake or the patients who undergo blood transfusion suffer from "iron overload" condition which further aids in the onset of the fungal infection (Haas et al. 2015). Hence, bioimaging technique is not effective in such cases.

The siderotyping could be widely applied in microbial diversity identification and taxonomy (Meyer 2010) and also as chemotaxonomic marker for the identification of organism types (Bultreys et al. 2006).

9.6 Conclusion

The future perspective is to augment scientific research on the relativity of low Fe bioavailability in the environments like ocean and some parts of soil region and siderophores, therefore to remove knowledge scarcity in this field. The metagenomics study would assist in analyzing the chemical structural diversity among different and within fungal species will be useful in developing the siderophore applications. Moreover, thorough understanding of siderophore biosynthesis and utilization mechanism of each individual fungal species would bring an insight to eradicate the pathogenic fungi from hiding and their replication in the host macrophages.

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Chapter 10
Bioinformatics Applications in Fungal Siderophores: Omics Implications

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Contents

10.1 Introduction

Nearly all known organisms exhibit a need for iron as an essential nutrient. However, the amount of soluble iron compound is too low and hence less accessible to both micro- and macro-organisms. (Lasocki et al. 2014; Schaible and Kaufmann 2004). Though there are exceptions such as *Lactobacillus plantarum* and *Borrelia burgdorferi* that do not require iron (Archibald 1983; Posey and Gherardini 2000), it is important for most organisms to utilize iron transport mechanisms for survival. At the same time, these mechanisms also need to strike a balance to avoid excess uptake and resultant toxicity (Schaible and Kaufmann 2004). Siderophores are one among such compounds that are released in cases of iron-deficient situations by microorganisms such as bacteria and fungi.

Siderophores help in dodging the issue of iron-limitation by forming water-soluble complexes with Fe^{3+} that has six co-ordination sites in the shape of octahedron to accommodate three bidentate ligands (Cornelis and Andrews 2010). Siderophores can be of different types such as catecholate, hydroxamate or alpha-hydroxy carboxylates, etc. Whereas bacteria produce siderophores containing a

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K. Dhusia et al. (eds.), *Fungal Siderophores*, Fungal Biology,
https://doi.org/10.1007/978-3-030-53077-8_10

variety of functional groups (Hider 1984), most of the discovered fungal siderophores belong to the hydroxamate classes (Renshaw et al. 2002). Nevertheless, exceptions, namely, the siderophores rhizzoferrin and pistillarin that belong to the carboxylate and catecholate groups, have also been discovered in certain fungal species (Haas 2014).

Almost all known fungal siderophores are synthesized by non-ribosomal peptide synthetases (NRPSs) (Haas et al. 2008). NRPSs are backbone enzymes facilitating the synthesis of secondary metabolites inclusive of compounds such as siderophores, pigments, toxins and antibiotics. These products are synthesized by a process independent of ribosomal machinery (Martínez-Núñez and López 2016). Again, the carboxylate siderophore rhizzoferrin is an exception and is synthesized in an NRPS-independent manner (Thieken and Winkelmann 1992). The 'omics' era has revolutionized the impact of computational tools and bioinformatics techniques on the discovery of secondary metabolites and their pre-cursors. In the past two decades, advances driven by genomic and metabolomic analysis provide powerful new methods to identify the occurrence of novel siderophores, their putative functions and biosynthetic pathways, albeit more in bacteria (Etchegaray et al. 2004). The objective of this chapter is to provide a brief overview of current methodologies and recent progress in the areas of bioinformatics related to the discovery and analysis of fungal siderophores using omics-based approaches.

10.2 Potential of Bioinformatics

The pace of research and development in the field of bioinformatics and computational biology skyrocketed with the advent of rapid genome sequencing techniques. The potential of bioinformatics analysis lies in the fact that with proper analysis, thousands of predictions can be made and tested for any organism. It can yield insight into gene/protein functions as well as metabolism in a manner that dramatically reduces the time of testing when using only the conventional tools in vitro. Additionally, with the large amount of data that is generated, there are bioinformatic applications to store, retrieve, share and compare the data. However, it is important to choose relevant data for analysis and hence to discriminate between meaningful and noisy data (Quatrini et al. 2007).

The omics analysis primarily includes the use of computational algorithms to predict genes, proteins and metabolic pathways, predominantly in sequenced genomes as part of high-throughput protocols. The study of omics involves a global assessment of a set of molecules such as the genome, proteome, transcriptome or metabolome. The potential of any study is tremendously increased with a meticulous integration of multiple omics analyses rather than a single one (Hasin et al. 2017; Zhang et al. 2010).

10.3 Bioinformatics Analysis in Natural Product Discovery Including Non-ribosomal Peptides

Natural products (NPs) have always been a subject of interest for research and development of bioactive molecules. Genome sequencing projects of fungi at large scales uncovered a huge realm of NPs useful for human use (Schueffler and Anke 2014). The continuously increasing load of fungal genomic data has incited the discovery of new NPs through identification of biosynthetic gene clusters (BGCs) that act as the source of these NPs. Such genes from which the fungal NPs are sourced include polyketide synthases and peptide synthetases. Fungal siderophores are secondary metabolites constructed from non-ribosomal peptide (NRP) biosynthetic machinery with the help of enzymes called non-ribosomal peptide synthetases (NRPS). These NRPSs are huge enzymes, generally multi-modular in nature that catalyse the peptide bond formation without the involvement of ribosomes (Haas et al. 2008).

Still, the number of such genes that produce them surpasses the number of known NP compounds so far, thus revealing a disproportion even in the current genomic era. Hence, it makes sense to further exploit the genomic data for mining the probable pathways to characterize novel NPs. (Stadler and Hoffmeister 2015). One of the major reasons for the disparity between the available genomic data and uncharacterized NPs including siderophores is attributed to the fact that the BGCs are either silenced at the transcriptional level or expressed only at low levels under laboratory conditions. The regulatory systems of BGCs function in such a way that they require specific environmental cues to be activated. Surpassing these challenges of the pre-genomic era, synthetic biology strategies are currently used for the activation of target BGCs followed by structural characterization of the NPs. However, it is imperative that the silent BGCs are correctly identified before these strategies. A typical workflow of modern NP discovery is initiated with the identification of silent BGCs using bioinformatics tools (Ren et al. 2017; Rutledge and Challis 2015; Zarin-Tutt et al. 2016). The major techniques devised and the recent developments in research for the *in silico*–based omics analyses of fungal siderophores are described below.

10.3.1 Genome Mining: Identification of BGCs

The catalytic domains of BGCs are highly conserved like the operons among bacteria. This supports the mining of genome sequences for identification of putative BGCs using homology searches using alignment techniques or Hidden Markov Models (HMMs) (Weber and Kim 2016). Once a matching domain is identified by similarity search, bioinformatic analysis of the neighbouring genes can then predict putative gene clusters (Rondon et al. 2004). Table 10.1 depicts the different web servers/tools and databases for BGC/NP discovery.

Table 10.1 Published computational implementations for NRPS analysis

Tool name	Web server address	Reference
SBSPKSv2/ NRPS-PKS	www.nii.ac.in/sbspks2.html	Khater et al. (2017)
antiSMASH	https://antismash.secondarymetabolites.org/	Blin et al. (2017)
Dereplicator	http://cab.spbu.ru/software/dereplicator/	Mohimani et al. (2017)
PRISM	http://magarveylab.ca/prism/	Skinnider et al. (2017) and Skinnider et al. (2015)
CASSIS/SMIPS	https://sbi.hki-jena.de/cassis	Wolf et al. (2015)
GARLIC	http://www.magarveylab.ca/garlic	Dejong et al. (2016)
GNPS	http://gnps.ucsd.edu/	Wang et al. (2016)
IMG-ABC	https://img.jgi.doe.gov/abc	Hadjithomas et al. (2015)
SEQL-NRPS	http://services.birc.au.dk/seql-nrps	Knudsen et al. (2015)
GNP	https://magarveylab.ca/gnp/	Johnston et al. (2015)
MIBiG	https://mibig.secondarymetabolites.org/	Medema et al. 2015)
ClusterMine360	http://www.clustermine360.ca/	Conway and Boddy (2012)
Pep2Path	http://pep2path.sourceforge.net/	Medema et al. (2014)
NRPSsp	www.nrpssp.com	Prieto et al. (2011)
NRPSpredictor2	https://bio.tools/NRPSpredictor2	Röttig et al. (2011)
SMURF	https://www.jcvi.org/smurf	Khaldi et al. (2010)

Genomic sequencing of representative strains with known and characterized BGCs can enable the characterization of the basic architecture of related NPRS coding genes (Bills et al. 2014). A single NRPS module in fungi follows certain uniformity in its domain architecture and is found to be consisting of three types of domains, namely:

(i) 'A' domain – The adenylation domain that recognizes and activates a substrate molecule via adenylation with ATP
(ii) 'T' domain – The thiolation domain that binds the substrate to the NRPS protein
(iii) 'C' domain – The condensation domain that helps in linking the two substrates through a condensation reaction.

The conserved nature of the NRPS modules thus aids in surveying a genome to identify the encoding genes of known NRPS sequences. This remains true for siderophore-producing NRPSs as well. Thus, the approaches generally used for NP and NRPS discovery and annotation are elaborated on to the identification of siderophores also.

Unlike other NRPSs, siderophore synthesizing NRPSs do not follow the colinear structure of the modules whose count and arrangement of domains could be used to characterize the final product (Mootz et al. 2002). Rather, the modules are placed iteratively with a single characteristic module with the domains in specific

orders used multiple times where required. Among the three families of siderophores, fusarinines and coprogens generally contain only a single module whereas ferrichromes might contain three or more of such modules. In any case, typically the final module or the only module present is found to be consisting of additional 'T' or 'C' modules (Schwecke et al. 2006). In addition, it is found that in the case of siderophore BGCs, genes homologous to ABC transporters – expected to be involved in secretion functions of siderophores – are often found clustered with core genes (Johnson et al. 2013; Munawar et al. 2013). Thus taking clues from these ideas, fungal genomes could be mined for potential siderophore synthesis genes.

Even though siderophore-specific prediction tools for fungi are not yet developed, identification of intact NRPS coding genes/individual NRPS domain from genomic data was first put forward as early as in 2004 from India via the interface NRPS-PKS (Ansari et al. 2004). The first version of the interface was developed by studying 22 BGCs that were experimentally characterized. The NRPS domains were manually curated and used to create a domain database. To identify similar domains in the input sequence, local alignments to the database using BLAST were conducted. In the past 15 years, the techniques for annotation have improved significantly. The implementation has since been updated and currently executed as a server called SBSPKSv2 that provides a platform for comprehensive analysis of secondary metabolite BGCs (Khater et al. 2017). With more than 130 characterized BGCs, HMM models for better searches and the inclusion of structural analysis, the server is a user-friendly interface for detection of putative NRPS/PKS BGCs and also provides clues on the putative products of uncharacterized NRPS/PKS clusters. Such a platform thus facilitates the identification and annotation of putative siderophore genes from fungal genomes.

Nevertheless, there are other popular bioinformatics tools developed afterwards based on similar principles but using better datasets in terms of number, specificity and diversity. The tools antiSMASH (Medema et al. 2011; Blin et al. 2017) and SMURF (Khaldi et al. 2010) are hence set off as the current state-of-the-art tools for BGC identification. The former is tailored to incorporate both bacterial and fungal genomes whereas the latter is specific to fungal genomes only. However, SMURF is only capable of gene prediction and hence must be used with other tools for the purpose of domain localization. Though these tools have been commonly used for prediction of BGCs producing various NPs and secondary metabolites in fungi, they have not been well exploited for siderophores.

As important as the localization of the BGC gene is the prediction of their substrate, particularly to isolate siderophore-producing genes. To facilitate the studies of "cryptic" NRPS clusters that are not linked to any NP, prediction of substrate specificity could be conducted (Verne Lee et al. 2015). This could be achieved by analysis of the substrate-binding adenylation domain 'A' in the NRPS module. Since domain architecture of siderophore-NRPS genes are not unified by a common pattern, database search approaches have been found useful (Etchegaray et al. 2004; Komaki et al. 2018; Aleti et al. 2015). Databases are developed based on sequence

information from siderophores whose substrates were experimentally determined or predicted from the domain architecture. Novel NRPSs are aligned against the database to predict their substrates. The analysis focuses mainly on the Stachelhaus code or the 10 residues in the active site of A domain that were identified to be specific for each substrate (Stachelhaus et al. 1999). For bacterial domain specificity prediction, this approach was found considerably efficient and was used by NRPS-PKS (Ansari et al. 2004). Other tools such as NRPSpredictor2 (Röttig et al. 2011) and NRPSsp (Prieto et al. 2011) were also developed based on similar principles but using machine learning techniques like support vector machines (SVM) and profile HMMs. Another prediction tool SEQL-NRPS (Knudsen et al. 2015) also claims to be able to decipher substrate specificity using machine learning but does not necessarily rely on active sites.

All the above concepts were found to perform well on bacterial NRPS prediction and have been used to predict novel siderophores from bacterial genomes (Etchegaray et al. 2004; Komaki et al. 2018; Aleti et al. 2015) however, their efficiency to predict substrate specificities in the case of fungal NRPSs is still debatable (Sørensen et al. 2014). The lack of characterized data of fungal origin for training is a major disadvantage to develop machine learning–based tools for the said purpose. It is important to obtain enough data primarily of fungal NRPS and more specifically of characterized fungal siderophores to train prediction models for fungal siderophore genes. To overcome the issue of lack of prior specificity data in fungi, a virtual screening approach was tested (Verne Lee et al. 2015) and found to be promising. Nevertheless for the approach to be efficiently exploited, the homology model build and assessment need to be enhanced.

Unlike the previously discussed tools that were developed based on domain homology, a tool called CASSIS predicts secondary metabolite gene clusters by taking into account co-regulation of cluster genes. However, it is not a genomic approach by itself but instead takes as input a given anchor/backbone gene under consideration. To facilitate the identification of such anchor genes from the genome, the SMIPS interface was developed along with CASSIS. Whole genome sequence is processed by SMIPS and the results could be loaded to CASSIS for gene cluster prediction. These servers are dedicated to mining eukaryotic genomes and hence are found useful for fungal genome mining (Wolf et al. 2015).

Generally, fungal NRPS domains put forward certain difficulties in substrate prediction due to its non-linear and iterative architecture. In such cases, the ‘A’ domain could not be mapped directly to a specific amino acid of the product as found in bacteria (Mootz et al. 2002). Additionally, post-translational modifications in the cyclic or branched peptides also make it difficult to determine the sequence of incorporation (Caboche et al. 2007). Ultimately, substrate specificity prediction of fungal NRPS and particularly of siderophore-producing genes is still largely unexplored leaving scope for collection of sufficient experimental data, appropriate curation, analysis and training.

10.3.2 Linking BGCs to NPs

Identification of corresponding BGCs is also an important task to discover the source of 'orphan' secondary metabolites. The availability of sequenced genomes can be exploited by mining to connect BGCs to compounds. Comparative analysis presents the easiest approach to tackle this. They could be used to identify similar BGCs from two or more species that produce same or highly similar compounds (Bok et al. 2015; Nielsen et al. 2013; Lambertz et al. 2014; Liu et al. 2015). Such techniques are called targeted approaches. The whole biosynthetic potential in one or more genomes could be assessed by an untargeted approach that analyses all detected BGCs in the genome to databases such as clustermine360 (Conway and Boddy 2012), IMG-ABC (Hadjithomas et al. 2015) and MIBiG (Medema et al. 2015) that contain information on fungal BGCs and their compounds. Nevertheless, the task of comparing and evaluating the similarities among BGCs in fungi for the purpose of clustering them is a not-so-straight-forward task due to obvious reasons. Large size, inaccurately described boundaries, re-arrangements and possible presence of non-relevant genes are all probable factors. Investigations of the similarities and uniqueness of BGCs among fungal species have been conducted minimally and concerning only a few major species (Nielsen and Nielsen 2017). Thus, the scope of using genomics to identify BGCs and corresponding NPs is by itself limited, particularly so in the case of siderophores. These limitations can be immensely overcome using integrated approaches merging computational genomics with the potentials of transcriptomics, proteomics and metabolomics.

10.3.3 Utilizing Integrated Omics Approaches for Revealing Natural Product Biosynthetic Pathways

With the revolution of high-throughput technologies in genome research, other omics technologies such as proteomics and metabolomics are often incorporated in genomic studies for the sake of better analysis. The identification of NRPS from fungal genomes provides only limited scope due to the requirement of prior homology data. Such bottlenecks in bioinformatic studies to identify novel clusters or to link known products to corresponding clusters are overcome by integrating information from proteomic, transcriptomic or metabolomic data. Consequent to the post-genomic era, integrating the omics approaches is becoming increasingly important in the discovery of NPs, making tremendous improvements in fungal characterization. The requirement of a genome a priori can also be bypassed using proteome-based approaches (Hillman et al. 2017). The discovery of siderophores in a bacterial species without a sequenced genome was also reported with the aid of proteomics (Chen et al. 2013).

One of the prime techniques in proteomics, mass spectrometry (MS) in compound detection has become highly sensitive with respect to NRP analysis and is

capable of sequencing a peptide even from small amounts of unpurified raw sample (Molinski 2010). MS-based approaches were used to investigate the secondary metabolomes of several microbes including fungi such as *Penicillium* and *Furcatum* (Krug and Müller 2014; Vansteelandt et al. 2012). The tandem mass spectroscopy (MS/MS) uses two mass spectrometers each to separate and identify fragments of compounds subject to investigation and is found very powerful to obtain characteristic spectra which can be compared to comprehensive libraries or databases to facilitate novel compound identification (Tautenhahn et al. 2012; Wang et al. 2016). Improved detection techniques are also being developed that includes spatial imaging to improve the sensitivity (Fang and Dorrestein 2014). MS-based proteomics can also be used to validate predicted compounds and identify their interactions (Smits and Vermeulen 2016) and modifications (Ribeiro et al. 2017).

With advances in computational technology, computational tools are available to connect gene clusters to its product based on MS/MS data analysis. Proteomic Investigation of Secondary Metabolism (PrISM) is a proteomics approach developed to detect NRPSs whose primary sequences were used to generate primers to amplify corresponding gene clusters (Bumpus et al. 2009). For unsequenced genomes, a strategy was developed that works based on grouping BGCs and MS/MS spectrums into corresponding families by similarities (Nguyen et al. 2013). Gene cluster families and molecular families are accordingly identified and correlated based on MS/MS techniques and networking by connecting them to gene cluster families of sequenced organisms. The original study was used in 60 unsequenced but related strains of bacteria but is easily adaptable to study other different organisms.

Addressing the issues present in the sequencing of non-linear peptides, the first NRP identification algorithm known as NRPquest was developed in 2014 (Mohimani et al. 2014). With a sequenced genome and MS dataset as inputs, the algorithm is implemented in four steps: (a) genome mining using NRPSpredictor2 and construction of putative genomic-NRP database, (b) matching of spectral data to database, (c) computation of statistical significance and ranking of peptide-spectrum matches and (d) construction of spectral network to enhance identified natural product sets and uncover related NRP families through dereplication. The same research group later implemented an extended tool known as Dereplicator that aims at the analysis of peptidic natural products including NRPs with modified algorithms using dereplication techniques (Mohimani et al. 2017). For a provided chemical database, the algorithm works by generating *in silico* mass spectra of compounds via prediction of their fragmentation during MS. The resultant spectra are then compared to experimental LC-MS/MS to identify similarities, based on which significant matches are reported.

With an enormous amount of research in MS-based analysis of NPs, it is vital to implement a platform for the sharing and management of data. Global Natural Products Social (GNPS) Molecular Networking was thus introduced as an open access knowledge base to allow researchers to share raw, processed or identified MS data. Crowd-sourced analyses and curation are supposed to assist improved annotation of the datasets (Wang et al. 2016).

Almost all the above implementations are aiming at the study of NPs in general. However, extension of MS/MS-based approaches to more specific compounds can happen in the near future. For the characterization of siderophores, in particular, a workflow based on high-resolution liquid chromatography (LC)-MS/MS was developed (Baars and Perlman 2016). The workflow mines LC-MS/MS data making use of a database of siderophore structures. An MS/MS auto-convolution technique is used with MS/MS siderophore networks to discover peptide monomers present in the siderophores under study. Corresponding families are thus identified, which is important in assigning structures to the siderophores by spectral reconstruction.

A peptidogenomics approach was established to mine organisms for novel metabolites establishing a field of research known as natural product peptidogenomics (Kersten et al. 2011). It uses MS/MS to get an amino acid sequence tag representing a part of the complete peptide and hence can be deduced based on the mass shift pattern. Subsequently, screening against predicted specificities of substrates is conducted. To automate the process and enable high-throughput detection of metabolites, it is necessary to match the identified mass spectra to the BGCs efficiently, both in terms of accuracy as well as time. The software package Pep2Path was introduced to address this automation problem and found to be paving the way towards high-throughput identification of peptide NPs. It works based on a Bayesian probabilistic approach for rapid matching of spectra to the clusters (Medema et al. 2014).

Parallel to the development of MS-based approaches, improvements in nuclear magnetic resonance (NMR) techniques are catching up with the objective of high-throughput detection of NPs. Introduction of miniaturized and cryogenic NMR probes, data-mining techniques and database management are narrowing down the gap between NMR and NP discovery (Halabalaki et al. 2014).

Similar to the widely used proteomics-based approaches, transcriptomics is also used to identify BGCs and particularly the conditions under which it is activated for NP production (Wang et al. 2015). RNA-seq is an efficient alternative to the classical microarray technique with its lowering costs, improvised technology and sequence-agnostic nature. For instance, investigations after exposing the fungi *Aspergillus niger*, *Penicillium chrysogenum* and *Trichoderma reesei* to competitive, co-cultured environments were found to be activating novel BGCs of uncharacterized NPs (Daly et al. 2017). Akin unexplored potentials of transcriptomics can be used in the identification of siderophores also. The tool FunGeneClusterS was developed for the identification of fungal BGCs and utilizes both genomic and transcriptomic data unlike the previously mentioned tools in Sect. 10.3.2 using genome sequence data alone (Vesth et al. 2016)

At the end of the omics string, metabolomics is a tool connecting NPs detected by MS to their corresponding BGCs. MS-based metabolomic approaches, largely overlapping the proteomic techniques, have helped to connect the missing links in the whole genome potential for the secondary metabolite production of an organism. The technique helps to provide clarity on the comparatively lower numbers of known compounds from the organism (Krug and Müller 2014). Multiple omics techniques of different NPs produced in an organism are studied together for the

metabolome analysis. Genome mining is conducted, the gene clusters and their NPs are identified with MS-based analysis and a spectral network is generated to compare the different products for the similarities and diversities. Thus, metabolomics techniques have assisted the structural characterization of new metabolites and also provided novel information on underlying pathways. Such an approach was conducted in analysing the siderophore metabolism of the bacteria *Azotobacter vinelandii* to reveal unreported derivatives of siderophores and their origins (Baars et al. 2016).

A next-generation automated pipeline developed for metabolomic analysis recently attempted to directly predict the products of BGCs including NRPS from genomes claiming to be a genomes-to-natural products (GNP) platform (Johnston et al. 2015). Consequent to the GNP platform, an implementation termed as 'Prediction Informatics for Secondary Metabolomes' (PRISM, not to be confused with PrISM – explained before) was also developed by the same group that uses genome mining to identify NRPSs and their corresponding substrates. (Skinnider et al. 2015, 2017). The implementation was later linked to an automated pipeline via two tools GRAPE, a retro-biosynthetic tool, and GARLIC, which compares the substrate prediction result of PRISM with GRAPE output to assess the production of a given compound (Dejong et al. 2016).

One of the major limitations of secondary metabolite study is the low amount of production compared to the primary metabolism. Mathematical representations of metabolism in genome-scale metabolic models (GEM) inclusive of secondary metabolism can be utilized to get around such limitations (Nielsen and Nielsen 2017). GEMs are useful models that assist the design of metabolic strategies in organisms. The functionality of GEM is based on connecting annotated genes to the biochemical reactions that are catalysed by the respective enzymes. This provides a subjective overview of the metabolic capabilities of the organism (Price et al. 2003; Agren et al. 2013). Further, along with primary metabolism, inclusion of secondary metabolism in genome-scale metabolic models of organisms could also help to optimize the production of fungal NPs such as siderophores.

Techniques of flux mode analysis and elementary flux mode analysis were commonly used in computational metabolic analysis to identify optimization techniques (Agren et al. 2013; Zanghellini et al. 2013). Whereas flux balance analysis deals with the simulation of flux distribution in the reconstructed GEM network, elementary flux mode analysis determines all the feasible and minimal pathway routes in the network (Lotz et al. 2014). The advantages of metabolic flux analysis in GEMs lie in the fact that only limited experimental data is required and the model can be associated with an iterative cycle of prediction and validation strategies for the rational design of engineering strategies. In recent years, a number of works using GEMs to focus on secondary metabolism in prokaryotes have been conducted to optimize the production of useful metabolites (Kim et al. 2016). However, such studies are lacking in fungi or are limited to only a few major species. The secondary metabolism of several actinomycetes were studied using GEMs. The study in *Streptomyces coelicolor* A3(2) GEM identified two complete secondary metabolism pathways including an NRP (Borodina et al. 2005). Recently, secondary the

metabolism of nine species of *Penicillium* was studied to identify thousands of gene clusters with immense potential (Nielsen et al. 2017).

With the sequence data expanding at a tremendous rate and new omics tools that are efficient and inexpensive to detect and analyse NP synthesis, systematic pipelines integrating omics studies are being developed using bioinformatics. Genome sequencing and mining are followed by detection and quantification of metabolites/transcripts or proteins after which products of interest are screened for biological activity (Hillman et al. 2017). QuantFung project makes use of similar ideologies for the production of novel bio-active compounds in fungi (Büttel et al. 2015). The utility of computational biology tools in NP detection in fungi are mainly concentrated on potential drug discovery. However, they could be adapted to siderophore identification and research as well with ease.

10.4 Conclusion

With the advent of genome sequencing technologies and concurrent omics analysis, there has been a vast increase in our knowledge of siderophore biosynthesis over the past two decades. The first step is the identification of gene clusters acting as the source of siderophores, which can be realized via bioinformatics too. Prediction of substrate specificity and combining the known information with algorithms parsing metabolomic-data to link the clusters to the corresponding compounds constitute the following steps. For each of the steps, multiple new techniques were developed in the last few years. *In silico* genome mining is an efficient high-throughput approach to uncover potential NRPS genes. As described in previous sections, analytical pipelines linking genomics with other omics data are being developed and can reveal immense information on the synthesis of such natural products. Additionally, with the advent of computational tools to mine the genome, the identification of BGCs largely surpasses their characterization. Nevertheless, emerging techniques of automated synthetic biology are hopeful of accomplishing such objectives.

Natural product research, as well as siderophore research, has been concentrated on bacterial species and there is an obvious bias in data availability as well as algorithm development for fungal research. Hence, it is important to consider the differences and test the relevance of already developed tools on fungal data before blind usage. To reorganize the use for fungal siderophore identification, it is essential to generate, collect and analyse fungal NRPS data – particularly siderophore-producing ones. The lack of such curated data is currently a shortcoming in developing and training prediction/classification models for fungal siderophores. It can be envisioned that algorithms for identification of siderophore-producing BGCs integrated with high-throughput proteomic and metabolomic product detection techniques can lead to the discovery and characterization of novel siderophores with novel biological significances.

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Chapter 11
Siderophores in Antifungal Drug Discovery: A Computational Approach

Anusuya Shanmugam, Vanathi Chithiravel, Arthi Gunasekar, and Anbazhagan Venkattappan

Contents

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K. Dhusia et al. (eds.), *Fungal Siderophores*, Fungal Biology,
https://doi.org/10.1007/978-3-030-53077-8_11

11.1 Introduction

Like bacteria and viruses, fungi also infect human beings and results in fungal diseases, also known as mycoses. Though millions of fungal species exist in nature, only few are harmful (Cdc.gov 2017). In humans, fungi generally reside in the skin, lungs and blood stream. Fungal infections in the skin are usually mild in nature and are observed as skin rashes. Fungal infections in lungs are similar to other lung infections such as a flu and tuberculosis. When fungus enters into the blood stream, it causes systemic fungal infections which are less common but life threatening, as they are associated with a higher mortality rate (Mavor et al. 2005). Aspergillosis, candidiasis, coccidioidomycosis (valley fever), blastomycosis, talaromycosis, sporotrichosis, histoplasmosis, ringworm, fungal eye infections and fungal skin infections are notable human fungal infections (Cdc.gov 2017).

Fungal infections are generally treated by antifungal drugs either as topical applications or given orally (Hay 2018). Clotrimazole, Ketoconazole, Miconazole, Oxiconazole, Sulconazole, Terbinafine, Terconazole, Tioconazole and Tolnaftate are few such drugs available to treat fungal diseases (Campoy and Adrio 2017). Despite the existence of many antifungal agents, fungal diseases are more common than previously realized. In addition, the newly emerging fungi are becoming increasingly problematic. A few fungal infections are life threatening, if they are not identified and treated in their initial stage itself (Cdc.gov 2017). Furthermore, recent outbreaks such as histoplasmosis in Illinois in 2013 and in the Dominican Republic in 2015, the fatal gastrointestinal mucormycosis in a premature infant in 2014, coccidioidomycosis (Valley fever) in California's Central Valley in 2013, fungal meningitis and other fungal infections associated with contaminated steroid injections in 2012, endophthalmitis in 2012, mucormycosis in 2011, blastomycosis in 2010–2011 (Cdc.gov 2017) insist on the need and importance of designing newer and more efficient antifungal agents.

Identifying and validating a potential drug target is a crucial step in drug discovery, as it determines the success of any drug designing process. Hence, it is essential to understand the existing drugs, its drug target, their mechanism of action and drawbacks before going to find novel drug targets for antifungal drug discovery. The current antifungal drugs primarily target and inhibit the enzymes involved in the biosynthetic pathways such as cell wall, cell membrane and DNA. In addition, the antifungal agents also target the essential components of the fungal cell wall such as ergosterol, lanosterol, chitin, glucans and several glycoproteins and inhibit them. Few antifungal agents disrupt mitotic spindles and inhibit fungal mitosis (Mazu et al. 2016). However, the current antifungal agents are associated with drawbacks such as: allergic reactions in humans; adverse reactions like altered oestrogen level and liver damage (Kyriakidis et al. 2017); significant side effects (HSE.ie 2019); association with many drug interactions (Doctor Fungus 2019); and the emergence of drug resistant and multidrug resistant strains (Cdc.gov 2019a, b). Hence, in order to overcome these obstacles, in recent years, much focus has been redirected towards siderophores, one of the most potent drug targets in bacteria and fungi (Balhara

et al. 2016). This chapter provides a brief discussion on fungal siderophores and then describes the possible ways the fungal siderophores are and can be targeted to overcome fungal diseases.

11.1.1 Siderophores

Iron is one of the essential elements for almost all living organisms, as it acts as a catalyst in a wide variety of important metabolic processes (Yun et al. 2000). In an iron-deficient condition, most of the plants, bacteria and fungi develop a unique iron uptake and transport strategies for uptake and utilization of iron from their environment even when it exists in trace quantity. One such unique iron uptake strategy includes the production and secretion of siderophores, small molecular weight compounds with high affinity for iron, especially ferric iron (Fe^{3+}) (Ahmed and Holmström 2014). Hence, siderophores act as ferric iron (Fe^{3+})–specific chelating agents and thus help fungi to scavenge iron from the environment, transport it across the cell membrane and thus make it available for the microbial cell.

Even though microbial siderophores are catecholates, and hydroxamates in nature, fungal siderophores are mainly of hydroxamates and very few are carboxylates, polycarboxylate and phenolate (Eck et al. 1999; Hissen et al. 2005; Schrettl et al. 2004; Tangen et al. 2007).

11.2 Iron Acquisition Mechanism

As discussed earlier, iron is required by almost all living organisms to perform normal cellular activities like distribution of oxygen, synthesis of genetic material and ATP Production. At neutral pH and in the presence of oxygen, the chemical nature of iron favours its oxidation from ferrous (Fe^{2+}) to ferric (Fe^{3+}). This rapid oxidation leads to the formation of ferric oxyhydroxide (FeO(OH)) which is insoluble in nature and this could not be taken up by the microbes (Heymann et al. 2002). Yet, microbes require a minimum of 10^{8}M concentration of iron for their optimal growth and development but the bioavailability of iron lies between 10^{-9} and 10^{-18} M (Hu et al. 2002). In order to overcome this iron shortage, microbes follow different iron acquisition mechanisms. Although fungus has different iron acquisition mechanisms, the first four are quite common in nature (Ahmed and Holmström 2014; Ismail et al. 1985). They are as follows:

1. Reductive iron acquisition
2. Siderophore biosynthesis
3. Utilization of siderophores by non-producers
4. Host molecule-specific iron acquisition
5. Acidification and mobilization

11.2.1 Reductive Iron Acquisition

In a reductive pathway, the extracellular ferric (Fe^{3+}) iron (either free or ligand-bound) is reduced into ferrous (Fe^{2+}) iron, and then reoxidized by ferroxidase which is linked to high-affinity permease. This reduction process usually takes place with the help of multiple enzymes. The membrane-bound reductases and secreted reductases play an important role in iron reduction (Howard 1999; Saikia et al. 2014; Schrettl et al. 2004). In addition, fungi are also expressing the high-affinity iron permeases like CaFtr1, CaFtr2, Cft1 and Cft2; multicopper ferroxidases; a copper transporter homolog Ccc2; and ferroxidase/permease homologs fetC and ftrA (Eck et al. 1999; Jung et al. 2008; Weissman et al. 2002). Studies showed that only Caftr1Δ and cft1Δ mutants displayed reduced virulence whereas the remaining exhibit either low virulence or no virulence in low iron environment (Jung et al. 2008). This ensures their significance in reductive iron acquisition mechanisms. However, when the reductive iron acquisition is blocked, fungi are compensating this loss by upregulating the non-reductive iron acquisition mechanisms to satisfy their demand for iron. Hence, these enzymes could not be a good choice as drug targets in antifungal drug design.

11.2.2 Siderophore Biosynthesis

Iron is essential for most of the important biological processes. When this iron is deficient in its environment, fungus synthesizes siderophores, ferric ion–specific chelating agents which can capture the iron with high affinity. As mentioned earlier, most of the fungal siderophores are hydroxamates and these are categorized into four families: rhodotorulic acid, fusarinines, coprogens and ferrichromes (Garnerin et al. 2017; Haas 2003).

Siderophore biosynthetic pathway starts with L-Ornithine, followed by the production of hydroxamate prosthetic groups which are linked together to form siderophores.

The first step in fungal siderophore biosynthesis is N^5-hydroxylation of L-Ornithine by L-Ornithine N^5-oxygenase. In *Ustilago maydis*, this enzyme is encoded by *Sid1* gene, expressed in an iron deficit condition to produce siderophores. The orthologs of this *Sid1* gene are observed in most of the fungus which shows the importance of this gene in fungal survival in various environmental conditions.

In the subsequent steps of the biosynthetic pathway, an acyl group is transferred to N^5-hydroxyornithine by N^5-transacetylase, whereas in coprogen and triacetylfusarinine siderophore families, further acetylation at N^2-amino group also takes place by N^2-transacetylase.

The final step is associated with the linking of hydroxamates by non-ribosomal peptide synthetases (NRPS). The simplest of fungal siderophore family,

rhodotorulic acid comprises only two such hydroxamates whereas the rest have three hydroxamates joined together by peptide or ester bonds. In the ferrichrome family of fungal siderophores, NRPS also incorporates three more amino acids: glycine, serine and alanine (Haas 2014; Haas 2003; Mercier and Labbé 2010). The genes sid2 and sidC encoding this synthetase enzyme are observed in *U. maydis* and *A. nidulans*, respectively (Bushley et al. 2008; Eichhorn et al. 2006). Studies also showed that the disruption of these genes blocks the siderophore biosynthesis. This ensures their significance in the survival of the pathogen in an iron-deficit condition. The schematic representation of the siderophore biosynthetic pathway is shown in Fig. 11.1.

Fungi such as *Aspergillus fumigates* and *H. capsulatum* synthesize siderophores and utilize them for acquiring iron from its host organism (Hilty et al. 2011; Hissen et al. 2005). In addition, these siderophores are also acting as iron storage molecules (Comensoli et al. 2017).

11.2.3 Utilization of Siderophores by Non-producers

In contrast to the siderophore synthesizing fungi, few of the fungi like *S. cerevisiae*, *C. albicans* and *C. neoformans* lack siderophore-synthesizing enzymes and/or their homologs in their genome. This ensures that these organisms could not synthesize siderophores (Blatzer et al. 2011; Tamayo et al. 2014). Instead, they have a very good siderophore-iron uptake system. Hence, they are consuming the siderophores

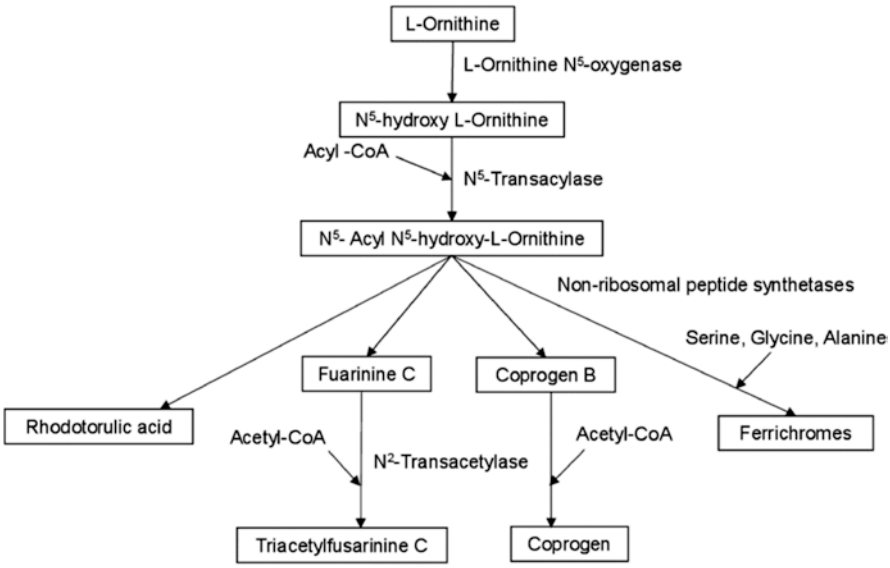

Fig. 11.1 Schematic representation of siderophore biosynthetic pathway in fungus

produced by the other organisms (Foster 2002; Haas 2003; Howard 1999; Hwang et al. 2008; Lesuisse et al. 2002; Philpott et al. 2002; Santos et al. 2003; Van Ho et al. 2002). The siderophore transporters such as CaSit1/CaArn1 and Sit1 present in these organisms help in this type of siderophore uptake (Heymann et al. 2002; Hu et al. 2002). Studies showed that the *CaSitD* mutant showed reduced invasion, which ensures the significance of these proteins (Tangen et al. 2007).

11.2.4 Host Molecule-Specific Iron Acquisition

The fungi also utilize some of the host molecules as their miscellaneous iron sources which include haem, haemoglobin, transferrin and ferritin.

Haem is a coordination complex of iron and porphyrin. It exits usually in the form of a complex with other proteins. However, only a trace quantity of haem is available in its free form. The oxidized form of haem is known as haemin. Fungi such as *H. capsulatum* and *C. albicans* utilize haemin as an iron source and acquire iron from it. The haem-binding protein Rbt5 and a haem oxygenase CaHmx1 play a significant role in acquiring iron from haemin (Foster 2002; Pendrak et al. 2004; Santos et al. 2003; Weissman and Kornitzer 2004). In *Cryptococcus neoformans*, CigI plays an important role in haem uptake. Mutants lacking CigI showed reduced virulence (Cadieux et al. 2013; Lian et al. 2005).

Haemoglobin, transferrin and ferritin are few iron-containing proteins present in humans, where haemoglobin acts as a transport protein, ferritin is a storage protein of iron and transferrin is a serum protein which has high affinity for iron.

In an iron-limiting condition, *C. albicans* utilizes all these proteins as their iron sources (Almeida et al. 2009; Bairwa et al. 2017; Fourie et al. 2018; Noble 2013). However, this iron acquisition process is dependent on several other proteins such as a haemin receptor Rbt5 (for acquiring iron from haemoglobin) (Bairwa et al. 2017), the cell-surface adhesin protein Als3, the high-affinity iron permease CaFtr1(for acquiring iron from ferritin) (Almeida et al. 2008; Liu and Filler 2011; Ramanan and Wang 2000), and ferric reductase Fre10 and the high-affinity permease Ftr1 (for acquiring iron from transferrin) (Bairwa et al. 2017; Heymann et al. 2002; Knight et al. 2005).

Similarly *C. neoformans* utilizes transferrin as an iron source. This iron acquisition process involves Cft1, Cfo1, Ftr1 (iron permease) and Fet3 (multicopper ferroxidase), where the ferrous iron is oxidized to ferric by Fet3 and transported by Cft1, Cfo1 and Ftr1. The mutants without these genes showed less virulence which ensures their importance (Jung et al. 2008). In contrast, *A. fumigatus* uses siderophores to extract iron from transferrin (Hissen and Moore 2005; Hissen et al. 2004).

11.2.5 Acidification and Mobilization

C. albicans and *C. neoformans* use an acidification process to acquire iron from the host (Almeida et al. 2009). They grow anaerobically and acidify their medium. In an acidic environment, the iron accumulates at the cell surface and is transported into the cell with the help of the excreted hydroxy acids (Howard 1999).

11.3 Siderophore-Mediated Iron Transport

The siderophore-mediated iron transport is an energy-dependent process and this transport process usually depends on the nature and type of siderophore, metal-ion coordination geometry and N-acyl residues surrounding the metal centre. Four different mechanism are associated with this siderophore-mediated iron uptake in fungi (Howard 1999; Renshaw et al. 2002). They are as follows:

1. Shuttle mechanism
2. Taxicab mechanism
3. Hydrolytic mechanism
4. Reductive mechanism

11.3.1 Shuttle Mechanism

In the shuttle mechanism, the entire siderophore-iron complex is transported into the cell. After the entire complex is moved into the cell, the iron is released and free siderophore is then excreted.

11.3.2 Taxicab Mechanism

In this mechanism, the iron from extracellular siderophores is transferred into intracellular ligands. Here, only the iron moves across the cell membrane and not the extracellular siderophores.

11.3.3 Hydrolytic Mechanism

In this transport, the entire siderophore-iron complex is transported into the cell and inside the cell by simultaneous action of reductase and esterases, and the iron is released.

11.3.4 Reductive Mechanism

In a reductive transport, the complex is not transported into the cell. Instead, the reduction takes place in the membrane and the reduced iron is taken into the cell.

11.4 Siderophore-Mediated Iron Storage

Iron is required by almost all living organisms for survival. Iron acquisition, uptake and storage are essential not only for surveillance and these also determine the virulence of an organism. Iron assimilation involves the conversion of insoluble ferric to ferrous iron by means of reduction or chelation. The unused internalized iron may generate free radicals, re-polymerize and become toxic. In order to avoid this, proper storage of iron is necessary. Various iron storage mechanisms are found in different organisms. In general, fungi use three iron storage mechanisms. They are (i) usage of iron storage protein like mycoprotein, (ii) vacuolar iron storage and (iii) siderophore-mediated iron storage (Garnerin et al. 2017).

Certain types of iron-rich proteins are found in the members of Zygomycota. They are mycoferritin and zygoferritin (a special type of ferritin found only in Zygomycetes) and are used as iron storage compounds. However, the phyla Ascomycota and Basidiomycota do not produce this kind of protein.

The vacuolar- and siderophore-mediated iron storage mechanisms are observed in most of the fungi. In *S. cerevisiae*, iron (Fe^{3+}) bound with polyphosphates are stored in vacuoles. *N. Crassa*, a widely studied member of the family *of Ascomycota*, produce two major hydroxamate-type siderophores coprogen and ferrichrocin. Among these, the (intracellular) ferrichrocin acts as an iron storage compound. In ascomycetes and basidiomycetes, hydroxamate-type siderophores act as iron storage molecules (Howard 1999).

11.5 Drug Discovery Strategies

Microbial siderophores not only function as chelating agents for minerals, especially iron, they also act as virulent factors (Haas et al. 2008). Hence, targeting microbial siderophores is one of the best options in overcoming the fungal infections as well as protecting humans from their virulent power. There are many ways by which siderophores can be targeted while designing a new drug. This includes targeting siderophore-biosynthetic pathways by inhibiting the catalytic enzymes involved in these pathways; targeting siderophore transport mechanisms; targeting siderophore-mediated iron storage; targeting iron acquisition mechanisms; conjugating the drugs with siderophores; and synthesizing siderophore analogues or mimics.

11.5.1 Siderophore–Drug Conjugates

Drug resistance is one of the major obstacles in the treatment strategies of most of the microbial infections. In spite of overcoming drug resistance, the drug discovery community has applied several approaches. One such approach is siderophore-mediated drug delivery also known as 'Trojan Horse strategy'. In this approach, the drug conjugated with siderophore is administered to a patient with microbial infection. The presence of siderophore facilitates its (siderophore-drug complex) binding with siderophore receptors and its transport across the cell membrane. Thus, the drug is successfully delivered into the microbial cell. As this approach of drug delivery is more successful in the treatment strategies of bacterial infection (Wencewicz et al. 2009), the same is also applied for fungal infections. The antifungal drug 13C-desketoneoenactin (DE) conjugated with siderophore was tested against various *Candida sp*. Results showed that the inhibitory activity of the siderophore-drug conjugates has increased 16-fold than that of the drug alone and thus ensures the success of the Trojan Horse strategy even for fungal diseases (Bernier et al. 2005). The siderophore-nucleoside analogue conjugates were also studied and the results showed an enhanced activity against *Candida albicans* (Balhara et al. 2016). The siderophore transporter, CaSit1/CaArn1 of *C. albicans*, is associated with this enhanced inhibition (Bernier et al. 2005).

11.5.2 Siderophore Mimics

As siderophores are crucially required for the survival of the pathogen in an iron limiting environmental condition in its human host, when siderophore analogues are designed as drugs and are administered, due to its structural resemblance, they would be readily taken up by the pathogen. Hence, the siderophore analogues (drugs) are easily transported across the fungal cell membrane, readily enter into the cell and reach their site of action. Due to this, medicinal chemists have gained an interest in designing and searching suitable siderophore analogues as potent antifungal agents.

In addition, the success of the siderophore-mediated drug delivery system (Trojan Horse strategy) has raised an interest over the biologist towards synthesizing siderophores and its analogues as vehicles of the drugs. As mentioned earlier, even though these techniques are focused with much interest for bacterial infections, a little focus has been started for fungal infections too.

VL-2397 is a cyclic hexapeptide and a natural compound isolated from the Malaysian leaf litter fungus, *Acremonium persicinium* MF-347833. Due to its structural resemblance with siderophore ferrichrome, it is readily taken up by the fungal cells via siderophore iron transporter 1 (Sit1) present in the cell membrane. This drug is currently under clinical trial (Nakamura et al. 2017).

11.5.3 Siderophore Biosynthesis Inhibitors

As the L-Ornithine N^5-oxygenase, N^5-transacetylase, N^2-transacetylase and non-ribosomal peptide synthetases play a crucial role in synthesizing the siderophores, one can target these enzymes to overcome the fungal infection.

11.5.4 Siderophore Transport Inhibitors

A number of proteins are involved in the siderophore-mediated iron transport system, which includes reductases (Fre1p–4p), permease-oxidase complex (Ftr1p and Fet3p), Sit1p (also called Arn3p), Arn1p and Enb1p (Heymann et al. 2002; Heymann et al. 2000; Lesuisse et al. 2001; Philpott and Protchenko 2008; Yun et al. 2001; Yun et al. 2000). These proteins can be targeted in an attempt to find a better antifungal agent.

11.5.5 Computational Approaches

An inhibitor can be designed against the siderophore biosynthetic pathway enzymes and siderophore transporter proteins by applying the principles of *structure-based drug designing* and/or *fragment-based drug designing*. The inhibitor is designed in a manner that it has complementary features with respect to the functional groups of the active site residues (amino acids). Hence, they can bind with greater affinity and thus can form a stable complex with the receptor. Similarly, the *structure-based pharmacophore* can also be predicted for these catalytic enzymes and the predicted pharmacophore can be searched against the chemical databases for suitable compounds as siderophore biosynthetic enzyme inhibitors. In contrast, competitive inhibitors of the biosynthetic pathway enzymes can also be identified using the shape and structural information of the enzyme substrates. By applying the *shape-based* and *fingerprint-based similarity search*, suitable competitive enzyme inhibitors can also be predicted from the chemical databases. In addition, the *drug repurposing approach* can also be applied to predict the existing drugs with antifungal activity. This approach reduces the drug discovery cost to a greater extent. In addition, the success rate in this approach is also higher than other approaches.

11.6 Conclusion

The recent outbreaks and emergence of multidrug-resistant fungal strains insists on the need for newer antifungal agents. As iron is essential for most of the biological processes, fungi have well-established iron acquisition mechanisms for its survival. Siderophores play an essential role in these iron acquisition mechanisms. This chapter briefs siderophores as antifungal drug targets and discusses various computational approaches to design a novel and potent antifungal agents.

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Chapter 12
A Summary on Up-To-Date Research on Fungal Siderophores on Disease, Treatment and Pathogenicity Based on Text Mining, Bioinformatics and Experts' Opinion

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K. Dhusia et al. (eds.), *Fungal Siderophores*, Fungal Biology,
https://doi.org/10.1007/978-3-030-53077-8_12

12.1 Introduction

Fungal siderophores are synthesized by fungus with low molecular weight and iron chelation properties. Most of them are hydroxamate siderophores (Renshaw et al. 2002) that possess beneficial or harmful effects. While the research work on bacterial siderophores are plenty, only fewer information is known about fungal siderophores. A comprehensive information on fungal siderophores based on literature resources in PubMed may benefit the microbiologists. This became the focus of the book volume "Fungal siderophores: From Mineral-Microbe Interactions to Antipathogenicity". The chapters are written by experts in the field of microbiology, pharmacology, pharmacognosy, biotechnology and bioinformatics. However, the main challenge reported by experts is finding the research articles related to fungal siderophores with a focus on their area of expertise. This motivated us to summarize the contents on fungal siderophores hidden within ~30 million PubMed articles.

Manual processing of PubMed articles to retrieve the ones related to fungal siderophores is not an effective approach. A Boolean query search ("fungal siderophore" OR "fungal siderophores") within the PubMed database retrieved only 51 articles. It is possible that we are missing many articles that include other representations for fungal siderophores. An automated approach to retrieve relevant articles from the PubMed database is mandatory for research focusing on fungal siderophores. Performing new research without the knowledge of existing information is a waste of time and hinders the progress in the field of microbiology. Alternatively, text mining is an automated approach meant for the retrieval and extraction of information from unstructured text (Raja et al. 2020). When the approach is applied to biomedical literature such as PubMed articles, it is known as "Biomedical Text Mining". When applied on patient's electronic health records and clinical notes, the approach is known as "Clinical Text Mining" (Raja et al. 2017).

Fungal siderophores are mainly involved in iron uptake, transport and storage of iron. It is crucial for various conditions such as iron starvation, antioxidative defence, microbial competition and virulence in humans. Recent studies identified the importance of siderophores and their biosynthetic pathways in the treatment and diagnosis of various fungal infections. Hence, gene and protein targets associated with fungal siderophore biosynthetic pathways are found to be the key components involved in the treatment of fungal diseases. For various fungal infections, we identified gene and protein targets, and constructed the network using Cytoscape. Despite the fact that there are a few studies investigating fungal siderophores of humans, the information remains scattered in the literature. Here, we have populated and integrated all the biological components to identify the therapeutic targets for fungal diseases using text mining of human fungal siderophore data. Text mining and data mining combined with network-based bioinformatics approaches (Prabahar and Natarajan 2017a, b, c) could unravel the mystery of hidden knowledge embedded in the literature resources and determine the novel targets involved in therapy.

In this chapter we used basic concepts of text mining to retrieve the relevant articles on fungal siderophores related to human. We applied bioinformatics technique to determine the genes and protein targets involved in siderophore biosynthetic pathways of various fungal organisms. Four experts with extensive knowledge in bioinformatics, physiology and pharmacology annotated the retrieved articles with human-related diseases, drugs and their association with fungal siderophores. Experts summarized the knowledge hidden within the retrieved articles and included the bioinformatics validation. We believe that this chapter will benefit the researchers in the field of microbiology by summarizing the contents available in the PubMed database.

12.2 Citation Retrieval

PubMed contains 12,679 articles related to siderophores (on 11 June 2019). Among these, 4795 articles are related to humans. However, Boolean query (i.e., "fungal siderophore" OR "fungal siderophores" AND human) retrieved only 10 articles for fungal siderophores related to humans. We observed that 811 PubMed articles (out of 4795) contain fungi or fungus in the title or abstract based on the Boolean query (siderophore OR siderophores) AND (fungi OR fungus). We suspect that many of these articles could be false positives with co-occurring terms, siderophore and fungi.

Our approach to retrieve relevant articles for the current chapter is simple. We used E-Utilities from NCBI (National Center for Biotechnology Information) to retrieve 4795 article IDs (PMID) related to siderophores and annotated with 'Human' in the Medical Subject Heading (MeSH) index. Our customized E-Utilities query is https://eutils.ncbi.nlm.nih.gov/entrez/eutils/esearch.fcgi?db=pubmed&term=("siderophore" OR "siderophores") AND humans[MeSH] &retmax = 20,000. All the retrieved PubMed articles may not contain abstracts. We filtered 3603 articles with abstracts and retrieved 106 PubMed articles related to human and fungal siderophores. We restricted the search to articles annotated with "Humans" and "Fungi" in the MeSH index. Our customized E-Utilities query for this retrieval is https://eutils.ncbi.nlm.nih.gov/entrez/eutils/esearch.fcgi?db=pubmed&term=("siderophore" OR "siderophores") AND humans[MeSH] AND fungi[MeSH] AND has abstract[Text] &retmax = 20,000. Our approach avoided many false positives. Thus, our approach obtained 10 times more PubMed articles when compared to the Boolean query search in the PubMed database.

12.3 Organisms Annotation

PubMed articles are annotated with organisms in the MeSH index. Though the retrieved articles are annotated with "Human", it would be interesting to know all the annotated organisms. We used the NCBI taxonomy to map the organisms and

their taxon for 106 PubMed articles. The resource includes an elaborated list of 1,757,316 taxon. Among these, seven taxon include "Homo sapiens" in their scientific names. We retrieved ~24 million PubMed articles with MeSH index and mapped organisms' scientific names or synonyms to the MeSH index. Our approach revealed many organisms other than humans for 106 articles (Table 12.1). Five articles did not include any annotations for organisms (PMID: 30816973, 30804121, 29458859, 29096192 and 28890395).

Table 12.1 Organism annotation for PubMed articles related to fungal siderophores and humans

PMID	Organisms	Taxon
29038255; 22869730	*Pseudomonas aeruginosa* | *Aspergillus fumigatus* | ***Homo sapiens***	287 | 746128 | 9606
28859314	*Viruses* | *Staphylococcus aureus* | *Pseudomonas aeruginosa* | *Aspergillus fumigatus* | ***Homo sapiens***	10239 | 1280 | 287 | 746128 | 9606
28730969; 28229977; 24312900; 22360467; 15328098; 6721470	*Fungi* | *Bacteria Latreille* et al. 1825 | ***Homo sapiens***	4751 | 629395 | 9606
28217776	*Metazoa* | *Cryptococcus neoformans* | *Aspergillus fumigatus* | ***Homo sapiens***	33208 | 5207 | 746128 | 9606
27797604; 24982320; 21399939; 18280720	*Metazoa* | *Fungi* | ***Homo sapiens***	33208 | 4751 | 9606
27243961	*Aspergillus* | *Cladosporium* | ***Homo sapiens***	5052 | 5498 | 9606
27060291	*Rattus sp.* | *Metazoa* | *Aspergillus* | ***Homo sapiens***	10118 | 33208 | 5052 | 9606
26974544; 26739764; 19453952; 14977945	*Aspergillus fumigatus* | ***Homo sapiens***	746128 | 9606
26960149;	*Arthrodermataceae* | ***Homo sapiens***	34384 | 9606
26712125; 17235665; 16003938; 6452038	*Fungi* | ***Homo sapiens***	4751 | 9606
26431675; 22393938; 7826025	*Metazoa* | *Fungi* | *Bacteria Latreille* et al. *1825* | ***Homo sapiens***	33208 | 4751 | 629395 | 9606
26195554	*Legionella pneumophila* | *Cunninghamella* | ***Homo sapiens***	446 | 4852 | 9606
25121733; 17194657; 3060947	*Mucorales* | ***Homo sapiens***	4827 | 9606
24984952	*Aureobasidium namibiae CBS 147.97* | *Aureobasidium subglaciale EXF-2481* | *Ascomycota* | ***Homo sapiens***	1043004 | 1043005 | 4890 | 9606
24416162; 19917080; 17082767	*Saccharomyces cerevisiae* | ***Homo sapiens***	4932 | 9606

(continued)

Table 12.1 (continued)

PMID	Organisms	Taxon
23968167	*Escherichia coli* | *Aspergillus fumigatus* | ***Homo sapiens***	562 | 746128 | 9606
23853581	*Mus sp.* | *Metazoa* | *Fusarium* | *Aspergillus fumigatus* | ***Homo sapiens***	10095 | 33208 | 5506 | 746128 | 9606
23818006	*Rattus sp.* | *Metazoa* | *Aspergillus fumigatus* | ***Homo sapiens***	10118 | 33208 | 746128 | 9606
23684655	*Metazoa* | *Fungi* | *Mucorales* | ***Homo sapiens***	33208 | 4751 | 4827 | 9606
23681725	*Trichoderma* | ***Homo sapiens***	5543 | 9606
23349056	*Saccharomyces cerevisiae* | *Penicillium* | ***Homo sapiens***	4932 | 5073 | 9606
22761575; 21053783; 12634327; 9423857; 1830280	*Candida albicans* | ***Homo sapiens***	5476 | 9606
22648507	*Embryophyta* | *Metazoa* | *Fungi* | ***Homo sapiens***	3193 | 33208 | 4751 | 9606
22144905; 20659583; 16113265; 16110789	*Mus sp.* | *Metazoa* | *Aspergillus fumigatus* | ***Homo sapiens***	10095 | 33208 | 746128 | 9606
21922920	*Avicennia* | *Fusarium* | ***Homo sapiens***	41377 | 5506 | 9606
21724450; 20974273; 15813678	*Metazoa* | *Aspergillus fumigatus* | ***Homo sapiens***	33208 | 746128 | 9606
21445236	*[Candida] glabrata* | ***Homo sapiens***	5478 | 9606
21341981; 8163646	*Mus sp.* | *Metazoa* | *Histoplasma* | ***Homo sapiens***	10095 | 33208 | 5036 | 9606
21143936	*Sporothrix* | ***Homo sapiens***	29907 | 9606
21067336; 19597710	*Scedosporium* | ***Homo sapiens***	41687 | 9606
18978530	*Metazoa* | *Mucorales* | ***Homo sapiens***	33208 | 4827 | 9606
18699866	*Mus sp.* | *Metazoa* | *Agrobacterium tumefaciens* | *Histoplasma* | ***Homo sapiens***	10095 | 33208 | 358 | 5036 | 9606
18042257	*Mus sp.* | *Metazoa* | *Cryptococcus neoformans* | ***Homo sapiens***	10095 | 33208 | 5207 | 9606
16928702; 1602914	*Mus sp.* | *Metazoa* | *Rhizopus* | ***Homo sapiens***	10095 | 33208 | 4842 | 9606
16302109; 2977619	*Paracoccidioides* | ***Homo sapiens***	38946 | 9606
15469520	*Saccharomyces cerevisiae* | *Candida albicans* | ***Homo sapiens***	4932 | 5476 | 9606

(continued)

Table 12.1 (continued)

PMID	Organisms	Taxon
14750563	*Actinobacteria* | *Brassicaceae* | *Fungi* | ***Homo sapiens***	201174 | 3700 | 4751 | 9606
12759789	*Mus sp.* | *Metazoa* | *Fungi* | ***Homo sapiens***	10095 | 33208 | 4751 | 9606
12518659	*Staphylococcus aureus* | *Mycobacterium* | *Pseudomonas aeruginosa* | *Burkholderia cepacia* | *Stenotrophomonas maltophilia* | *Fungi* | *Bacteria Latreille* et al. *1825* |*Haemophilus influenzae* | ***Homo sapiens***	1280 | 1763 | 287 | 292 | 40324 | 4751 | 629395 | 727 | 9606
12183576	*Mus sp.* | *Metazoa* | *Candida albicans* | ***Homo sapiens***	10095 | 33208 | 5476 | 9606
11870856	*Metazoa* | *Saccharomyces cerevisiae* | *Candida albicans* | ***Homo sapiens***	33208 | 4932 | 5476 | 9606
11709340	*Rattus sp.* | *Metazoa* | *Pneumocystis* | ***Homo sapiens***	10118 | 33208 | 4753 | 9606
11154409	*Candida* | ***Homo sapiens***	1535326 | 9606
11136762	*Stenotrophomonas maltophilia* | *Fungi* | *Candida albicans* | ***Homo sapiens***	40324 | 4751 | 5476 | 9606
10831457	*Metazoa* | *Stachybotrys* | ***Homo sapiens*** | *Ovisaries*	33208 | 74721 | 9606 | 9940
10678997	*Mus sp.* | *Metazoa* | *Penicillium* | ***Homo sapiens***	10095 | 33208 | 5073 | 9606
10569756; 7486926	*Histoplasma* | ***Homo sapiens***	5036 | 9606
10398672	*Metazoa* | *Geotrichum* | *Fungi* | *Saccharomyces cerevisiae* | *Histoplasma* | *Cryptococcus neoformans* | *Candida albicans* | ***Homo sapiens***	33208 | 43987 | 4751 | 4932 | 5036 | 5207 | 5476 | 9606
10377984	*Eukaryota* | *Metazoa* | *Fungi* | ***Homo sapiens***	2759 | 33208 | 4751 | 9606
8852350	*Pseudomonas* | *Fungi* | *Bacteria Latreille* et al. *1825* | ***Homo sapiens***	286 | 4751 | 629395 | 9606
8204101; 8316265; 1526205	*Rhizopus* | ***Homo sapiens***	4842 | 9606
8067783	*Mus sp.* | *Rattus sp.* | *Metazoa* | *Pneumocystis* | ***Homo sapiens***	10095 | 10118 | 33208 | 4753 | 9606

(continued)

Table 12.1 (continued)

PMID	Organisms	Taxon
8486769	*Cavia* | *Metazoa* | *Rhizopus* | *Candida albicans* | ***Homo sapiens***	10140 | 33208 | 4842 | 5476 | 9606
8259828	*Embryophyta* | *Ascomycota* | ***Homo sapiens***	3193 | 4890 | 9606
1818180	*Pneumocystis* | ***Homo sapiens***	4753 | 9606
1831796	*Staphylococcus* | *Candida albicans* | *Escherichia coli* | ***Homo sapiens***	1279 | 5476 | 562 | 9606
2530602	*Eukaryota* | *Embryophyta* | *Metazoa* | *Fungi* | *Bacteria Latreille* et al. *1825* |***Homo sapiens***	2759 | 3193 | 33208 | 4751 | 629395 | 9606
2702092; 3293856; 3662280	*Rhizopus* | ***Homo sapiens***	4842 | 9606
3514055	*Mus sp.* | *Eukaryota* | *Metazoa* | *Fungi* | *Bacteria Latreille* et al. *1825* | ***Homo sapiens***	10095 | 2759 | 33208 | 4751 | 629395 | 9606
6398589	*Mycobacterium leprae* | ***Homo sapiens***	1769 | 9606
43597	*Trichophyton* | ***Homo sapiens***	5550 | 9606
108388	*Mus sp.* | *Rattus sp.* | *Metazoa* | *Rhodotorula* | *Macaca fascicularis* | ***Homo sapiens*** | *Canis lupus familiaris*	10095 | 10118 | 33208 | 5533 | 9541 | 9606 | 9615

12.4 Genes and Protein Targets Associated with Fungal Siderophores

Siderophores play an essential role in fungal virulence and iron metabolism. An extensive literature survey was performed to identify the genes associated with the siderophore pathway for each of the fungal organisms. The data were collected from publicly available databases for the analysis. Table 12.2 gives the information of fungal organisms with their associated genes and siderophores activity. Organisms such as aspergillus, candida, etc., are known to be responsible for iron homeostasis. From the available information, a network of fungal organisms was constructed using Cytoscape 3.7.1 with genes and protein targets present in siderophore biosynthetic pathways. These interactome networks with fungal organism, genes and proteins are shown in Figs. 12.1, 12.2, 12.3, 12.4, 12.5, 12.6 and 12.7, respectively. Network and tabulated information of *Aspergillus sp.* is reported in our chapter 3 "Association of Fungal Siderophores in Human Diseases: Roles and Treatments" within the same book.

Table 12.2 Fungi, associated genes and their siderophores activity

Organisms	Associated Genes	Siderophores activity
Candida albicans	CAALFM_C405770CA, CFL1 FRE1, CaO19.8848, CaO19.1263	Ferric-chelate reductase 1 (ferric reductase transmembrane component 1)
	SIT1 orf19.2179, CAALFM_C208050CA	Siderophore transporter
	CFL1	Ferric-chelate reductase (probable ferric reductase transmembrane component)
	Cd36_22250, CD36_22250	Siderophore iron transporter, putative
Pseudomonas aeruginosa	pfeA_6 pfeA_2, C0044_20230, CAZ10_23360, DZ934_23610, EQH76_25155	Ferric enterobactin receptor (siderophore Enterobactin receptor PfeA)
	fhuA_1 fhuA_2, IPC669_23975, PAERUG_E15_ London_28_01_14_00259, RW109_RW109_02973	Ferric hydroxamate uptake (ferrichrome-iron receptor)
	bfrD_1 bfrD_3, CAZ10_20740, E4V10_32270, IPC669_11240, PAERUG_E15_ London_28_01_14_01048	Putative TonB-dependent receptor BfrD
	pfeA PA14_29350, EIP97_12335	Ferric enterobactin receptor (siderophore enterobactin receptor PfeA)
	PSPA7_4579	Siderophore receptor protein
	piuA PA14_58570, EIP97_24705	Putative outer membrane ferric siderophore Receptor (TonB-dependent siderophore receptor)
	CGU42_00025, DZ934_23635, IPC3_06205	TonB-dependent siderophore receptor

(continued)

Table 12.2 (continued)

Organisms	Associated Genes	Siderophores activity
Escherichia coli	cueOyacK, b0123, JW0119	Blue copper oxidase CueO (copper efflux oxidase) Iron(3+)-hydroxamate-binding protein FhuD (ferric hydroxamate uptake protein D) (ferrichrome-binding periplasmic protein) (iron(III)-hydroxamate-binding protein FhuD)
	fhuD b0152, JW0148, LCN1, VEGP, fiu Z1026, ECs0883, fiu c0890	Catecholate siderophore receptor Fiu (ferric iron uptake protein) (TonB-dependent receptor Fiu)
	fiuybiL, b0805, JW0790	Neutrophil gelatinase-associated lipocalin, NGAL (lipocalin-2) (oncogene 24p3, 24p3) (SV-40-induced 24p3 protein) (siderocalin LCN2) (p25)
	Lcn2	Lipocalin-1 (tear lipocalin, Tlc) (tear prealbumin, TP) (von Ebner gland protein, VEG protein)
	Fiu UTI89_C0808	Siderophore export accessory protein MmpS4 (PGB14T-X)
	mmpS4 Rv0451c, MTV037.15c	ABC-type cobalamin/Fe3 + − siderophores transport systems, ATPase components
	entF	EnterobactinsynthasesubunitF,EC2.7.7.-
Staphylococcus aureus	fepG NCTC13131_02773, feuB_2 NCTC13131_04898, fecD_1 NCTC7887_00508, feuC_2 NCTC13131_04897, fecD_2 NCTC7887_00509	Cobalamin Fe3 + −siderophores ABC transporter Permease
Saccharomyces cerevisiae	FRE1 YLR214W, L8167.2, CFL1 FRE1, CAALFM_C405770CA, CaO19.1263, CaO19.8848	Ferric/cupric reductase transmembrane component 1
	FRE2 YKL220C	Ferric/cupric reductase transmembrane component 2
	FRE3 YOR381W, O6754	Ferric reductase transmembrane component 3
	FIT1 YDR534C, D9719.37	Facilitator of iron transport 1
	FRE5 YOR384W, O6765	Ferric reductase transmembrane component 5

(continued)

Table 12.2 (continued)

Organisms	Associated Genes	Siderophores activity
Histoplasma capsulatum	SRE1	Siderophore uptake regulator SRE1
	SID1 LOM1	Siderophore biosynthesis cluster protein 1
	NPS1	Siderophore biosynthesis cluster protein NPS1
	ABC1	Siderophore biosynthesis cluster protein ABC1
	SID4	Siderophore biosynthesis cluster protein SID4
	OXR1	Siderophore biosynthesis cluster protein OXR1
	NIT22	Siderophore biosynthesis cluster protein NIT22
	SID3	Siderophore biosynthesis cluster protein SID3
	RTA1	Siderophore biosynthesis cluster protein RTA1
	SID5	Siderophore biosynthesis cluster protein SID5
Gibberellazeae	SID1 FG05371, FGRAMPH1_01T17749	Siderophore biosynthesis protein A
	FET3 FG05159, FGRAMPH1_01T17241	Cell surface ferroxidase FET3
	NPS2 FG05372, FGRAMPH1_01T17751	Intracellular siderophore synthetase
	NPS6 FG03747, FGRAMPH1_01T13607	Extracellular siderophore synthetase
	MIR1 FG00539, FGRAMPH1_01T01375	MFS siderochrome iron transporter 1

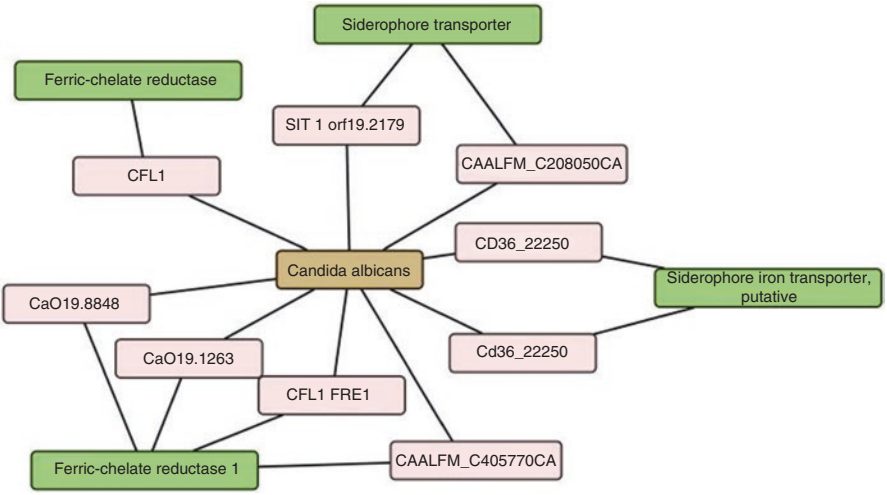

Fig. 12.1 *Candida albicans* network with genes and protein targets involved in biosynthetic pathway. (Pink nodes represent genes, green nodes represent protein targets and brown node represents the fungal organism)

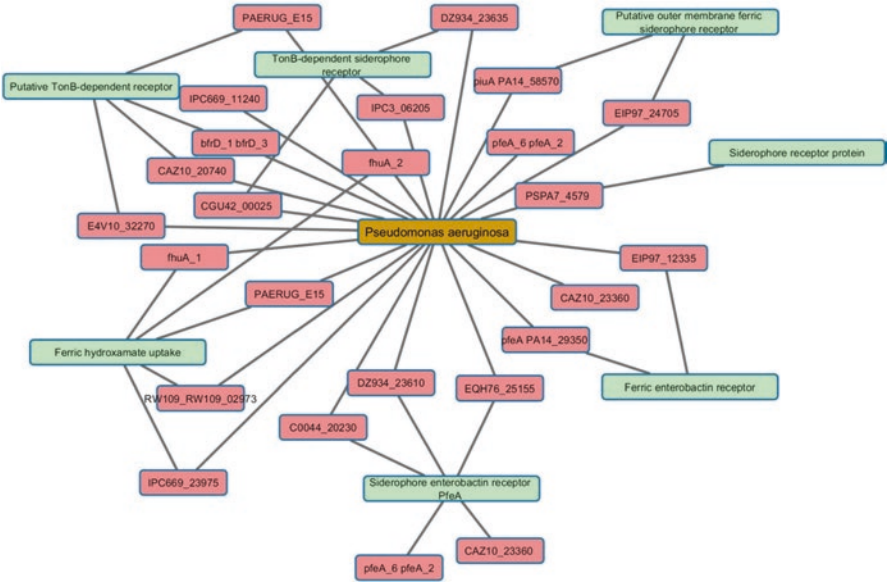

Fig. 12.2 *Pseudomonas aeruginosa* network with genes and protein targets involved in biosynthetic pathway

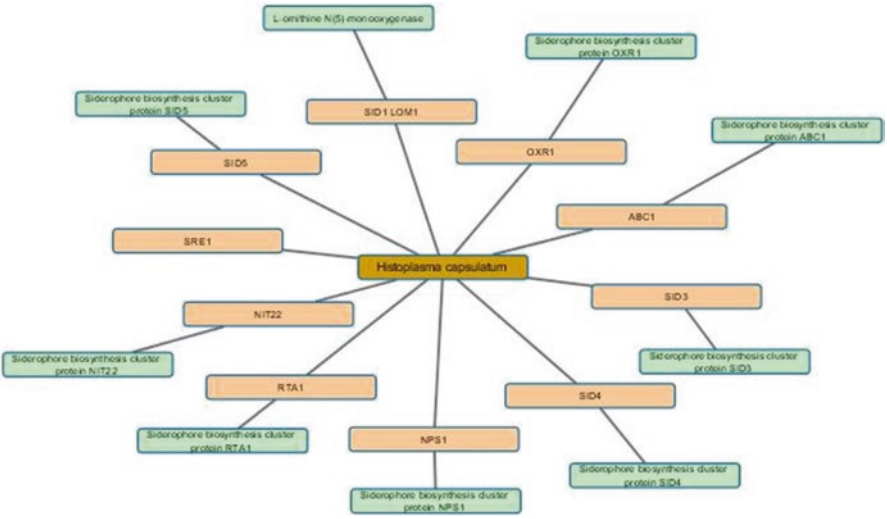

Fig. 12.3 *Histoplasma capsulatum* network with genes and protein targets involved in biosynthetic pathway

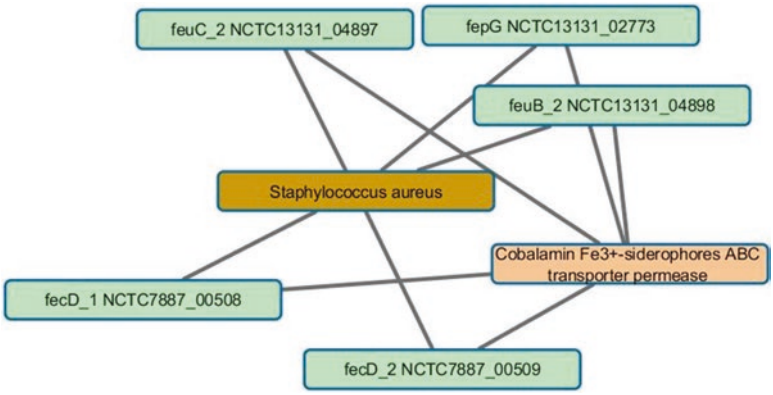

Fig. 12.4 *Staphylococcus aureus* network with genes and protein targets involved in biosynthetic pathway

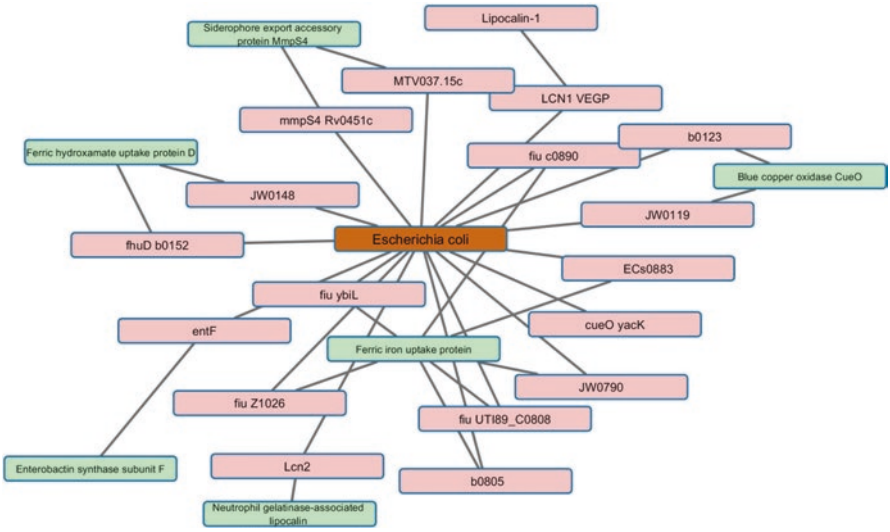

Fig. 12.5 *Escherichia coli* network with genes and protein targets involved in biosynthetic pathway

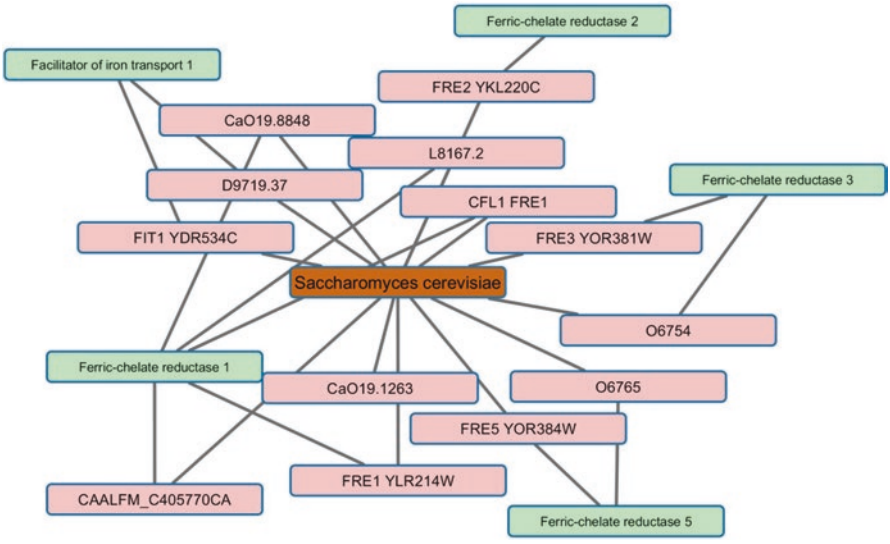

Fig. 12.6 *Saccharomyces cerevisiae* network with genes and protein targets involved in biosynthetic pathway

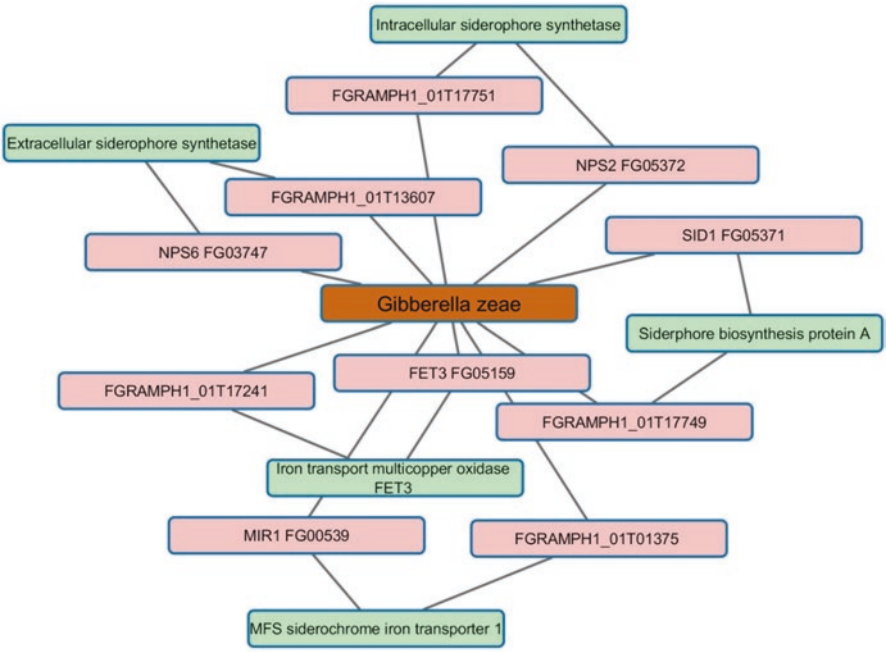

Fig. 12.7 *Gibberella zeae* network with genes and protein targets involved in biosynthetic pathway

12.5 Fungal Siderophores and Human Diseases

Metals act as a key player in the progression of infection. Iron is an essential nutrient for the growth and development of many living organisms and is non-toxic. In humans, Fe is the main constituent of haemoglobin that carries oxygen from lungs to various cells and tissues, and it is required for the synthesis of red blood cells. Fe deficiency leads to anaemia and defect in oxygen transport for tissue respiration, and its excess leads to oxidative stress. Fe binds with plasma proteins such as ferritin, transferrin and lactoferrin. In the microbial environment, Fe is necessary for growth, and it is an important cofactor for various biological processes such as respiration, DNA synthesis, RNA synthesis and many more (Weinberg 1989). Since Fe is required for the host (e.g., human) as well as the invading microorganism, there exists a heavy competition between the host and the invading organism for the acquisition of Fe.

Fungi are the group of eukaryotic organisms occupying a considerable space in the environment around us. They usually infect the cutaneous surface, gastrointestinal tract, oral and other mucosal surfaces of the human body. The fungal cells are larger, and their metabolic functions are characteristically unique when compared to other microorganisms. They possess many beneficial and harmful effects that have a significant impact on both plants and animals. Fungi mainly depend on Fe to pro-

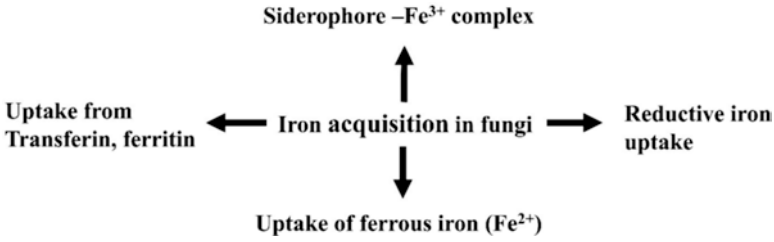

Fig. 12.8 Schematic representation of iron acquisition in fungi

duce the effects because it acts as the coenzyme in many biochemical reactions. In aerobic condition, Fe is converted into a ferric form (Fe^{3+}) which is highly insoluble and decreases the bioavailability of Fe to the fungi. However, the fungi exhibit certain strategies to obtain Fe. The major ways through which the fungi acquire iron for their metabolic needs are (Fig. 12.8): (i) by producing iron-binding substances called as "siderophores", (ii) through the uptake of Fe from the iron-binding proteins in the host, (iii) by reductive iron uptake and (iv) through the uptake of ferrous forms of Fe (Fe^{2+}).

In humans, many destructive diseases are caused by fungal pathogens, but these are less focused than other microbial infections. The ignorance is due to poor knowledge and diagnostic procedures, and the availability of fewer clinical studies. Recently, the number of patients with immune deficiency kept increasing, and the fungal infections were being recognized as clinically important. Although many fungal pathogens are involved in severe infections, the candida, *Aspergillus*, *Histoplasma capsulatum, Cryptococcus* species and *Trichophyton species* are the most common fungi causing severe fungal diseases. The cutaneous infections are commonly caused by *Trichophyton species.* The mucosal cell injury is caused by *Candida albicans* during the phagocytosis by the endothelial cells (Fratti et al. 1998). The fungal infections are controlled and treated by the immune cells. Thus, the infections caused by fungi are common in immunosuppressed patients who have acquired cancer or AIDS, and in patients who have undergone organ transplant or immune-modulatory therapy. Table 12.3 summarizes the various diseases caused by fungi in humans.

12.5.1 Allergy and Histoplasmosis through Conidia

The allergic reactions and other occasional diseases in humans are commonly caused by *A. fumigatus*, a saprophytic fungal pathogen, and various fungal species including *Alternaria*, *Penicillium* and *Cladosporium* (Żukiewicz-Sobczak 2013). Fungi cause the allergy by forming an asexual fungal spore called conidia. The conidia produced by *A. fumigatus* often spreads in the air and is being inhaled con-

Table 12.3 Human diseases caused by fungi

Name of the fungus	Disease	Literature evidence (PMID)
Aspergillus fumigatus	Invasive aspergillosis	26974544
	Respiratory tract infections and mycotoxicosis	15813678
	Cystic fibrosis	30174658, 29038255
Candida albicans	Mucosal infection, epithelial invasion, and endothelial cell injury	21776285
Histoplasma capsulatum	Histoplasmosis	10569756
	Pneumonia, respiratory failure and mediastinitis	17223625, 29744231
Cladosporium sphaerospermum	Allergy and other occasional diseases	27243961
L. corymbifera	Mucormycosis	25121733
Aureobasidium pullulans	Opportunistic infections	24984952
Melanized fungi (black yeast)	Phaeohyphomycosis, chromoblastomycosis and mycetoma	24982320
Filamentous fungi-aspergillus and fusarium strains	Fungal infection of the cornea	23853581
Candida glabrata	*Candida glabrata* infection in HIV infection and tuberculosis	21445236
H. capsulatum strain G217B	Pulmonary histoplasmosis	21341981
Scedosporium apiospermum	Cystic fibrosis, scedosporiosis	21067336
Cryptococcus neoformans	Meningoencephalitis	22144905
Zygomycetes	Zygomycosis	16003938
Candida dubliniensis and *Candida albicans*	APECED (autoimmune polyendocrinopathy–candidosis–ectodermal dystrophy) and chronic candidosis	11154409
Stachybotrys chartarum	Pulmonary haemorrhage and hemosiderosis	10831457
Rhizopus microsporus	Iron overload in dialysis and mucormycosis	8204101
Cryptococcus yeast	Neoplastic disease	2530602
Cunninghamella bertholletiae	Mucormycosis (diabetic patients or patients receiving chemotherapy treatment) *C. bertholletiae* infection during deferoxamine therapy for iron overload unrelated to haemodialysis patients	3060947
Rhizopus species	Mucormycosis	2702092
	Rhinocerebral rhizopus infections	3662280

stantly by humans. In a healthy human, the immune system rapidly removes it to protect the lungs. In an immunocompromised patient, the inhaled conidia infiltrate the lungs and sinuses and cause invasive aspergillosis (Haas 2014). Apart from allergy and aspergillosis, *Histoplasma capsulatum* causes histoplasmosis through conidia or mycelial fragmentation (Timmerman and Woods 1999). Conidia requires Fe for its growth. Fungi secrete Fe^{3+} chelating hydroxamate siderophore during mycelial growth to acquire Fe from the host. Thus, the siderophore-iron complex plays a vital role in allergic reactions and other diseases in humans.

12.5.2 Mucormycosis and Zygomycosis

Many fungi cause life-threatening diseases in patients with decreased immunity. *C. albicans* and *Cryptococcus neoformans* are the common fungal pathogens causing superficial mucosal infections, candidiasis and meningoencephalitis in humans (Sloan and Parris 2014; Caza and Kronstad 2013; Moyes and Naglik 2011). Mucormycosis is a fatal fungal infection caused by the *Rhizopus* species, *Lichtheimia* species, *Cunninghamella bertholletia* and *A. fumigatus* in patients with renal failure, diabetes and in patients under chemotherapy or steroid therapy (Schwartze et al. 2014; Boelaert et al. 1988; Nakamura et al. 1989; Rex et al. 1988; Boelaert et al. 1988). The disease is common in renal failure patients under dialysis with iron overload and receiving chelation therapy with the deferoxamine (Boelaert et al. 1993). Xenosiderophores such as *S. cerevisia*, *Candida spp.* and *C. neoformans* also cause mucormycosis disease. Zygomycosis is caused by *R. rhizopodiformis* that shows increased growth rate during desferrioxamine therapy (Boelaert et al. 1994).

12.5.3 Fungal Diseases in Cystic Fibrosis

Cystic fibrosis (CF) is caused by the mutation in the CF Transmembrane conductance Regulator (CFTR) gene which is an autosomal recessive disorder. CF mainly affects the respiratory system and the digestive system (Williams et al. 2016). Nearly 50% of CF patients are reported with invading *A. fumigatus* and *Candida spp*. Recently, *Scedosporium apiospermum* is reported in CF patients. It causes fatal infection during lung transplant without any clinical signs. A common fungal disease in CF patients is the allergic bronchopulmonary aspergillosis and its prevalence is high. The fungi form a biofilm that is resistant to antimicrobial drugs and the host defence system, and produce a defect in lungs and respiratory function (Boelaert et al. 1993; Sass et al. 2019). The formation of biofilm requires Fe (Lin et al. 2012). During the initial stages of CF, Fe availability is highly limited for the infecting microorganism. In the host body, Fe is present in the bound form of ferritin, transferrin and lactoferrin. *A. Fumigatus* acquires Fe from transferrin by producing siderophores. *C. albicans* acquires Fe directly from the ferritin and transferrin by

releasing different ferric reductases (Tyrrell and Callaghan 2016). *Scedosporium apiospermum* acquires Fe by producing two types of siderophores, dimerumic acid and Nα-methyl coprogen B (Bertrand et al. 2009).

12.5.4 Miscellaneous Fungal Infections

Rhinocerebral rhizopus infection is a rare fungal infection that occurs in some dialysis patients receiving deferoxamine for the metal overload (Ammon et al. 1992). The deferoxamines bind with iron and form iron a feroxamine complex which acts as in vitro siderophore for the fungal organism. The fungal species *Rhizopus rhizopodiformis* and *R. microsporus var.* are believed to utilize Fe from ferric-DFO complex in dialysis patients (De Locht et al. 1994). *R. oryzae* has the ability to extract and utilize Fe from deferoxamine and grow in the host body. Fe permease transports the iron released from the deferoxamine into the fungi (Ibrahim et al. 2008).

Arthroderma benhamiae is a fungal pathogen that spreads through infected animals. In humans, they cause infection in the keratinized structures like skin, nails and hair. Generally, these infections are mild and not fatal (Kröber et al. 2016). The spores of the filamentous fungal species, *Aspergillus* and *Fusarium*, cause corneal ulcer that leads to visual injury and loss of vision (Karthikeyan et al. 2011). According to the World Health Organization estimation, the corneal ulcer is one of the major causes for blindness and 65% is caused by fungal infections. *S. schenckii* is a fungus in soil and plants that causes a cutaneous infection called sporotrichosis. *P. carinii* pneumonia (PCP) is a life-threatening disease caused by the opportunistic fungal pathogen *Pneumocystis carinii* (Clarkson et al. 2001; Weinberg and Shaw 1991). Onychomycosis, a fungal infection of the nails, and keratitis are mainly caused by *Trichophyton rubrum*, *Trichophyton Mentagrophytes* and *Candida* species (Piraccini and Alessandrini 2015). *Trichophyton* species also causes stromalysis which leads to visual defects (Jin et al. 2014). Fungal infections such as hemosiderosis and pulmonary haemorrhage are caused by *Stachybotrys chartarum* (Vesper et al. 2000). *Aureobasidium pullulans* is a black-yeast-like fungus that grows at any climatic condition. It causes opportunistic infections in humans (Gostinčar et al. 2014).

The availability of essential nutrients is the factor that influences the organisms' pathogenesis. Fe is a vital nutrient that has a key role in the development of infections caused by many pathogens. Fe is necessary for the growth and maturation of an organism. Availability of Fe is one of the major virulent determinants in microbes (Brock et al. 1991). During iron scarcity, a greater amount of siderophore has been observed during in vivo growth of microorganisms (Brock et al. 1991). Hence, the virulence of the fungal pathogen depends on in vivo siderophores (Kröber et al. 2016; Schrettl et al. 2004). Certain evidence showed that siderophores produced by the yeast-like microorganism induce the growth of bacterium such as *M. leprae* in leprous lesions. The therapeutic inhibition of fungal Fe acquisition with Fe chelators decreases the fungal growth. The widespread infectious fungal pathogens like *Aspergillus*, *Candida* and *Cryptococcus* make use of the siderophores fusarinine C (FsC), triacetyl fusarinine C (TAFC) and ferricrocin to acquire Fe either for germi-

nation, virulence or to prevent in vivo oxidative stress during infection (Blatzer et al. 2011). The growth of *C. albicans* mainly depends on Fe availability. The endothelial cell injury caused by *C. albicans* shows more adherence to the endothelial cell when pretreated with Fe chelators. In addition, Fe chelators upregulate the receptors of *C. albicans* on the endothelial cells (Fratti et al. 1998). The studies in the mouse model showed that N, N′, N″-triacetyl fusarinine C (TAFC) produced within the host organism is required for fungal growth and virulence of *A. fumigatus* in invasive aspergillosis. The increased TAFC concentration in the plasma is considered as an early biomarker in the invasive aspergillosis. TAFC is a cyclic tripeptide consisting of three N 2-acetyl-N 5-cis-anhydromevalonyl-N 5-hydroxyornithine residues linked by ester bonds. L-ornithine-N 5-monooxygenase catalyses the first step in the biosynthesis of TAFC which causes virulence (Schrettl et al. 2004). *A. fumigatus* and *H. capsulatum* show a severe defect in virulence when siderophore genes are mutated or deleted. The siderophores produced by *A. fumigatus* can also extract Fe from the host iron-binding protein transferrin. Tear lipocalin is a secretary protein that inhibits the microbial growth by removing the siderophore when it is used topically in humans (Fluckinger et al. 2004). In corneal infection caused by *Aspergillus* and *Fusarium*, the elevated level of the siderophore binding protein and the mutations in the siderophore production pathway leads to inhibition of fungal growth (Hazlett et al. 2016). Thus, the siderophores are essential for the growth, conidiation and virulence of fungus under iron-restricted condition. At the same time, defective siderophore biosynthesis leads to decreased growth and virulence in fungi.

12.6 Fungal Siderophores in Cancer Therapy

Fe demand is high in cancer cells to sustain the proliferation. Many studies have been carried out to understand the role of Fe in cancer cells by modulating the genes responsible for Fe regulation or by using siderophores. The fungal siderophores produce their effect by the inhibition of DNA synthesis, and arrest G1-S-phase cell cycle, attenuate Epithelial-mesenchymal transition (EMT), modify the epigenetic signatures of malignant tumour cells and also promote the apoptosis of cancer cells. Three natural compounds, NBRI16716A, NBRI16716B and NBRI16716C, isolated from a fungal species, *Perisporiopsis melioloides* Mer-f16716, are found to be siderophores through the colour reaction to FeCl3 (Frederick et al. 1981). The compounds possessed anti-tumour activity and gave promising results on prostate cancer (Kawada et al. 2010). Among the three compounds, NBRI16716B exhibits strong selectivity as well as activity in vitro and NBRI16716C exhibits comparatively weak activity.

Fe is necessary for cell proliferation and growth. It is harmful to the redox abilities of a cell because the formation of free radical may worsen the oxidative stress and DNA damage. Fe plays an important role in tumour progression and metastasis. Tumour-associated macrophages (TAMs) are the main source for iron. By targeting

TAM with fungal siderophores, it is possible to transport and utilize Fe for various functions of the cells. The pathways of Fe acquisition, export and storage are often disturbed in cancer. Hence, targeting Fe metabolic pathways should be a novel approach in cancer drug discovery. Although there is close connectivity between Fe homeostasis and cancer biology, our knowledge remains descriptive and is based only on the in vitro experimental models or from the in vivo animal models that employ xenogeneic tumour cell transplantation. The potential role of Fe in cancer and tumour microenvironment are not completely addressed in human cancer or patient cohorts. There is toxicity in the excess labile Fe as it acts as a catalyst in the formation of reactive oxygen species (ROS) via Fenton-/Haber-Weiss chemistry (Pfeifhofer-Obermair et al. 2018).

Fungal siderophores are useful in cancer therapy: desferrioxamine, a natural siderophore, deferasirox, a synthetic Fe chelator and thiosemicarbazone show promising anti-cancer activity (Kalinowski and Richardson 2005). These siderophores are validated through clinical trials (Hatcher et al. 2009). Unfortunately, serious side effects such as abnormalities in the hearing, nephropathy, optic neuropathy and retarded growth in children are observed as a side effect of cancer treatment with fungal siderophores and their use is hampered (Nadkarni et al. 2008; Dayani et al. 2004). Several studies target Fe in tumour cells, but there are fewer approaches that address Fe chelation. Metal chelation therapy is one of the challenges in cancer treatment although it has systemic iron depletion ability.

12.7 Fungal Siderophores and Pathogenicity

A. fumigatus adjusts to iron limitation by the process of siderophore biosynthesis through iron upregulation and down regulation of pathways involved in iron consumption (Schrettl and Haas 2011). *A. fumigatus* causes invasive infection in ImC populations and is considered to be a serious life-threatening infection worldwide. Genes such as sidA present in *A. fumigatus* are found to be involved in hydroxamate siderophore biosynthesis. The gene sidA causes virulence in *A. fumigatus* and hence inhibition of this gene could be a target to treat fungal infection in humans (Hissen et al. 2005; Wasylnka et al. 2005). *A. fumigatus* employs extra- and intracellular siderophores to maintain adequate supply of iron. This helps fungal growth even during iron starvation. Hence, the fungal siderophore system is highly required for interaction with the host immune cells and extracellular growth (Schrettl and Haas 2011). Siderophores are also required for storage of iron, resistance to oxidative stress, asexual/sexual development, iron-induced toxicity protection and virulence in some fungal organisms (Johnson 2008).

Candida glabrata is a life-threatening human fungal pathogen that causes infections in a number of ImC individuals who are exposed to cancer treatment, HIV

infections, tuberculosis and other organ transplants. Iron acquisition occurs and the microbial virulence determinant causes iron overload in humans and have been found to be correlated with increased infection (Nevitt and Thiele 2011). The dimorphic fungal pathogen *Histoplasma capsulatum* causes intracellular growth of yeasts and may be the causative agent for pulmonary histoplasmosis. Several human and murine experiments demonstrate that SID1 (coding for L-ornithine-N(5)-monooxygenase) expression is required for siderophore biosynthesis by *H. capsulatum* and hence inhibition of SID1 expression would reduce the virulence of *H. capsulatum* yeasts (Hilty et al. 2011). Cryptococcus neoformans controls the iron levels using the polysaccharide capsule and melanin virulence factors. This pathogen is known to cause meningoencephalitis in ImC people by acquiring iron through the reductants from the cell surface and secretion. In addition, permease/ferroxidase uptake system and transporters of siderophores are also involved in causing infection (Jung and Kronstad 2008). *Rhizopus oryzae* causes mucormycosis in several patients. Patients who undergo deferoxamine treatment are susceptible to mucormycosis. Deferoxamine also serves as a siderophore that delivers free Fe to *R. oryzae* and remains as a major cause of mucormycosis. Other iron chelators, such as deferiprone, do not deliver free ion and hence are used in iron-chelation therapy to treat mucormycosis (Ibrahim et al. 2006).

Siderophores are the virulence factors for iron-transport functions because of their iron acquisition property. Siderophore biosynthesis and its iron uptake suggests possible therapeutic targets to treat fungal infection (Nyilasi et al. 2005). The most life-threatening fungal pathogens such as aspergillus, candida and Cryptococcus utilize the siderophore-mediated iron uptake mechanism for various purposes like propagation or resistance to oxidative stress, survival and virulence that occur in vivo during infection. Pathogens release siderophores in order to prevent the loss of iron. Hence, the fungal siderophore biosynthetic pathways along with their biological mechanisms serve as a target for antifungal therapy (Balhara et al. 2016).

12.8 Conclusion

Fungal siderophores are less studied when compared to bacterial siderophores. They possess both beneficial and harmful effects on humans. Knowledge on fungal siderophores could be useful to advance the current treatments on fungal infections and fungal diseases. Interestingly, fungal siderophores are useful in cancer therapy. Text mining helped us retrieve relevant articles on fungal siderophores. Bioinformatics approaches helped us identify the genes and protein targets. We also discussed on the pathogenicity of fungal siderophores. Our summary on fugal siderophores is based on PubMed articles retrieved by our simple text-mining approaches. We believe that our finding will be useful to microbiologists to carry out several future studies on fungal siderophores.

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K. Dhusia et al. (eds.), *Fungal Siderophores*, Fungal Biology,
https://doi.org/10.1007/978-3-030-53077-8