Metal Transport

Microbial siderophore-mediated transport

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Abstract

Microbial iron chelates, called siderophores, are synthesized by bacteria and fungi in response to low iron availability in the environment. The present review summarizes structural details of siderophore ligands with respect to their transport properties. This presentation is largely centred on the occurrence and function of siderophores in the various bacterial and fungal genera.

Introduction

Siderophores are defined as low-molecular-mass microbial compounds with a very high affinity for iron. Their function is to mediate iron uptake by microbial cells. For a more comprehensive review of the structures and functions of siderophores the reader is directed to reviews on iron transport [1] and microbial transport systems [2]. Although most siderophores are water soluble and are excreted into the environment, there are some siderophores that are not excreted at all, such as the mycobactins, synthesized by mycobacteria, that are located within the cell envelope [3,4]. This is in contrast to the carboxymycobactins and exochelins that represent the real extracellular siderophores of the mycobacteria. Fungal siderophores may also be divided into extracellular and intracellular siderophores, as found in spores and mycelia of Neurospora and Aspergillus [5].

Also, extremely lipophilic siderophores have been found in marine bacteria that do not readily diffuse into the surrounding medium, but which form vesicles [6]. Thus the environmental distribution of siderophores may vary to some extent. However, their general iron-transport function is obvious and has been documented by radioactive labelling experiments in a variety of microbial organisms. Although a number of transport mechanisms are based on non-destructive shuttle systems, some of the ligands may be degraded by esterases after iron delivery to the cells. In general, most siderophore transport systems are highly specific for certain siderophores, although some broad-range siderophore-recognition systems have been described. Several novel siderophore-transport systems have recently been proposed based on ligand-exchange mechanisms [7,8].

Functions of siderophores

Although their main function is to acquire iron from insoluble hydroxides or from iron adsorbed to solid surfaces, siderophores can also extract iron from various other soluble and insoluble iron compounds, such as ferric citrate, ferric phosphate, Fe-transferrin, ferritin or iron bound to sugars, plant flavone pigments and glycosides or even from artificial chelators like EDTA and nitrilotriacetate by Fe(III)/ligand-exchange reactions. Thus, even if siderophores are not directly involved in iron solubilization, they are required as carriers mediating exchange between extracellular iron stores and membrane-located siderophore-transport systems.
The efficiency of siderophores in microbial metabolism is based mainly on three facts. (1) Siderophores contain the most efficient iron-binding ligand types in Nature, consisting of hydroxamate, catecholate or \(\alpha\)-hydroxy-carboxylate ligands that form hexadentate \(\text{Fe(III)}\) complexes, satisfying the six co-ordination sites on ferric ions. Moreover, siderophores possessing three bidentates in one molecule (iron-to-ligand ratio \(=1:1\)) show increased stability due to the chelate effect. (2) Regulation of siderophore biosynthesis is an economic means of spending metabolic energy, but it also allows for the production of high local concentrations of siderophores in the vicinity of microbial cells during iron limitation. This kind of overproduction may also be operating in host-adapted bacterial and fungal strains, leading to increased virulence. (3) Besides their ability to solubilize iron and to function as external iron carriers, siderophores exhibit structural and conformational specificities to fit into membrane receptors and/or transporters. This has been amply demonstrated by modifying siderophore chemical structure, i.e. using derivatives, enantiomers, metal-replacement studies or by genetic and mutational analysis of receptors and membrane transporters [8-10].

**Biosynthetic and structural aspects**

The biosynthetic pathways of siderophores are tightly connected to aerobic metabolism involving molecular oxygen activated by mono-, di- and N-oxygenases and the use of acids originating from the final oxidation of the citric acid cycle, such as citrate, succinate and acetate. Moreover, all siderophore peptides are synthesized by non-ribosomal peptide synthetases and in the case of fungal siderophores are mainly built up from ornithine, a non-proteinogenic amino acid. Thus, siderophore synthesis is largely independent from the primary metabolism. Most siderophores contain one or more of the following simple bidentate ligands as building blocks: (1) a dihydroxybenzoic acid (catecholate) coupled to an amino acid, (2) hydroxamate groups containing \(N^2\)-acyl-\(N^2\)-hydroxyornithine or \(N^4\)-acyl-\(N^4\)-hydroxylysine and (3) hydroxycarboxylates consisting of citric acid or \(\beta\)-hydroxyaspartic acid.

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

A further aspect of siderophore evolution is the optimization of chelate conformation. Although linear di-, tetra- and hexadentate siderophores have been found in all siderophore classes, there is a tendency for cyclization in the final biosynthetic end products (Figure 1). Examples are enterobactin or corynebactin [11] in the catecholate class, fusigen, triacyltetrasarnine and ferrioxamines E and G, as well as the ferrichromes and asperchromes, in the hydroxamate class [12]. Cyclization enhances complex stability, chemical

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**Figure 1**

Cyclic siderophores

(a) Ferrichrome, (b) triacyltetrasarnine C and (c) enterobactin.
stability and improves resistance to degrading enzymes. Cyclization is regarded as a common feature of secondary metabolism and is found in microbial peptides, polyketides, macrocyclic antibiotics and other bioactive compounds. Cyclization might also be advantageous for diffusion-controlled transport processes across cellular membranes. Moreover, due to a reduction of residual functional groups, the surface of the siderophores becomes non-reactive or inaccessible to modifying enzymes.

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

Siderophores are also involved in mycorrhizal symbiosis, as found in all terrestrial plant communities. One of the major types of mycorrhizum are the ectomycorrhiza, typically formed by almost all tree species in temperate forests. So far, only a few siderophores have been described due to the difficulties with cultivating the mycorrhizal fungi in pure culture under iron limitation. However, siderophores from three ericoid mycorrhizal fungal species, Hymenoscyphus ericae, Oidiodendron griseum and Rhodothamnus chamaecistus, and an ectendomycorrhizal fungus Wilcoxina and an ectomycorrhizal fungus Cenococcum geophilum, have been isolated which all produce hydroxamate siderophores of the ferrichrome and fusigen class [13].

Zygomycetes produce solely aminocarboxylates based on citric acid and amines, which show optimal iron-binding activity at a weakly acidic pH [14]. Although phenolate and catecholate pigments have been detected in higher fungi, defined structures of catecholate-based siderophores have never been reported in fungi. The concomitant production of organic acids by most fungi probably prevents the use of ferric catecholates that are unstable at acidic pH, while ferric hydroxamates are generally stable down to pH 2. Characterization of siderophore classes based on microbial groups, however, is not always possible. The phylogenetic distance between catechol- and hydroxamate-producing genera can be very small and occasionally both siderophore types have been observed in the same genus, and indeed in at least one case in a single siderophore [15]. We reported earlier that both catecholate- and hydroxamate-type siderophores have been isolated from the Erwinia/Enterobacter/Hafnia group, representing closely related genera of the family of Enterobacteriaceae [16,17]. Fluorescent Pseudomonads and the related non-fluorescent Burkholderia group, which are well-known producers of linear peptide siderophores [18], might profit from the generally neutral environment of soil, where acid stability is of minor importance. The alternating α- and γ-configuration of peptidic amino acids also makes these siderophores very resistant to microbial proteases.

When we look more deeply into the large group of marine Vibrios, we notice that a broad range of structurally different siderophores is produced [12]. Thus, catecholate siderophores have been detected in Vibrio cholerae, Vibrio vulni'cus and Vibrio fluvialis. A mixed-type catecholate-thiazoline-hydroxamate siderophore, named anguibactin, has been isolated from Vibrio anguillarum and the citrate-based hydroxamate, aerobactin, has been described in certain marine Vibrios. Also the occurrence of ferrioxamine G has been reported in Vibrio species and we have recently identified the structurally related dihydroxamate, bisucaberin, in the fish pathogen Vibrio salmonicida [19]. This broad range of structurally different siderophores in the family Vibrionaceae may reflect the existence of a large pool of siderophore biosynthetic genes and may also indicate that the different genera of the family are more heterogeneous than previously assumed. Vibrios are widespread in marine water, but this does not necessarily mean that they are really free-living bacteria. We may assume that most Vibrios are somehow associated with particles in marine coastal water. Siderophores have also been isolated from several other marine bacteria, like Alteromonas, Halomonas and Marinobacter, indicating that siderophore production in the marine environment is widespread [6].
The role of citric acid

With respect to the diversity of siderophores, it is interesting to note that citrate is the starting point of a variety of siderophores. There is evidence that citrate-containing siderophores are present in a variety of bacterial and fungal genera. Some of these siderophores, like aerobactin, arthrobactin, schizokinen, acinetoferrin and nannochelin, contain citrate linked to hydroxamate residues, while others, like rhizoferrin, staphyloferrin and vibrioferrin, represent aminocarboxylate siderophores [12].

Citric acid may be linked amidically to the α-amino group of ornithine or lysine, as found in staphyloferrin A and aerobactin. On the other hand, citric acid may be bound to an amine group, as found in rhizoferrin, or to the terminal amine group in staphyloferrin A. Thus, carboxylic groups may combine with both α-amino groups and terminal amine groups. Condensation of succinic acid with amines is common among all ferrioxamines, suggesting that decarboxylation may occur prior to condensation. However, while succinic acid forms both amide and hydroxamate groups, citric acid has never been shown to form hydroxamic acyl residues. A functionally analogous hydroxycarboxylate donor is provided by β-hydroxyaspartic acid that is inserted in the peptide backbone of some pyoverdines and ornibactins, representing siderophores of the genus Pseudomonas and the non-fluorescent genus Burkholderia respectively [20].

Stereochemistry and recognition

An interesting finding is the occurrence of rhizoferrin in both fungal and bacterial genera. The fungal rhizoferrin was isolated from Rhizopus strains and other Mucorales of the Zygomycetes, while the bacterial rhizoferrin was isolated from Ralstonia picketti [21]. However, as we have reported earlier, the configuration of the chiral centre of citric acid residues in the rhizoferrins is different (Figure 2). While the fungal rhizoferrin has an R,R-configuration, the bacterial enantio-rhizoferrin has an S,S-configuration, suggesting different biosynthetic pathways [21]. This also indicates that the rhizoferrin molecule must have arisen twice in Nature. The stereochemistry of the corresponding iron complexes has also been identified by CD spectroscopy, being Δ for R,R-rhizoferrin and Δ for S,S-rhizoferrin. The bacterial staphyloferrin A is structurally very similar to rhizoferrin, but possesses d-ornithine instead of putrescine, which results in a third chiral centre at the C-α atom [22]. Although the chirality of the citryl residues in staphyloferrin A has never been determined, there is evidence from analogous synthetic compounds for an S,S-configuration identical to enantio-rhizoferrin from R. picketti (H. Drechsel and G. Winkelmann, unpublished work). A growth-promotion test with Staphylococcus strains confirmed this observation by showing that staphyloferrin A and enantio-rhizoferrin were both functionally active, while the fungal rhizoferrin was inactive. We therefore suggest that the two bacterial ferric carboxylate complexes staphyloferrin A and enantio-rhizoferrin are stereochemically equivalent and that their iron complexes are recognized by the same transport system in Staphylococcus.

We had previously shown that the chirality of siderophores is an important structural feature for recognition and transport in fungi [23,24]. The first observation of the functionally inactive enantio-ferrichrome in filamentous fungi like Neurospora, Penicillium and Aspergillus was recently confirmed when studying siderophore transporters of the major facilitator superfamily (MFS) in yeast [25,26]. Enantio-ferrichrome was not recognized by the SIT1 and ARN1 transporters in the yeast Saccharomyces cerevisiae [26]. A function
Figure 3
Siderophore iron transporters and routes of entry of siderophores in S. cerevisiae

of SIT1 in ferrioxamine/ferrichrome transport had previously been proven by mutational analysis of yeast cells [27]. Using a functional genomics strategy by starting from known genome sequence data, we were able to disrupt six open reading frames previously assigned to unknown MFS transporters in S. cerevisiae. Of the four siderophore transporter proteins identified (Figure 3), we found specificities for ferrioxamines/ferrichromes (Sitlp), triacetylfusarine C (Taflp) [24], anhydromevalone-ferrichromes (Arnlp) [26] and enterobactin (Enblp) [28]. The expression of all siderophore transporters is iron-regulated by the transcription factor Aft1p [29]. The relatively large number of siderophore transporters in S. cerevisiae is surprising, since this fungus is unable to synthesize its own siderophores. However, iron-regulated siderophore transporters in yeast, now collectively called siderophore iron transporter (SIT), are of high environmental value, since they enable yeast cells to survive in a low-iron environment by using external siderophores produced by various accompanying bacteria and fungi.

References
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Siderophore production by *Fusarium venenatum* A3/5

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**Abstract**

*Fusarium venenatum* A3/5 was grown in iron-restricted batch cultures and iron-limited chemostat cultures to determine how environmental conditions affected siderophore production. The specific growth rate in iron-restricted batch cultures was 0.22 h⁻¹, which was reduced to 0.12 h⁻¹ when no iron was added to the culture. The specific growth rate in iron-restricted chemostat culture was 0.1 h⁻¹. Siderophore production was correlated with specific growth rate, with the highest siderophore production occurring at 0.08 h⁻¹ and the lowest at 0.03 h⁻¹. Siderophore production was greatest at pH 4.7 and was significantly reduced at pHs above 6.0. Siderophore production could be enhanced by providing insoluble iron instead of soluble iron in continuous flow cultures.

**Introduction**

Siderophores are produced by micro-organisms to chelate and take up ferric iron from the environment, in which it is typically available in an insoluble form [1]. The majority of filamentous fungi produce hydroxamate-type siderophores, derived from *N*-acyl-*N*-hydroxynormithine, with the hydroxamic acid forming the functional group [2]. Although it is possible to produce siderophores by chemical synthesis, the processes are often slow and expensive [3], and there is continued interest in producing and studying siderophores from biological systems. Therefore it is important to establish conditions under which optimal siderophore production will take place. This paper describes the effect of iron-restricted growth on the specific growth rate of *Fusarium venenatum* A3/5 and the production of siderophores by *F. venenatum* A3/5 in iron-restricted cultures. The effects of iron concentration, dilution rate and pH were assessed.

**Materials and methods**

*F. venenatum* A3/5 was obtained from Mr T. W. Naylor (Marlow Foods, Billingham, Cleveland, U.K.). The defined medium contained (per litre) 10 g of glucose, 3.3 g of (NH₄)₂SO₄, 0.3 g of KH₂PO₄, 0.2 g of MgSO₄·7H₂O, 0.1 g of CaCl₂·2H₂O, 5 mg of citric acid, 5 mg of ZnSO₄·7H₂O, 0.26 mg of CuSO₄·5H₂O, 0.05 mg of MnSO₄·4H₂O, 0.05 mg of MgCl₂·6H₂O, 0.05 mg of NaMoO₄·2H₂O and 0.05 mg of biotin. For continuous flow cultures, the same medium was used at half-concentration. Iron was provided as FeCl₃·6H₂O or as a suspension of FeO(OH). All media were prepared using MilliQ-purified water.

Batch cultures were grown in 250 ml shake flasks containing 50 ml of medium, buffered to pH 5.8 with Mes and incubated on rotary shakers at 200 rev./min. Chemostat cultures were grown in either a Braun Biostat M or an Infors IFS100 (working volumes 2.1–2.3 l) as described by Wiebe and Trinci [4]. The pH was kept constant by addition of 0.5 M NaOH and foaming was controlled by addition of polypropylene glycol. FeCl₃·6H₂O was added to the glucose/mineral salts medium, but FeO(OH) was suspended in 0.15 % (w/v) sodium polyacrylate and supplied to the cultures in a separate feed line (4 ml · h⁻¹) in order to avoid problems with insoluble particles settling in the medium reservoir.

Maximum specific growth rates were measured by increase in attenuance using a Cecil Ce7200 spectrophotometer. For bioreactor cul-