

**Microbial Siderophores in Rhizosphere Interactions in Heavy
Metal-Containing Environments**

Dissertation

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GENERAL INTRODUCTION

1. Overview

The soil is replete with diverse populations of bacteria, fungi and other microbes, which, together with plant roots, constitute the denizens of the rhizosphere community. Of the bacterial species, *Pseudomonas*, *Bacillus* and *Streptomyces* are especially known to possess prolific rhizosphere competences, stemming from their individual survival ability under normal conditions, but also in specific niches such as heavy metal contaminated sites. This ability has been attributed to certain features including, but not limited to the possession of cell wall components with metal-binding properties (Beveridge *et al.*, 1982); production of antimicrobial substances to fend off competition from other microbes; extrusion of metal-chelating substances into the environment, and ability to utilize compounds released by plant roots as their main nutrient sources. In return for the use of plant exudates for their growth, microbes provide plants with certain growth-promoting substances.

Trace or heavy metals occur naturally as components of bulk soil, with an increased occurrence resulting from several anthropogenic mechanisms, especially from industrial activities. Thus, in addition to the recognized toxic metals with no known biological functions, metals such as Fe, Zn, Mn, and to a lesser extent, Ni, though essential micro-nutrients, become toxic when present in excessive amounts. An elevated level of these metals will exert different forms of plant growth inhibition; however, in general, such growth impediment can take the form of inhibition of enzyme functions; prevention of water and mineral uptake, and obstruction of photosynthetic as well as nitrogen metabolic processes (Di Toppi and Gabbrielli, 1999, and references therein). In contrast to metal toxicity, the deficiency in the environment of essential metals such as Fe poses a different kind of problem, as the latter is involved in several important biological processes. For instance, in plants, Fe is utilized in chlorophyll biosynthesis; hence its deficiency results in chlorosis, which is characterized by yellowing of leaves. It is also involved in critical enzymatic processes where it serves as co-factors. In aerobic microbes such as *Streptomyces*, Fe plays a role in the reduction of oxygen for ATP synthesis, reduction of ribonucleotide precursors of DNA, as well as in the formation of heme. As such, Fe levels of at least 1 μM (Neilands, 1995) and 66 $\mu\text{g g}^{-1}$ (Marschner, 1995), respectively, are considered critical for optimum growth in aerobic microorganisms and plants. In order for

the microbes to survive environmental iron deficiency, which is a common feature of most aerobic environments, they secrete into the environment a variety of siderophores that perform an iron scavenging role (Figure 1).

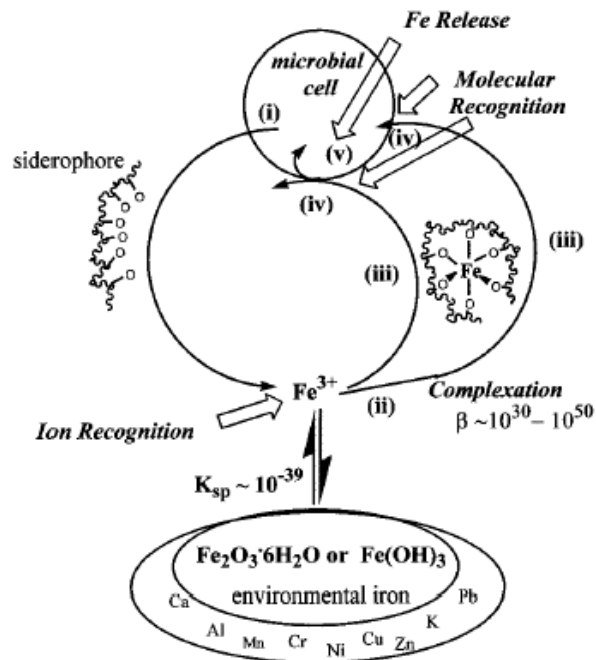


Fig. 1. Schematic representation of siderophore-mediated acquisition of environmental iron by microbial cells: (i) siderophore ligand synthesis and release by the cell; (ii) Fe^{3+} ion recognition and complexation; (iii) diffusion to the cell surface, (iv) siderophore complex molecular recognition by cell surface receptor; (v) iron release to the cell interior and recycling of free siderophore. Other metals are also present in the environment (modified from Boukhalfa and Crumbliss, 2002).

By virtue of this important role, siderophores possess a high affinity for ferric iron, which, nevertheless, varies among the different siderophore types, depending on their structure (Boukhalfa and Crumbliss, 2002). Similar to the goal in microbes, graminaceous plants release (phyto)siderophores to enhance Fe uptake, a mechanism referred to as strategy II (Römheld and Marschner, 1986; Crowley *et al.*, 1991). However, many other plant species, namely, dicotyledonous and non-grass monocotyledonous plants lack this feature, utilizing, instead, a separate mechanism for Fe uptake (strategy I). For these plant species, microbial siderophores have, however, proven to be a source of Fe (Kloepper *et al.*, 1980; Cline *et al.*, 1984; Crowley *et al.*, 1988). In spite of the fact that siderophores are secreted essentially as a response to environmental Fe deficiency, they can also chelate other metals, albeit with considerably reduced affinity (Evers *et al.*, 1989; Martell *et al.*, 1995; Hernlem *et al.*, 1996).

Despite their greater preference for Fe, some metals (e.g., Al) can, nevertheless, compete with Fe for siderophore binding (Hu and Boyer, 1996b; Greenwald *et al.*, 2008; Dimkpa *et al.*, 2009). In this regard, the presence of such competitive metals can affect siderophore-mediated iron uptake by microbes and plants, since they will reduce free siderophore pool (Hu and Boyer, 1996b; Gilis *et al.*, 1998; Dimkpa *et al.*, 2008a, b). There is, however, a flip side to the role of siderophores in the presence of metals other than Fe. Metals are generally known to cause the formation of free radicals, thereby elevating oxidative stress conditions in affected biological systems (Cakmak & Horst, 1991; Avery, 2001; Shanmuganathan *et al.*, 2004; Gratão *et al.*, 2005; Sharma *et al.*, 2006; Stobrawa *et al.*, 2007; Dimkpa *et al.*, 2009). Therefore, in such metal-contaminated conditions as pictured above, siderophores bind available metals, thus altering free metal concentrations and, ultimately, alleviating metal-induced oxidative stress (Dimkpa *et al.*, 2009)

1.1. *Streptomyces* spp and siderophores

Streptomyces are ubiquitous, gram positive, free living, but also symbiotic (Tokala *et al.*, 2002) soil bacteria renowned for their exceptional ability to elaborate diverse types of secondary metabolisms. Indeed, up to 80 % of all known microbial antibiotics, in addition to a number of other secondary products of pharmaceutical and agricultural importance, are produced by members of the actinomycetes to which streptomyces belong (Kieser *et al.*, 2000; Doumbou *et al.*, 2002; Hopwood, 2006; Haferburg *et al.*, 2008). This exceptional secondary metabolic capability has been described as a contingency mechanism to survive competition from other soil bacteria (Challis and Hopwood, 2003). Most *Streptomyces* species studied so far have been shown to produce siderophores of diverse structural backgrounds (Challis and Hopwood, 2003). Siderophore production is one of the mechanisms by which *Streptomyces* can exert beneficial effects on plant growth (Tokala *et al.*, 2002; Dimkpa *et al.*, 2008a, b, 2009).

1.1. 1. Genetics of siderophore production in *Streptomyces*

1. 1.1.a. Biosynthesis of siderophores in *Streptomyces*

In bacteria and fungi, there are several hundreds of compounds identified as siderophores, with diverse structural variations. This variation determines the selectivity of these molecules for ferric iron, depending mainly on the metal binding group, as well as on the number (denticity) of binding units. Thus, most siderophores possess hydroxamate,

catecholate and/or α -hydroxycarboxylic acid binding groups; however, hydroxamates are multi-dentate siderophores (Boukhalfa and Crumbliss, 2002). Many of these siderophores are peptides synthesised by members of the non-ribosomal peptide synthetase (NRPS) multi-enzyme family, which also directs the synthesis of majority of the microbial peptide antibiotics (Barona-Gomez *et al.*, 2004). An NRPS-dependent mechanism implies that mRNAs are not involved in the biosynthetic process. However, a number of others, including many of the hydroxamate-type siderophores, are synthesised by several *Streptomyces* species based on mechanisms that are NRPS-independent. Hydroxamate siderophores of *Streptomyces* are hexadentate molecules consisting of alternating dicarboxylic acid and diamine building blocks linked by amide or ester bonds (Challis, 2005). A well known group of hydroxamate siderophores that have been reported in *Streptomyces* are the desferrioxamines, comprising mainly of desferrioxamine B (DFOB), desferrioxamine E (DFOE) and desferrioxamine G (Yang and Leong, 1982; Imbert *et al.*, 1995; Barona-Gomez *et al.*, 2004, 2006). Of them, however, DFOB and DFOE are the most important (figure 2).

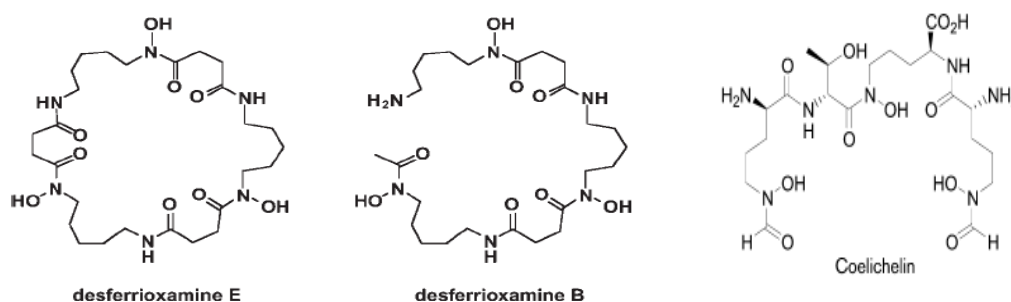


Fig. 2. Structures of desferrioxamines E (cyclic) and B (linear), and coelichelin (linear), 3 hydroxamate siderophores produced by *Streptomyces* species (Yamanaka *et al.*, 2005; Lautru *et al.*, 2005).

Though of different structures, DFOE and DFOB are encoded by the same operon (*des* operon, Figure 3a), which consists of four genes (*desABCD*). The structural difference (cyclic versus linear) between DFOE and DFOB arises depending on whether acylation of the molecule was achieved with succinyl-CoA, as in the case of DFOE, or with acetyl-CoA, as with DFOB (Barona-Gomez *et al.*, 2004, 2006; Tunca *et al.*, 2007). Briefly, the biosynthesis of desferrioxamine siderophores begins with the decarboxylation of lysine by DesA, yielding cadaverine. The latter is then hydroxylated by DesB, forming N-hydroxycadaverine. Next is the structure-differentiation step in which DesC catalyses an acylation reaction either by the addition of succinyl-CoA or acetyl-CoA. Finally, the

hydroxamic acid is formed by an oligomerization reaction of the acylated molecule which is catalyzed by DesD (Barona-Gomez *et al.*, 2006; Figure 4).

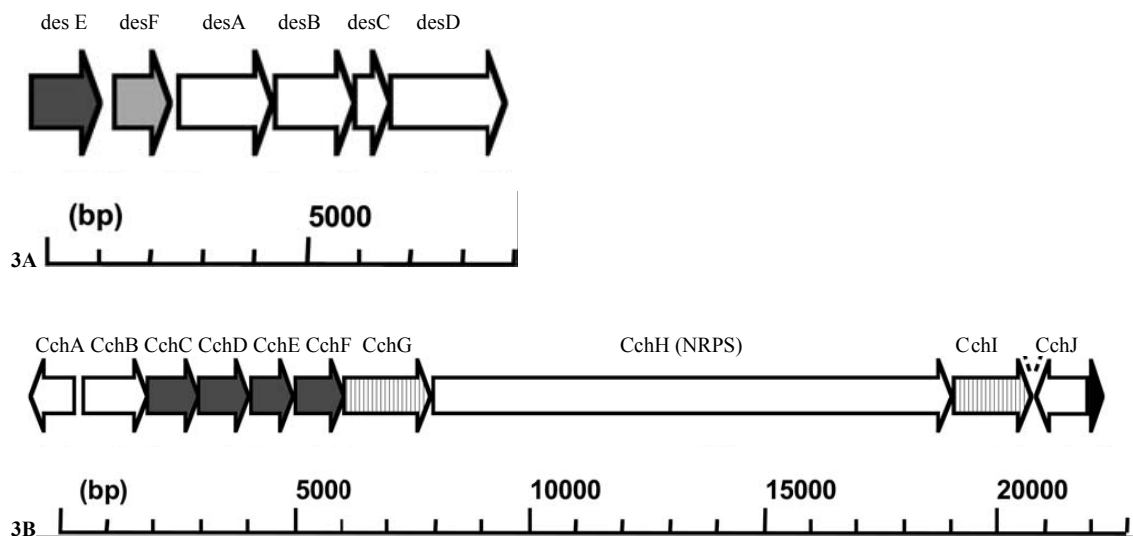


Fig. 3. (A). Organization of the desferrioxamine siderophores biosynthetic operon in *Streptomyces* spp. Dark grey arrow represents siderophore uptake genes, while light grey and white arrows represent siderophore utilization and biosynthetic genes, respectively. (B) Organization of the coelichelin biosynthetic gene clusters in *Streptomyces* spp. Genes represented by black arrow are of unknown function, while striped arrows indicate genes involved in siderophore export (modified from Barona-Gomez *et al.*, 2006).

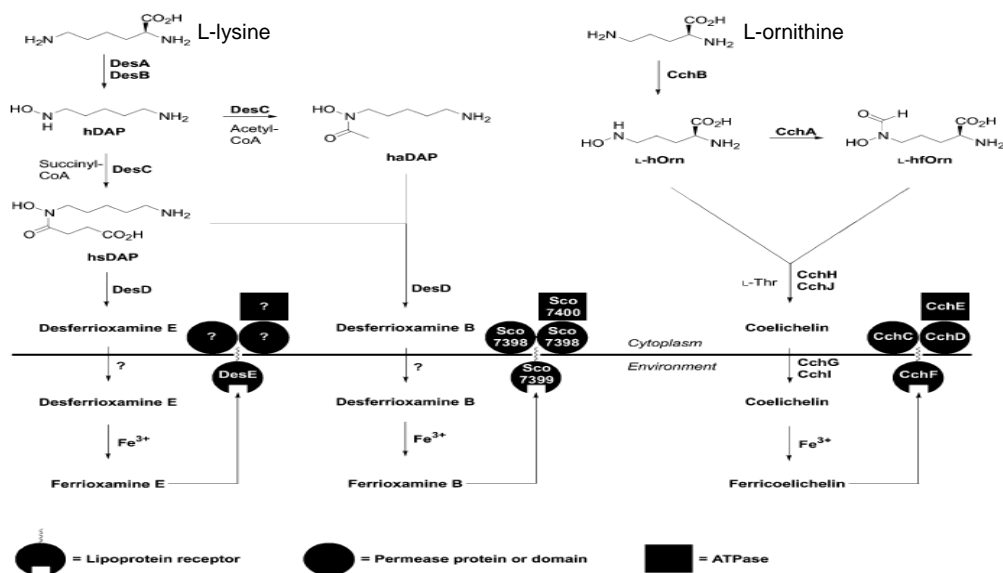


Fig. 4. Schematic representation of the biosynthesis, export and uptake of desferrioxamine E, desferrioxamine B and coelichelin by *Streptomyces* spp. Abbreviations for intermediates in coelichelin and desferrioxamine biosynthesis are as follows: hDAP, N-hydroxy-1,5-diaminopentane; haDAP: N-hydroxy-N-acetyl-1,5-diaminopentane; hsDAP: N-hydroxy-N-succinyl-1,5-diaminopentane; LhOrn; L-N5-hydroxyornithine; L-N5-hydroxy-N5-formylornithine (adapted from Barona-Gomez *et al.*, 2006).

In addition to the NRPS-independent desferrioxamine siderophores, a gene cluster (Figure 3b) encoding an NRPS-dependent hydroxamate siderophore, coelichelin (Cch; Figure 2), had been identified in the genome sequence of *Streptomyces coelicolor* A3(2), based initially on genome mining and structure deduction from the translated sequence of the identified gene (Challis and Ravel, 2000), and subsequently confirmed in this strain, as well as in *S. ambofaciens* ATCC 23877, *S. acidiscabies* E13 and *S. tendae* F4, by gene functional analysis, nuclear magnetic resonance (NMR) spectroscopy, and electrospray ionization mass spectrometry (ESI-MS), respectively (Lautru *et al.*, 2005, and online supplementary data; Barona-Gomez *et al.*, 2006; Dimkpa *et al.*, 2008a,b). Unlike desferrioxamine siderophores, however, the biosynthesis of Cch involves the assemblage of one L-threonine molecule and two non-proteinogenic amino acids. This rather unusual pathway begins with the conversion of the non-proteinogenic ornithine to L-N5-hydroxyornithine by a monooxygenation reaction catalyzed by CchA. L-N5-hydroxyornithine is then formylated by CchB, yielding L-N5-formyl-N5-hydroxyornithine. The NRPS, CchH, then catalyzes the condensation of these two intermediate products, which together with a molecule of L-Thr, yields the final linear hydroxamate siderophore. The Cch siderophore is subsequently released from CchH by a differently encoded thioesterase, CchJ (Lautru *et al.*, 2005; Barona-Gomez *et al.*, 2006). Previous investigations have shown that certain *Streptomyces* species are capable of producing siderophores other than hydroxamates. For instance, metabolite profiling techniques (HPLC, MS and NMR), did show that strains of *Streptomyces tendae* can secrete catecholate siderophores which are characteristic for enterobacteria such as *E. coli* (Fiedler *et al.*, 2001). Catecholate siderophores are cyclic trimers composed of 2, 3-dihydroxy-N-benzolserine (Payne, 1988). Thus, there exists in *Streptomyces* a strong diversity in the siderophore biosynthetic pathways and subsequent modifications to produce the final molecules.

1. 1.1.b. Transport of siderophores in *Streptomyces*

Based on their homology to iron uptake genes, three gene clusters (SCO0494-0497, SCO 1785-1787, and SCO7398-7400) with putative involvement in iron transport were identified in the genome sequence of *Streptomyces coelicolor* (Bentley *et al.*, 2002). Subsequently, Bunet *et al.* (2006) analyzed cluster 7398-7400 (*cdtABC*) for its involvement in siderophore-Fe transport. Mutational analysis indicated that there was no difference between the *cdtABC* mutant and wildtype, in the uptake of radiolabelled ferrioxamine, supporting the existence of other siderophore transporters in *S. coelicolor*. Thus, since the

cdtABC mutant could not transport salmycin, which is structurally similar to ferrioxamines, it was thought that *cdtABC* is most likely, but not the sole ferrioxamine transporter (Bunet *et al.*, 2006). Considering that genes SCO0494-0497 are located in the Cch biosynthetic cluster (Barona-Gomez *et al.*, 2006), the possibility of their participation in Fe transport, especially of Cch-Fe, is, thus, likely. Nevertheless, the specificity of these other potential siderophore transporters remains unclarified (Bunet, *et al.*, 2006).

1. 1.1.c. Regulation of siderophore production in *Streptomyces*

As indicated in Figure 1, following iron acquisition from the environment, intact siderophore-Fe(III) complexes are taken up by the bacteria, the Fe is reduced intracellularly, and the Fe-free siderophores are recycled for subsequent rounds of Fe scavenging. On attainment of adequate cellular Fe levels, the production of siderophores is repressed; the extent of repression being a function of available Fe concentration. As demonstrated by Flores and co-workers (2003, 2004, 2005) and Tunca *et al.* (2007), the regulation of desferrioxamine siderophores in *S. pilosus* and *S. coelicolor* is mediated by the ferric iron-dependent repressor proteins, DmdRs (divalent metal dependent repressors). Two different DmdR proteins (DmdR1 and DmdR2) have been identified in *S. coelicolor*. In the presence of ferrous iron or, to a lower efficiency, other divalent metal ions, both DmdRs were shown to be capable of binding to the *desA* iron-box sequence (TTAGGTTAGGCTCACCTAA) located in the promoter region of *desABCD*, forming two different complexes. This interaction results in the cessation of siderophore production. However, the addition of a ferrous iron chelator could reverse this inhibition (Flores *et al.*, 2003; Flores and Martin 2004). Of the two *dmdRs*, *dmdR1* appears to be the default repressor gene, since *dmdR2* was silent during normal expression of *dmdR1*, but was expressed in a *dmdR1*-disrupted mutant (Flores *et al.*, 2005). So far, iron-regulated repressors of Cch biosynthesis have not been unequivocally identified, although both DmdRs have been shown to be capable of binding to several other iron boxes related to siderophore biosynthesis, some of which, indeed, are part of some of the genes (*CchA*, *CchB*, *CchE*, *CchF* and *CchJ*) in the Cch biosynthesis cluster (Flores and Martin, 2004; Barona-Gomez *et al.*, 2006).

1.2. Ecology of siderophores with special reference to environmental pH

Despite the fact that iron is the fourth most abundant element in the soil, its bioavailability is limited by the formation of insoluble ferric complexes [Fe(III)] which, unfortunately, occurs at biologically relevant pH values. Given that siderophores are elicited primarily as

an Fe deficiency response, pH is, therefore, an important ecological consideration in the biosynthesis of microbial siderophores, as well as in their stability in the environment (Winkelmann, 2007). Despite this, the production of siderophores by different microbes appears to controvert the commonly acknowledged pH-dependence of Fe deficiency. Acid soils are rich in hydroxamate siderophores produced mainly by fungi, but also by *Streptomyces*, which also reflects in the optimal stability of their ferric complexes at low pH. In contrast, neutral to alkaline soils support the production of both hydroxamate and catecholate siderophores. Especially for catecholate siderophores, this situation is said to be based on the fact that catecholates are designed for optimum iron binding at neutral conditions, with a tendency to lose ferric iron at low pH conditions (Winkelmann, 2007, 2008). Thus, the effect of pH on siderophore production is both species and structure-dependent. Just to give a few examples, Federspiel *et al.* (1991) showed that the fungus *Hymenoscyphus ericae* produced hydroxamate siderophores at a pH range of 3.5 and 5.5, with optimum production obtained at 4.5. Similarly, *Escherichia coli* strain Nissle 1917 produced the catecholate siderophore, aerobactin maximally at pH 5.6. In contrast, the production of two other catecholate siderophores (salmochelin and yersiniabactin) by this strain was achieved maximal at pH 7 and above (Valdebenito *et al.*, 2005). Clearly, siderophore production is influenced to a large extent by the pH range of the niche of a given microorganism, which determines the redox state of environmental Fe.

1. 3. Role of siderophores in agriculture, human health and soil remediation

Two kinds of benefits can be provided to plants by soil free-living plant growth promoting bacteria (PGPB): indirect and direct. The indirect promotion of plant growth involves the prevention or reduction of the harmful effects of one or more bacteria, in this case, a pathogen, by another bacterium through several mechanisms. In contrast to this, direct promotion of plant growth entails providing the plant with a bacterially-synthesized substance, or enhancing the uptake of nutrients from the environment (Glick, 1995). Such provisions include but not limited to siderophore and phytohormone production (Dimkpa *et al.*, 2008a, b, 2009), phosphate solubilization, as well as specific enzymes involved in plant stress alleviation mechanisms (see for example, Belimov *et al.*, 2005; Rajkumar *et al.*, 2006). In addition to scavenging for iron in the soil for microbial use, siderophores are also known to function as virulence factors in pathogenic microorganisms by enabling the competitive acquisition of iron from their hosts, to the detriment of the latter (Dellagi *et al.*, 1998, 2005; Greenshields *et al.*, 2007). However, on the positive side, siderophore-

producing bacteria can serve as biocontrol agents against pathogenic bacteria or fungi if they have a better siderophore competitive ability than the pathogens, thereby starving them of iron. This is especially true for certain *Pseudomonas* spp which are popular biocontrol agents. Furthermore, the use of microbial siderophores to supply Fe to plants growing under Fe deficiency has been investigated by several workers. Indeed, enhanced plant growth mediated by Fe acquisition by hydroxamate and other classes of siderophores have been reported in a variety of food and industrial crops, including Potato (Kloepper *et al.*, 1980), sunflower and sorghum (Cline *et al.*, 1984; Bar-Ness *et al.*, 1991), Oat (Crowley *et al.*, 1988), peanut and cotton (Bar-Ness *et al.*, 1991; Jadhav *et al.*, 1994), cucumber (Wang *et al.*, 1993), as well as in peas (Tokala *et al.*, 2002; Dimkpa *et al.*, 2008a, 2009).

With regard to human (and indeed, other animals) health, siderophores are a critical component of virulence and pathogenicity of several pathogens, especially those of fungal origin. Ferrichrome, a well-known siderophore commonly produced by many fungi was detected in the human pathogenic fungi *Microsporium* and *Trichophyton* (Mor *et al.*, 1992), indicating a possible iron binding activity during bacterial growth in the epithelium. Subsequently, ferrichrome was demonstrated to be required during human epithelial invasion by *Candida albicans* (Heymann *et al.*, 2002). On the other hand, owing to their affinity for a range of metals, siderophores can potentially be applied to eliminate or reduce the unwanted presence of specific metals from particular environments. For example, desferrioxamine B is used in medicine as a drug for treating not only Fe, but also Al overload in human (Brown *et al.*, 1982; Malluche *et al.*, 1984; Allain *et al.*, 1987). Furthermore, as metal contamination in soil is a particularly serious environmental concern, the possibility of the use of biological (and thus, environmentally-sustainable), instead of synthetic chelators, to help clean up metal-polluted environments has been emphasised (White, 2001). In this regard, the use of microbial siderophores has received attention. In one instance, Cd, Zn and Pb bioavailability could be increased by siderophore solubilization of these metals, and their subsequent adsorption to biomass, which was then separated from soil slurry by flocculation, thus decreasing their concentrations in the soil (Diels *et al.*, 1999). Another elegant example involved the use of a siderophore-overproducing strain of *Kluyvera ascorbata* to alleviate Ni toxicity in plants grown in a Ni-contaminated soil. Although bacteria-treated plants showed enhanced growth in the presence of Ni than untreated plants, similar levels of Ni was taken up by both plants. However, by allowing the acquisition of enough Fe in the presence of inhibitory levels of Ni, plant growth was promoted (Burd *et al.*, 1998, 2000; Dimkpa *et al.*, 2008a). Yet

another demonstration of the possible application of siderophores for environmental remediation is illustrated in the study of Braud *et al.* (2008), in which an immobilized siderophore-producing strain of *Pseudomonas aeruginosa* augmented with skimmed milk enhanced Cr and Pb uptake by maize. Thus, metal contaminated soil hitherto considered unsuitable, can be remediated for plant growth through the application of plant growth-promoting bacteria (Glick *et al.*, 2003), many of which are considered siderophore-producing. Recently, the application of a novel hydroxamate siderophore, AS7, for inhibiting metal corrosion in the oil industry has also been potentiated (Perez-Miranda *et al.*, 2007).

1.4. Production of auxins by *Streptomyces* spp.

In addition to siderophores, plant-associated bacteria may provide their hosts with microbially-synthesized phytohormones, especially auxins. Although not all bacteria produce auxins (Kamnev *et al.*, 2005; Rajkumar *et al.*, 2006; Kuffner *et al.*, 2008), a wide range of rhizosphere bacteria are able to do so using tryptophan contained in plant root exudates (El-Sayed *et al.*, 1987; Sarwar *et al.*, 1992). Auxins from specific bacteria have been experimentally demonstrated to promote plant growth (Patten and Glick, 2002). In *Streptomyces* spp., microbially-produced auxins are thought to contribute to bacterial development, particularly in the regulation of spore formation and differentiation (Efremenkova *et al.*, 1985; El-Shanshoury, 1991), and possibly, in the pathogenicity of scab-producing strains (Lapwood, 1973). Beginning from L-tryptophan, Manulis *et al.*, (1994) traced the pathway of auxin synthesis in *Streptomyces*, which was shown to be the indole-3-acetamide pathway. Subsequently, Aldesuquy *et al.* (1998) demonstrated plant growth promotion by auxin-containing culture filtrates of *Streptomyces* spp.

Despite these possibilities, there is a dearth of scientific investigations on how specific bacterially-produced secondary metabolites interact with each other, and with metals in the environment. So far, the effect of the direct application of cell-free siderophores in metal clean up in the environment has not been fully elucidated, since previous studies have been conducted using siderophore-producing bacteria, and not the isolated siderophores. Moreover, the biochemical and physiological mechanisms by which siderophores enable plant growth in obviously stressful metal contaminated soil is yet to be investigated to any great length. A near-complete inhibition of auxin synthesis was observed in *Streptomyces* spp. under iron-sufficient conditions that prevented siderophore production (Dimkpa *et al.*,

2008a, b), initiating the link in the concomitant production of both substances, and, thus, their co-application in microbe-assisted phytoremediation of metal pollution in soil. In a nutshell, some of these issues, including siderophore-mediated plant growth and uptake of Fe and Ni *in vitro*, siderophore alleviation of metal-induced inhibition of auxins synthesis by *Streptomyces*, as well as siderophore mitigation of metal-induced oxidative stress, biomass production and augmented metal uptake from soil obtained from a heavy metal contaminated site, were investigated and reported in the enclosed papers (Dimkpa *et al.*, 2008a, b, 2009). Furthermore, results on siderophore production in solid and liquid media by *Streptomyces acidiscabies* E13 and *S. tendae* F4, and the effect of heavy metals in the process; the effects of *S. acidiscabies* E13 and *S. tendae* F4 on plant growth and metal uptake; siderophore-mediated uptake of Cd by *S. tendae* F4, and investigation on the production of a stress ethylene-alleviating enzyme by *S. acidiscabies* E13 and *S. tendae* F4 are presented. The overall objective of the study was to evaluate the role of siderophores produced by heavy metal resistant strains of *Streptomyces* (*S. acidiscabies* E13 and/or *S. tendae* F4) in “rhizosphere” interactions involving *Streptomyces* and plants growing under heavy metal contamination. Both live bacterial cells and cell-free culture filtrates containing siderophores were tested for plant growth promoting efficiency under metal contamination. Ultimately, results obtained were discussed as to the potential application of microbial siderophores for chelator-assisted phytoremediation of metal polluted soils.

The above overall objective was sub-divided into the following sub objectives:

1. To study siderophore production in *Streptomyces* and the effect of metals in this process.
2. To assess the effect of siderophores on the production of auxins by *Streptomyces* in the presence of heavy metals.
3. To investigate the effect of siderophores on growth and abiotic stress related physiological changes in plants growing under metal stress conditions.
4. To evaluate the effect of microbial siderophores in the uptake of metals from contaminated environments by plants.

SUMMARY OF MANUSCRIPTS

MANUSCRIPT I

Dimkpa, C., Merten, D., Svatoš, A., Büchel, G., and Kothe, E. (2008) Hydroxamate siderophores produced by *Streptomyces acidiscabies* E13 bind nickel and promote growth in cowpea (*Vigna unguiculata* L.) under nickel stress. Canadian Journal of Microbiology, 54:163-172.

Summary

The manuscript describes the production of siderophores in the presence of nickel by nickel-resistant *Streptomyces acidiscabies* E13 and the role of the elicited siderophores in promoting plant growth under nickel stress. It was shown that three different hydroxamate siderophores simultaneously produced by the bacterium in the presence of nickel bound both nickel and iron, the latter being present as trace contaminants in the medium. Use of cell-free supernatants containing the released siderophores and nickel in *in vitro* plant growth could promote cowpea growth by enhancing Fe uptake, while minimizing the uptake of nickel by plants. The study indicates that depending on the metals involved, siderophores can both enhance and prevent uptake of metals by plants.

Author contribution

The experiments were conceived and designed by me, in concert with Erika Kothe. Bacterial culture, auxin and siderophore measurements (except electro spray ionization mass spectrometric {ESI-MS} aspects), plant growth, Fe-reduction assay, ESI-MS data collection, preparation of plant tissues for elemental measurements and statistical analyses were done by me. I wrote the paper with some input on ESI-MS and spectroscopic methods from Aleš Svatoš and Dirk Merten, respectively.

SUMMARY OF MANUSCRIPTS

MANUSCRIPT II

Dimkpa, C.O., Svatoš, A., Dabrowska, P., Schmidt, A., Boland W., and Kothe, E. (2008) Involvement of siderophores in the reduction of metal-induced inhibition of auxin synthesis in *Streptomyces* spp. *Chemosphere*, 74:19-25.

Summary

Both auxins and siderophores are well known microbially-produced plant growth promoting substances. The manuscript describes the concomitant production of auxins and siderophores by several strains of *Streptomyces* under stress induced by a range of metals. It was shown that all the metals inhibited auxin levels in the cultures, but the presence of siderophores alleviated the inhibition. At the same time, siderophore production was stimulated by the metals, with the exception of iron, and the siderophores were shown to bind cadmium and nickel. It was, therefore, concluded that siderophores reduced the inhibition imposed on bacterial auxin synthesis by metals by binding the metals, which resulted in a reduction in free metal concentrations, thereby increasing the plant growth promoting potentials of the phytohormone.

Author contribution

The experiments were conceived and designed by me, in concert with Erika Kothe. Bacterial culture, auxin and siderophore measurements (except aspects on gas chromatography mass spectrometry {GC-MS} and ESI-MS), ESI-MS data collection, and statistical analyses were fully done by me. I performed IAA extraction and GC-MS analysis together with Paulina Dabrowska. I wrote the article, with input on materials and method for GC-MS contributed by Paulina Dabrowska.

SUMMARY OF MANUSCRIPTS

MANUSCRIPT III

Dimkpa, C.O., Merten, D., Svatoš, A., Büchel, G., and Kothe, E. (2009) Metal-induced oxidative stress impacting plant growth in contaminated soil is alleviated by microbial siderophores. *Soil Biology and Biochemistry*, 41:154-162.

Summary

The manuscript describes the role of microbial siderophores in promoting plant growth and augmenting metal uptake in soil contaminated with different toxic metals. Whereas the metals impacted negatively on plant growth by increasing oxidative stress levels, siderophores lowered the impact and reduced the effects of oxidative stress on plants. Together with auxins, which activity was, apparently, rendered ineffective by the presence of the metals in control treatments, the addition of siderophores augmented metal uptake, thereby potentiating the simultaneous application of auxin and siderophore-containing bacterial culture filtrates for assisted phytoremediation of metal pollution.

Author contribution

The experiments were conceived and designed by me, in concert with Erika Kothe. Bacterial culture, auxin and siderophore measurements, soil preparation, plant growth, plant enzyme and other biochemical assays, siderophore competition assay, preparation of soil and plant tissues for elemental measurements and data analyses were done by me. I wrote the paper, with method on soil elemental content measurements provided by Dirk Merten.

SUMMARY OF MANUSCRIPTS

MANUSCRIPT IV

Dimkpa, C.O., Merten, D., Svatoš, A., Büchel, G., and Kothe, E (in press) Siderophores mediate reduced and increased uptake of cadmium by *Streptomyces tendae* F4 and sunflower (*Helianthus annuus*), respectively (Journal of Applied Microbiology).

Summary

The manuscript describes the kinetics involved in siderophore production by *Streptomyces tendae* F4 in the presence and absence of cadmium. Thereafter the effect of the siderophores on the uptake of cadmium by the releasing bacterium was investigated, followed by the application of the siderophores to study sunflower plant growth and Cd uptake from a soil amended with cadmium. Real-time siderophore production revealed that production peaks three days after culture establishment. The released siderophores reduced Cd uptake by the bacteria, while promoting sunflower growth via solubilization of Fe in soil; however, they also increased Cd uptake by the plant, both of which were slightly better than the effect of EDTA. Thus, it was concluded that siderophores would be a better chelant than EDTA, for assisted phytoremediation, considering their biological origin, and the reported negative effects of EDTA on the environment.

Author contribution

The experiments were conceived and designed by me, in concert with Prof. Kothe. Bacterial culture, auxin and siderophore (except ESI-MS aspects) measurements, soil preparation, plant growth, ESI-MS data collection, preparation of bacterial and plant samples for elemental measurements, ACCD enzyme assay and data analyses were done by me. I also wrote the manuscript.

Manuscript I

Hydroxamate siderophores produced by *Streptomyces acidiscabies* E13 bind nickel and promote growth in cowpea (*Vigna unguiculata* L.) under nickel stress.

Hydroxamate siderophores produced by *Streptomyces acidiscabies* E13 bind nickel and promote growth in cowpea (*Vigna unguiculata* L.) under nickel stress

Christian Dimkpa, Aleš Svatoš, Dirk Merten, Georg Büchel, and Erika Kothe

Abstract: The siderophore-producing ability of nickel-resistant *Streptomyces acidiscabies* E13 and the role of the elicited siderophores in promoting plant growth under iron and nickel stress are described. Siderophore assays indicated that *S. acidiscabies* E13 can produce siderophores. Electrospray ionization mass spectrometry (ESI-MS) revealed that the bacterium simultaneously produces 3 different hydroxamate siderophores. ESI-MS showed that in addition to iron, all 3 siderophores can bind nickel. In vitro plant growth tests were conducted with cowpea (*Vigna unguiculata*) in the presence and absence of the elicited siderophores. Culture filtrates containing hydroxamate siderophores significantly increased cowpea height and biomass, irrespective of the iron status of the plants, under nickel stress. The presence of reduced iron was found to be high in siderophore-containing treatments in the presence of nickel. Measurements of iron and nickel contents of cowpea roots and shoots indicated that the siderophore-mediated plant growth promotion reported here involves the simultaneous inhibition of nickel uptake and solubilization and supply of iron to plants. We conclude that hydroxamate siderophores contained in culture filtrates of *S. acidiscabies* E13 promoted cowpea growth under nickel contamination by binding iron and nickel, thus playing a dual role of sourcing iron for plant use and protecting against nickel toxicity.

Key words: *Streptomyces acidiscabies* E13, siderophores, cowpea, iron, nickel.

Résumé : La capacité de production de sidérophores de *Streptomyces acidiscabies* E13 résistante au nickel, et le rôle joué par les sidérophores produits dans la promotion de la croissance des plantes sous un stress en fer ou en nickel sont décrits. Un essai de détection de sidérophores a indiqué que *S. acidiscabies* E13 peut produire des sidérophores. La spectrométrie de masse par ionisation en mode électrospray (ESI-MS) a révélé que la bactérie produit trois hydroxamates sidérophores simultanément. L'ESI-MS a montré qu'en plus du fer, les trois sidérophores peuvent lier le nickel. Des tests de croissance de plantes in vitro ont été réalisés avec la dolique à œil noir (*Vigna unguiculata*) en présence ou non des sidérophores produits. Des filtrats de culture contenant les hydroxamates sidérophores ont augmenté significativement la hauteur et la biomasse des plants de dolique à œil noir, sans égard au statut en fer de la plante, sous un stress au nickel. La quantité de fer réduit était élevée lors des traitements en présence de sidérophores et de nickel. Des mesures des contenus en fer et en nickel des racines et des pousses de doliques à œil noir ont indiqué que la promotion de la croissance des plants assurée par les sidérophores décrite ici implique l'inhibition simultanée de la captation de nickel et la solubilisation et l'alimentation en fer des plants. Nous concluons que les hydroxamates sidérophores contenus dans les filtrats de culture de *S. acidiscabies* E13 font la promotion de la croissance de la dolique à œil noir lors d'une contamination en nickel en liant le fer et le nickel, jouant ainsi un double rôle en permettant l'utilisation de fer par la plante et en la protégeant de la toxicité du nickel.

Mots-clés : *Streptomyces acidiscabies* E13, sidérophores, dolique à œil noir, fer, nickel.

[Traduit par la Rédaction]

Introduction

Heavy metals exert different, mostly deleterious, effects on plants growing in contaminated environments. Apart from pH-induced insolubility and thus lack of iron (Fe) in calcareous soils, Fe deficiency symptoms in plants have also been linked to the presence of heavy metals. In this regard, nickel (Ni), for example, could affect Fe uptake in dicotyledonous plants by either inhibiting Fe(III) reduction or by competing with Fe(II) at root uptake sites (Alcántara et al. 1994). Moreover, it was hypothesized that Fe can form nonusable polymers with specific contaminating heavy metals (Davis et al. 1971). To circumvent Fe deficiency, dicotyledonous plants release phenolic compounds and extrude

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protons, thereby acidifying the rhizosphere (Schmidt 1999; Jin et al. 2006). On the other hand, microorganisms and graminaceous plants elicit low-molecular-mass, high-affinity, Fe-scavenging compounds — siderophores (Schwyn and Neilands 1987; Crowley et al. 1991). Taken together, these mechanisms represent the 2 currently recognized strategies (strategy I and II, respectively) for Fe acquisition in plants. Hydroxamates are a class of siderophores produced by many *Streptomyces* species (Yang and Leong 1982; Meiwes et al. 1990; Imbert et al. 1995; Barona-Gómez et al. 2004, 2006; Yamanaka et al. 2005). Although they show the highest affinity towards Fe, with association constants of $K_B \approx 10^{30}$ and 10^{32} for the hydroxamates desferrioxamine B (DFOB) and E (DFOE), respectively (Crumbly 1991; Fernández and Winkelmann 2005), siderophores can also chelate other metals with lower binding strength (Evers et al. 1989; Hernlem et al. 1996). Binding of the siderophore to a metal dramatically alters the free metal concentration. Thereafter, the siderophore-metal complex can either be assimilated by microbes and strategy II plants (as in the case of Fe), or rejected, as has been observed at high (and hence toxic) aluminum (Hu and Boyer 1996a, 1996b; Poschenrieder et al. 2005) and cadmium (Gilis et al. 1998; Hill and Lion 2002) concentrations. In spite of their importance, not all microbes produce siderophores, as some microbes use xeno-siderophores instead. Nonetheless, one way to alleviate the toxicity of heavy metals to plants may involve the use of siderophore-eliciting rhizobacteria. The literature is replete with reports of plant growth enhancement by rhizobacteria. In particular, microbial siderophores have been reported to enhance growth in strategy I plants (Kloepper et al. 1980; Cline et al. 1984; Becker et al. 1985; Bar-Ness et al. 1991; Crowley et al. 1991; Tokala et al. 2002) by serving as a source of Fe to plants. In an attempt to expand the frontier of knowledge regarding siderophore sequestration of metals other than Fe, and the implication in heavy metal contaminated environments, we investigated the siderophore-producing ability of a nickel-resistant, pigment-producing strain, *Streptomyces acidiscabies* E13. Following this, we examined the involvement of siderophores in promoting plant growth and possibly inhibiting Ni uptake in plants using cowpea (*Vigna unguiculata*), a strategy I plant. We hypothesized that siderophores can either inhibit plant uptake of nickel by chelating and immobilizing the metal, thus making it unavailable for uptake by the plant, or influence increased Fe uptake in the presence of Ni by solubilizing and making Fe available to the plant. Our long-term aim is to apply results from this in vitro study to plant growth in heavy metal contaminated field sites in the former uranium mining site in Thuringia, Germany. To the best of our knowledge, the present work is the first report of in vitro nickel binding and promotion of plant growth in cowpea by hydroxamate siderophores produced by a streptomycete.

Materials and methods

Bacterial strain and growth conditions

Streptomyces acidiscabies E13 (Amoroso et al. 2000) was used. The strain is capable of growth in the presence of Ni at a concentration of $5 \text{ mmol}\cdot\text{L}^{-1}$ (Schmidt et al. 2005;

Haferburg et al. 2006). It was maintained at 28°C on solid minimal actinomycete medium containing, per litre, L-asparagine (0.5 g), $\text{KH}_2\text{PO}_4\cdot 3\text{H}_2\text{O}$ (0.65 g), $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$ (0.2 g), $\text{FeSO}_4\cdot 7\text{H}_2\text{O}$ (0.01 g), glucose (10 g), and agar (16 g). For siderophore production in solid medium, the modified chrome – azurol S (CAS) agar plate method (Milagres et al. 1999) was applied. For detecting siderophores in liquid medium, the strain was cultured under Fe and Ni interplay in liquid cultures. For this, spores were collected from 3- to 5-day-old agar plates and diluted to 10^6 colony-forming units (CFUs). These were then cultured in siderophore-inducing medium (100 mL) prepared as previously described (Alexander and Zuberer 1991). If indicated, the medium was amended with or without $2 \text{ mmol}\cdot\text{L}^{-1}$ Ni (as NiCl_2), in the absence or presence of added Fe ($50 \text{ }\mu\text{mol}\cdot\text{L}^{-1}$; as $\text{FeSO}_4\cdot 7\text{H}_2\text{O}$). Thus, 4 types of Fe and Ni interplay were set up as follows: iron deficient, nickel containing (–Fe, +Ni); iron deficient, nickel deficient (–Fe, –Ni); iron containing, nickel containing (+Fe, +Ni); and iron containing, nickel deficient (+Fe, –Ni). Each set-up was inoculated with 2 mL of 10^6 CFU bacterial spore suspension and grown in the dark at 28°C for 24–96 h under agitation ($150 \text{ r}\cdot\text{min}^{-1}$). Glasswares used were treated overnight with $6 \text{ mol}\cdot\text{L}^{-1}$ HCl to reduce Fe contamination, but no defferation of the medium was carried out. Cultures from –Fe, +Ni treatments were centrifuged (Heraeus Instrument, Osterode, Germany) at $4000g$, and the supernatant was collected and filter-sterilized using a $0.22 \text{ }\mu\text{m}$ membrane filter (and hereafter referred to as siderophore-containing culture filtrate (SCF)).

In vitro detection of siderophores in *S. acidiscabies* E13

The presence of siderophores in the culture media was detected using the CAS assay of Schwyn and Neilands (1987). Briefly, bacteria culture was centrifuged ($4000g$) and the supernatant was collected, filter-sterilized using a $0.22 \text{ }\mu\text{m}$ membrane filter (SCF), and mixed with CAS assay solution (1:1, v/v), which was prepared as described by Alexander and Zuberer (1991). After incubation for 30–60 min, the absorbances of the solution and a reference sample from uninoculated siderophore-inducing medium were measured spectrophotometrically (Thermo Electron Corp.) at 630 nm. Purified DFOE (EMC Microcollections, Tuebingen, Germany) was used to prepare a standard curve. The type of siderophore elicited by *S. acidiscabies* E13 was determined by the Csáky and Arnou assays, which were performed as described by Tokala et al. (2002). Hydroxylamine was used as a standard for the Csáky assay.

Confirmation of siderophore type

To validate results from the Csáky assay, electrospray ionization mass spectrometry (ESI-MS) was carried out with culture filtrates as described by Bednarek et al. (2005). Briefly, culture supernatants were centrifuged and $20 \text{ }\mu\text{L}$ aliquots were loaded onto a Micromass Quattro II tandem quadrupole mass spectrometer (Micromass, Manchester, UK) equipped with an electrospray ionization source. The capillary and cone voltages in ESI mode were 3.8 kV and 25 V, respectively. Nitrogen for nebulization was applied at $15 \text{ L}\cdot\text{h}^{-1}$ and drying gas at $250 \text{ L}\cdot\text{h}^{-1}$. Source and capillary were heated at 80 and 150°C , respectively. The mass spec-

trometer was operated in conventional scanning mode using the first quadrupole. Positive-ion full-scan mass spectra were recorded from m/z 150 to 900 (scanning time 1.5 s). Fixed precursor ion (MS/MS) spectra (daughter-ion scan) and fixed product spectra (parent-ion scan) were recorded by standard methods. Argon was used for collision-induced dissociations (CID) at 1.5×10^{-3} mbar and the collision energy was varied from 12 to 50 eV for fragmentation. Separation was achieved on a reverse-phase column (5 μm C18 phase, 100 mm \times 2.1 mm i.d., Supelco) equipped with a pre-column (Supelco) using a gradient of 0.1% aqueous formic acid (A) and acetonitrile (B): 0–5 min, 1.5%–45% B; 5–16 min, 45%–100% B; 16–19 min, hold at 100% B (flow rate 0.25 mL \cdot min $^{-1}$, column temperature 30 $^{\circ}\text{C}$, UV detection at 450 nm). Purified DFOE (EMC microcollections, Tuebingen, Germany) with or without the addition of Fe(III) or Ni(II) ions was used as standards. On the basis of the molecular masses of DFOB (Winkelmann et al. 1999) and coelichelin (Cch) — a linear tri-hydroxamate siderophore recently discovered in *Streptomyces coelicolor* M145 (Barona-Gómez et al. 2006) and *S. ambofaciens* ATCC 23877 (Lautru et al. 2005) — m/z values for complexed DFOB and Cch were calculated using the formula $[\text{M}-2\text{H}^+ + \text{Fe}^{3+}]^+$ for siderophore–Fe complexes, and $[\text{M}-\text{H}^+ + \text{Ni}^{2+}]^+$ for siderophore–Ni complexes, where M is the molecular mass of specific siderophore contained in SCF analysed.

Plant material and growth conditions

Cowpea seeds were sterilized in 3% H_2O_2 for 10 min, followed by further sterilization in 70% ethanol for 30 s, and rinsing several times in autoclaved distilled water. Sterilized seeds were germinated in 1.5% plant agar (Sigma, Steinheim, Germany) in the dark. Five days later, a subset of uniformly germinated seedlings, one per beaker, was aseptically transferred into acid-treated, sterile glass beakers containing 100 mL of SCF (100 $\mu\text{mol}\cdot\text{L}^{-1}$ of the detected hydroxamate siderophores + 2 $\text{mmol}\cdot\text{L}^{-1}$ Ni^{2+}). A second subset of the seedlings was similarly transferred to 100 mL of uninoculated siderophore-inducing medium (non-SCF), which had been supplemented with 2 $\text{mmol}\cdot\text{L}^{-1}$ Ni^{2+} , as already contained in SCF. Both treatments were further amended with or without 50 $\mu\text{mol}\cdot\text{L}^{-1}$ Fe^{3+} (as FeCl_3). Thus, 4 treatments were involved, each replicated 5 times. Under these conditions, an excess of Ni^{2+} was present in the system. The pH of the solutions was 7.5. Each plant in a beaker was partially anchored in agar block moulded in sterile open-ended falcon tubes, to prevent logging, but roots were fully submersed in the solutions. Plants were grown in a plant growth chamber for 21 days. The growth chamber was set on a 12 h light (23 $^{\circ}\text{C}$) : 12 h dark (18 $^{\circ}\text{C}$) photoperiod, 70% relative humidity. A similar but independent plant-growth experiment was established concurrently. Data from both experiments were combined for statistical analysis.

Detection of auxins in *S. acidiscabies* E13 under Fe and Ni interplay

Streptomyces acidiscabies E13 was cultured under Fe and Ni interplay as described above. No L-tryptophan was added to the culture medium. The production of auxins by *S. acidiscabies* E13 under the different Fe and Ni interplay

scenarios was assayed using the Salkowski assay as described by Patten and Glick (2002). Indole-3-acetic acid (IAA; ROTH, Karlsruhe, Germany) was used to prepare a standard curve.

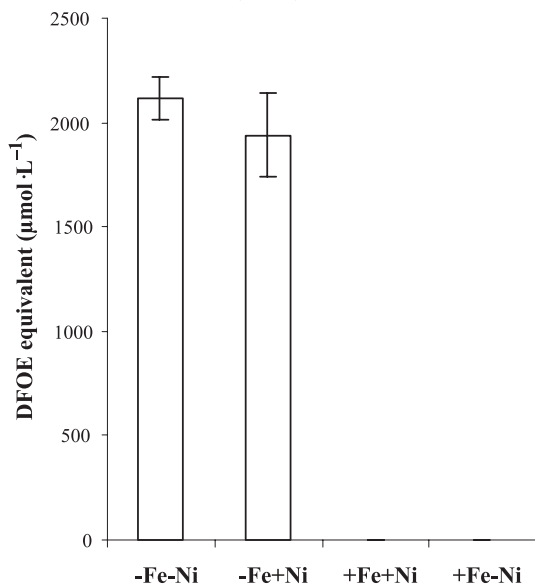
In vivo detection of Fe(II) in cowpea roots

Ferrozine (SERVA, Heidelberg, Germany) was used to detect the presence of Fe(II) in intact cowpea plant cultures. This was performed as previously described (Alcántara, et al. 1994), with some modifications. Briefly, seeds were sterilized and germinated as described earlier. Uniform seedlings were transferred to standard nutrient solution (Alcántara, et al. 1994). After 3 days, plants were transferred to standard nutrient solution without added Fe^{3+} . Cotyledons were removed and 0.3 $\text{mmol}\cdot\text{L}^{-1}$ ferrozine was added to the plant cultures to capture any reduced Fe and thus enhance Fe deficiency. Two days later, plants were rinsed repeatedly in sterile water to eliminate the ferrozine, and a subset of the plants was transferred to 25 mL of SCF (pH 6) containing 100 $\mu\text{mol}\cdot\text{L}^{-1}$ of the detected siderophores and Ni^{2+} (2 $\text{mmol}\cdot\text{L}^{-1}$), with or without added Fe^{3+} (100 $\mu\text{mol}\cdot\text{L}^{-1}$). Additionally, the SCF contained about 2.6 $\mu\text{mol}\cdot\text{L}^{-1}$ auxins elicited by the bacteria during growth under Ni stress. As siderophore and auxin control, a second subset of the plants was transferred to Ni-containing uninoculated siderophore-inducing medium (pH 6) with and without Fe^{3+} . This medium was then amended with 2.6 $\mu\text{mol}\cdot\text{L}^{-1}$ IAA, the equivalent of auxins produced by *S. acidiscabies* E13 under Ni^{2+} and siderophore-inducing conditions. Similar to plant growth conditions, an excess of Ni was present in the system. After 3 days, the presence of Fe(II) was assayed by adding 0.3 $\text{mmol}\cdot\text{L}^{-1}$ ferrozine to 1 mL of each cowpea growth solution in disposable cuvettes. The mixture was incubated for 2 h and the presence of Fe(II) was determined by measuring the absorbance (562 nm) of the Fe(II)–ferrozine complex and using an extinction coefficient of 29 800 $\text{mol}\cdot\text{L}^{-1}\cdot\text{cm}^{-1}$ (Lucena et al. 2006).

Plant metal contents measurement

Plants from the growth experiment were subjected to heavy metal analyses. Plant samples were washed 3 times using deionized water (PureLab Plus, USF Elga, Germany) in an ultrasonic bath (Sonorex RK31H, Bandelin, Germany). The cleaned samples were then separated into above-ground biomass and roots using a ceramic knife. Afterwards, the plant samples were dried at room temperature until a constant mass was achieved, and subsequently crushed using an ultra-centrifugal mill (ZM 100, Retsch, Germany) for 1 min to a size below 500 μm . Samples of 32–209 mg were weighed with an accuracy of 0.1 mg and allotted to pre-cleaned polymer vessels and 5 mL of nitric acid (65%, sub-boiled quality) was added. The vessels were closed and the mixture was allowed to stand overnight. Following this, the sample vessels were subjected to a microwave-assisted pressure digestion (Mars 5, CEM, Germany) using a microwave of up to 1200 W. The samples were heated to 180 $^{\circ}\text{C}$ within 15 min and the temperature was maintained for another 15 min. After the digestion, the samples were allowed to cool for at least 90 min before the vessels were opened. The completely digested plant samples were transferred to

Fig. 1. Chrome–azurol S (CAS) assay-based quantitative estimation of siderophore production by *Streptomyces acidiscabies* E13 grown under Fe and Ni interplay. CAS-reactive colour change was not observed within the incubation time in Fe-containing treatments. Error bars show standard deviation ($n = 5$).



calibrated polymer flasks and adjusted to a final volume of 25 mL using deionized water. The solutions were analyzed for their Fe content using inductively coupled plasma optical emission spectrometry (ICP-OES; SpectroFlame, Spectro, Germany); and for their Ni content using inductively coupled plasma mass spectrometry (ICP-MS; XSeries II, Thermo Electron, Germany). Quantification was performed by calibration against dilutions of mixtures of single-element standards (1 g each, Merck, Germany).

Statistical analysis

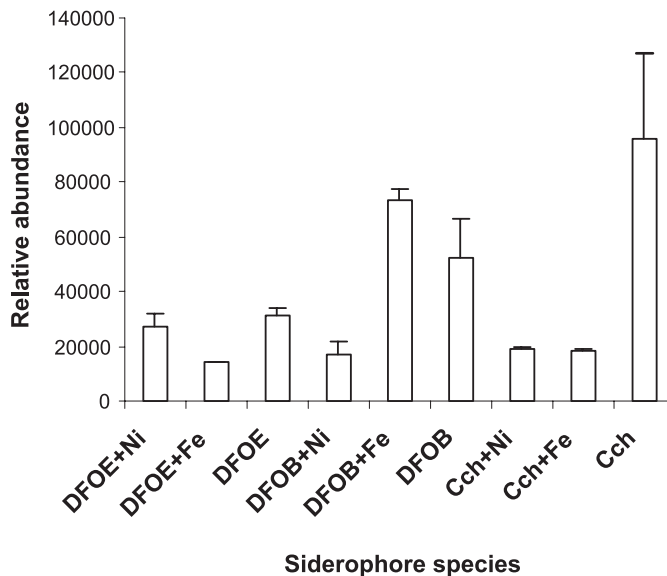
Data were analyzed using simple statistics, including analysis of variance (ANOVA) and, where significant, treatments were separated by least-significant difference or Duncan's multiple range tests.

Results

In vitro production of siderophores by *S. acidiscabies* E13

A modified CAS agar plate test was used for the detection of siderophore production in solid medium. In addition, siderophores were detected in liquid medium after cultivation under siderophore-inducing conditions. After 3–4 days, the presence of siderophores in the culture supernatants was shown with high contents in –Fe, –Ni culture filtrates, while –Fe, +Ni showed slightly weaker results. Fe-containing media, irrespective of Ni status, did not indicate the presence of siderophores. These results indicate that *S. acidiscabies* E13 produces siderophores under Fe limitation in the presence or absence of Ni. Use of purified DFOE to prepare a standard curve enabled quantitative measurement of the substance (Fig. 1). The Cšáky assay for detecting hydroxamate siderophores was positive for culture filtrates of *S. acidiscabies*

Fig. 2. Electrospray ionization mass spectrometry analysis of *Streptomyces acidiscabies* E13 culture filtrates showing the production of desferrioxamine E (DFOE), desferrioxamine B (DFOB), and coelichelin (Cch) and their chelation to Fe and Ni in medium without added Fe, but amended with Ni. Error bars show standard deviation ($n = 3$).

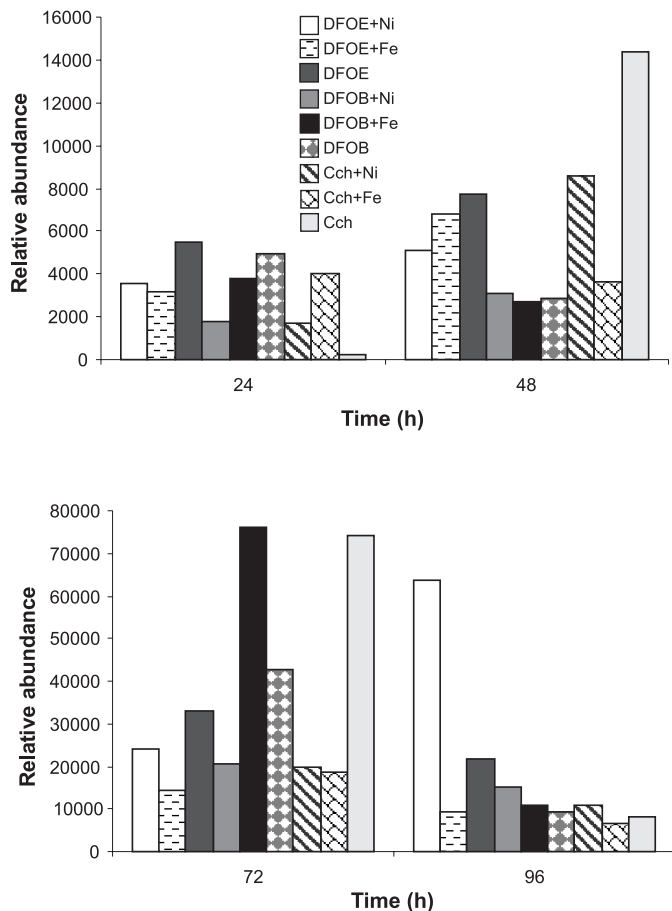


E13, while the Arnow assay for detecting catechololate siderophores was negative. To validate results from the Cšáky assay, culture supernatants were analysed by ESI-MS, revealing that *S. acidiscabies* E13 can produce at least 3 structurally different hydroxamate siderophores, namely DFOE, DFOB, and Cch. The highest overall (free and metal-chelate species) abundance was observed for DFOB, followed by Cch. It was also shown that DFOE and DFOB chelated high amounts of Ni and Fe, respectively. There was a more or less equal binding of both metals by Cch; however, Cch was found predominantly in its free state. Siderophores are known to have the highest affinity for Fe. However, in the presence of excess Ni, there was no obvious binding preference for Fe as compared with Ni, especially by DFOE or Cch. Also, quite high abundances of free siderophores, particularly of DFOB and Cch, were detected in spite of the overwhelming presence of Ni presumably available for binding (Fig. 2).

Siderophore production and metal binding over time

Streptomyces acidiscabies E13 was cultured over a 4 day period in Fe-deficient, Ni-amended medium. Sampling was conducted after every 24 h for detection of siderophore production and metal chelation over time. ESI-MS showed that production of all siderophores could be detected simultaneously after 24 h. There was a progressive increase in siderophore production up to 72 h, after which most siderophores except DFOE+Ni were detected at lower abundances. An early binding of metals by the different siderophores was also observed. A high degree of preference for Ni by DFOE and for Fe by DFOB was shown, especially at the peak production times. Indeed, the highest abundance of any siderophore–Ni complex was a DFOE–Ni species, observ-

Fig. 3. Real-time (24–96 h) siderophore production in *Streptomyces acidiscabies* E13. Metal chelation by the released siderophores in Fe-deficient medium amended with Ni is shown. Experiments were repeated several times with similar results, and representative values of one experiment are shown. DFOE, desferrioxamine E; DFOB, desferrioxamine B; and Cch, coelichelin.



able at 72 and, especially, 96 h. On the other hand, DFOB chelated more Fe than Ni over time, especially at 24 and 72 h, while more or less similar levels of metals were chelated at 48 h. However, at 96 h, DFOB was found to bind more Ni than Fe. The random time-dependent response of Cch to the presence of Ni was similar to those of DFOE and DFOB. Production of Cch increased quite sharply between 48 and 72 h after incubation, with significant amounts of Fe chelated by this siderophore at 48 h. However, compared with the other siderophores, relatively low amounts of metals were shown to be chelated by Cch. Indeed, most of the Cch produced at the highest production time (72 h) remained metal free (Fig. 3).

Plant growth under nickel stress

In short-term (21 days) growth experiments of cowpea plants in the presence of Ni, culture filtrate from *S. acidiscabies* E13 (SCF) promoted plant growth significantly, both in terms of plant height (Fig. 4) and biomass (Fig. 5), irrespective of Fe status. In contrast, growth was not promoted in plants grown in uninoculated media, irrespective of Fe status. Most plants showed chlorosis in the

Fig. 4. Photograph of 21-day-old cowpea plants grown in uninoculated siderophore-inducing medium and filter-sterilized *Streptomyces* culture filtrate (SCF) obtained from a similar medium type, both containing nickel. ±Fe, representative plants grown with and without added Fe; SCF±Fe, representative plants grown with *Streptomyces acidiscabies* E13 SCF, with and without added Fe.

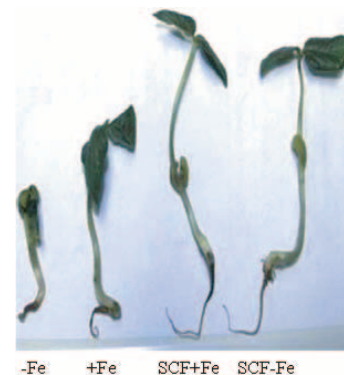
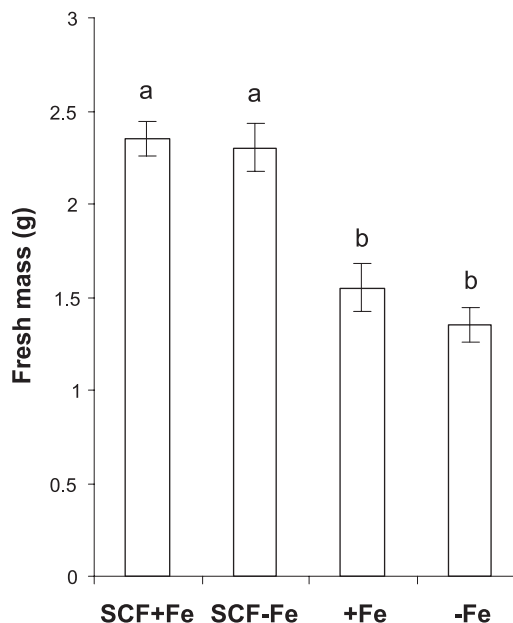


Fig. 5. The effect of *Streptomyces acidiscabies* E13 culture filtrates on cowpea fresh biomass in the presence of Ni, with and without added Fe. Data are the means of 10 replicates, and treatments labelled with the same letters are not different at $P = 0.01$.



presence of Ni, much more pronounced in nonculture filtrate treatments of both Fe-deficient and Fe-sufficient plants. Growth of non-Fe-treated plants was slightly lower than growth of those treated with Fe. In all cases, better shoot than root growth was observed. To show that plant growth promotion was indeed linked to the presence of siderophores, and not due to concurrent production of auxins by the bacterium, we tested for the production of auxin phytohormones. The Salkowski assay indicated that *S. acidiscabies* E13 produces auxins at low levels in vitro, in the absence of the IAA precursor, L-tryptophan. Auxin production was shown to occur in both Fe-limited and non-limited conditions in the absence of Ni. The presence of Ni significantly inhibited auxin production in Fe-deficient

Table 1. Effects of Fe and Ni interplay on auxin and siderophore production by *Streptomyces acidiscabies* E13.

Treatment	Auxin ($\mu\text{mol}\cdot\text{L}^{-1}$) (IAA equivalent)	Siderophore ($\mu\text{mol}\cdot\text{L}^{-1}$) (DFOE equivalent)
-Fe, -Ni	14.9 \pm 1.90a	77.5 \pm 11.5
+Fe, -Ni	5.23 \pm 1.10b	nd
-Fe, +Ni	2.6 \pm 1.23c	71.6 \pm 7.64
+Fe, +Ni	nd	nd

Note: Values are the means \pm standard deviation of 4 replicates. Means followed by different letters are statistically significantly different at $P = 0.01$. IAA, indole-3-acetic acid; DFOE, desferrioxamine E; nd, not detected.

medium, and completely abolished auxin production when Fe was present (Table 1). Thus, the sterile SCF derived from medium containing Ni (-Fe, +Ni) that had been used to show plant growth promoting effects did not contain measurable auxin concentrations.

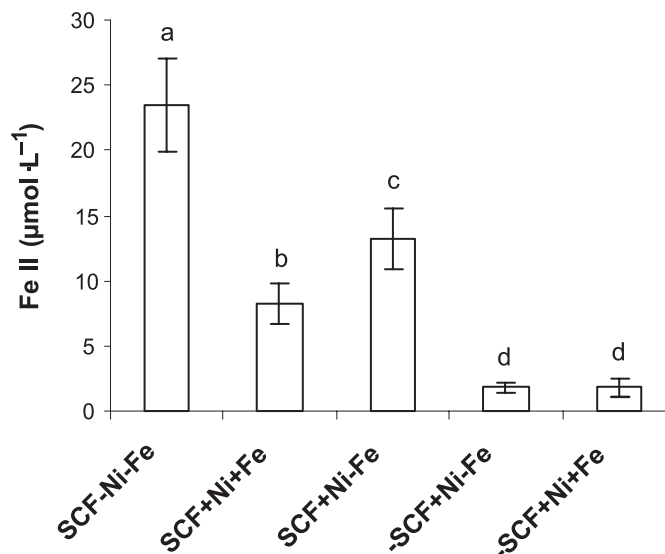
Detection of reduced Fe in cowpea roots

Intact cowpea plants were used to detect the presence of reduced Fe in Ni-stressed plants. Results indicated that in comparison with the absence of Ni, a significantly lower concentration of reduced Fe(II) was detected (Fig. 6). In these treatments, a clear Ni-mediated reduction of Fe(II) levels was observed in the absence of siderophores. In addition, the presence of 2.6 $\mu\text{mol}\cdot\text{L}^{-1}$ IAA did not enhance Fe(II) levels in treatments without siderophores. In contrast, results from plants assayed in the presence of siderophores showed high levels of Fe(II), which was significantly higher in Fe-starved plants.

Iron and nickel contents in cowpea roots and shoots

Roots of plants grown in Fe-deficient conditions were supplied with more Fe (by over 57%) when siderophores were present. Similarly, in Fe-containing treatments, it was observed that the presence of siderophores increased Fe concentration in plant root by more than 19%. Although more Fe was found in +Fe roots lacking siderophores than in -Fe plants treated with siderophores, overall there was significantly more Fe in roots of SCF \pm Fe treatments than in non-SCF treated roots (Table 2). One interesting observation in shoot Fe concentration is that more Fe was measured in shoots of -Fe + SCF than +Fe. The addition of siderophores in +Fe plants further increased the shoot Fe content significantly. High Ni concentration was observed in root samples of all treatments (Table 2). This was not surprising, considering that Ni was by far the dominant metal species in our system. Higher Ni contents were obtained in roots of -Fe plants than their siderophore-containing counterparts. Nickel contents of roots were higher in Fe-sufficient than in Fe-deficient conditions, but the addition of siderophores reduced Ni contents considerably in Fe-sufficient treatments. Highly significant reduction in Ni concentration was recorded in plant shoots, where the presence of siderophores resulted in only a very slight reduction in Ni content under Fe deficiency. However, a more marked reduction of shoot Ni was observed in Fe-sufficient plants in the presence of siderophores.

Fig. 6. Detection of reduced Fe in roots of Ni-stressed cowpea plants. Data are the means of 4 replicates, and differences were significant at $P = 0.01$, except for treatments with the same letters.



Discussion

Streptomyces acidiscabies E13 produces hydroxamate siderophores, namely DFOB, DFOE, and Cch, in Fe-deficient media irrespective of Ni content. Two of these siderophores, DFOB and DFOE, are encoded by the *des* operon comprising 4 genes, *desA-desD* (Barona-Gómez et al. 2004, 2006). Depending on the mechanism of acylation of the cadaverine backbone of these molecules, the final product can be cyclic (e.g., DFOE) or linear (e.g., DFOB; Barona-Gómez et al. 2006). The third compound, Cch, a nonribosomally synthesized molecule encoded by a different operon, had been recently discovered in 2 *Streptomyces* species (Lautru et al. 2005; Barona-Gómez et al. 2006). Thus, this work is only the third report of the biosynthesis of Cch in any siderophore-producing bacteria, and the first report of siderophore production in *S. acidiscabies*. Moreover, we present here the first evidence of Ni sequestration by DFOE and Cch.

In terms of kinetics, siderophores were detected as early as 24 h. Siderophore production increased progressively, peaking after 72 h, and decreasing thereafter. The general siderophore production trend observed agrees with results from Oliveira et al. (2006). No clearly defined preferential biosynthesis of any individual siderophore was observed over time, since high abundances of all siderophores, free or metal-complexed, were found at different times. Taken together, Figs. 2 and 3 indicate that estimating the abundance of specific siderophore types in a multisiderophore-producing species may provide underestimated results for specific siderophores.

Under Fe deficiency induced by high Ni levels, DFOB appears to be involved in more Fe sequestration than other siderophores. Similarly, more DFOE-Ni chelate species were detected. We are tempted, therefore, to speculate that in our SCF system, DFOB and DFOE, compared with Cch, were more dedicated to Fe and Ni binding, respectively.

Table 2. Effect of siderophore-containing culture filtrate (SCF) on plant Fe and Ni contents ($\mu\text{g}\cdot(\text{g dry mass})^{-1}$) in the absence and presence of added Fe.

Response variable	-Fe	-Fe, +SCF	+Fe	+Fe, +SCF
Root Fe	44.6±9.6a	78±12.0b	106±15.7c	131±19.4d
Shoot Fe	47±7.0a	69±14.2b	60.1±9.9c	83±20.6d
Root Ni	2441±100.0a	2137±138.0b	3221±184.4c	2902±195.8d
Shoot Ni	261±59.4a	259±32.7a	222±21.5b	184±18.4c

Note: Values are the means \pm standards deviations of 5 replicates. Means followed by a different letter between columns are significantly different at $P = 0.05$.

Siderophores generally have the highest affinity for ferric iron. In this regard, we could not help noticing the ability of the compounds to scavenge for and chelate the very trace amounts of Fe normally present as impurities in media components. The stability constant for the DFOB–Ni complex is estimated at 10^{10} (Keberle 1964), far below the value for a stable association with Fe ($K_B \approx 10^{30}$) (Crumbliss 1991). This being the case, free siderophores will have to scavenge for and chelate Fe, since their primary role is to solubilize and supply Fe to microorganisms and associated plants.

The plant growth promoting effects of SCF are indicative of a possible role for siderophores in plants growing under heavy metal stress conditions. The culture filtrate used in this study contained both cyclic and linear hydroxamates, which complement each other in high affinity and redox potential (E_0). For example, the DFOE–Fe complex, with a greater stability constant, has an E_0 of -477 mV compared with a higher value of -468 mV for DFOB–Fe (Boukhalfa and Crumbliss 2002). Thus, although DFOE was shown to have chelated significant amounts of Ni, it scavenged Fe in relation to its low starting concentration ($50 \mu\text{mol}\cdot\text{L}^{-1}$) with higher relative proportions. We therefore suppose that in the presence of high Ni concentrations, obtaining even greater plant yield would require dramatic siderophore concentrations (Cline et al. 1984; Bar-Ness et al. 1991) to supply Fe to the plants, and simultaneously counteract the toxic effects of Ni. For plants grown in the absence of siderophores (i.e., \pm Fe), growth was inhibited.

As expected, plants showed signs of chlorosis. All plant growth was conducted at a starting pH of 7.5, where added Fe(III) may remain slightly insoluble and therefore cannot be sufficiently acquired by cells. The culture pH did not fall below 6.8 during the experiment. This can also explain why +Fe plants grew only slightly better than –Fe plants, and further emphasizes the role of pH and siderophores in Fe solubilization. It is important to mention that a siderophore-inducing medium (uninoculated) was used for plant growth (i.e., \pm Fe), since the SCF used for the alternative treatment was obtained from a similar medium.

Auxins are well known for their involvement in plant growth and development, and reports of their production by bacteria, including *Streptomyces*, exist (Manulis et al. 1994; Patten and Glick 2002; Jin et al. 2006; Rajkumar et al. 2006). We therefore investigated the role, if any, of auxins produced by the bacterium in the observed cowpea growth promotion under Ni stress. *Streptomyces acidiscabies* E13 was shown to produce auxins in vitro at low concentrations. However, the production of auxin was drastically repressed in the presence of Ni. Nickel repression of auxin synthesis

correlates with heavy metal inhibitory effects on auxin synthesis by rhizobacteria (Kamnev et al. 2005). Therefore, based on several related observations, namely (i) Ni inhibition of auxin production; (ii) adventitious root growth (typically the function of auxins at high enough levels) not being especially promoted, and (iii) the low presence of reduced Fe in –SCF, +Ni, \pm Fe treatments, even with the addition of $2.6 \mu\text{mol}\cdot\text{L}^{-1}$ IAA (Fig. 6), we can conclude that auxins produced by *S. acidiscabies* E13 cultured in the presence of Ni have not played a major role in cowpea growth promotion in our system (SCF).

Although a component of essential enzymes, at elevated concentrations Ni can inhibit root elongation, cause chlorosis and foliar necrosis, hinder nutrient absorption by roots, affect root metabolism, and inhibit photosynthesis and transpiration (Khalid and Tinsley 1980; Woolhouse 1983). Indeed, there were signs of chlorosis in some of the plants, specifically those without SCF treatment. In addition, roots of Ni-treated plants became darkened and showed reduced elongation and side roots formation, unlike plants tested in the absence of Ni (not shown).

We used ferrozine to determine the concentration of ferrous iron in Ni-stressed cowpea root cultures. Ferrous iron chelators such as ferrozine bind Fe^{2+} (Alcántara et al. 1994; Bohórquez et al. 2001; Chang et al. 2003; Lucena et al. 2006) with a characteristic colour change, enabling the spectrophotometric determination of Fe(II) concentrations. Based on that, we could show that the presence of Ni decreased the availability of ferrous Fe in cowpea root culture, but the addition of siderophores resulted in increased Fe(II) detection. The difference in Fe(II) content observed in SCF plants growing under Ni stress (i.e., SCF, +Ni, +Fe versus SCF, +Ni, –Fe; Fig. 6) reflects the dire Fe need of Fe-starved plants. In this regard, more ferric iron reduction is expected in Fe-starved plants, as previously observed (Chang et al. 2003; Jin et al. 2006; Lucena et al. 2006; Meda et al. 2007). The presence of higher Fe(II) in SCF treatments lacking added Fe also demonstrates the power of siderophores to efficiently solubilize Fe and avail it to plants, even in the presence of inhibitory levels of heavy metals. Iron absorption by plants is known to be decreased in the presence of heavy metals such as Ni (Burd et al. 1998, 2000). The reduction of Ni toxicity and resulting enhancement of plant growth under Ni stress by a strain of *Kluyvera ascorbata*, a siderophore-producing rhizobacterium, was reported in canola. However, the effect was linked to the release of bacterial ACC deaminase rather than to siderophores (Burd et al. 1998).

Having established that siderophores from *S. acidiscabies*

E13 can directly bind Ni, on release into a nickel-replete environment, it was thus important to assess the effect of these metal chelators on Ni uptake in cowpea seedlings. In this work, plants grown in 2 mmol·L⁻¹ Ni were visually compared to those grown in nonstressed conditions. The results indicated that the former were stunted and chlorotic. The growth deficiency and chlorosis observed can thus be linked to the presence of Ni in the plant environment. Both root and shoot iron contents in the Fe-deficient treatments were significantly lower than in the +Fe treatments. The addition of cell-free culture filtrate in each case enhanced the iron content, indicating a positive effect of SCF on plant iron nutrition. This resulted in the interesting scenario that shoot Fe was better (69 versus 60 µg·(g dry mass)⁻¹) with the addition of SCF to Fe-deficient treatments, as compared with the presence of Fe without SCF. At the same time, the experiments could show that siderophore-containing cell-free supernatant from *S. acidiscabies* E13 had a protective effect on Ni accumulation, thus alleviating Ni stress. Lower Ni contents were obtained both in roots and shoots, independent of Fe status, when the plants were treated with culture filtrate. It would appear then that in the absence of added Fe, the siderophores in the culture filtrate were more involved in sourcing for Fe for plant use than in Ni uptake inhibition. In contrast, the addition of Fe resulted in significantly lower total Ni, as well as higher Fe contents of SCF plants, in comparison with those without siderophores. Considering that hexadentate hydroxamate siderophore–metal interaction is a 1:1 stoichiometry (Boukhalfa and Crumbliss 2002), and that Ni concentration in the experimental system was 20 times higher than that of siderophores, a vast concentration of the uncomplexed metal could have been freely taken up by the roots. This explains the general high root Ni contents of the plants. Nevertheless, the fact that Fe(III) is more competitive for siderophore chelation than Ni resulted in better plant Fe nutrition, despite excess Ni. Therefore, based on the total higher Fe and lower Ni levels found in siderophore-treated plants than in their untreated counterparts, independent of Fe status, we propose a dual role for siderophores in promoting plant growth in our system. The siderophores helped in the acquisition of sufficient Fe for growth in the presence of levels of Ni that might otherwise hinder Fe acquisition. Moreover, we could show that the siderophores contained in the cell-free supernatants are active Ni chelators. We have synthesized results obtained from mass spectrometric analysis of siderophores, plant growth, Fe(II) detection assay, and metal contents measurement to conclude that microbial hydroxamate siderophores contained in *S. acidiscabies* E13 culture filtrates promoted cowpea growth under heavy nickel contamination by binding both iron and nickel, thus playing a dual role of sourcing iron for plant use and protection from nickel toxicity.

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Manuscript II

Involvement of siderophores in the reduction of metal-induced inhibition of auxin synthesis in *Streptomyces* spp.



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Involvement of siderophores in the reduction of metal-induced inhibition of auxin synthesis in *Streptomyces* spp.

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ABSTRACT

Unlike synthetic metal chelators, microbe-assisted phytoremediation provides plants with natural metal-solubilizing chelators which do not constitute a potential source of environmental pollution. Concurrently with microbial chelators, plant growth promotion can be enhanced through bacterially-produced phytohormones. In this work, the simultaneous production of siderophores and auxins by *Streptomyces* was studied to gain insight for future application in plant growth and phytoremediation in a metal-contaminated soil. Standard auxin and siderophore detection assays indicated that all of the investigated *Streptomyces* strains can produce these metabolites simultaneously. However, Al³⁺, Cd²⁺, Cu²⁺, Fe³⁺ and Ni²⁺, or a combination of Fe³⁺ and Cd²⁺, and Fe³⁺ and Ni²⁺ affected auxin production negatively, as revealed by spectrophotometry and gas chromatography–mass spectrometry. This effect was more dramatic in a siderophore-deficient mutant. In contrast, except for Fe, all the metals stimulated siderophore production. Mass spectrometry showed that siderophore and auxin-containing supernatants from a representative *Streptomyces* species contain three different hydroxamate siderophores, revealing the individual binding responses of these siderophores to Cd²⁺ and Ni²⁺, and thus, showing their auxin-stimulating effects. We conclude that siderophores promote auxin synthesis in the presence of Al³⁺, Cd²⁺, Cu²⁺ and Ni²⁺ by chelating these metals. Chelation makes the metals less able to inhibit the synthesis of auxins, and potentially increases the plant growth-promoting effects of auxins, which in turn enhances the phytoremediation potential of plants.

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1. Introduction

Trace or heavy metals such as aluminum (Al³⁺), cadmium (Cd²⁺), copper (Cu²⁺), nickel (Ni²⁺), zinc (Zn²⁺) and lead (Pb²⁺), which are commonly found in contaminated soils, can enhance Fe deficiency symptoms in microbes (Huyer and Page, 1988; Hu and Boyer, 1996b; Baysse et al., 2000) and plants (Alcántara et al., 1994; Yoshihara et al., 2006), thus affecting their growth negatively. Currently, one of the strategies for removing these metals from the soil is based on uptake of the metals by plants, facilitated by metal solubilization through the addition of synthetic chelators such as EDTA (White, 2001; Lopez et al., 2005, 2007; Liphadzi et al., 2006). Nevertheless, on their own, free EDTA and other synthetic chelators are not easily degraded in the soil, thereby constituting a new source of environmental pollution (White, 2001). In conjunction with EDTA, an external application of phytohormones was recently shown to increase metal uptake (Lopez et al., 2005, 2007; Liphadzi et al., 2006; Dimkpa et al., unpublished) due to enhanced plant

root growth that resulted in more roots being available for metal uptake. However, the use of purified indole acetic acid (IAA) may be expensive, and therefore unsustainable for large-scale phytoremediation, especially in resource-poor countries.

Many bacterial species can synthesize secondary metabolites that can potentially be useful in phytoremediation, thus providing a cheap and environmentally-friendly alternative to the use of synthetic chelators. Siderophores are metal chelators which bind Fe³⁺ with a high affinity (Boukhalfa and Crumbliss, 2002; Fernández and Winkelmann, 2005), but which can also interact with metals other than Fe³⁺, albeit with reduced affinity (Martell et al., 1995; Hernlem et al., 1996). In addition to siderophores, microbially-produced auxins can enhance root growth dramatically (Patten and Glick, 2002). Therefore, bacteria that can produce siderophores and auxins simultaneously can be potential candidates for microbe-assisted phytoremediation of metal contamination. Unfortunately, some metals have been reported to inhibit auxin synthesis in bacteria (Kamnev et al., 2005; Dimkpa et al., 2008). Auxin-producing rhizosphere microbes in metal-polluted soils may, thus, become less efficient in promoting plant growth, which affects their phytoremediation efficiency.

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Streptomyces are among the most important microbes in the rhizosphere renowned for their exceptional ability to produce diverse secondary compounds (Doubou et al., 2002; Challis and Hopwood, 2003), some of which can be applied in phytoremediation. We are interested in siderophore-mediated use of heavy metal-resistant, siderophore and auxin-producing *Streptomyces* to facilitate the phytoremediation of metals from the soil of a contaminated field site, and our hypothesis is that siderophores can, through chelation, influence metal effects on auxin synthesis. Nevertheless, to be able to apply this strategy successfully requires knowledge of how the two microbial metabolites can interact in the presence of diverse metals, since there is, apparently, a lack of literature describing the ability of *Streptomyces* to simultaneously produce auxins and siderophores in the presence of diverse metals. The present study, therefore, evaluated (i) the simultaneous production of siderophores and auxins under heavy metal stress by strains of *Streptomyces*, and (ii) the effect of the siderophores on the synthesis of auxins in the presence of toxic metals.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Metal-resistant *Streptomyces tendae* F4 and *S. acidiscabies* E13 (Amoroso et al., 2000; Schmidt et al., 2005), *S. avermitilis*, *S. coelicolor* A3(2), *S. tanashiensis*, and *S. griseus* were obtained from our laboratory strain collection. *S. coelicolor* W13, a strain of *S. coelicolor* A3(2) genetically-modified by deletion of the siderophore biosynthetic operons, *des* and *cch* (Barona-Gómez et al., 2006), was a kind gift from Dr. Gregory Challis (University of Warwick, UK). *S. mirabilis* P10A-3, *S. mirabilis* K7A-1, *S. chromofuscus* P4B-1, *S. chromofuscus* P10A-4, *S. prunicolor* P6A-1, and *S. naganishii* P9A-1 were recently isolated by us, from a field site contaminated with heavy metals located near Ronneburg, in the eastern part of Germany (Schmidt et al., 2008). The strains were maintained as previously described (Dimkpa et al., 2008). To investigate the co-production of siderophores and auxins in these strains, Fe³⁺-deficient siderophore-inducing medium (Alexander and Zuberer, 1991) was supplemented with L-tryptophan (20 µg mL⁻¹). Tryptophan was added in the culture to induce auxin production, similar to the effect in rhizosphere plant-microbe interaction. The medium (100 mL) was inoculated with spores (10⁶ CFUs) of the strains collected from 5 to 7 d-old agar plates. To produce auxins and siderophores simultaneously in liquid medium in the presence of Fe³⁺ (as FeCl₃), Al³⁺ (as AlCl₃), Cd²⁺ (as CdCl₂), Cu²⁺ (as CuSO₄·5H₂O) and Ni²⁺ (as NiCl₂), spores were collected from *S. acidiscabies* E13, *S. tendae* F4, *S. coelicolor* A3(2), and *S. coelicolor* W13, as described above, and cultured in Cu²⁺-deficient, siderophore-inducing medium supplemented or not supplemented with the respective metals (100 µM each), and with or without tryptophan (20 µg mL⁻¹). Cultures were grown for 3–5 d, with appropriate controls. In the experiment involving the effect of a combination of metals on auxin production in the absence of tryptophan, 100 µM each of Fe³⁺ and Cd²⁺ on the one hand, and Fe³⁺ and Ni²⁺, on the other, were applied to tryptophan-deficient medium and inoculated with spores of *S. tendae* F4 and *S. acidiscabies* E13, respectively. For the investigations involving Cd²⁺ and Ni²⁺ interactions with siderophores released by *S. tendae* F4, the medium was respectively amended with 100 µM Cd²⁺ and 1 mM Ni²⁺, under a starting Fe³⁺ concentration of 100 µM in each case, and also in the absence of added Fe³⁺. For this, four types of Fe³⁺ and Cd²⁺/Ni²⁺ treatments were set-up as follows: –Fe – Cd/Ni; –Fe+ Cd/Ni; +Fe – Cd/Ni, and +Fe+ Cd/Ni. Each set-up was inoculated with 10⁶ CFU of the bacterial spore suspension and cultured as described above. All culture conditions and glassware treatments were as previously described (Dimkpa et al., 2008).

2.2. Detection of auxins in *Streptomyces*

The production of auxin by *Streptomyces* was determined by means of the Salkowski assay as described by Patten and Glick (2002). IAA (ROTH, Karlsruhe, Germany) was used to prepare a standard curve. To validate the production of auxin and its inhibition by metals, IAA was extracted and quantified according to a modified protocol from Stelmach et al. (1999). Briefly, 2 mL each of culture filtrates of *S. tendae* F4 and *S. acidiscabies* E13, in which no tryptophan was added, but which contained 100 µM combinations of Fe and Cd, and Fe and Ni, respectively, were placed in centrifuge tubes. Next, [¹³C₆]-IAA (0.5 µg; Cambridge Isotope Laboratories, Andover, MA, USA, 99% isotopic enrichment) was added as an internal standard, to enable quantification. Samples were then acidified to pH 2 with 0.1 M HCl (2 mL), and water phase was quantitatively extracted three times with ethyl acetate. Phase separation was facilitated by centrifugation. Combined organic layers were subsequently passed through pre-conditioned (methanol, 5 mL; ethyl acetate, 5 mL) Chromabond NH₂ cartridges (3 mL/0.5 g, Macherey-Nagel, Düren, Germany). Cartridges were washed with *i*-propanol:dichloromethane (5 mL, 2:1, v:v) and eluted with diethyl ether:formic acid (10 mL, 98:2, v:v). Eluting solvent was then removed under a gentle stream of argon. The residue was treated with an ethereal solution of diazomethane and re-dissolved in 45 µL of dichloromethane, after removal of the diazomethane. Under split mode (1:10), samples were analyzed on a Finnigan Trace Instrument (Thermoelectron, Bremen, Germany), equipped with Zebron DB-5 column (15 m × 0.25 mm × 0.25 mm with 10 m guard column, Phenomenex, Aschaffenburg, Germany). Elution was performed under programmed conditions from 60 °C followed by 15 °C min⁻¹ to 140 °C, 5 °C min⁻¹ to 210 °C and 15 °C min⁻¹ to 300 °C. Helium, served as a carrier gas, at a flow rate of 1.5 mL min⁻¹. The GC injector, transfer line, and ion source were set at 220 °C, 280 °C, and 280 °C, respectively. Spectra were taken in the total-ion-scanning (TIC) mode at 70 eV. Quantification was based on ion traces for *m/z* = 189 (IAA–Me) vs *m/z* = 195 ([¹³C₆]-IAA–Me). Calibration curve was obtained by adding known amounts of IAA to 2 mL of pure bacterial medium and following the extraction procedure. Control experiments to exclude complex formation between the metals and IAA were performed: Fe³⁺ Cd²⁺ and Ni²⁺ (two repeats with 100 µM and 200 µM concentrations) were added to 2 mL of un-inoculated bacteria medium containing 1 µg of pure IAA. This was followed by the previously described extraction procedure. The recovery rate was not affected in any of these experiments.

2.3. Detection of siderophores

The presence of siderophores in the culture media was detected using the chrome-azurol S (CAS) assay of Schwyn and Neilands (1987), performed as described (Dimkpa et al., 2008) with some modifications: the CAS assay solution-culture filtrate mix was incubated for 2 h in the absence of metals, and for 12 h in the experiments involving Fe³⁺, Al³⁺, Cd²⁺, Cu²⁺ and Ni²⁺. Electrospray ionization mass spectrometry (ESI-MS) was carried out with culture filtrates of *S. tendae* F4. This was performed as previously described (Dimkpa et al., 2008). The mass of purified hydroxamate siderophore, desferrioxamine E (DFOE; 601 Da), and published molecular masses of desferrioxamine B (DFOB [561.0 Da]; Winkelmann et al., 1999) and coelichelin (Cch [566.3 Da]; Lautru et al., 2005) were used as references for detecting and calculating molecular masses of protonated [M+H] and metal-siderophore complexes, using the formula [M–2H⁺+Fe³⁺]⁺ for siderophore–Fe³⁺ complexes, [M–H⁺+Cd²⁺]⁺ for siderophore–Cd²⁺ complexes, and [M–H⁺+Ni²⁺]⁺ for siderophore–Ni²⁺ complexes; where *M* is the molecular mass of the specific siderophores analyzed.

2.4. Statistical analysis

Data were analyzed for variance (ANOVA) and where significant, treatments were separated by the Tukey Test.

3. Results

3.1. Diverse *Streptomyces* strains produce auxins and siderophores concurrently

In order to investigate the auxin-producing ability of 13 different *Streptomyces* strains, we supplemented siderophore-producing medium with tryptophan ($20 \mu\text{g mL}^{-1}$). All *Streptomyces* strains produced auxins with dramatically varied intensities of the auxin-reactive color changes in the Salkowski assay. At the same time, we analyzed the 13 *Streptomyces* strains for their ability to produce siderophores under auxin-inducing conditions. Except for *S. coelicolor* W13, a siderophore-deficient mutant of *S. coelicolor*, all other strains showed strong CAS-reactivity, indicative of siderophore production (Table 1).

3.2. The inhibitory effect of metals on auxin production is less pronounced in siderophore-producing *Streptomyces*

S. tendae F4 and *S. acidiscabies* E13 are known to be resistant to Cd^{2+} and Ni^{2+} , respectively; however, these strains were also found to be resistant to a range of other metals (results not shown). Therefore, they were considered suitable for studying auxin and siderophore production in the presence of the metals. Growth of *S. coelicolor* A3(2) and *S. coelicolor* W13 were also evaluated in the presence of Fe^{3+} , Al^{3+} , Cd^{2+} , Cu^{2+} and Ni^{2+} . It was observed that the metals (with the exception of Fe^{3+}) affected the growth of both *S. coelicolor* strains negatively in relation to *S. tendae* F4 and *S. acidiscabies* E13. However, there was no difference in growth between *S. coelicolor* A3(2) and *S. coelicolor* W13 (result not shown). This finding, thus, enabled the use of both *S. coelicolor* strains for testing the effect of siderophores and metals on auxin production, in addition to *S. tendae* F4 and *S. acidiscabies* E13. To determine how metals affect auxin production in *Streptomyces* under siderophore-inducing conditions, the above strains were cultured in siderophore-inducing medium supplemented with tryptophan, and with Fe^{3+} , Al^{3+} , Cd^{2+} , Cu^{2+} and Ni^{2+} . Relatively high amounts of auxins were detected in control treatments in all strains. However, in all strains, the presence of the metals significantly reduced the

levels of detectable auxins, with Fe^{3+} being by far, the most potent inhibitor, and Al^{3+} being the least, in most of the strains. Interestingly, the level of auxins in the presence of the metals was more severely affected in the siderophore-deficient mutant, *S. coelicolor* W13, than in other strains (Fig. 1). Following the observed effect of the individual metals on auxin production by the bacteria, a different but related experiment was conducted involving a combination of metals. This was done to simulate heterogeneous soil contamination by metals, which is the case in our contaminated field site; however only two metal combinations were randomly selected in order to simplify the experiment. In this case, the inclusion of Fe^{3+} as one of the metals was considered important, to inhibit siderophore production and thereby show its effect on auxin production. For this, gas chromatography–mass spectrometry (GC–MS) was used to quantify IAA levels in tryptophan-deficient cultures of *S. tendae* F4 and *S. acidiscabies* E13, containing a combination of Fe^{3+} and Cd^{2+} , and Fe^{3+} and Ni^{2+} , respectively. Almost no auxins could be detected in the combined presence of the metals (Fig. 2). Thus, in addition to the effect shown for individual application of Fe^{3+} , Cd^{2+} and Ni^{2+} (Fig. 1), this result confirmed that Fe^{3+} , Cd^{2+} and Ni^{2+} can dramatically inhibit the production of auxins in *Streptomyces*.

Table 1

Co-detection of auxins and siderophores in different *Streptomyces* strains grown in Fe-deficient medium supplemented with L-tryptophan

Strain	Auxin ^a	Siderophore ^b
<i>S. acidiscabies</i> E13	+++	+++
<i>S. griseus</i>	++	+++
<i>S. avermitilis</i>	++++	+++
<i>S. tendae</i> F4	+++	+++
<i>S. tanashiensis</i>	++++	+++
<i>S. coelicolor</i> A3(2)	++++	++++
<i>S. mirabilis</i> K7A-1	+	++++
<i>S. chromofuscus</i> P4B-1	+	++++
<i>S. mirabilis</i> P10A-3	+	++++
<i>S. chromofuscus</i> P10A-4	+	++++
<i>S. prunicolor</i> P6A-1	+	++++
<i>S. naganishii</i> P9A-1	+	++++
<i>S. coelicolor</i> W13	++++	n.d. ^c

^a +: weak reaction; ++: intermediate reaction; +++: strong reaction; ++++: strongest reaction.

^b +++: strong reaction; ++++: stronger reaction.

^c N.d, not detected.

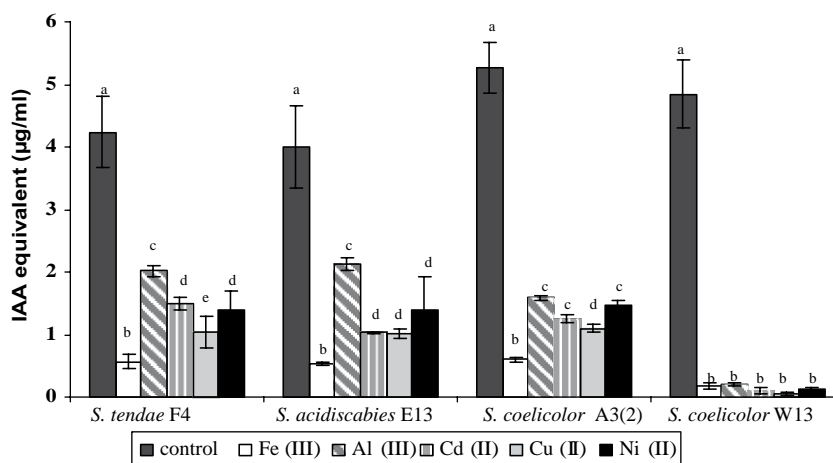


Fig. 1. Influence of $100 \mu\text{M}$ of Fe(III) , Al(III) , Cd(II) , Cu(II) and Ni(II) on the production of auxins by *Streptomyces tendae* F4, *S. acidiscabies* E13, *S. coelicolor* A3(2), and *S. coelicolor* W13 grown under siderophore-inducing conditions in the presence of L-tryptophan ($20 \mu\text{g mL}^{-1}$). Bars denote standard deviation and different letters show significantly different results ($P=0.05$) within each strain.

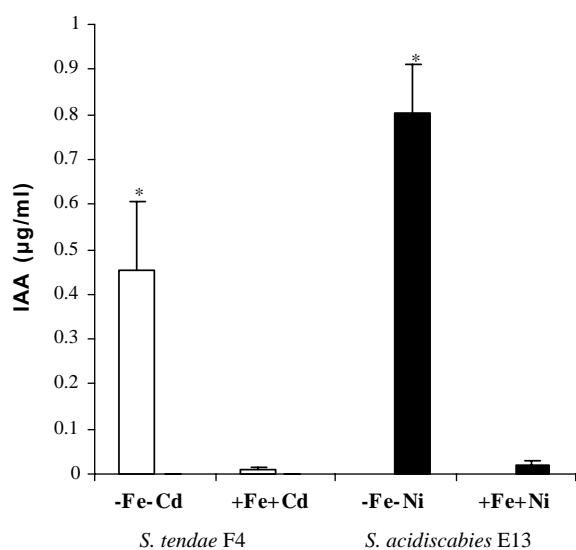


Fig. 2. Gas chromatography–mass spectrometry analysis of culture filtrates of *S. tendae* F4 and *S. acidiscabies* E13 validating IAA production in the absence of tryptophan, and its inhibition by metals. Fe³⁺, Cd²⁺ and Ni²⁺ were each added at 100 µM. Asterisks indicate significant differences at P=0.01 (n=3).

3.3. Metals stimulate siderophore production in *Streptomyces*

Three representative *Streptomyces* species (*S. acidiscabies* E13, *S. tendae* F4, and *S. coelicolor* A3(2)) were cultured in a tryptophan-containing siderophore-inducing medium amended with the respective metals. After 72h of growth, the presence of siderophores was assayed by incubating the samples together with the CAS assay solution for 12h, during which time varying intensities of CAS-reactive color changes were observed. Not surprisingly, almost no siderophores were produced in any of the strains when treated with a starting Fe³⁺ concentration of 100 µM. In contrast, all the other metals significantly up-regulated siderophore production in all strains, compared to the control (Fig. 3).

3.4. *S. tendae* F4 produces three hydroxamate siderophores that bind Cd

Since no previous report exists on siderophore production in *S. tendae* F4, added to its metal-resistance capability, which makes it a potential candidate for application in bioremediation, the strain was selected for further analysis in terms of siderophore produc-

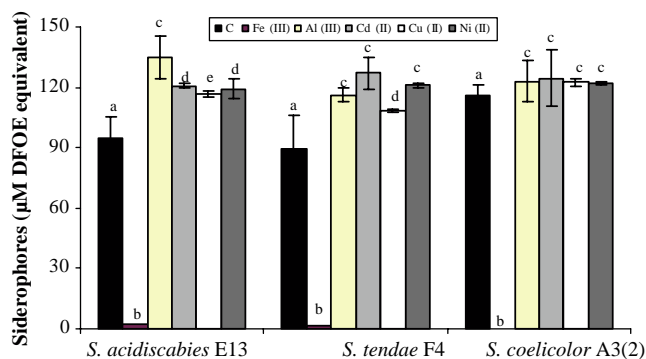


Fig. 3. CAS assay demonstrating the influence of 100 µM of Fe(III), Al(III), Cd(II), Cu(II) and Ni(II) on siderophore production by *Streptomyces acidiscabies* E13, *S. tendae* F4, and *S. coelicolor* A3(2) grown for 72 h in siderophore-inducing medium amended with (20 µg mL⁻¹) L-tryptophan. Bars with different letters in each strain are significant from each other at P=0.05 (n=6).

tion. *S. tendae* F4 was treated with Cd²⁺ (100 µM), in the presence of high (100 µM), low (35 µM) or no added Fe³⁺. After incubation for 72 h, cultures were assayed for siderophore production, using the CAS method, and then ESI-MS analysis. As no CAS-reactive color change was observed in treatments with high levels of Fe³⁺ (100 µM) irrespective of their Cd²⁺ status (result not shown), no further analyses were conducted. However, ESI-MS confirmed that 3 structurally different tri-hydroxamate siderophores, DFOE, DFOB and Cch, were simultaneously produced by *S. tendae* F4. In addition, consistent with the CAS assay, Cd²⁺ was confirmed to stimulate siderophore production in the absence of Fe³⁺. At the same time, Cd²⁺ was shown to override the repression of siderophore production by low Fe³⁺ (35 µM) concentrations. Without addition of Fe³⁺, irrespective of Cd²⁺ status, desferrioxamine siderophores (DFOE and DFOB) were mainly produced. When Cd²⁺ was absent, the presence of low concentrations of Fe³⁺ resulted in low abundance of all three siderophores. In contrast, all three siderophores were significantly up-regulated when Cd²⁺ was added. Overall, in addition to chelating Fe³⁺, where present, all three hydroxamates bound Cd²⁺ at varying abundances (Fig. 4).

3.5. Nickel overrides the repression of siderophore production by iron in *S. tendae* F4

As with Cd²⁺, ESI-MS confirmed that Ni²⁺ stimulates siderophore production in *S. tendae* F4. In addition, the presence of a low level of Fe³⁺ (35 µM) decreased siderophore production; however,

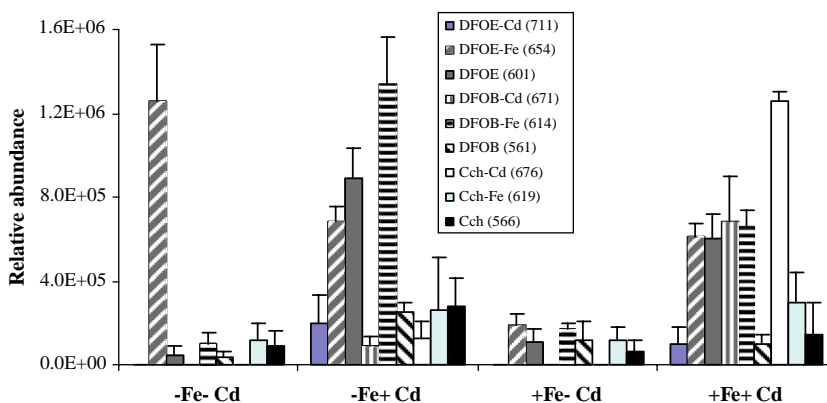


Fig. 4. Identification of DFOE, DFOB and Cch, and the detection of their metal chelate species in culture supernatants of *Streptomyces tendae* F4 grown under Fe³⁺ and Cd²⁺ set-ups. The relative abundances of the released siderophores and their respective metal chelate species are indicated. Bracketed legend numbers are measured m/z values used for siderophore identification (ESI-MS; n=3).

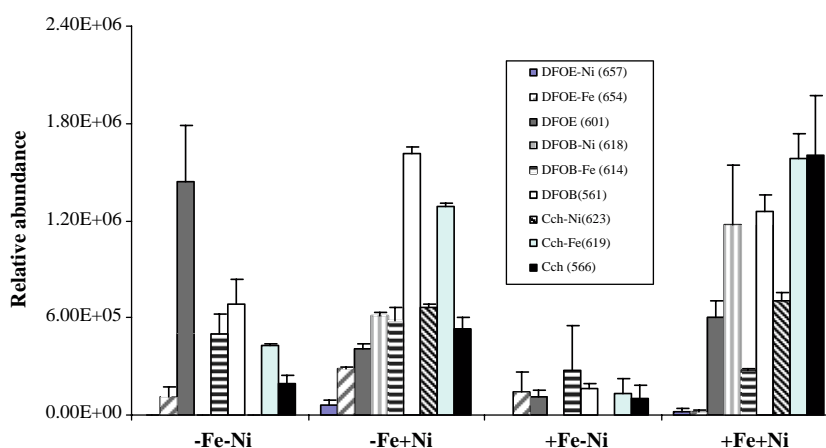


Fig. 5. Stimulation of siderophore production in *S. tendae* F4 by high concentration of Ni^{2+} after 120 h growth. The relative abundances of the released siderophores and their respective metal chelate species are indicated (ESI-MS; $n=3$).

siderophore production was clearly stimulated when a high concentration of Ni^{2+} (1 mM) was added to the treatment containing low Fe^{3+} . In the presence of Ni^{2+} , Cch and DFOB were up-regulated, both of which, in comparison with DFOE, showed considerable abundances of Fe^{3+} and Ni^{2+} chelate species (Fig. 5).

4. Discussion

Auxin-producing rhizobacteria can utilize tryptophan contained in plant root exudates for the synthesis of auxins (Sarwar et al., 1992; Manulis et al., 1994; Patten and Glick, 2002). Beginning with L-tryptophan, Manulis et al. (1994) elucidated the pathway for auxin biosynthesis in *Streptomyces*, which is based on the indole-3-acetamide pathway; however, more than one pathway can be found in one bacteria strain. All *Streptomyces* strains investigated in the current study produced measurable amounts of auxins, either at low concentrations ($20 \mu\text{g mL}^{-1}$), or in the absence of added tryptophan. We had previously shown that *S. acidiscabies* E13 can produce auxins in the absence of added tryptophan, and that Ni^{2+} , as well its combination with Fe^{3+} , affected this ability significantly (Dimkpa et al., 2008). Thus, we tested these findings in Cd-resistant *S. tendae* F4. To this end, GC-MS was used to quantify specific auxin (IAA) production by tryptophan-deficient cultures of both strains (Fig. 2). Although optimal production of auxins by bacteria is achieved under tryptophan induction, the possibility to produce auxins under low or outright absence of added inducing substances is of interest for subsequent application of these bacteria in our metal-contaminated test field site, where plant growth, and, thus, release of auxin-inducing root exudates, is limited by metal stress and attendant poor soil fertility.

In line with previous studies (Sarwar et al., 1992; Patten and Glick, 2002; Kravchenko et al., 2004; Kamnev et al., 2005), the amount of auxins produced by soil bacteria varied, depending on the level of available exogenous tryptophan, even within one genus. Clearly, *Streptomyces* strains can produce ecologically relevant (Patten and Glick, 2002) concentrations of auxins, even under low tryptophan conditions. Consistent with previous reports (Kamnev et al., 2005; Dimkpa et al., 2008), the presence of metals (Fe^{3+} , Al^{3+} , Cd^{2+} , Cu^{2+} and Ni^{2+} , as well as a combination of Fe^{3+} and Cd^{2+} , or Fe^{3+} and Ni^{2+} significantly affected auxin production in *S. tendae* F4, *S. acidiscabies* E13, and *S. coelicolor* A3(2). Interestingly, auxin production was almost completely abolished by the metals in the siderophore-deficient mutant, *S. coelicolor* W13. In order to demonstrate that low auxin recovery was not due to the addition of metals but that the results truly showed lower auxin

levels with the metals in the culture medium, control experiments were performed in which adding Fe, Cd and Ni to medium containing synthetic IAA did not affect the recovery of IAA. These results exclude the possibility that the formation of IAA-metal complexes (Oota and Tsudzuki, 1971) leads to decreasing amounts of free IAA. Instead, the reduction in auxin concentrations in the presence of metals can be attributed either to lower biosynthesis induced by the metals, or to auxin degradation by IAA peroxidases which are themselves up-regulated by metal-catalyzed free radical formation, as reported for plants (Potters et al., 2007, and references therein). The dramatically reduced levels of auxins in the absence of siderophores indicate that the latter played a significant role in auxin production under toxic metal influence.

In *Streptomyces*, different siderophores are produced with structurally different molecules, even among strains of the same species (Fiedler et al., 2001; Lautru et al., 2005; Barona-Gómez et al., 2006; Dimkpa et al., 2008). Here, we provide the first evidence that *S. tendae* F4 produces the hydroxamate siderophores, desferrioxamine B, desferrioxamine E, and coelichelin. In addition to confirming previous reports of siderophore production by *S. griseus* (Yamanaka et al., 2005), *S. coelicolor* (Barona-Gómez et al., 2006), and *S. acidiscabies* (Dimkpa et al., 2008), we also report the production of siderophores in more strains of *Streptomyces*.

Although some metals have been shown to stimulate siderophore production and interact with the released siderophores in some other bacteria (see for e.g., Huyer and Page, 1988; Visca et al., 1992; Hofte et al., 1993; Hu and Boyer, 1996b; Dao et al., 1999; Sinha and Mukherjee, 2008; Wichard et al., 2008), this phenomenon has, surprisingly, not been investigated in *Streptomyces* until now. In the current study, the increased production of siderophores by *Streptomyces* in the presence of metals can be explained by the fact that metal ions compete for siderophore binding with the trace amounts of iron present, necessitating increased siderophore production to obtain equivalent levels of iron to circumvent, or at least alleviate, metal-induced Fe deficiency. The presence of metals during siderophore production often underestimates CAS-based measurement of siderophore concentrations (Hu and Boyer, 1996a; Dimkpa et al., 2008), since free, but not metal-bound siderophores are required for this assay (Schwyn and Neilands, 1987). This being the case, we subjected the siderophore-CAS solution mix to a long incubation time (12 h). This resulted in a more accurate estimation of siderophore production by allowing the dissociation of the metals from their siderophore complexes, and in their place, the binding of Fe released from the CAS complex.

By means of mass spectrometry, we confirmed that siderophore production by *Streptomyces* is stimulated by a range of metals, and also showed to what relative extents the production of individual hydroxamate siderophores (DFOB, DFOE and Cch) were affected by Cd²⁺ and Ni²⁺, in the presence or absence of Fe. Whereas previous reports of metal-hydroxamate siderophore interactions have mainly been based on the titration of purified siderophores with the metals, the current study, together with our recent work (Dimkpa et al., 2008) demonstrates *in situ* binding of Cd and Ni by these siderophores, upon release by *Streptomyces*. A high occurrence of Cch–Cd complex was observed, especially in Fe-containing cultures, in contrast to those containing Ni²⁺, where high abundances of the Cch remained Ni-free (Dimkpa et al., 2008; this study). Thus, we postulate that, in *Streptomyces*, the biosynthesis of multiple siderophores illustrates the benefit of producing chelators with varying metal affinities and preferences: bacteria producing more than one type of siderophore may better survive environments that are heterogeneously contaminated with different toxic metals.

The repression of auxin production by the metals was more severe in the absence of siderophores, both in Fe-replete treatments lacking measurable presence of siderophores, and, especially, in the siderophore biosynthetic mutant, *S. coelicolor* W13. Of the metals which showed siderophore stimulation activity in this study, Al has by far the highest affinity for siderophore binding (Martell et al., 1995; Fernández and Winkelmann, 2005). Indeed, the high affinity of Al³⁺ for siderophore binding was experimentally demonstrated recently, in which pyoverdine produced by *Pseudomonas aeruginosa* was shown to bind Al³⁺, and the complex recognized by the siderophore receptor (Greenwald et al., 2008). This strong affinity may, thus, explain why Al showed overall less inhibition of auxin synthesis in the strains, given that bound Al will be prevented from interfering with auxin production. Since the only plausible mechanism by which siderophores can affect auxin production under metal stress condition is by chelating the metals, we therefore speculate that binding of the metals by siderophores lowers free toxic metal concentrations that otherwise interferes with auxin synthesis. On the basis of two observations: (i) the severe inhibition of auxin synthesis by the metals under both genetic (*S. coelicolor* W13) and Fe-replete conditions which also precluded siderophore production, and (ii) the *in situ* binding of two representative metals (Cd and Ni) by the released siderophores, we postulate a relationship whereby metals other than Fe will both stimulate and diminish siderophore and auxin production. The stimulated siderophores will, in turn, help alleviate the inhibition of auxin production induced by the metals. From an applied point of view, metal-resistant, auxin-producing rhizobacteria may not be as effective in promoting plant growth or in microbe-assisted phytoremediation of soils polluted with metals other than Fe, if they do not simultaneously produce siderophores, preferably of more than one type. Our results indicate that *Streptomyces* spp are suitable candidates for the biofertilization and microbe-assisted phytoremediation of metal-contaminated soils. In the case of pollution by Fe (especially in acidic soil) and its consequent inhibition of siderophore production in microorganisms, supernatants of *Streptomyces* containing siderophores and auxins can potentially achieve similar results. We anticipate that future plant growth and metal uptake experiments in soil from the heavy metal-contaminated field sites at the former uranium mining site in Thuringia, Germany, will shed light on these possibilities.

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Manuscript III

Metal-induced oxidative stress impacting plant growth in contaminated soil is alleviated by microbial siderophores.



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Metal-induced oxidative stress impacting plant growth in contaminated soil is alleviated by microbial siderophores

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ABSTRACT

High levels of metals impede plant growth by affecting physiological processes. Siderophores are microbial Fe-chelators that, however, bind other metals. This study evaluated plant growth in a soil containing elevated levels of metals, including Al, Cu, Fe, Mn, Ni, and U, using *Streptomyces*-derived cell-free supernatant containing siderophores and auxins. Cowpea plants in the soil were treated with the culture filtrate. Growth was measured and biochemical analyses such as chlorophyll contents, RNA and protein quantification, lipid membrane peroxidation, and anti-oxidative responses were conducted to evaluate oxidative stress in the plants. Liquid chromatography–mass spectrometry was used to simulate competition for siderophore binding, and metal content of plants was determined spectroscopically. Whereas the metals inhibited plant growth, addition of siderophores improved growth. There was evidence of lipid peroxidation, an enhanced superoxide dismutase activity, and lowered chlorophyll, RNA, protein, carotenoid and residual indole acetic acid contents, especially in control plants. Siderophore competition assays between Al and Fe, and Fe and Cu suggested that trivalent metals are more competitive for siderophore binding than divalent ones. Compared to control plants, higher amounts of metals were obtained in siderophore-treated plants. Siderophores were able to supply plants with Fe in the presence of levels of metals, mainly Al, Cu, Mn, Ni and U that otherwise inhibit Fe acquisition. This led to enhanced chlorophyll content, circumventing lipid peroxidation effects on leaves. Siderophores lowered the formation of free radicals, thereby protecting microbial auxins from degradation and enabling them to enhance plant growth which in turn resulted in augmented metal uptake.

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1. Introduction

Soils contain a repertoire of diverse heavy and trace metal elements which, at high concentrations, negatively affect plant cellular and physiological processes, leading to reduced growth. Al and Fe are the two most abundant metals in soil (Feng, 2005). However, unlike Al, Fe is an essential element required by most biological systems, albeit at low concentrations. In calcareous soils of alkaline pH, Fe is usually present as insoluble ferric iron. At most pH values below 7, or a redox potential of around 100 mV (Patrick and Jugsujinda, 1992), ferric iron is reduced to bioavailable iron, leading to the uptake of toxic concentrations of the metal (Schmidt, 1999). Like Fe, Al occurs either as insoluble aluminosilicates (Al_2SiO_5) or as aluminosilicates (Al_2SiO_5), which at low pH, are mobilized as highly plant-toxic cations (Kochian, 1995). Excess of the metals induces elevated levels of reactive oxygen species (ROS). ROS-

induced oxidative stress adversely affects plant growth and yield (Cakmak and Horst, 1991; Gratão et al., 2005; Stobrawa and Lorenc-Plucińska, 2007).

Since Fe is essential, plants have evolved strategies for its acquisition, which, in dicotyledonous plants such as cowpea (*Vigna unguiculata* (L.) Walp), is based on strategy I. Unlike strategy II found in grass monocotyledonous plants, strategy I does not involve the release of phytosiderophores. Rather, it is characterized by an enhanced Fe(III) reductase activity, release of reductants such as phenolics, and acidification of the rhizosphere (Römheld and Marschner, 1986). Furthermore, in strategy I plants, microbial siderophores—low-molecular-mass Fe chelators—have been reported to promote growth under Fe deficiency (Cline et al., 1984; Bar-Ness et al., 1991; Crowley et al., 1991). One of such siderophores, desferrioxamine B (DFOB), has been used for Fe and Al detoxification in human (Brown et al., 1982; Malluche et al., 1984; Allain et al., 1987). Yet, aside from our recent in vitro study (Dimkpa et al., 2008), there are hardly any reports of the application of microbial siderophores to alleviate metal toxicity in plants in contaminated soil.

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Like siderophores, microbially-produced auxins also promote plant growth (Patten and Glick, 2002), and we have recently observed that auxin production in *Streptomyces* is enhanced under siderophore-producing conditions; but the presence of metals, including Fe, inhibits auxin production (Dimkpa et al., in press). Auxin-producing rhizosphere microbes in metal-polluted soil may, therefore, become less efficient in promoting plant growth in such soil. Thus, we hypothesize that siderophore and auxin-containing bacteria culture filtrates, instead of the bacteria themselves, may enhance plant growth in heavy metal-contaminated soil, where, on the one hand, high presence of bioavailable Fe prevents microbial siderophore production, and, on the other, the presence of metals generally affect microbial auxin synthesis (Dimkpa et al., in press).

The aim of the current study was, thus, to evaluate the role of siderophores elicited in culture filtrates of *Streptomyces* in promoting growth and alleviating metal toxicity in cowpea plants grown in soil from a metal-contaminated field located at the former uranium mining site in Ronneburg, Germany.

2. Materials and methods

2.1. Bacterial strain and growth conditions

Nickel-resistant *Streptomyces acidiscabies* E13 (Amoroso et al., 2000; Schmidt et al., 2005) was obtained from our laboratory strain collection. The strain was maintained as earlier described (Dimkpa et al., 2008).

2.2. Production of siderophores in *Streptomyces acidiscabies* E13

To induce siderophore production, *Streptomyces acidiscabies* E13 was cultured in Fe-deficient siderophore-inducing medium (Alexander and Zuberer, 1991) as earlier described (Dimkpa et al., 2008). The produced siderophores were quantified spectrophotometrically (Genesys 10uv, Thermo Electron Corporation, USA) based on a standard curve of purified desferrioxamine E (DFOE; EMC microcollections, Tübingen, Germany), following the method of Schwyn and Neilands, 1987. Cell-free supernatants were pooled, concentrated to required concentration for subsequent plant growth experiment, and hereafter referred to as siderophore-containing culture filtrate (SCF).

2.3. Detection of auxins in *Streptomyces acidiscabies* E13

Since *Streptomyces acidiscabies* E13 has been shown to produce auxins (Dimkpa et al., 2008); the presence of the latter in the culture filtrate (SCF) of the strain was confirmed by means of the Salkowski assay as described (Patten and Glick, 2002). Indole acetic acid (IAA; Roth, Karlsruhe, Germany) was used to prepare a standard curve. Briefly, a 1 ml aliquot of the supernatant was mixed vigorously with 4 ml of Salkowski's reagent consisting of 150 ml of concentrated H₂SO₄, 250 ml of distilled H₂O, and 7.5 ml of 0.5 M FeCl₃ · 6H₂O, and allowed to stand at room temperature for 20 min before the absorbance (Genesys 10uv, Thermo Electron Corporation, USA) was measured at 535 nm.

2.4. Determination of elemental composition of metal-contaminated soil

Metal-contaminated soil was obtained from our test field located in the former uranium mining site in Ronneburg, Eastern Germany, while normal agricultural soil was collected from a conventional farm field at Jena-Isserstedt also in Eastern Germany. Both soil samples were collected by digging into the soil about 10 cm deep, in three distinct locations of the respective field sites. According to Carlsson and Büchel, 2005, the contaminated

soil collected from this depth is clay. In contrast, the farm soil is dark loamy sand. Samples were taken to the laboratory, stored in plastic bags at room temperature, and analyzed for their elemental composition within 1 month of collection. For this, a pressure digestion system (DAS, PicoTrace, Germany) was used for total digestion of the soil samples. One hundred milligrams of the ground samples were dispensed into polymer vessels. To each were added 4 ml of 40% hydrofluoric acid and 4 ml of 70% HClO₄. The mixture was incubated overnight in closed vessels, after which the vessels were heated to 180 °C for 12 h, and allowed to cool. To enable evaporation of the acids, the system was again heated to 180 °C with the aid of a special evaporation hood. After 12 h, the remaining solid sample was brought to solution by adding 2 ml of HNO₃ (65%, sub-boiled), 0.6 ml of HCl (30%), and 7 ml of pure water (Pure Lab Plus, USF, Germany). To dissolve, the mixture was heated to 150 °C for 10 h. The cooled samples were transferred to calibrated polymer flasks (25 ml; Vitlab, Germany). The solution was increased to 25 ml with pure water and then analyzed with inductively coupled plasma-optical emission spectroscopy (ICP-OES; Spectroflame, Spectro, Germany) for Al, Fe, K, Mg, Mn and P; and with inductively coupled plasma-mass spectrometry (ICP-MS; PQ3-S, Thermo Elemental, UK) for Cd, Cu, Cr, Ni, Pb, Zn and U. To determine bioavailable fractions of the metals, the first step of sequential extraction based on the method of Zeien and Brümmer (1989) was performed. Briefly, 2 g of air-dried soil (<2 mm) was weighed into acid-washed centrifugal cups. To this was added 50 ml of NH₄NO₃ (1 M), and the suspension was shaken (25 rpm, ELU safety lock, Edmund Bühler, Germany) for 24 h. Following the extraction, solution was obtained by centrifugation (2500 rpm) for 15 min. The solution was filtered (cellulose acetate filter, pore size 0.45 µm, Sartorius, Germany) in an acid-washed polypropylene bottle and stabilized with 0.5 ml HNO₃ (65%, sub-boiled). The stabilized extracts were then analyzed for their metal contents using ICP-OES or ICP-MS. The C and N contents of ground soil samples were determined by means of a combustion CNS Analyzer (Vario El, Elementar Analysesyteme GmbH, Hanau, Germany) according to the manufacturer's protocol. Soil pH was determined using an inoLab pH 720 meter (WTW GmbH, Weilheim, Germany), from aliquots of the soil mixed in deionized water (2:1 w/v) while stirring at room temperature.

2.5. Plant material and growth conditions

Approximately 1 month after soil sampling, 1 kg of heavy metal contaminated soil was loaded into dark plastic pots and subsequently supplemented with 200 ml of SCF containing ≈200 µM and 14.5 µM of concurrently-produced hydroxamate siderophores (DFOB, DFOE and coelichelin (Cch)) and microbial auxins, respectively. The control treatment consisted of 200 ml of uninoculated siderophore-inducing medium supplemented with 14.5 µM pure IAA, to account for the determined auxin content of SCF. Since no bacteria were added to this medium, this treatment lacked the siderophores contained in SCF. For a better appreciation of the effects of metal contamination on plant growth, the soil collected from normal farm field was simultaneously potted. However, this soil was not given any further treatment, in contrast to the contaminated soil. Subsequently, cowpea seeds, one per pot, were sown in the potted soils. Each treatment was replicated 10 times. The pots were placed on plant growth platforms in a conventional green house and grown for a period of 3 months. Watering was done when necessary and no artificial lighting was provided since the growth period was during summer, with sufficient sunlight, and daily temperatures often reaching as high as 30 °C. In addition, SCF (as well as control) treatments were re-administered every 2 weeks (6 times in total) by sub-surface incorporation of the respective solutions (200 ml) into the soil around the plant roots.

Ultimately $\approx 0.72 \text{ mg g}^{-1}$ soil and 0.02 mg g^{-1} soil respectively, of siderophores (DFOE equivalent) and auxins (IAA equivalent) were supplied. The plants grown in the farm soil are hereafter referred to as metal stress-free (MSF) plants.

2.6. Determination of chlorophyll content in plants

Fresh samples were harvested from the two youngest leaves of the plants for chlorophyll content determination. Chlorophyll extraction and calculation of total chlorophyll contents of the leaves were performed as previously described (Tait and Hik, 2003).

2.7. Determination of lipid peroxidation in plants

The level of lipid peroxidation in plant roots and leaves was analyzed by the thiobarbituric acid (TBA) method which determines malondialdehyde (MDA) as the end-product of lipid peroxidation. Briefly, 1 g of fresh plant material was ground in 3 ml of trichloroacetic acid (1% w/v). The homogenate was centrifuged at $14,000 \times g$ for 15 min to clarify the solution, and 0.5 ml of the supernatant was mixed in 3 ml of TBA (0.5% w/v) prepared in 20% trichloroacetic acid and incubated at 95°C for 50 min. Absorbances were measured at 532 nm and 600 nm (Genesys 10uv, Thermo Electron Corporation, USA), with values from the later subtracted from the former to eliminate non-specific absorption. The concentration of MDA was calculated using an extinction coefficient of $155 \text{ mM}^{-1} \text{ cm}^{-1}$ (Cakmak and Horst, 1991).

2.8. Determination of RNA content of plant leaves

The upper second fully-expanded leaves of 45-day-old experimental plants were harvested for RNA isolation using the protocol described in the RNeasy Mini Handbook (2006) for the purification of total RNA from plants (Qiagen, Hilden, Germany). The purified RNA was subsequently quantified using a NanoDrop[®] ND-1000 UV-Vis spectrophotometer (NanoDrop Technologies, Wilmington, USA).

2.9. Total protein determination from cowpea leaves

Simultaneously with RNA isolation, 250 mg of the harvested leaves were analyzed for their protein contents based on the procedure described in the Plant Total Protein Extraction Kit (Sigma, Steinheim, Germany). Protein concentrations were determined by the method of Bradford (1976).

2.10. Measurement of superoxide dismutase activity

Superoxide dismutase (SOD) activity in cowpea plants was assayed using protein samples isolated as described above. This was performed based on the method described by McCord (1999) with slight modifications. Briefly, 75 μl of cytochrome *c* solution (26 mg cytochrome *c*, Sigma, Steinheim, Germany; 10 ml sodium phosphate buffer [50 mM Na_2HPO_4 :50 mM NaH_2PO_4 , 3.98:1 v/v; pH 7.8]) was transferred to a 2.5 ml cuvette. To this was added 100 μl xanthine solution (3 mg xanthine, Sigma, Steinheim, Germany; 10 ml of sodium phosphate buffer, pH 7.8; 1–2 drops of NaOH solution), followed by 500 μl of the protein sample and 500 μl of sodium phosphate buffer. The mixture was placed on a spectrophotometer (Ultrospec 2100[®] pro, Amersham Biosciences, Cambridge, UK). To start the reaction, 500 μl of xanthine oxidase solution (50 μl xanthine oxidase, Serva, Heidelberg, Germany; 10 ml sodium phosphate buffer) was added. The reaction was followed using a software program (SWIFT II) executed on a PC connected to the spectrophotometer. A control reaction having all the components of the enzyme reaction except the protein sample was

simultaneously performed. SOD activity (Units) was calculated as follows: control slope – sample slope/(control slope/2).

2.11. Determination of carotene content in plants

The carotenoid content in leaves of fresh plants was determined as described by Sprecher et al. (1998).

2.12. IAA oxidase assay in cowpea

The oxidation of IAA in cowpea was determined by measuring the IAA content of a protein reaction mixture following the method of Johri et al. (2005) with slight modifications. Briefly, 100 μl of IAA (1 mM) was added to glass vials containing 100 μl of 2,4-dichlorophenol (2,4-DCP; 1 mM) and 100 μl of MnCl_2 (1 mM). To this was added 67 μl of 0.1 M acetate buffer (1.098 g^{-1} acetic acid + 11.12 g^{-1} sodium acetate; pH 5.4), and the reaction was started by adding 5 μl of protein samples from the respective experimental plants. The vials were incubated at 30°C simultaneously with a control assay which contained no protein sample. After 50 min, Salkowski reagent (660 μl) was added to each vial, and the samples were further incubated in the dark for 35 min to allow for color development. Absorbances of the samples were measured at 525 nm, and IAA oxidase activity was determined by calculating residual IAA in each treatment based on a standard curve of purified IAA.

2.13. Plant metal contents measurement

Plants from growth experiments were prepared for metal contents analysis. To this end, shoots were separated from roots and the latter were then rinsed in water and washed twice in EDTA solution, followed by repeated rinsing in distilled water, to remove adsorbed metals. Root and leaf metal contents were subsequently determined as previously described (Dimkpa et al., 2008).

2.14. Detection of metals competitively bound to siderophores

Liquid chromatography–electrospray ionization mass spectrometry (LC–ESI–MS) was performed with SCF amended with Al, Fe and Cu to study competition for these metals by siderophores. To 1 ml of SCF (pH 5) Fe, Al or Cu was added to a final concentration of 100 μM . The solutions were shaken briefly but vigorously, and the formation of siderophore–metal complexes was allowed to proceed at room temperature. After 24 h, 100 μM of Fe was added to the Al treatments, and vice versa. Similarly, 100 μM of Fe was added to the Cu treatments, and vice versa. The mixtures were incubated for a further 24 h, after which LC–ESI–MS was performed to detect metal-bound siderophores. Mass peaks were determined based on purified DFOE and the molecular masses of DFOE–Al/Cu/Fe complexes as described previously (Dimkpa et al., 2008).

2.15. Statistical analysis

Data were analyzed for variance (ANOVA) and where significant, treatments were separated using the Tukey test (Tukey, 1951).

3. Results

3.1. Determination of elemental composition of metal-contaminated soil

As indicated in Table 1, Al, Fe, Cu, Mn, Ni, U and K were more present in the contaminated soil than in the farm soil. In contrast, Cd content was similar in both soils, while more C, Cr, Mg, N, P, Pb and Zn were detected in the farm soil. However in terms of metal

Table 1

Concentrations ($\mu\text{g g}^{-1}$ dry mass) of elements and pH of soils used in plant growth experiments.

Element	Total content		Bioavailable fraction		
	Farm soil	Contaminated	Farm soil	Contaminated	
Heavy/trace metals	Al	30183 \pm 1539	61855 \pm 692	1.07 \pm 0.06	22.6 \pm 0.30
	Fe	16411 \pm 1016	36410 \pm 574	3.82 \pm 0.24	0.50 \pm 0.02
	Cu	22.3 \pm 1.73	50.1 \pm 1.9	0.025 \pm 0.002	0.025 \pm 0.003
	Ni	20.0 \pm 0.88	48.9 \pm 1.1	0.26 \pm 0.04	4.87 \pm 0.10
	Cd	0.45 \pm 0.036	0.45 \pm 0.01	0.04 \pm 0.004	0.09 \pm 0.001
	Pb	42.5 \pm 5.59	18.5 \pm 1.7	0.053 \pm 0.004	0.028 \pm 0.002
	Zn	180 \pm 13.65	72.1 \pm 2.0	1.97 \pm 0.106	1.15 \pm 0.13
	Cr	42.5 \pm 16.30	38.0 \pm 2.0	0.025 \pm 0.003	0.025 \pm 0.002
	Mn	436 \pm 32.4	569 \pm 24	33.0 \pm 1.0	63.0 \pm 1.0
	U	1.56 \pm 0.10	5.54 \pm 0.20	0.0025 \pm 0.0006	0.015 \pm 0.0007
Nutrient elements (total)	P	1838 \pm 186	576 \pm 4.0		
	K	14526 \pm 885	21499 \pm 183		
	Mg	5302 \pm 188	4428 \pm 91		
	C	8.65 \pm 0.2	0.13 \pm 0.025		
	N	0.045 \pm 0.021	0.01 \pm 0.003		
pH		7.35 \pm 0.07	5.28 \pm 0.12		

Values represent means \pm SD ($n = 3$).

bioavailability, Al, Cd, Mn, Ni and U were more bioavailable in the contaminated soil, whereas more bioavailable Fe, Pb and Zn were detected in the farm soil. Contrary to these results, Cr and Cu showed similar levels of bioavailability in both soils. The soil pH measurements indicated that the contaminated soil is acidic while the farm soil is alkaline (Table 1).

3.2. Siderophore-containing filtrates promote plant growth in metal contaminated soil

After 3 months, cowpea plants in metal contaminated soil amended with siderophore and auxin-containing culture filtrates (SCF) of *S. acidiscabies* E13 showed dramatic growth differences than control plants which lacked siderophores, but which were, nevertheless, treated with pure IAA to compensate for the auxin content of SCF. Growth of control plants was dramatically impeded, with obvious loss of leaf pigmentation (Fig. 1a). Consequently, there was significantly better plant height, greater root mass and total biomass in SCF plants than in their control counterparts, both of which, however, showed less overall growth in comparison to metal stress-free (MSF) plants (Fig. 1b).

3.3. Effect of siderophores in SCF on chlorophyll, MDA, RNA and protein contents of cowpea

With respect to chlorophyll contents, leaves of SCF plants were substantially greener than control plants, which showed clear loss of green pigment. However, the chlorophyll contents of siderophore-treated plants were similar to MSF plants (Fig. 2). Plant lipid peroxidation was measured as a function of TBA reactivity, which releases MDA as a final product. MDA contents in roots and leaves of plants growing under metal stress were significantly elevated in control treatments lacking siderophores. Leaves of SCF-treated plants showed only slightly elevated MDA levels as indicated by comparison with MSF plants (Fig. 2). RNA and protein isolation from leaves of the experimental plants indicated higher total contents per gram fresh weight in MSF plants. In comparison to this, there was approximately 60% and 17.5% reduction, respectively, in the total RNA and protein contents of SCF plants, and further 20% and 75% reductions, respectively, in control plants (Fig. 2).

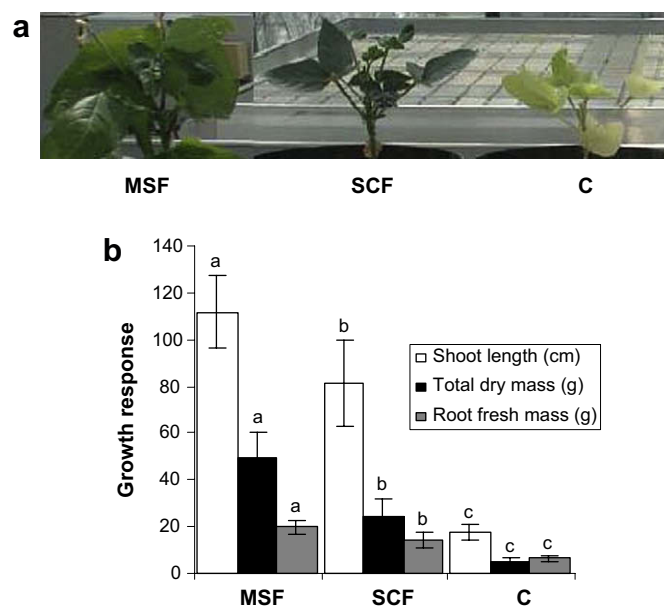


Fig. 1. Photograph of metal-free, siderophore-treated and control plants and growth response of cowpea in metal contaminated soil. (a) Photograph of metal stress-free (MSF), siderophore-treated (SCF), and control (C) cowpea plants showing the influence of microbial hydroxamate siderophores on cowpea growth under metal toxicity. (b) Growth response of cowpea in metal contaminated soil in the presence (SCF) and absence (C) of siderophores. Values represent means \pm SD of 10 replicates and bars with different letters for each growth parameter are significantly different at $P = 0.05$.

3.4. Effect of siderophores on SOD and carotenoid content of cowpea

In agreement with MDA contents, SOD activities showed almost no effect of metal stress in SCF-treated leaves while significantly higher SOD activity was obtained from control plants. In contrast to SOD, however, carotenoid content was significantly higher in SCF (similar to that in MSF plant) than in control treatment (Fig. 3).

3.5. Effect of siderophores on IAA oxidation in cowpea

The oxidation of IAA resulting from oxidative stress was assayed using protein samples from cowpea shoots and roots. As indicated from residual IAA concentrations, significantly more IAA was oxidized by peroxidases in the protein samples derived from both leaves and roots of control treatments, as compared to SCF plants. Oxidation of IAA was reduced to levels observed in MSF plants in leaf but not root protein samples of SCF plants (Fig. 4).

3.6. Metal contents of plant root and shoot

The analysis of metal uptake by plants indicated that roots generally contained by far higher amounts than shoots, of all the metals analyzed, with the exception of Mn, in both SCF and control treatments, and Ni in control treatment. Furthermore, aside from Ni, we consistently observed very high amounts of the metals in roots of SCF plants, relative to their control counterparts. In contrast to these results, shoot Mn and Fe contents were higher than other metals, and metal contents were generally statistically similar in shoots of SCF and control treatments, with the exception of Fe, which was significantly higher in SCF plants. On the other hand, although shoot U and Ni contents were both higher in control than SCF plants, U, but not Ni, was significantly higher (Fig. 6). However, when total plant biomass is considered, there was, by far, a higher uptake of all metals in SCF, than in control plants (Table 2).

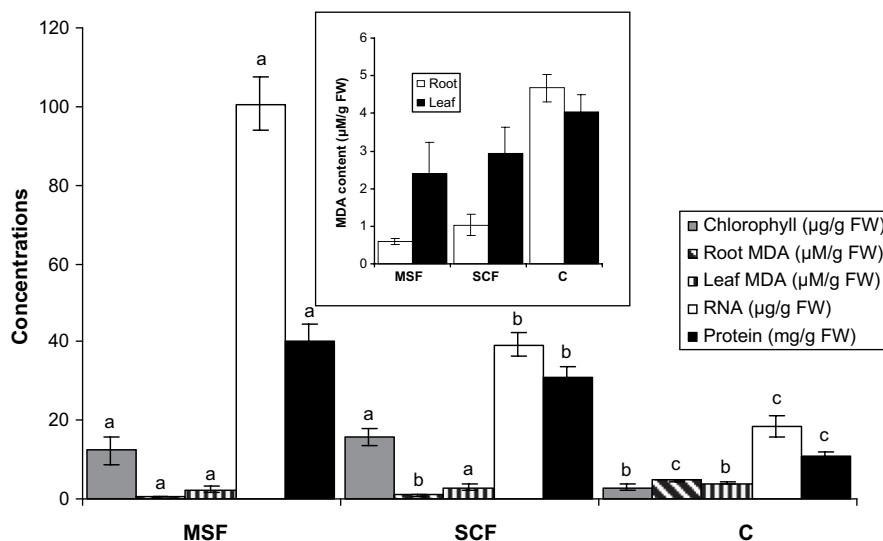


Fig. 2. Effect of siderophore-containing culture filtrate (SCF) on chlorophyll, root and leaf MDA, RNA, and protein contents of cowpea under metal stress. Values represent means \pm SD of five replicates, and bars with different letters in each parameter indicate significant differences at $P = 0.05$. Insert is an enlarged representation of MDA data.

3.7. Trivalent, but not divalent metals are competitive for siderophore binding

Since siderophores in SCF have a protective function on plant growth under metal stress, their ability to sequester toxic metals was tested. Therefore, metal competition assays were conducted to demonstrate competitive siderophore binding between trivalent cations (Fe and Al), on the one hand, and between a trivalent (Fe) and a divalent cation (Cu), on the other. LC-ESI-MS indicated that as much as 27% of bound Al was displaced by Fe from a DFOE-Al complex. Al, in turn, was shown to displace about 18% of DFOE-bound Fe. In contrast, Cu showed poor affinity towards DFOE, as more than 80% of the DFOE remained free of Cu, in comparison to the mass peak of unbound DFOE. Moreover, the available DFOE-Cu complex was completely displaced by Fe, while the addition of Cu had no effect on the stability of DFOE-Fe complex. Instead, Fe formed complexes with a large abundance of DFOE that initially could not bind Cu (Fig. 5).

4. Discussion

4.1. Siderophore-containing filtrates promote plant growth in metal contaminated soil

In this work, the pH of the contaminated soil was slightly acidic, unlike the farm soil which was calcareous (Table 1); an indication that Fe and, indeed, other metals present in the former soil should be in a more soluble state. Despite the significantly higher amount of total Fe present in the contaminated soil than in the farm soil, however, bioavailable Fe was greater in the latter, a condition which might arise considering the hypothesis on the possible formation of un(bio)available polymers between Fe and other metals (Davis and Byers, 1971). Moreover, it is known that dependent on specific metals, clay soils are a good adsorbent for metals, thereby affecting their solubility and bioavailability (Owojori et al., 2009, and references therein), possibly even during chemical extraction procedures. This may, therefore, explain the low bioavailability of some of the metals in the contaminated soil. Thus, taking into account the

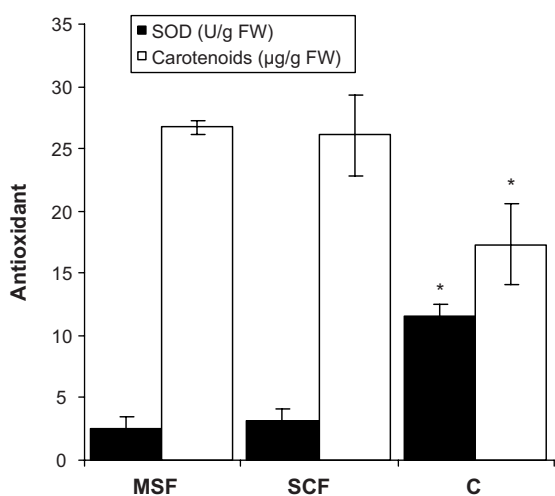


Fig. 3. Antioxidative response of metal-stressed cowpea, and the effect of SCF on superoxide dismutase (SOD) activity and carotenoids content of cowpea under metal stress. Values represent means \pm SD of three (SOD) and five (carotenoids) replicates, and asterisked bars in each parameter indicate significant differences at $P = 0.05$.

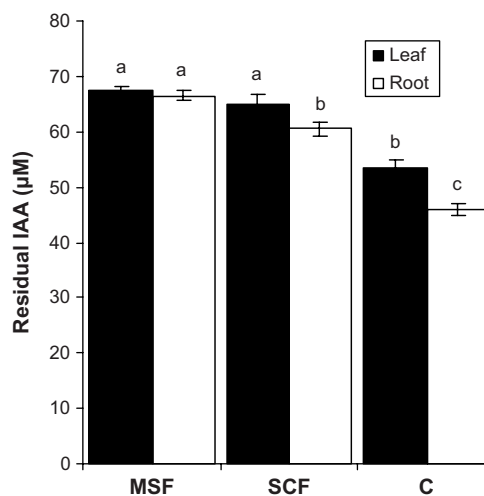


Fig. 4. Effect of siderophore-containing culture filtrate (SCF) on IAA oxidation in leaves and roots of cowpea under metal stress. Values represent means \pm SD of three replicates, and bars with different letters in each organ are significantly different at $P = 0.05$.

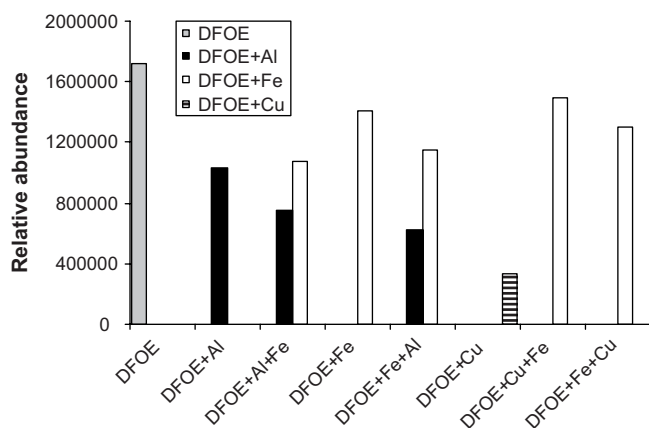


Fig. 5. Metal competition assays showing the relative abundances of desferrioxamine E (DFOE)-bound Al, Fe and Cu as determined from LC-ESI-MS measurements. Representative values of one experiment are shown.

bioavailable concentrations of the individual metals in both soil types, it might be reasonable to conclude that the retarded growth in cowpea reported here was due to the toxic effects mainly of Al, Mn, Ni and U. To test how metal-chelating microbial products can influence the toxic effects of these metals, an external application of siderophores was made using *Streptomyces* cell-free culture filtrate, which showed a dramatic alleviation of the plant growth impediment imposed by the metals.

4.2. Effects of siderophores contained in bacterial culture filtrate on the metabolism of metal-stressed cowpea

In human, siderophores are known to alleviate metal-induced oxidative stress by lowering the formation of free radicals (Brown et al., 1982; Malluche et al., 1984; Allain et al., 1987). In line with this, siderophores contained in SCF produced dramatic effects in several physiological components of the cowpea plants, thus contributing to the observed growth response. The first evidence for the involvement of oxidative stress is loss in leaf pigmentation (Fig. 1a). Thus, chlorophyll and carotenoid reduction under the experimental condition indicate metal-mediated ROS activation as previously reported (Krupa et al., 1996; Panda et al., 2003; Sinha and Saxena, 2006; Gajewska and Sklodowska, 2007). Addition of siderophores, however, resulted in greener leaves than even in MSF plants, the soil of which contained higher amounts of bioavailable Fe (Table 1) than the contaminated soil. This reflects the power of siderophores to solubilize Fe for plant use, even in the presence of metals that otherwise inhibit Fe uptake (Burd et al., 2000; Dimkpa et al., 2008).

Loss of chlorophyll usually correlates with lipid peroxidation, and since the latter is a marker for cellular oxidative damage (Cakmak and Horst, 1991; Bueno and Piqueras, 2002; Sinha and Saxena, 2006; Stobrawa and Lorenc-Plucińska, 2007), its level in the plants was assessed. As indicated by the high MDA content, significant lipid peroxidation occurred in plants under metal stress conditions, with dramatic effects in the control treatment. In the case of SOD, its response in metal-stressed plants has been controversial (Gratão et al., 2005); however, our result agrees with previous reports (Shainberg et al., 2000; Bueno and Piqueras, 2002; Ghanati et al., 2005; Guo et al., 2007; Stobrawa and Lorenc-Plucińska, 2007). Apparently, the activation of specific antioxidant enzymes (for example, SOD) may be insufficient to confer tolerance to long exposure to metal-induced oxidative stress, as the rate of formation of free radicals may overwhelm the plant's antioxidant capacity (Becana et al., 1998), resulting in such observation as in the control plants, and even to cell death (Boscolo et al., 2003).

In further support of the role of cellular oxidative damage, we observed a significant reduction in total RNA and protein concentrations in the metal-stressed plants, which was more substantial in the control treatment lacking siderophores. Metals such as Al have been shown to decrease the rate of RNA synthesis by binding to DNA (Matsumoto and Morimura, 1980), which, invariably, reflects on protein contents (Burd et al., 2000; Guo et al., 2007; this study). Since, in comparison with MSF plants, growth was reduced in metal contaminated soil treated with either microbial or pure auxins, it was necessary to determine the role, if any, of IAA oxidation in our system. To this end, IAA oxidase assay showed less residual IAA in the control treatment, indicative of a higher expression of stress-inducible IAA oxidase enzyme(s). The oxidative degradation of IAA has been reported in metal-stressed plants, and is known to be catalyzed by several isoforms of peroxidases. Some of these peroxidases with high activity under oxidative stress conditions (Chaoui and El-Ferjani, 2005; Johri et al., 2005; Potters et al., 2007) are capable of degrading IAA using 2,4-DCP and Mn ions as cofactors (Johri et al., 2005).

4.3. Metal contents of plant root and shoot

Plant root activities, which can be influenced by local rhizosphere soil conditions, will affect actual metal uptake by plants. Thus, the statistically similar shoot Fe contents obtained for control and MSF plants (224 versus 208 $\mu\text{g g}^{-1}$ shoot dry mass, respectively) underscores the role of low pH in the solubilization and uptake of Fe, as was also shown by Bar-Ness et al. (1991); despite the inhibition in Fe acquisition which other metals might impose. Nevertheless, unlike SCF plants, the impact of the pH-enhanced Fe absorption by control plants was, apparently, not reflected in the chlorophyll content of these (control) plants, due to the counteracting effect of lipid peroxidation on chlorophyll content (Cakmak and Horst, 1991; Bueno and Piqueras, 2002; Sinha and Saxena, 2006). Depending on the presence or not of siderophores in the contaminated soil, the plant root contents of Cd, Cr, Cu, Fe, and Ni, are more or less than reported for plants grown in a polluted soil (Stobrawa and Lorenc-Plucińska, 2007). But, in particular, the Mn content reported in the current study is higher by far than reported by these workers. It should be noted, however, that the soil and plants used in both studies are different.

On the basis of overall high metal content in root than shoot of SCF plants, it seems that solubilization of metals by siderophores resulted in the metals becoming concentrated in the root apoplast (Vansuyt et al., 2003); however, apart from Fe, release of the metals into the symplast was somehow affected, resulting in a lowered concentration of toxic metals translocated to the shoots of these plants. This effect may be due to differential transport of metals from root to shoot, which depends on their differential mobility and loading into xylem vessels (Page and Feller, 2005). Nevertheless, the addition of siderophores to contaminated soil resulted in an overall higher metal contents than in control plants (Table 2), due mainly to the significantly larger biomass of SCF plants, which in turn had a dilution effect on shoot metal contents, thereby reducing the toxic effects of the metals. Because metals adsorbed to root surfaces were removed by treatment with EDTA, it is certain that the root metal contents reflect true amounts that had been absorbed by the plant. From a practical point of view, a higher overall amount of metals in the biomass of siderophore-treated plants indicates a potential for the use of microbial siderophores and auxins as cheaper and more sustainable alternatives to synthetic chelators (e.g., EDTA) and purified IAA, both of which are currently used for assisted phytoremediation of metal pollution in soil (White, 2001; Lopez et al., 2005, 2007; Liphadzi et al., 2006). This application will be subject to an optimization of siderophore concentrations, followed by comparative tests of both types of

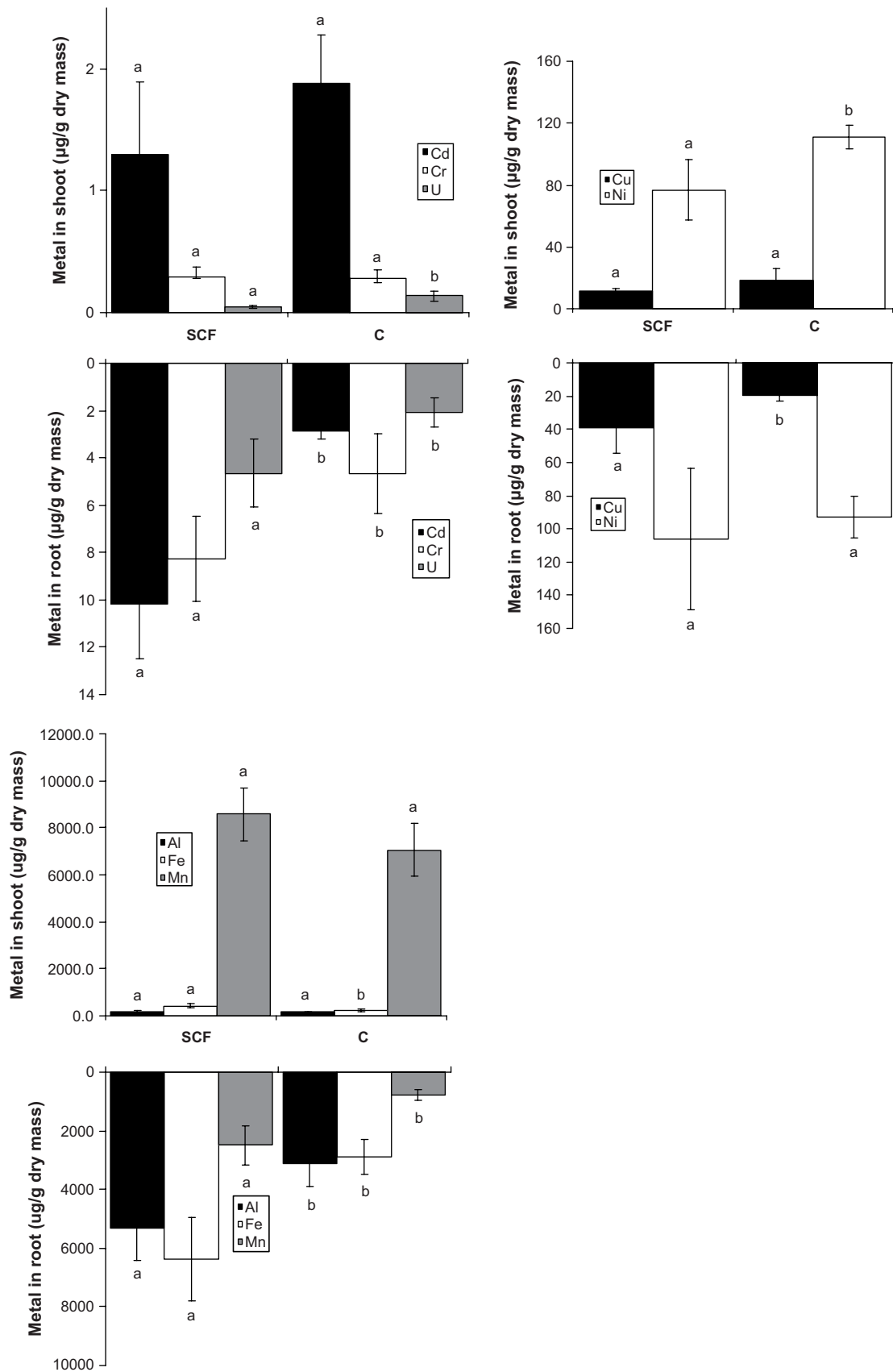


Fig. 6. Effect of siderophore-containing culture filtrate (SCF) on metal contents of shoot and root of cowpea. Values represent the means \pm SD of four replicates, and bars having different letters indicate significant differences among treatments ($P = 0.05$). Graph was separated (in three places) based on values on Y axis, to enhance the visualization of metals with low values.

Table 2

Effect of siderophore-containing culture filtrate (SCF) on total (combined μg of metals in root and shoot) uptake of metals by cowpea.

Plant metal content (Root + shoot)	Treatment	
	SCF	Control
Al	77898.3	19668.6
Cd	162.0	18.1
Cr	121.3	29.5
Cu	717.4	122.7
Fe	95865.9	18339.3
Mn	155805.0	4792.4
Ni	2580.6	585.5
U	66.2	16.1

Values were determined as the product of average metal concentration ($n = 4$) and average weight of plants ($n = 10$).

chelators, and the selection of appropriate metal-accumulating plants; and is a subject of future research.

4.4. Trivalent, but not divalent metals are competitive for siderophore binding

Chelation of Al and Cd by phytosiderophores released by stressed maize has been suggested as a metal toxicity-prevention strategy (Hill and Lion, 2002; Poschenrieder et al., 2005). Cowpea being a strategy I plant, and hence, phytosiderophore non-releasing, microbially-produced metal chelators exogenously applied were investigated for their role in alleviating soil metal toxicity in plants. Of the three hydroxamate siderophores produced by *S. acidiscabies* E13 (Dimkpa et al., 2008), we chose DFOE as a representative siderophore for studying metal competition based on its exceptionally strong affinity for metals (Fernández and Winkelmann, 2005). As indicated, despite possessing the highest affinity towards Fe, Al can compete with Fe for siderophore binding. In contrast, Cu showed formation of a weak complex with DFOE, such that the DFOE–Cu complex was completely displaced by Fe. Cu was used to study metal competition because its complex formation with a related siderophore (DFOB) is already known (Hernlem et al., 1996). Though relatively low, the pH of the contaminated soil (Table 1) is not sufficiently acidic to disallow a reversible redox process, which promotes Fe(III) complexation by siderophores (Boukhalfa and Crumbliss, 2002). Thus, in support of existing reports on the comparative affinities of hydroxamate siderophores to metals, both trivalent and divalent (Evers et al., 1989; Martell et al., 1995; Hernlem et al., 1996; Fernández and Winkelmann, 2005), this *in vitro* competition study suggests that DFOE may also preferentially bind to trivalent cations in the soil, driven by prevailing soil chemical conditions.

4.5. Conclusion

In conclusion, the role of siderophores in the current study can be viewed from their ability to supply plants with adequate Fe, in the presence of inhibitory levels of other metals. This resulted, for instance, in enhanced chlorophyll content. However, a major effect of the siderophores is its binding of metals in the immediate vicinity of the roots which apparently lowered free radical formation, protecting auxins from oxidative degradation. Since pure auxins in the control plants lacking siderophore treatment were more severely affected by oxidative degradation, the implication is that siderophore-treated plants were kept as healthy as possible, enabling the microbial auxins to function in the development of roots. This role was negated in the absence of siderophores, seemingly due to enhanced oxidative stress. This resulted in superior root growth, which consequently augmented metal uptake in siderophore-treated plants.

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Manuscript IV

Siderophores mediate reduced and increased uptake of cadmium by *Streptomyces tendae* F4 and sunflower (*Helianthus annuus*), respectively.

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1 **Siderophores mediate reduced and increased uptake of cadmium by *Streptomyces tendae* F4**
2 **and sunflower (*Helianthus annuus*), respectively**

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22 **ABSTRACT**

23 **Aims:** As a toxic metal, cadmium affects microbial and plant metabolic processes, thereby
24 potentially reducing the efficiency of microbe or plant-mediated remediation of Cd polluted soil. The
25 role of siderophores produced by *Streptomyces tendae* F4 in the uptake of Cd by bacteria and plant
26 was investigated to gain insight on the influence of siderophores on Cd availability to
27 microorganisms and plants.

28 **Methods and Results:** The bacterium was cultured under siderophore-inducing conditions in the
29 presence of Cd. The kinetics of siderophore production and identification of the siderophores and
30 their metal-bound forms were performed using electrospray ionization mass spectrometry.
31 Inductively-coupled plasma spectroscopy was used to measure Fe and Cd contents in the bacterium
32 and in sunflower plant grown in Cd-amended soil. Siderophores significantly reduced Cd uptake by
33 the bacterium, while supplying it with iron. Bacterial culture filtrates containing three hydroxamate
34 siderophores secreted by *S. tendae* F4 significantly promoted plant growth and enhanced uptake of
35 Cd and Fe by the plant, relative to the control. Furthermore, application of siderophores caused
36 slightly more Cd, but similar Fe uptake, compared to EDTA. Bio-inoculation with *Streptomyces*
37 caused a dramatic increase in plant Fe content, but resulted only in slight increase in plant Cd
38 content.

39 **Conclusion:** It is concluded that siderophores can help reduce toxic metal uptake in bacteria, while
40 simultaneously facilitating the uptake of such metals by plants. Also EDTA is not superior to
41 hydroxamate siderophores in terms of metal solubilization for plant uptake.

42 **Significance:** The study showed that microbial processes can indirectly influence the availability and
43 amount of toxic metals taken up from the rhizosphere by plants. Furthermore, although EDTA is
44 used for chelator-enhanced phytoremediation, microbial siderophores would be ideal for this
45 purpose.

46 **keywords:** α -aminocyclopropane-1-carboxylic acid deaminase, cadmium, biochelator-assisted
47 phytoremediation, siderophores, *Streptomyces tendae* F4

48 **INTRODUCTION**

49 Cadmium (Cd) is a soil pollutant which affects the biodiversity and activity of soil microbial
50 communities (McGrath, 1994) and causes oxidative stress (Guo et al. 2004; Chaoui and Ferjani,
51 2005), reduced enzyme activity (Chang et al. 2003; Yoshihara et al., 2006), and consequent cell-
52 damaging and growth-retardation effects in plants (Di Toppi and Gabbrielli, 1999). Microbes have
53 evolved several strategies to circumvent heavy metal toxicity, among which efflux pumping systems,
54 sequestration and immobilization in cellular compartments have been described (Prasad, 2001). Plant
55 response against the toxic effects of Cd mainly involves the modification of various antioxidant
56 responses, including the regulation of super oxide dismutases, catalases and various kinds of
57 peroxidases (Guo et al. 2004; Chaoui and Ferjani, 2005; Qui et al. 2008), as well as modulation of
58 transport proteins such as P-type ATPases or multidrug transporters (see for e.g., Hall and Williams,
59 2003, and references therein). Bio-augmentation of Cd-stressed plants with bacteria may influence
60 Cd uptake from contaminated soil in two ways: (i) if the bacteria adsorb the metal (Andreoni et al.
61 1991; Valentine et al. 1996), the concentration of metals available for plant uptake may be reduced
62 (Sinha and Mukherjee, 2008); and (ii), if siderophores produced by the bacteria solubilize the metals,
63 the bioavailability of Cd may be enhanced (Dimkpa et al., 2009). Siderophore-mediated uptake of
64 metals by bacteria and plants depends on the recognition or not, of the siderophore-metal complex by
65 an uptake receptor (Wang et al. 1993; Vansuyt et al., 2007; Greenwald et al. 2008).

66 *Streptomyces tendae* F4 is a Cd-resistant bacterium (Schmidt et al., 2005), which can
67 simultaneously produce a variety of hydroxamate siderophores that bind Cd, the production of which
68 is up-regulated by the metal, in the absence or presence of (low) Fe concentrations (Dimkpa et al.
69 2008a). The increased production of siderophores in the presence of Cd by *S. tendae* F4 might be
70 interpreted as a resistance mechanism against Cd.

71 The use of synthetic metal chelators for assisted-phytoremediation is a common practice, with
72 EDTA being the most commonly used (see for example, Kirkham, 2000; Chen and Cutright, 2001;
73 Liphadzi et al. 2003; Wu et al. 2004; Lopez et al. 2005; Luo et al. 2005; Wenger et al. 2005;).

74 However, in place of synthetic chelators, the use of biologically-originated hydroxamate
75 siderophores contained in culture filtrates of bacteria has been recommended (Dimkpa et al. 2009).
76 Hydroxamate siderophores are readily degradable by rhizosphere bacteria (Winkelmann et al. 1999),
77 and, thus, do not constitute a subsequent source of environmental pollution. EDTA, on the other
78 hand, is not readily degraded, which causes metal leaching to distant soils (Bucheli-Witschel and
79 Egli, 2001; White, 2001; Wu et al. 2004; Nowack et al. 2006). Fe-deficient cultures of *Streptomyces*
80 spp, including *S. tendae* F4, are known to contain three different hydroxamate siderophores, namely
81 desferrioxamine B (DFOB), desferrioxamine E (DFOE) and coelichelin (Cch) (Barona-Gomez et al.
82 2006; Dimkpa et al. 2008a, b), which, together with concomitantly-produced auxins, enhance plant
83 growth and uptake of metals from a heterogeneously contaminated soil (Dimkpa et al. 2009). In
84 order to test our recommendation, the effect of siderophore production on the bioavailability of Cd to
85 *S. tendae* F4 was investigated. Subsequently, siderophores excreted into culture solutions of the
86 bacterium was compared with EDTA for Cd and Fe uptake by sunflower (*Helianthus annuus*) grown
87 in soil amended with Cd.

88 **MATERIALS AND METHODS**

89 **Bacterial strain and growth conditions**

90 *Streptomyces tendae* F4 was cultured under Cd (100 μ M CdCl₂) stress and, where applicable, in the
91 presence of Fe (100 μ M FeCl₃) as previously described (Dimkpa et al. 2008a). Depending on specific
92 experiments, the following treatments were administered: -Fe-Cd, -Fe+Cd, +Fe-Cd and +Fe+Cd.
93 Cells were grown for 3 days and harvested via centrifugation at 4000g. Bacterial biomass from -
94 Fe+Cd, +Fe-Cd and +Fe+Cd treatments were subsequently treated with 0.2 M EDTA to eliminate
95 unbound Cd and Fe; washed several times in de-ionized water, and stored at -20 °C until used for
96 metal content analyses. The supernatants were used for siderophore quantification. All cultures were
97 replicated 3 times.

98 **Quantification of siderophore production in *S. tendae* F4**

99 Siderophores produced by *S. tendae* F4 was quantified by the CAS assay of Schwyn and Neilands
100 (1987) with the modification previously described (Dimkpa et al. 2008a). As with our previous
101 studies (Dimkpa et al. 2008b, 2009), the siderophores contained in this culture filtrate are
102 subsequently referred to as SCF (siderophore-containing culture filtrate).

103 **Kinetics study of siderophore production in *S. tendae* F4**

104 To study the kinetics of siderophore production by *S. tendae* F4, the strain was cultured in iron-
105 deficient (siderophore-inducing) medium between 24 h and 120 h. Cells were harvested by
106 centrifugation, and the culture filtrates were analyzed using electro spray ionization mass
107 spectrometry (ESI-MS) for their hydroxamate siderophore content, as previously described (Dimkpa
108 et al. 2008a, b).

109 **Estimation of auxin content of bacterial culture**

110 The presence of auxins in 72 h-old, Fe-deficient bacteria cultures (namely -Fe-Cd) grown in the
111 absence of added tryptophan was estimated as previously described (Dimkpa et al. 2009).

112 **ACC deaminase assay in *S. tendae* F4 under different metal interplay**

113 α -Aminocyclopropane-1-carboxylic acid deaminase (ACCD) assay is used to determine the ability of
114 bacteria to degrade plant ACC, the precursor of ethylene, thereby lowering stress ethylene levels
115 which otherwise affect plant growth (Glick and Penrose, 1998). Thus, ACCD activity was assayed on
116 *S. tendae* F4 according to the method described by Penrose and Glick (2003), with slight
117 modifications: instead of the medium used above, the siderophore-inducing medium of Alexander
118 and Zuberer (1991) was used. However, NH_4Cl in the siderophore-inducing media recipe was
119 replaced with 3 mM α -aminocyclopropane-1-carboxylic acid (ACC) as the sole nitrogen source. The
120 medium was then differentially amended as follows: -Fe-Cd/-Ni; -Fe+Cd/+Ni; +Fe-Cd/-Ni, and
121 +Fe+Cd/+Ni, at 100 μM each of Fe, Cd and Ni, where present. Cultures were incubated for 72 hours
122 and the ACCD assay was conducted thereafter, using a serially-diluted α -ketobutyrate (2-oxobutyric
123 acid; Sigma-Aldrich, Schnellendorf, Germany) solution to prepare a standard curve.

124 **Soil characteristics and treatments**

125 The soil used in the plant growth experiment, which dark and loamy sand in texture, was obtained
126 from farmer's field in Jena-Isserstedt, Thuringia, Eastern Germany, and had been analyzed for its
127 relevant chemical composition, showing among other elements, a total Cd concentration of 0.45
128 $\mu\text{g}\cdot\text{g}^{-1}$ and a bio-available concentration of 0.04 $\mu\text{g}\cdot\text{g}^{-1}$ (Dimkpa et al. 2009). The soil was sterilized
129 by autoclaving twice at 121 °C for 30 min to eliminate native bacteria; no further aseptic measures
130 were taken thereafter. Soil pH was then measured as described (Dimkpa et al., 2009). Two kg of soil
131 in dark plastic pots were spiked with 27.5 $\mu\text{g}\cdot\text{g}^{-1}$ of Cd by dissolving the Cd in 100 mL of distilled
132 water and mixing the solution into the soil. Each soil subsequently received the following differential
133 treatments: 7.6 $\mu\text{g}\cdot\text{g}^{-1}$ of microbial siderophores (SCF from -Fe-Cd treatment; bacteria were cultured
134 in siderophore-inducing medium); 7.6 $\mu\text{g}\cdot\text{g}^{-1}$ of EDTA dissolved in uninoculated siderophore-
135 inducing medium; spores of *S. tendae* F4 containing 10^7 CFUs and diluted in siderophore-inducing
136 medium, and uninoculated siderophore-inducing medium as a control. Additionally, EDTA and
137 control treatments received $\approx 0.29 \mu\text{g}\cdot\text{g}^{-1}$ of indole acetic acid (IAA), being the equivalent of
138 microbial auxins contained in the SCF. The various treatments were mixed thoroughly into the soil to
139 achieve even distribution in the amended soil. No auxins were administered in bacterial treatments,
140 since bacterium would produce auxins during the period of plant-microbe interaction. All treatments
141 were replicated 7 times.

142 **Plant material and plant growth conditions**

143 Seeds of sunflower were soaked in water, sterilized and pre-germinated as previously described
144 (Dimkpa et al. 2008b). Seedlings of uniform height and weight were then sown in the potted soil at
145 one seedling per pot. Pots were placed in a growth chamber and watered when needed. The growth
146 chamber was set at 12 h light and dark regimes; 70 % relative humidity; and 23 °C and 18 °C day
147 and night temperatures, respectively (Dimkpa et al. 2008b).

148 **Measurement of Cd and Fe uptake in bacteria and plant**

149 The amounts of Cd and Fe in growing cells of *S. tendae* F4, as well as in shoots of 31-d old
150 sunflower plants grown in Cd-treated soil were determined by inductively-coupled plasma optical

151 electron spectroscopy (ICP-OES) for Fe, and inductively coupled plasma mass spectroscopy (ICP-
152 MS) for Cd, as previously described (Dimkpa et al. 2008b).

153 **Statistical analysis**

154 Data were analyzed for variance (ANOVA) and where significant, treatments were separated
155 using the Tukey Test (Tukey, 1951).

156 **RESULTS**

157 **Quantification of siderophore production in *S. tendae* F4**

158 Siderophore quantification by the CAS assay indicated that *S. tendae* F4 produced siderophores
159 under Fe deficiency and Cd-supplemented conditions, with the presence of Cd increasing the
160 amount of siderophores significantly. No siderophores were detected in the presence of 100 μ M
161 Fe, irrespective of Cd status (Table 1), as the expected CAS-reactive color change from blue to
162 orange was not observed in these treatments.

163 **Kinetics of siderophore production by *S. tendae* F4 in the absence and presence of Cd**

164 ESI-MS analysis of filtrates of *S. tendae* F4 obtained from 24 h to 120 h-old iron-deficient cultures
165 in the absence (-Fe-Cd) and presence (-Fe+Cd) of Cd showed the production of the hydroxamate
166 siderophores, DFOB, DFOE and Cch, as well as the relative abundances of free siderophores and
167 their Fe- and Cd-bound forms (Figure 1A and 1B, respectively). Moreover, siderophore production
168 trend over time could be followed. In general, for both treatments, the presence of siderophores in
169 the cultures could be detected as early as 24 h after establishment and the abundance increased
170 rather steeply up to 72 h, when maximum production was observed. The levels of siderophore
171 declined thereafter, up to 120 h, although the abundance of siderophores detected at 120 h was
172 greater than at the beginning of biosynthesis in the absence, but not in the presence, of Cd. The
173 presence of Cd resulted in a stimulation of siderophore production at all times.

174 An in-depth analysis of specific siderophore production revealed that in the absence of Cd,
175 less DFOB was produced than were other siderophores, at all time points, and its complex with Fe
176 could only be detected at 72 and 120 h. However, the overall abundance of this siderophore was

177 more or less similar at these time points, if its free and Fe-bound forms are taken together. DFOE
178 was next in abundance, with overall highest production at 72 h, which, however, is only slightly
179 greater than at 96 h, both of which vary by at least 43%, relative to its abundance after 48 h and
180 120 h. Unlike DFOB, however, the Fe chelates of DFOE could be found at all time points. Cch
181 had, by far, the overall highest abundance, increasing by as much as 96% from 24 h, relative to the
182 peak time point of 72 h, and decreasing by more than 88% after 120 h, in relation to its peak
183 production time. Although most of the Cch at these times were detected as free siderophores,
184 higher abundance of its Fe chelates could be detected at 96 and 120 h (Figure 1A). With respect to
185 Cd-treated cultures, DFOB was by far the most abundant siderophore detected, being present at all
186 times; however, its Cd complexes were low, in relation to the free compounds. Although Cd-free
187 DFOB was not detected after 24 h, there was an increase of more than 55% after 72 h, in
188 comparison to its abundance after 48 h. However, relative to 72 h time point, the abundance of free
189 DFOB decreased by 47% after 96 h, and dipped by 92% after 120 h Figure (1B).

190 **Estimation of auxin production by *S. tendae* F4**

191 During siderophore production, *Streptomyces tendae* F4 produces auxins which can contribute in
192 plant growth promotion and, thus, can potentially obscure the effect of the co-produced
193 siderophores. Therefore, to account for microbial auxins in non-microbial plant treatments, the
194 production of auxins by *S. tendae* F4 in -Fe-Cd treatments lacking added tryptophan was
195 estimated, showing a concentration of $8.22 \pm 1.2 \mu\text{M}$ ($1.44 \mu\text{g.mL}^{-1}$).

196 **Uptake of Cd and Fe by growing cells of *S. tendae* F4**

197 Cd and Fe differentially-treated mycelia were harvested after 72 h, a period corresponding to peak
198 siderophore production in streptomycetes. The Cd and Fe contents of the cells were determined. It
199 was observed that the production of siderophores by the bacterium affected Cd uptake significantly
200 in Cd-amended cultures (Figure 2). At the same time, it could be shown that the uptake of Fe was
201 slightly higher in the presence of Cd in Fe-treated samples (Figure 2). It was also observed that

202 bacterial growth was severely affected by a combined stress effect of Fe deficiency and Cd toxicity
203 (-Fe+Cd), compared to the stress of Cd alone (+Fe+Cd; Table 1).

204 **Effects of chelators and bacterial inoculation on the biomass of Cd-treated sunflower**

205 Siderophore-containing culture filtrates from -Fe-Cd treatments, EDTA, as well as bacterial
206 spores, were used to evaluate sunflower growth and uptake of Cd from soil. The addition of equal
207 amounts of IAA to EDTA and control treatments ruled out the possibility that microbial auxins in
208 the culture filtrate gave any growth advantage to siderophore-treated plants. After 31 d of growth,
209 plants were harvested, washed, separated into shoots and roots and dried to a constant weight. The
210 root mass of plants treated with siderophore culture filtrates was significantly greater than those of
211 other treatments, whereas EDTA and bacterial treatments did not show any root enhancement
212 effect. In addition, the shoot mass of siderophore-treated plants was highest, followed closely by
213 EDTA treatment, both differing significantly from the control. Bacterial treatment showed an
214 insignificant shoot growth-promoting effect, compared to other treatments (Figure 3).

215 **Non-production of ACCD by *S. tendae* F4**

216 Since the degradation of plant ACC by bacteria is known to contribute to plant growth under
217 abiotic stress conditions, the ability of *S. tendae* F4 to produce ACCD was investigated. It could be
218 shown that *S. tendae* F4 did not produce detectable amounts of ACCD, at least in the
219 recommended standard range (Penrose and Glick, 2003), as spectrophotometric measurements of
220 all treatments were similar to the control (0 μ M α -ketobutyrate) in the standard assay. In our assay,
221 the presence or absence of Fe or Cd, and indeed, Ni, had no effect on ACCD production by *S.*
222 *tendae* F4 (Table 2).

223 **Cadmium and iron concentrations in sunflower shoot**

224 The effect of siderophores on Cd and Fe contents in shoot biomass were investigated.
225 Measurement of Cd in plant shoots indicated that siderophores significantly increased Cd uptake.
226 EDTA and bacteria-treated plants had similar Cd contents; however, this effect was only slightly

227 different (Figure 4). In contrast to Cd, Fe contents were by far greater in bacteria-inoculated plants
228 than other treatments (Figure 4).

229 **DISCUSSION**

230 Heavy metals are known to stimulate siderophore production in bacteria. Thus, the increased
231 production of siderophores by *S. tendae* F4 in the presence of Cd is consistent with recent reports
232 showing a stimulation of bacterial siderophore production by Cd (Dimkpa et al. 2008a; Sinha and
233 Mukherjee, 2008). The revelation of the presence of DFOB, DFOE and Cch, further validated the
234 production of these siderophores as previously reported (Dimkpa et al. 2008a).

235 A similar trend of hydroxamate siderophore production kinetics, comparable to that
236 observed in *S. acidiscabies* E13 (Dimkpa et al. 2008b), and, more or less, in *Saccharopolyspora*
237 *erythraea* (Oliveira et al. 2006), was also observed in *S. tendae* F4. This suggests an analogous
238 siderophore production dynamics in actinomycetes, although the presence of Ni at high
239 concentrations has been shown to alter this trend in *S. acidiscabies* E13 (Dimkpa, 2009). The up-
240 regulation of DFOB over DFOE and Cch in the presence of Cd was also observed in our previous
241 study (Dimkpa et al. 2008a). In contrast to DFOB, DFOE and Cch were detected in more or less
242 equal abundance at all time points. So far, changes in the production of specific hydroxamate
243 siderophores over time have only been tracked in *S. acidiscabies* E13, albeit in the presence of
244 nickel (Dimkpa et al. 2008b). A comparison of that study with the current one, as well as with
245 results from Dimkpa et al. (2008a), shows that, Cch is a prominent siderophore of *Streptomyces*
246 spp, which, along with desferrioxamines, are produced prominently at peak siderophore production
247 times. The reason for multiple siderophore production in *Streptomyces* may not be obvious;
248 however, given the interaction of these siderophores with different metals at different
249 concentrations (Dimkpa et al. 2008a, b), it would appear that there is a benefit in producing
250 chelators with varying metal affinities and preferences, enhancing bacterial survival in
251 environments that are heterogeneously contaminated with different toxic metals, thereby
252 contributing to their usefulness in remediating such soils.

253 *S. tendae* F4 has previously been shown to produce auxins (Dimkpa et al. 2008a). In the
254 current study, the production of auxins by the strain in the absence of any metals, with no added
255 tryptophan was slightly less than was reported for *S. acidiscabies* E13 grown under similar
256 conditions (Dimkpa et al. 2008b). However, considering that the present result includes IAA, as
257 well as other auxins detected by the Salkowski's assay, the amount was comparable to previous
258 measurements with the strain (Dimkpa et al. 2008a).

259 Binding of Cd by siderophores produced by *S. tendae* F4 has been previously demonstrated
260 (Dimkpa et al., 2008a). Thus, these results support previous data regarding a preventive role for
261 siderophores in metal uptake and toxicity in bacteria; a function which has previously been shown
262 for Al and Cd, and appears to be directed by metal concentrations (Hu and Boyer, 1996; Gilis et al.
263 1998). Nevertheless, considering that slightly more siderophores were produced than the added Cd
264 concentration, it is assumed that the relatively lower affinity of hydroxamate siderophores for Cd
265 (Martell et al. 1995) resulted in the occurrence of a high abundance of Cd-free siderophores (see
266 also Dimkpa et al. 2008a). This is apparent from the non-negligible concentration of Cd detected in
267 cells producing siderophores (Figure 2). Although Fe(III), and not Fe(II), was used for bacterial
268 growth, the starting pH of the medium (6.8) was probably sufficient to permit a reversible Fe redox
269 process, resulting in the formation of soluble Fe (FeII) which can diffuse into cells via porins
270 (Boukhalfa and Crumbliss, 2002). This may, in part, explain the high Fe content observed in the
271 bacterium in the absence of siderophores. Moreover, it has been shown that certain bacteria
272 possess Fe uptake systems unrelated to siderophore production. For example, Kammler et al.
273 (1993) and Mey et al. (2008) have shown that *E. coli* and *Vibrio cholerae* take up Fe via a ferrous
274 iron-specific uptake system involving a ferrous iron transporter, Feo, and an inner-membrane
275 protein, VcIB, respectively. Interestingly, these and other homologous uptake systems also take up
276 metals other than Fe (see for e.g., Makui et al. 2000). Given the amount of Cd in the cells, it
277 would, thus, appear that such alternative metal transport systems could also be present in
278 *Streptomyces*. Nevertheless, a role for siderophores in the observed reduction of Cd uptake in

279 *Streptomyces* is supported by the fact that no evidence could be found in the literature indicating
280 that such alternative metal transport systems will operate under Fe-replete, and not Fe-deficient
281 conditions, in the same bacterium. The identification of the range of proteins involved in these
282 processes is the subject of an on-going study.

283 Sunflower has previously been used in metal accumulation studies (Chen and Cutright,
284 2001; Liphadzi et al. 2003). Chen and Cutright (2001) have reported significantly reduced biomass
285 production by Cd-treated sunflower when EDTA was added. Similarly, Liphadzi et al. (2003) have
286 reported that shoot dry mass of Cd-treated sunflower was either unaffected, or was reduced by
287 different EDTA concentrations. These results suggest that EDTA may be detrimental to plant
288 growth, independent of the effects of Cd on the plants. In contrast to the well-reported negative
289 effects of Cd on plants (Di Toppi and Gabbrielli, 1999), the addition of Cd shows positive effects
290 on barley growth (Wu and Zhang, 2002; Guo et al. 2004). Though surprising at first glance, these
291 results are, nevertheless, supported by a recent report in which the presence of low amounts of Cd
292 (22 to 89 μM) stimulates the growth of a Cd hyper-accumulator plant, while growth of the same
293 plant is dramatically suppressed when Cd concentration is increased to 178 μM (Qui et al. 2008).
294 In these studies, the stimulation of plant growth by low amounts of Cd is linked to the possibility
295 that Cd may, after all, be useful to plants, as previously observed in the marine diatom,
296 *Thalassiosira weissflogii* (Lane et al. 2005). Although Cd-resistant bacteria have been
297 demonstrated to enhance the growth of plants under Cd stress, in the current study, however,
298 *Streptomyces tendae* F4 did not show a clear plant growth-promoting effect on Cd-stressed
299 sunflower. Nevertheless, Cd-chelating siderophores produced by the bacterium significantly
300 increased root and shoot biomass. In relation to the current observation, siderophores from *S.*
301 *acidiscabies* E13 promote growth of cowpea, both *in vitro*, and in soil (Dimkpa et al. 2008b,
302 2009). In addition, plant growth promotion has been observed with siderophore and/or auxins-
303 producing bacteria under Cd stress (Belimov et al. 2005; Madhaiyan et al. 2007; Kuffner et al.
304 2008; Sinha and Mukherjee, 2008).

305 In addition to siderophores and auxins, bacterial ACCD has been implicated in plant
306 growth promotion under metal stress conditions. A number of plant growth promoting bacteria
307 (PGPB) are able to alleviate the effect of stress ethylene on plant growth during abiotic stress by
308 the enzymatic deamination of the ethylene precursor, ACC, into NH₃ and α -ketobutyrate. (Burd et
309 al. 1998; Belimov et al. 2005; Rajkumar et al. 2006; Sheng et al. 2008). Belimov et al. (2005)
310 have obtained a positive correlation between ACCD activity and plant root elongation,
311 emphasizing the importance of this enzyme in bacterial plant growth enhancement under stress
312 conditions (Glick et al. 1998). However, the presence of ACCD may obscure siderophore-related
313 effects. Based on the observation that *S. tendae* F4 does not show ACCD activity, we speculate
314 that the bacterium, though a siderophore and auxin producer, could not clearly enhance plant
315 growth due, probably, to the occurrence of high levels of stress ethylene induced by Cd (Pennasio
316 and Reggero, 1992), which, in this case, might not have been neutralized owing to the absence of a
317 bacterial ACCD. Possibly, the amount of siderophores produced in soil might have been
318 insufficient to counter the inhibitory effect of Cd on auxins, which seemed apparent from the fact
319 that most of the siderophores were involved in plant Fe uptake - hence higher Fe content in these
320 plants - rather than in interacting with Cd.

321 The uptake and translocation of Cd to plant shoots has been described to be less efficient,
322 as compared to other metals such as nickel and zinc (Page and Feller, 2005). However, it has been
323 previously demonstrated that the uptake of metals (including Cd) by plants can be augmented by
324 siderophores (Dimkpa et al. 2009). In this work, a significantly high concentration of Cd was
325 obtained in sunflower shoots in the presence of siderophores. Cadmium concentration in willows is
326 enhanced by the application of a non siderophore-producing *Streptomyces* AR17 (Kuffner et al.
327 2008); whereas Cd uptake is prevented in pumpkin and mustard by the addition of a siderophore-
328 producing strain of *Pseudomonas aeruginosa* (Sinha and Mukherjee, 2008). Thus, the contrasting
329 effects showed by different rhizosphere bacteria on plants stressed by specific metals (Burd et al.
330 1998; Rajkumar et al. 2006; Safronova et al. 2006; Madhaiyan et al. 2007; Dimkpa et al. 2008b,

331 2009; Kuffner et al. 2008) suggest that the mechanisms underlying metal uptake are largely plant-
332 dependent. This might be related to siderophore-metal complexes; and thus, a function of the
333 uptake or exclusion of different xenosiderophores.

334 Hydroxamate siderophores such as one of those produced by *S. tendae* F4, namely DFOB,
335 has previously been shown to facilitate iron uptake in unstressed sunflower (Cline et al. 1984). In
336 the presence of nickel, as well as a combination of several metals, these siderophores also
337 enhanced Fe uptake in cowpea (Dimkpa et al. 2008b, 2009). Low Fe availability in the
338 environment, aggravated by Cd-induced Fe deficiency (Yoshihara et al. 2006), could result in
339 increased microbial siderophore production (Dimkpa et al, 2008a), despite the soil pH of $6.97 \pm$
340 0.036 . In plants, microbial siderophores enhance Fe uptake in the presence of metals that would
341 otherwise inhibit its uptake (Burd et al. 1998, 2000; Dimkpa et al. 2008b, 2009); however, in
342 bacteria, not much is known about the co-interaction of Fe and Cd with siderophores. Given the
343 reduced Cd content in bacteria in the presence of siderophores (i.e., -Fe+Cd versus +Fe+Cd;
344 Figure 2), the non-significantly higher Fe content in +Fe+Cd could be explained as the effect of an
345 increased cell requirement for Fe to counteract the toxic effect of Cd, since such metals can inhibit
346 Fe uptake not only in plants, but also in bacteria (Hu and Boyer, 1996; Bayse et al., 2000). At the
347 same time, the presence of microbial siderophores in the rhizosphere benefits plants via provision
348 of Fe, despite the presence of other metals which could inhibit Fe acquisition by plant (Burd et al.
349 1998, 2000; Dimkpa et al. 2009). Apparently, in metal-contaminated soils, concentration-
350 dependent metal competition for siderophore binding (the consequence of which is reduced
351 dedication of siderophores to Fe scavenging) will determine how much of each metal is
352 accumulated by plants or bacteria. Synthesizing data from Cd uptake in *S. tendae* F4 and
353 sunflower, illustrating microbial mechanisms that reduce the bioavailability of toxic metals in the
354 environment for rhizosphere bacteria, as well as provide plants with improved access to metals, we
355 suppose that it is more informative to conduct studies of rhizosphere bacteria-mediated metal

356 mobility simultaneously in plants, since bacteria are often associated with plant roots, and are,
357 thus, likely to contribute to the efficiency of phytoremediation under natural conditions.

358 Poor solubility of metals is a serious set-back to their extraction from contaminated soil by
359 means of plants. Despite good results obtained using EDTA to enhance metal solubility, EDTA is not
360 readily degraded and, therefore, considered a secondary pollutant. Thus, the use of biodegradable
361 metal chelators, such as microbial siderophores, is recommendable, preceded by comparative studies
362 on the ability of EDTA versus siderophores in the uptake of specific metals. To this end, the effect of
363 both chelators on Cd uptake by sunflower was investigated. It could be shown that the addition of
364 siderophores resulted in slightly more Cd in sunflower than did EDTA; nevertheless, the slightly
365 greater biomass of siderophore-treated plants led to a final higher Cd extraction. Considering the two
366 main factors critical in the use of siderophores or EDTA for assisted phytoremediation, (i) good
367 effect on the uptake of metals, and (ii) subsequent degradation of the chelator compound, it would
368 appear, from current results, that siderophores are favored over EDTA. Along these lines, Parra et al.
369 (2008) have tested the metal extractability of a variety of metal-chelating substances, with purified
370 (and hence, expensive) DFOB showing relatively good performance for uranium extraction from
371 soil. However, our proposal for the use of siderophores in biochelator-assisted phytoremediation is
372 based on crude culture filtrates containing DFOB and other hydroxamate siderophores of
373 *Streptomyces*, the production of which is much less expensive. Given its 1:1 stoichiometry with
374 metals, higher siderophore concentrations should result in better metal extractability. This will
375 require further investigations, for example, with increased siderophore-expressing strains, and to test
376 the expressed siderophores on different metals and metal-accumulating plants.

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561 **Table and Figure Legends**

562 Table 1: CAS-assay based siderophore quantification in *S. tendae* F4 grown in different Fe and Cd
563 set ups, in relation to bacterial cell growth. Values represent averages \pm SDs (n=3).

564 Table 2: Activity assay indicating non-production of α -aminocyclopropane-1-carboxylic acid
565 deaminase by *Streptomyces tendae* F4 under Fe, Cd and Ni interplay. Experiments were repeated
566 twice with similar outcome, and one representative data is presented.

567 Figure 1: Kinetics of siderophore production and the detection of specific hydroxamate siderophores
568 (DFOB, DFOE and Cch) and their respective Fe (A) and Cd (B) complexes in culture filtrates of *S.*
569 *tendae* F4 grown in the absence and presence of Fe and Cd over a 120 h period. The relative
570 abundances of the siderophores in the absence and presence of Cd are shown. Siderophore
571 measurement was conducted with 3 independent samples with similar results and representative
572 values are presented.

573 Figure 2: Effect of siderophore production on cadmium and iron uptake by growing cells of
574 *Streptomyces tendae* F4. Values represent means \pm SD of 3 replicates, and bars with different
575 letters in each parameter indicate significant differences at $P=0.05$. The -Fe-Cd treatment was not
576 included in the analysis, since it is not informative for both Cd and Fe uptake.

577 Figure 3: Effect of siderophores (SCF), EDTA and *Streptomyces tendae* F4 application on root and
578 shoot biomass of sunflower plants grown in Cd-amended soil. Bars represent SDs (n=7) and
579 different letter on bars indicate statistical differences ($P=0.05$).

580 Figure 4: Effect of siderophores (SCF), EDTA and *Streptomyces tendae* F4 application on shoot Cd
581 and Fe contents in sunflower plants grown in Cd-amended soil. Values represent means \pm SD of 7
582 replicates, and different letters on bars indicate statistical differences ($P=0.05$) for each parameter.

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587 Table 1

Treatment	Siderophore (μM)*	Biomass ($\text{g}\cdot\text{L}^{-1}$ DCW)
-Fe-Cd	74.9 ± 6.7	3.6 ± 0.37
-Fe+Cd	121.2 ± 12.7	2.7 ± 0.28
+Fe-Cd	-	5.8 ± 0.45
+Fe+Cd	-	5.3 ± 0.4

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*not detected.

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608 Table 2

Standard curve	Abs	<i>S. tendae</i> F4	Abs*
(μM α -ketobutyrate)	(540 nm)	Treatment	(540 nm)
0.0	0.128	-Fe-Cd	0.111
0.1	0.303	-Fe+Cd	0.115
0.2	0.614	+Fe-Cd	0.129
0.3	0.958	+Fe+Cd	0.123
0.4	1.305	-Fe-Ni	0.098
0.5	1.613	-Fe+Ni	0.098
0.6	1.764	+Fe-Ni	0.097
0.7	1.934	+Fe+Ni	0.103
0.8	1.992		
0.9	2.112		
1.0	2.234		

609 *Since absorbances (abs) are comparable to absorbance of 0.0 μM α -ketobutyrate in standard,
610 sample absorbances did not account for those of assay reagent + ACC, and bacterial extract +
611 assay reagent in the absence of ACC.

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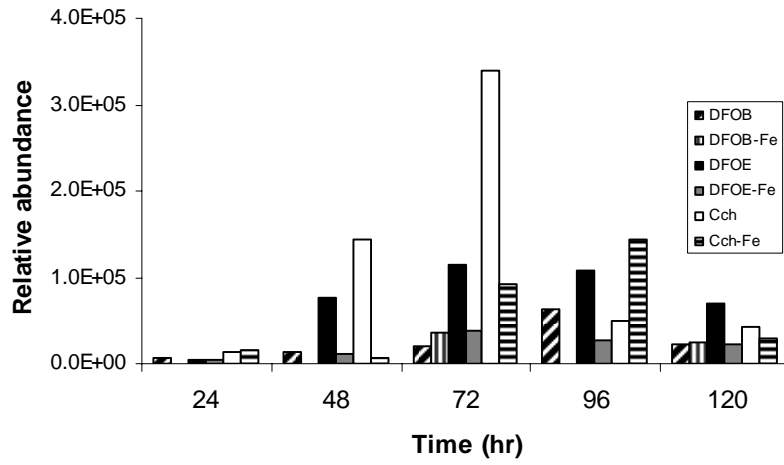
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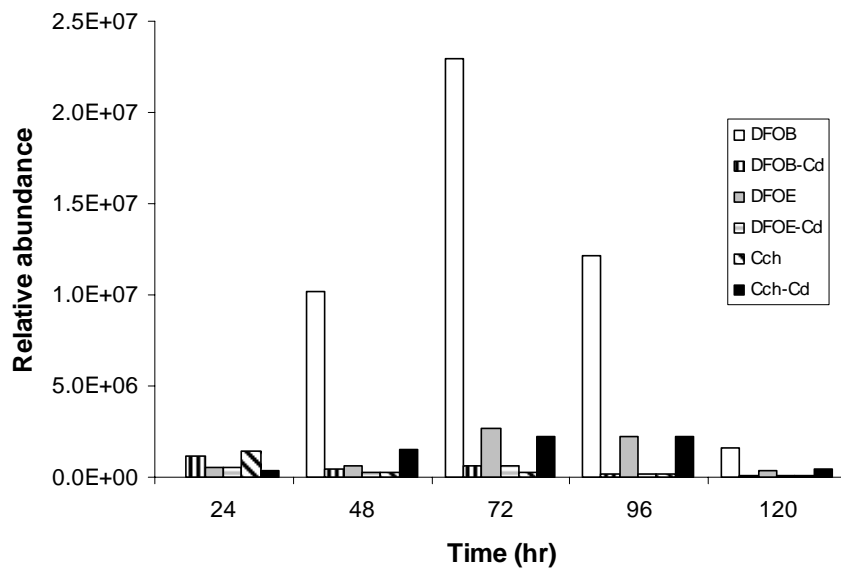
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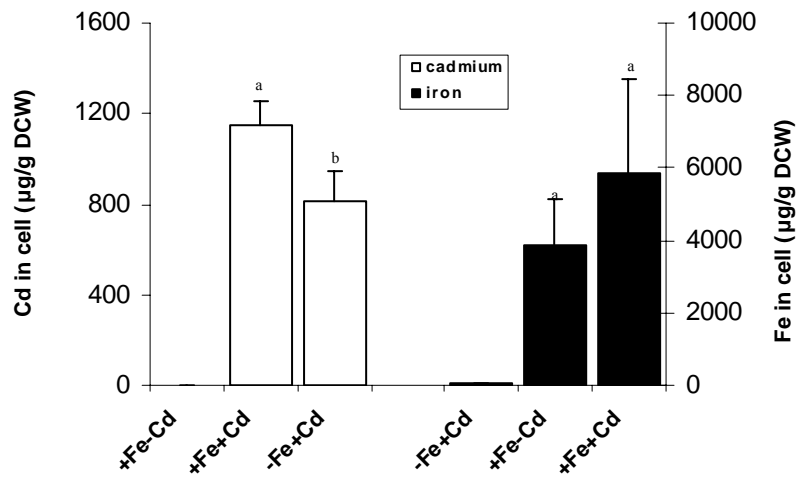
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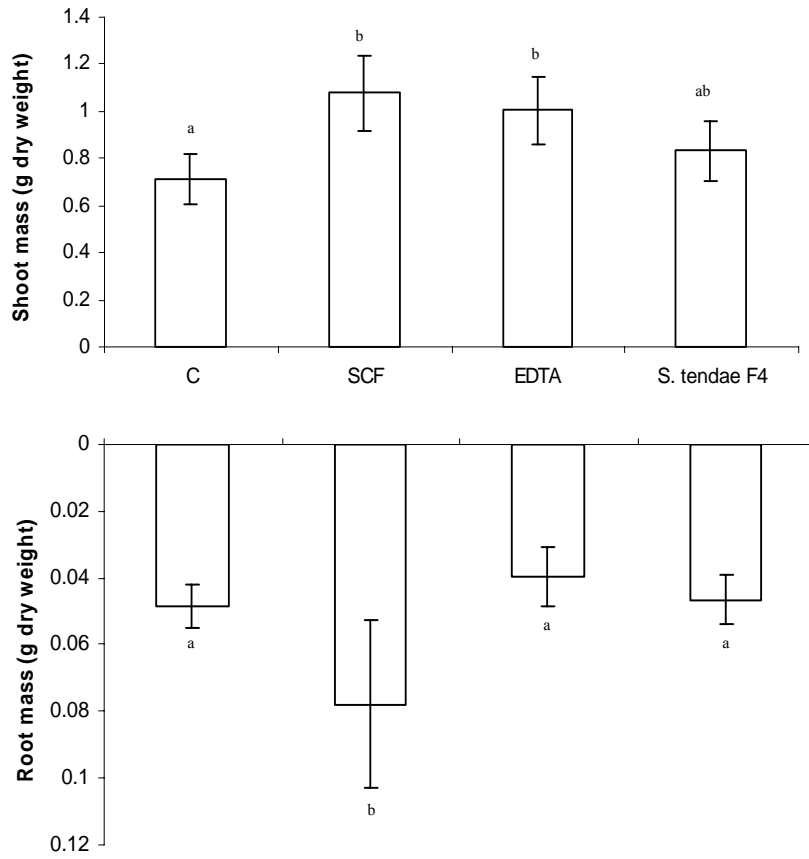
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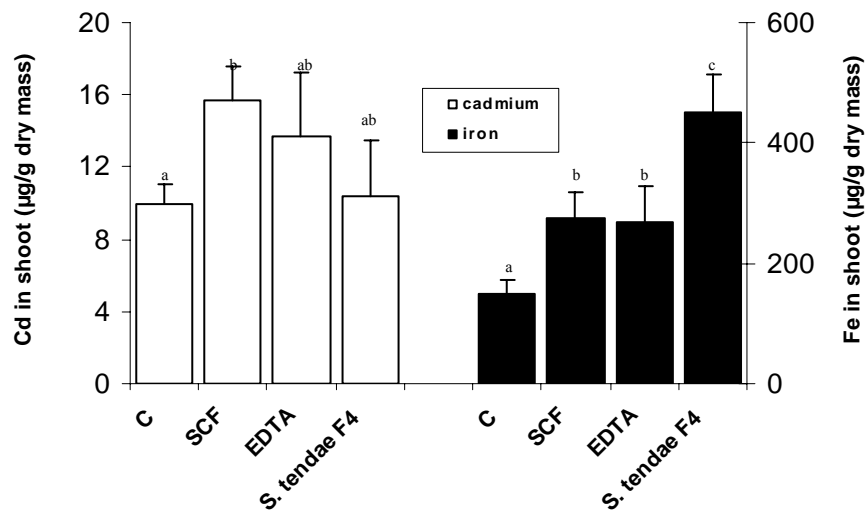
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ADDITIONAL UNPUBLISHED RESULTS

CHAPTER 1

SIDEROPHORE PRODUCTION BY *STREPTOMYCES*

1.1. Introduction

From available reports, many *Streptomyces* species produce hydroxamate-type siderophores. *Streptomyces acidiscabies* E13 and *Streptomyces tendae* F4 are metal-resistant strains (Amoroso *et al.*, 2000; Schmidt *et al.*, 2005) with potential application for soil remediation. Since siderophores can bind a range of metals, thereby determining environmental concentrations of free metal species, its use for bioremediation of metal pollution, therefore, becomes potent. Thus, investigating siderophore production in both strains will be a prelude to their possible application in the bioremediation of a metal-polluted field site located in the former uranium mining site in Ronneburg, east of Germany.

1.2. Materials and Methods

Siderophore production and detection in *S. acidiscabies* E13, *S. tendae* F4 and other *Streptomyces* species used in this study was performed as described (Dimkpa *et al.*, 2008a, b). Csáky's assay for the detection of hydroxamate siderophores was performed as previously described (Tokala *et al.*, 2002); and where applicable, the detection of catecholate siderophores was performed following the method of Arnow (1936). Briefly, to 1 mL of Fe-deficient culture supernatant was added 1 mL 0.5 M HCl. The mixture was shaken briefly and 1 mL of nitrite-molybdate reagent (10 g sodium nitrite + 10 g sodium molybdate dissolved in 100 mL ddH₂O) was added, followed by 1 mL of 1 M NaOH solution. The mixture was incubated at room temperature for 5 minutes, and if necessary, quantification can be performed afterwards by measuring the absorbance at 500nm. Catechol and uninoculated siderophore inducing medium were used as positive and negative controls, respectively.

1.3. Results and Discussion

1.3.1. Siderophore production by *Streptomyces* spp in agar-plates

Streptomyces acidiscabies E13 and *S. tendae* F4 can produce siderophores in solid medium. This was shown using the modified CAS agar plate method of Milagres *et al.* (1999). After more than one week of growth, it could be shown that the strains produce CAS-reactive

substances, as indicated by a colour change in the blue detection agar (Figure 1). The produced colour changes were similar to those previously reported (Milagres *et al.*, 1999; Oliveira *et al.*, 2006)

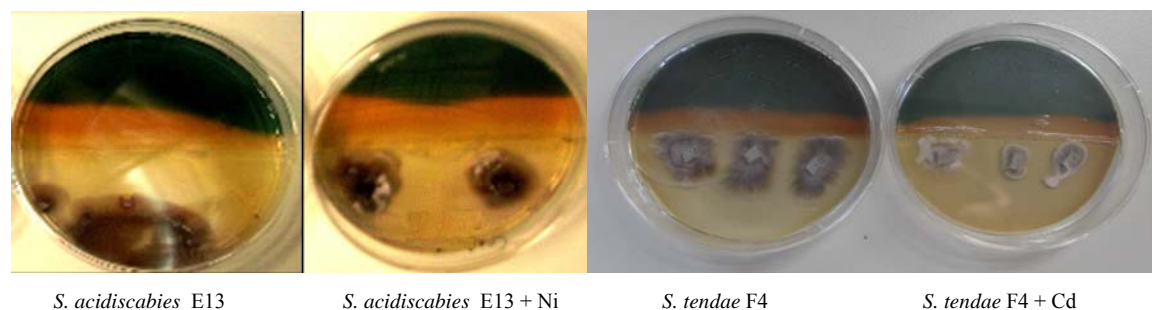


Fig. 1. Modified chrome azurol S (CAS) agar plates showing color change in blue detection agar indicative of the exudation and diffusion of siderophores into the blue agar from iron-deficient and nickel or cadmium-containing nutrient agar halves inoculated with *S. acidiscabies* E13 and *S. tendae* F4. Color change is seen starting from the boundary between the two agar types.

1.3.2. Siderophore production in liquid cultures and identification of siderophore types

Siderophore production and quantification was also conducted in liquid medium as described, with positive results (Dimkpa *et al.*, 2008a, b). However, since the CAS assay does not differentiate between siderophore types, C�áky and Arnow assays were performed in *S. acidiscabies* E13 and *S. tendae* F4 to distinguish between hydroxamate and catecholates siderophores, respectively. Both strains were positive for hydroxamate siderophores, but negative for the presence of catecholates (not shown). The near-colorless mixture turned pink in Fe-deficient samples, after hydrolysis of the hydroxamic acids to hydroxylamine by means of heat and acid treatments. Fe (100 μ M)-containing samples remained colorless. When compared to hydroxylamine (standard), the intensity of the pink color indicated the concentration of hydroxamate siderophores present in the Fe-deficient sample from *S. acidiscabies* E13 (Figure 2)



Fig. 2. Detection of hydroxamate siderophores in Fe-deficient culture filtrate of *S. acidiscabies* E13. No siderophores were detected in iron-containing cultures. Uninoculated siderophore-inducing medium and hydroxylamine were used as negative (–ve) and positive (standard) control, respectively.

Following the detection of hydroxamate siderophores by Csáky's assay, the specific production of DFOB, DFOE and Cch by *S. acidiscabies* E13 and *S. tendae* F4 was unequivocally confirmed by ESI-MS (Dimkpa *et al.*, 2008a, b). In addition to this, the kinetics involved in the production of the siderophores was shown in *S. acidiscabies* E13 cultures grown over a 120 h period in the absence of Fe. In *S. acidiscabies* E13, the highest production time was after 72 h. At 120 h, siderophore production was as low as during the beginning of cell growth, similar to bacterial growth trend, as estimated from the dry cell weight (DCW) (Fig 3). These results very likely represent normal cell growth and siderophore production dynamics in actinomycetes, whereby decreased biosynthesis of siderophores implies reduced cell growth (Oliveira *et al.*, 2006).

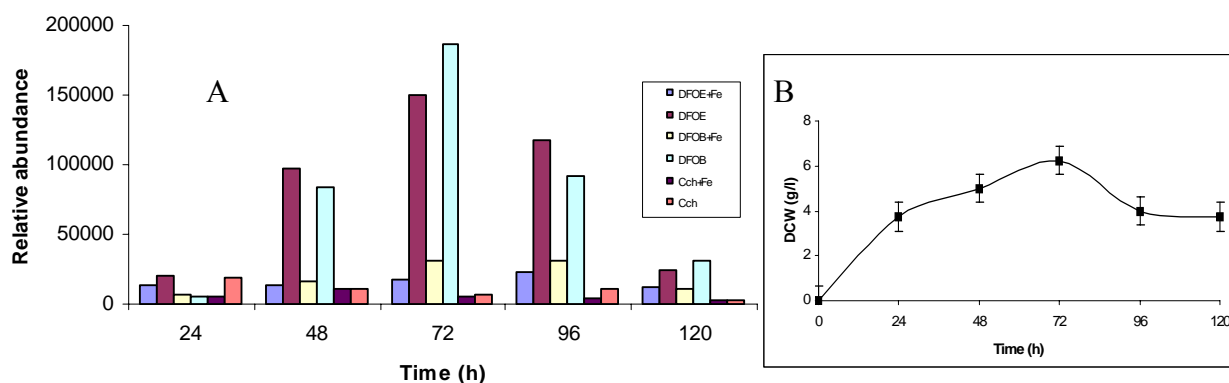


Fig. 3. Relative abundances of the hydroxamate siderophores DFOE, DFOB and Cch and their respective Fe chelates (a) in culture filtrates of *S. acidiscabies* E13 grown in the absence of Fe, in relation to cell growth (dry cell weight-DCW) over a 120 h period (b). Siderophore measurements were conducted with 3 independent samples with similar results, and one representative values are presented. N= 3 for DCW.

The production of desferrioxamine siderophores has been described in a number of *Streptomyces* species, including *Streptomyces pilosus* (Bickel *et al.*, 1960), *S. spp.* strain Wak. A-305 (Yang and Leong, 1982), *S. olivaceus* T \ddot{U} 2718 (Meiwes *et al.*, 1990), *S. ambofaciens*, *S. coelicolor*, *S. lividans*, *S. viridosporus* (Imbert *et al.*, 1995, Barona-Gomez *et al.*, 2006), *S. lydicus* WYEC108 (Tokala *et al.*, 2002), and *S. griseus* (Yamanaka *et al.*, 2005). In addition to this, non-desferrioxamine siderophores, namely, coelichelin (Lautru *et al.*, 2005; Barona-Gomez *et al.*, 2006) and enterobactin (Fiedler *et al.*, 2001), have also been reported in *Streptomyces ambofaciens* and *S. coelicolor*, and *S. tendae* T \ddot{U} 901/8c and T \ddot{U} 6125, respectively. Thus, the production of DFOB, DFOE and Cch in *S. acidiscabies* E13 and *S. tendae* F4 (Dimkpa *et al.* 2008a, b) are in agreement with previous studies.

Further to this, the production of siderophores (though untyped) was evaluated in several other strains of *Streptomyces*, including *S. avermitilis*, *S. tanashiensis*, *S. tanashiensis* IAM0016 (previously described as a probable siderophore deficient strain; Yamanaka *et al.*, 2005), *S. mirabilis* P10A-3, *S. mirabilis* K7A-1, *S. chromofuscus* P4B-1, *S. chromofuscus* P4B-1, *S. chromofuscus* P10A-4, *S. prunicolor* P6A-1, and *S. naganishii* P9A-1 with positive outcome (Dimkpa *et al.*, 2008b). With regards to siderophore production in *S. tanashiensis* IAM0016, preliminary evidence from Cšaky's and Arnow's assays suggested that the siderophores elicited are of the catechol type (Figure 4), of which further analyses are required to confirm which specific catechol siderophore is produced. Arnow's assay is based on the fact that catechol, when combined with nitrous acid, gives a yellow color, which becomes an intense orange-red in the presence of excess sodium hydroxide (Arnow, 1936). As with most colorimetric assays, after all components have been added, the assay is allowed to incubate at room temperature, in this case, for approximately 5 minutes to allow for color development, followed by absorbance measurement. A positive reaction is indicated by the production of a pink to deep-red color, the intensity of which depends on the amount of catechol present.

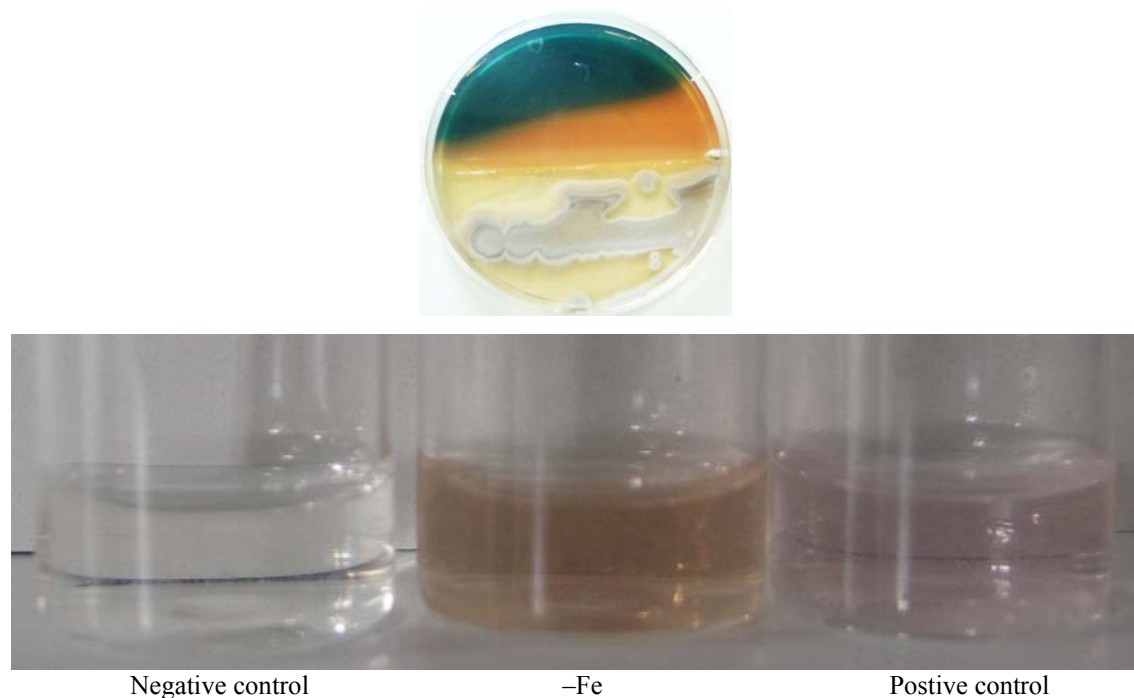


Fig. 4. (Up): Modified chrome azurol S assay of *S. tanashiensis* IAM0016 indicating siderophore-induced color change from blue to orange in the detection agar from an iron-deficient nutrient agar. (Down): Arnow's assay for the detection of catechol siderophores in iron-deficient *S. tanashiensis* IAM0016 cultures. Uninoculated siderophore-inducing medium and 50 μ M catechol were respectively used as negative and positive controls.

1.3.3. Effect of nickel on the production of siderophores over time

As reported previously (Amoroso *et al.*, 2000; Schmidt *et al.*, 2005, 2008), *Streptomyces acidiscabies* E13 and *S. tendae* F4 are capable of growth in the presence of a variety of metals. This capability was, thus, tested for siderophore production in both solid and liquid media supplemented with Ni (2 mM) and Cd (0.1 mM), in *Streptomyces acidiscabies* E13 and *S. tendae* F4, respectively. As seen in Figure 1, slightly more siderophores were excreted by *S. acidiscabies* E13 in the presence of Ni. This was, however, not the case with Cd-treated *S. tendae* F4. The presence of high amounts of ferric and/or ferrous iron prevents siderophore production in bacteria, as also indicated (Dimkpa *et al.*, 2008b). Nevertheless, investigating Ni or Cd-induced siderophore production by *S. acidiscabies* E13 and *S. tendae* F4, respectively, in liquid cultures supplemented with or without low amount of iron (36 μ M), could show the presence of secreted hydroxamate siderophores that are excreted at high rates, with a stimulating effect by these metals (Dimkpa *et al.*, 2008b). Furthermore, ESI-MS analysis of culture filtrates from *S. acidiscabies* E13 indicated a normal cell growth pattern under 2 mM Ni stress, similar to cell growth dynamics in the absence of Ni (Figure 5).

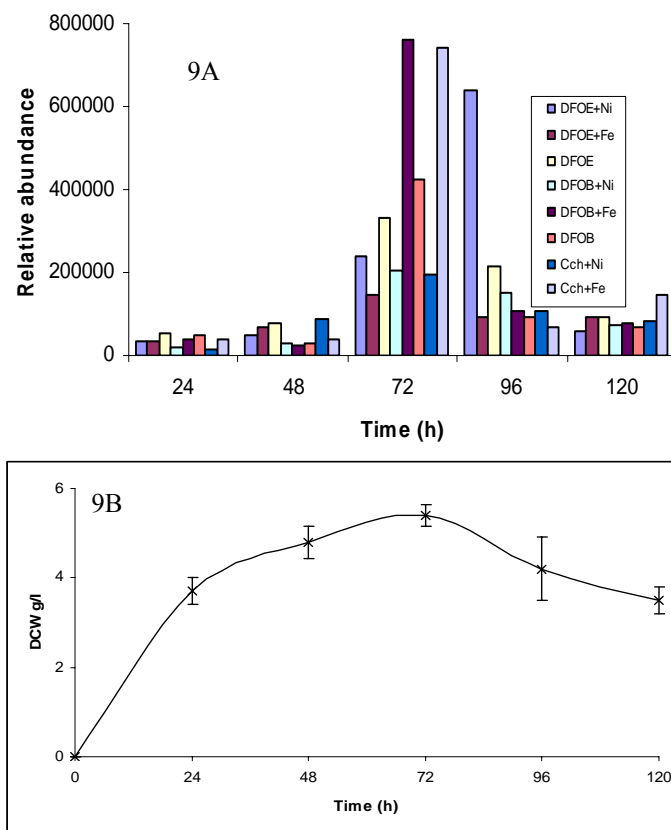


Fig. 5. Relative abundances of the hydroxamate siderophores DFOE, DFOB and Cch and their respective Fe chelates (a) in culture filtrates of *S. acidiscabies* E13 grown in the presence of 2 mM Ni, in relation to cell growth (DCW) over a 120 h period (b). Siderophore measurements were conducted with 3 independent samples with similar results, and one representative values are presented. N= 3 for DCW.

A different cell growth kinetics was, however, observed in the presence of excessive amounts (5 mM) of Ni. In this case, a delay in the release of the compounds was observed, which pushed maximum siderophore production to 120 h, contrary to results obtained at 72 h, either under Ni-deficiency, or at 2 mM Ni concentration. At 5 mM Ni concentration, Cch, and to a lesser extent DFOB, were the main siderophores detected (Figure 6). The siderophore production kinetics was coincident with cell growth (DCW) changes under Ni stress, with overall siderophore production at peak time (120 h) significantly higher than in the absence or lower Ni content. A similar siderophore production trend was previously reported in aluminum-treated *Bacillus megaterium* (Hu and Boyer, 1996b), which indicates that increased siderophore exudation is a strategy for the continued survival of these bacteria when faced with toxic levels of metals, since the compound helps to acquire Fe in the presence of high levels of other metals which otherwise impede Fe acquisition.

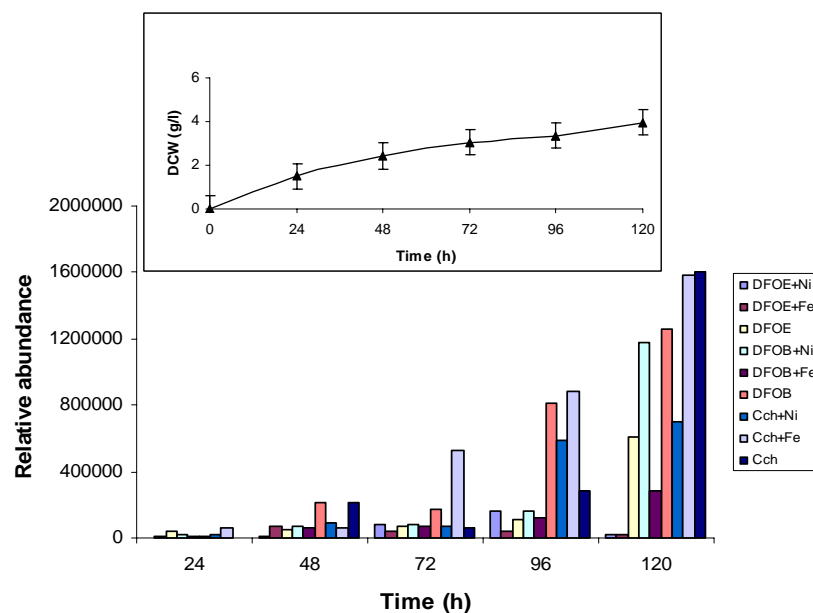


Fig. 6. Effect of high level of Ni (5 mM) on the production of desferrioxamine siderophores (DFOE and DFOB) and coelichelin (Cch) by *Streptomyces acidiscabies* E13; and the detection of their respective Fe and Ni complexes over a 120 h period, in relation to cell growth changes (DCW). Siderophore measurements were conducted with 3 independent samples with similar results, and representative values of one are presented. N= 3 for DCW.

1.3.4. High nickel concentration prevents binding of iron by desferrioxamine E

In the course of comparative ESI-MS analysis of Ni-differentially treated cultures of *S. acidiscabies* E13 growing under severe Fe stress, we found that increasing the Ni concentration to 5 mM significantly prevented the binding of Fe by DFOE. In this instance, whereas, almost one-half of the siderophores remained as DFOE, free of any metal, all metal-bound forms were detected as DFOE-Ni complexes, which we have tentatively termed “nickeloxamine” (NOE). No Fe-bound forms- ferrioxamines (FOE) were detected. In the absence of Ni, however, more than half of the DFOEs detected were free, while the rest were detected as FOE (Figure 7).

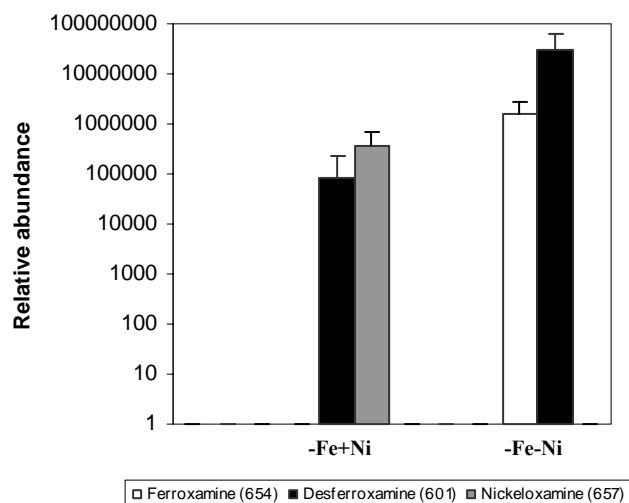


Fig. 7. Electrospray ionisation mass spectrometry analysis of desferrioxamine E (DFOE) siderophore contained in culture filtrates of *S. acidiscabies* E13 grown under iron deficiency in the presence and absence of nickel (5 mM). The prevention of DFOE-Fe complex formation by high Ni concentrations and the relative abundances of the Fe-free hydroxamates (desferrioxamine), Fe-bound hydroxamates (ferrioxamine), and Ni-bound hydroxamates (nickeloxamine) are indicated (n=3).

Of all characterized desferrioxamines, DFOE has the greatest affinity for Fe, by virtue of its cyclic structure (Crumbliss, 1991; Boukhalfa and Crumbliss, 2002; Fernández and Winkelmann, 2005). It could, thus, be imagined that DFOE should be better suited to scavenge for Fe, in the event of dire Fe limitation inducible by the presence of excess levels of other metals. On this basis, the above investigation was conducted to evaluate how this compound might function in its supposed primary role of Fe-scavenging, in the presence of an elevated level of another metal, in this case, Ni. The results indicate that under severe Fe scarcity, DFOE can be effectively prevented from Fe scavenging by excessive levels of Ni. Furthermore, it appears to support previous argument that in addition to affinity, the differential concentrations of competing metals may indeed drive the binding of siderophores to the metals (Hu and Boyer, 1996b). Thus, it would seem that in the presence of high Ni contamination, bacteria producing only one type of siderophore (in this case, DFOE) may be less efficient in scavenging for Fe.

CHAPTER 2

***STREPTOMYCES*-PLANT INTERACTIONS IN METAL CONTAMINATED ENVIRONMENTS**

2.1. Introduction

Whereas the growth-enhancing effect of *Streptomyces* spp. in plants in the absence of metal stress is documented (see for example, Doumbou *et al.*, 2002, and references therein; Tokala *et al.*, 2002), not much is known about such interactions with plants growing under metal stress conditions, except, perhaps, the recent work of Kuffner *et al.* (2008) in which two of four reported *Streptomyces* spp. produced siderophores. Surprisingly, however, the siderophore-producing strains could neither enhance plant growth nor metal uptake. Instead, uptake of Zn and Cd was enhanced by a non siderophore-producing strain, which incidentally, did not also promote plant growth. Thus, following the confirmation that *S. acidiscabies* E13 and *S. tendae* F4 can produce siderophores, *in vitro* (using *S. acidiscabies* E13) and soil experiments (both species) were conducted to investigate the effect of inoculating these bacteria on the growth of metal-stressed plants, namely cowpea and maize.

2.2. Materials and Methods

2.2.1. Plant material and growth conditions

2.2.1.a. *In vitro* experiments

Cowpea seeds were sterilized and germinated as described (Dimkpa *et al.*, 2008a). Uniformly-germinated seedlings were aseptically transferred into acid-treated, sterile glass beakers containing 100 mL of uninoculated siderophore-inducing medium supplemented with 2 mM NiCl₂. The medium was then inoculated with and without 0.5 mL 10⁶ CFU bacterial spore suspension. Each treatment was replicated five times. In order to maintain a balance between the acidophilic nature of *S. acidiscabies* E13 and the appropriate pH for siderophore production, the plant cultures were maintained at a pH of 6.8. Plant anchorage and subsequent growth conditions were as previously described (Dimkpa *et al.*, 2008a).

2.2.1.b. Soil-based experiments

Heavy metal contaminated soil (0.9 kg) was loaded into dark plastic pots and mixed thoroughly with 0.1 kg of endomycorrhiza derived from the fungus *Glomus intraradices* (Amykor, Bitterfeld-Wolfen, Germany), giving a total of 1 kg soil medium per pot. Next,

maize or cowpea seeds were differentially dipped in 1 mL of spore suspensions of *S. acidiscabies* E13, *S. tendae* F4, or a spore mixture of both strains containing 10^7 spores. The seeds were then sown in the plastic pots (one seed per pot). The bacterial suspension was further mixed with the soil around the seeds. Each treatment was replicated 10 times. The pots were placed on plant growth platforms in a conventional green house and grown between the period of mid-June and mid-September. Watering was done when necessary and no artificial lighting was provided since the growth period coincided with the summer season with sufficient sunlight, and daily temperatures often reaching as high as 30 °C.

2.2.2. Detection of siderophore and auxin during *in vitro* *S. acidiscabies* E13-cowpea interaction

Siderophore production by *S. acidiscabies* E13 during its interaction with cowpea was detected using the CAS assay as previously described (Dimkpa *et al.*, 2008a). The production of auxins by *S. acidiscabies* E13 in the presence of Ni under siderophore-inducing conditions was assayed using the Salkowski assay described by Patten and Glick (2002). Indole-3-acetic acid (IAA) was used to prepare a standard curve.

2.2.3. Measurement of iron and nickel contents of maize tissues

The uptake of Fe and Ni in maize shoot and root was determined spectrometrically, as previously described (Dimkpa *et al.*, 2008a).

2.3. Results and Discussion

2.3.1. Effect of *Streptomyces acidiscabies* E13 on *in vitro* growth of Ni-stressed cowpea under siderophore-producing conditions

Having established that *S. acidiscabies* E13 produces siderophores in the absence of Fe, the next goal was to evaluate the potential of the strain to promote plant growth in a metal contaminated environment in which Fe is lacking. To this end, an *in vitro* plant growth study involving cowpea (*Vigna unguiculata* L.) under Fe deficiency and Ni contamination was conducted with the strain. The result indicated that *S. acidiscabies* E13 applied at 10^6 CFUs only slightly promoted cowpea growth compared to control plants (Figure 1). This was in contrast to a concurrent experiment with cell-free culture filtrates containing siderophores produced by the strain, which showed significant plant growth promotion (Dimkpa *et al.*, 2008a).

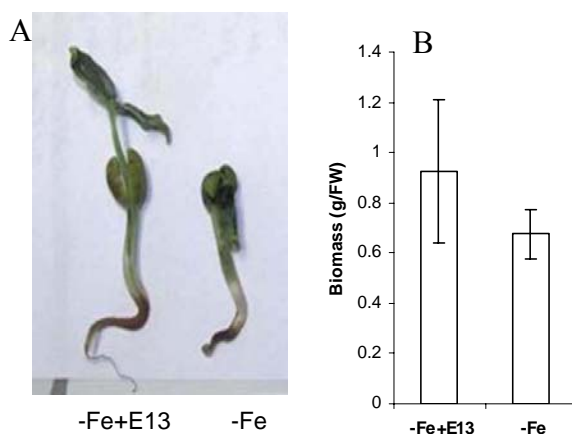


Fig. 1. (A): Photograph of representative 21-day-old Fe-stressed inoculated (10^6 CFU spore suspension of *S. acidiscabies* E13) and uninoculated (-Fe) cowpea grown in the presence of 2 mM nickel. (B) Measured biomass of cowpea plants (n=5).

2.3.2. Microbial siderophore and auxin production during *S. acidiscabies* E13-cowpea interaction

During *S. acidiscabies* E13-cowpea interaction, siderophore detection assay indicated that the bacterium produced siderophores, which was estimated at 80 μ M. In contrast, no Salkowski's assay-detectable level of auxins was observed. The weak growth promoting effect of *S. acidiscabies* E13 on cowpea by the strain was somewhat surprising, especially in view of previously reported dramatic effects of another strain of *Streptomyces* (*S. lydicus* WYEC108) on pea growth, albeit in the absence of metal stress (Tokala *et al.*, 2002), as well as other positive reports of probable siderophore-mediated microbial plant growth promotion in the presence of toxic metals (Burd *et al.*, 1998, 2000; Rajkumar *et al.*, 2006). However, the absence of auxins in the culture medium could also have contributed to the results. Heavy metals such as Ni are reported to inhibit auxin production in bacteria (Kamnev *et al.*, 2005; Dimkpa *et al.*, 2008b).

A number of reasons can be advanced for the lack of plant growth promotion by *S. acidiscabies* E13. Firstly, it is possible that there was a strong bacteria-plant competition for Fe during cowpea growth in the presence of Ni. Siderophores are intrinsic to bacteria, and, by being able to directly assimilate Fe-siderophore chelates, bacteria circumvent the Fe reduction step which is a main feature of strategy I plants. Thus, bacteria would have a greater competitive advantage than plants in terms of siderophore-mediated iron uptake, especially in the presence of inhibitory levels of a toxic metal. Indeed, it has been shown that rhizosphere microorganisms effectively compete for Fe with roots of plants (von

Wirén *et al.*, 1995; Yang and Crowley, 2000). Secondly, certain strains of *S. acidiscabies* have been demonstrated to infect tap-rooting plants (Lambert, 1991), of which cowpea is one. Furthermore, a role was found for DFOE in iron acquisition during plant pathogenicity in bacteria (Dellagi *et al.*, 1998; 2005). Synthesizing these possibilities, future molecular analyses of *S. acidiscabies* E13-cowpea interaction under siderophore-producing conditions would be useful to provide more insight.

2.3.3. Effect of *Streptomyces* spp. on maize and cowpea growth in heavy metal contaminated soil

After 50 days of growth in contaminated soil, no clear difference could be observed in maize plants treated with strains of *Streptomyces*, compared to the control treatment. As can be observed (Figure 2), green pigmentation of the maize plants was affected and all plants appeared stunted (Figure 3), despite mycorrhization.

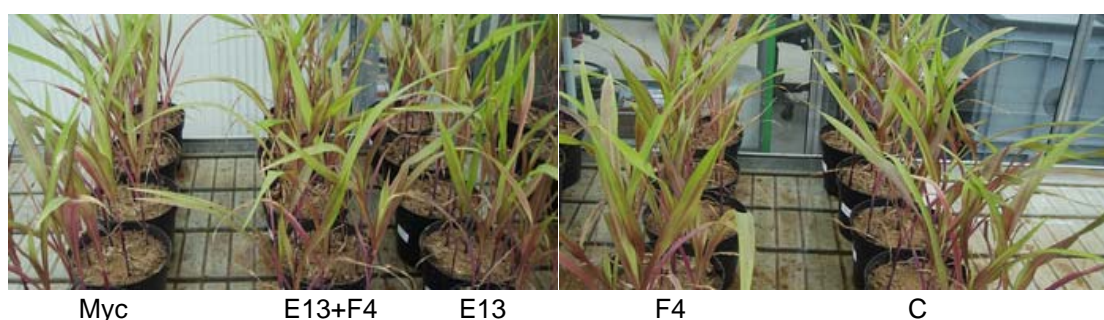


Fig. 2. Photograph of 50-day old maize plants showing the effect of metal toxicity (similar to the “bronzing” effect of Fe toxicity on rice leaves) on leaves. C= control; Myc = mycorrhized plants; E13 = bacterial spores of *S. acidiscabies* E13; F4 = bacterial spores of *S. tendae* F4; E13+F4 = mixed bacterial spores.

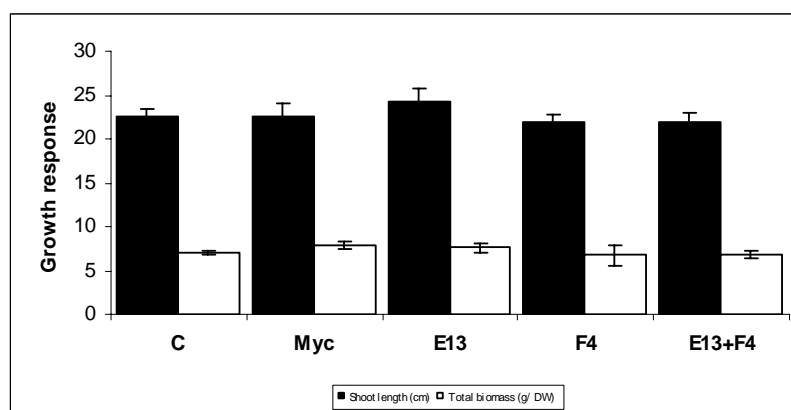


Fig. 3. Effect of endomycorrhization and treatment with spores of *Streptomyces acidiscabies* E13 and *S. tendae* F4 on shoot length and total biomass of maize plants grown in metal contaminated soil (C = no spores, no mycorrhization; n =5).

In view of the apparent loss of green pigmentation by the plants, the chlorophyll contents of the plants were, thus, low (Figure 4). The low chlorophyll contents, which was in spite of the fact that in most cases, the iron contents in the leaves were far higher than previously reported in maize (Meda *et al.*, 2007), can be attributed to loss of chlorophyll which is generally known to result from lipid peroxidation of plants (Sinha and Saxena, 2006; Dimkpa *et al.*, 2009), and which also reflected in the visualizable non-green coloration of the leaves. Iron uptake was not improved by bacterial treatments. Indeed, a significant difference was observed in the shoot iron content of the control plants, compared to treated plants, while all root iron contents were similar. Nickel contents of plants were not affected by bacterial treatments, both in root and shoot (Figure 5), similar to previous report (Madhaiyan *et al.*, 2007)

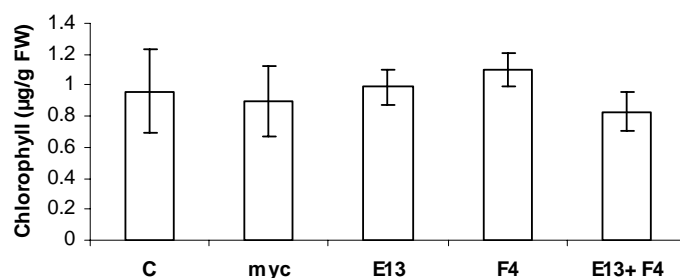


Fig. 4. Effect of endomycorrhization and treatment with spores of *Streptomyces acidiscabies* E13 and *S. tendae* F4 on the chlorophyll contents of maize plants grown in a metal contaminated soil (C = no spores, no mycorrhization; n=5).

In addition to other metals, the experimental soil was shown to contain a high level of Fe (Dimkpa *et al.*, 2009) which, due to the acidic condition of the soil, would be present as plant-toxic ferrous iron (Schmidt, 1999). Not only would this high level of ferrous iron prevent siderophore production by soil bacteria, it would, together with other metals present, also cause metal toxicity in plants. The toxic effect of Fe seemed to have been shown as symptoms similar to leaf bronzing (Figure 2), which is symptomatic for Fe-catalyzed oxidative stress in the monocotyledonous plant, rice (Asch *et al.*, 2005; Becker and Asch, 2005). Indeed, measurements of Fe, Ni and Cd contents in these plants indicated high Fe, but not Ni and Cd contents (Figure 5), in comparison with control cowpea plants grown in the same soil (Dimkpa *et al.*, 2009).

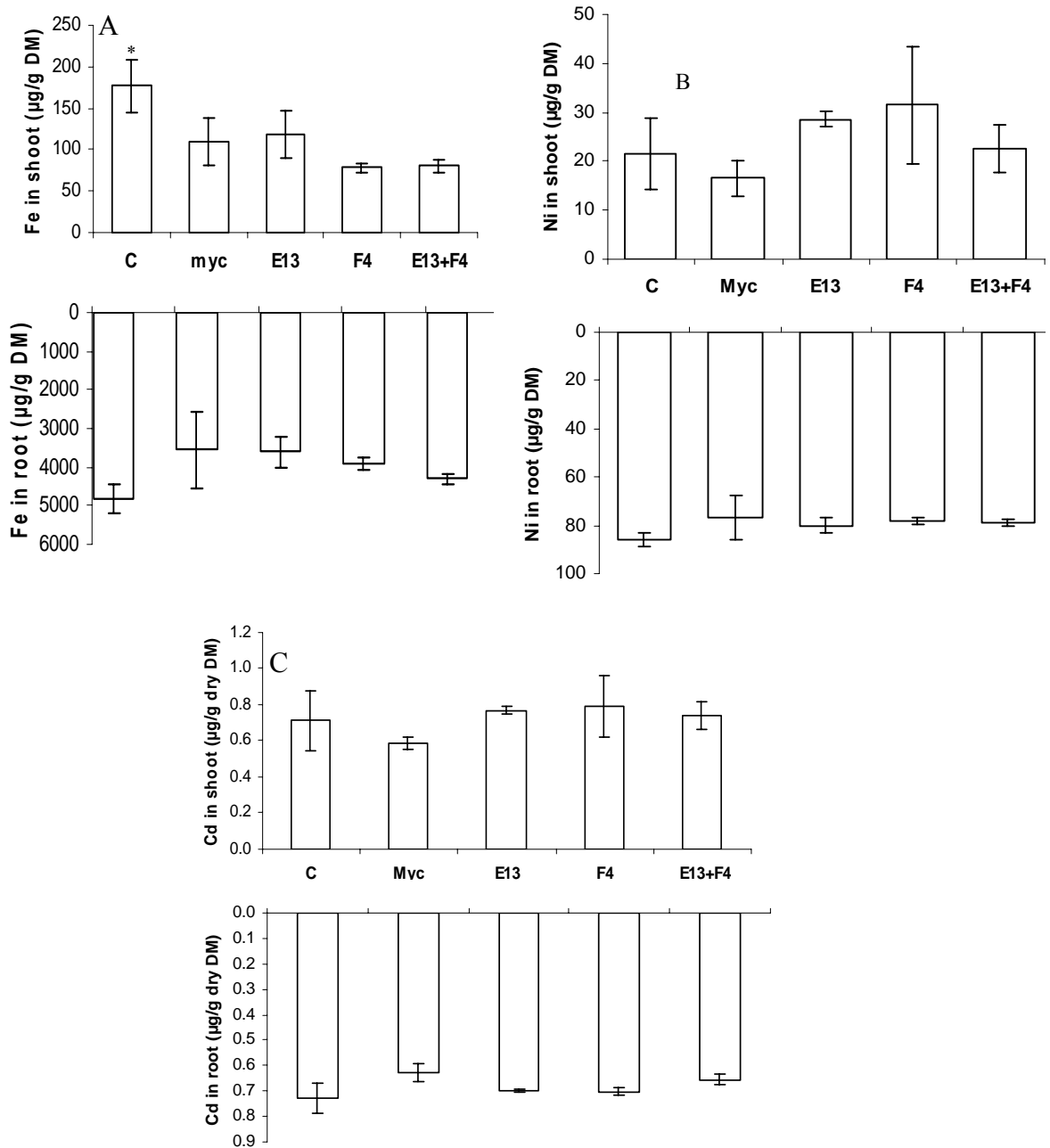


Fig. 5. Effect of endomycorrhization and treatment with spores of *Streptomyces acidiscabies* E13 and *S. tendae* F4 on the uptake of Fe (A); Ni (B) and Cd (C) by maize in metal contaminated soil (C = no spores, no mycorrhization; n=3).

For the cowpea experiments in the contaminated soil, bacterial and mycorrhizal treatments were performed as for maize. After 3 months, the application of mycorrhizae to the soil did not result in positive effect on plant growth, in comparison with control (no mycorrhization and no bacterial treatment). However, mycorrhization and inoculation with bacterial spores showed slightly improved growth in cowpea shoot. Similarly, slightly positive, but statistically insignificant differences (specifically in treatments of *S. acidiscabies* E13, and

in its mixture with *S. tendae* F4) were observed in shoot length of the treated plants. In addition to these results, chlorophyll contents of all metal-stressed plants remained low, irrespective of inoculation (Fig. 6).

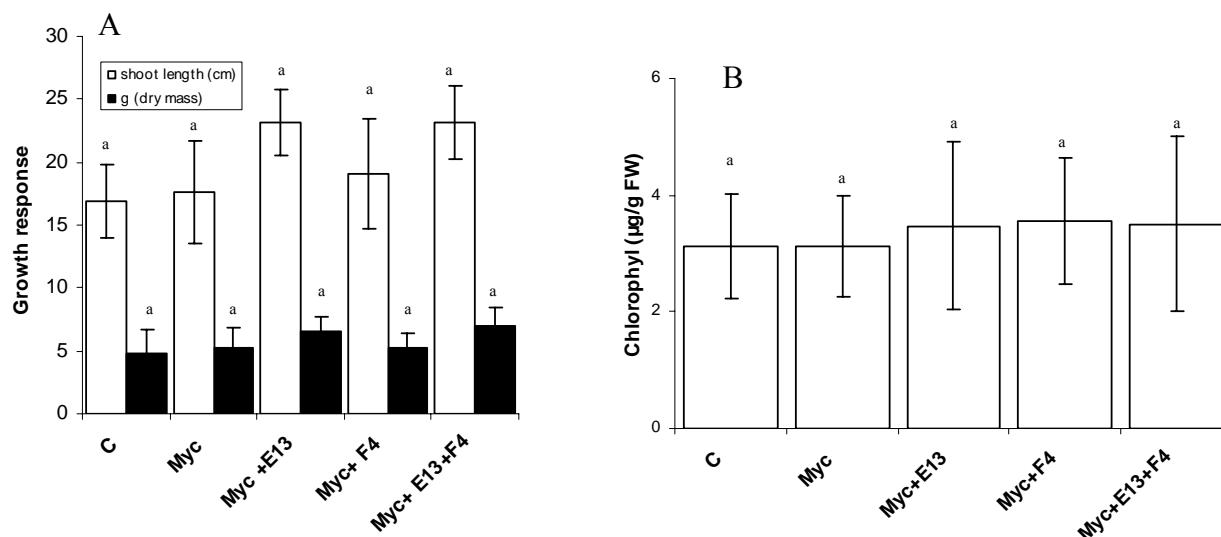


Fig. 6. (A): Effect of mycorrhization (myc) and spore inoculations of *S. acidiscabies* E13 and *S. tendae* F4 on (A) growth, and (B) chlorophyll contents of cowpea grown in soil containing several metals. Values represent means \pm SD of 10 (A) and 5 (B) replicates, and similar letters on the bars indicate lack of statistical differences at $P=0.05$.

In the above soil-based plant growth studies, a subset of the plants were mycorrhized on the basis of previous reports that mycorrhization can protect plants from metal toxicity by either enhancing plant P nutrition, which results in increased plant growth, thereby diluting the toxic effects of metals; or by immobilizing the metals via binding to fungal mycelia (Joner *et al.*, 2000; Chen *et al.*, 2001). In comparison with control treatment, however, the positive effect of mycorrhization on the inhibition of metal uptake was slightly observed in the Fe, Ni and Cd contents of maize, although co-inoculation of the plants with mycorrhizae and bacterial spores tended to increase the metal contents, but not significantly. In all cases, plant growth was positively unaffected. In contrast to mycorrhization, the inability (in the case of maize) or slight ability (in the case of cowpea) of *Streptomyces* to promote plant growth in the contaminated soil can be attributed to the poor C and N status of the soil, which may have affected optimum survival of the bacteria. Furthermore, although Fe bioavailability in the soil appears to be low in terms of a soil unit (g) (Dimkpa *et al.*, 2009), this is actually not the case, when viewed from the total mass of soil used (1 kg). *Streptomyces*, as indeed most other microbes, require far less concentrations of Fe for

their metabolism than is totally bioavailable, limiting the need for siderophore biosynthesis. In addition to the inhibition of siderophore production, excess of the different metals may have also negatively affected microbial auxin production (Dimkpa *et al.*, 2008a, b). Like siderophores, microbially-produced auxins can enhance plant growth (Aldesuquy *et al.*, 1998; Patten and Glick, 2002). Apparently, retarded plant growth in the contaminated soil was due to the presence of toxic levels of several metals, and although siderophore-producing *Streptomyces* could not alleviate the growth inhibition, the external application of siderophore-containing *Streptomyces* cell-free culture filtrate, as with the *in vitro* experiment, did alleviate the growth impediment imposed on cowpea by the metals (Dimkpa *et al.*, 2009).

CHAPTER 3

INVESTIGATION OF α -AMINOCYCLOPROPANE-1-CARBOXYLIC ACID DEAMINASE PRODUCTION BY *S. ACIDISCABIES* E13

3.1. Introduction

In addition to IAA and siderophores, the production of α -aminocyclopropane-1-carboxylic acid deaminase (ACCD) by plant-associated bacteria has frequently been associated with plant growth promotion under metal stress conditions (Burd *et al.*, 1998; Belimov *et al.*, 2005; Rajkumar *et al.*, 2006; Sheng *et al.*, 2008). A number of plant growth promoting bacteria (PGPB) are able to alleviate the effect of stress ethylene on plant growth during pathogen attack, abiotic stress, or during seed germination by the enzymatic deamination of the ethylene precursor, ACC, into NH₃ and α -ketobutyrate. The NH₃ is subsequently utilized as a nitrogen source (Hao *et al.*, 2007). Thus, ACCD activity is an important feature of efficient PGPBs, especially for plants faced with heavy metal stress conditions, and has contributed in plant growth promotion under metal stress according to several reports (see for example, Burd *et al.*, 1998; Belimov *et al.*, 2005; Rajkumar *et al.*, 2006; Sheng *et al.*, 2008). In order to provide further insight on the observed inability of *Streptomyces acidiscabies* E13 to clearly promote plant growth in the presence of heavy metals, the ability of the strain to produce ACCD in the presence and absence of toxic metals was tested.

3.2. Materials and Methods

3.2.1. Bacterial strain, growth and ACC deaminase assay conditions

ACC deaminase activity was assayed on *S. acidiscabies* E13 according to the method described by Penrose and Glick (2003) with slight modification: instead of DF medium, the siderophore-inducing medium of Alexander and Zuberer (1991) was used. However, NH₄Cl in the siderophore-inducing media recipe was replaced with 3 mM ACC as the sole nitrogen source. The medium was then differentially amended as follows: -Fe-Cd/Ni; -Fe+Cd/Ni; +Fe-Cd/Ni, and +Fe+Cd/Ni, at 100 μ M each of Fe, Cd and Ni, where present. Cultures were incubated for 72 hours and the ACCD assay was conducted thereafter, using a serially-diluted (0.1 μ M and 1.0 μ M) α -ketobutyrate (2-oxobutyric acid; Sigma-Aldrich, Schnellendorf, Germany) to prepare a standard curve.

3.3. Results and Discussion

When the absorbances of the different bacterial treatments were compared with those of the standard, it could be shown that *S. acidiscabies* E13 did not produce detectable amounts of ACCD, at least in the recommended standard range (Penrose and Glick, 2003). Moreover, the color of all treatment solutions remained light-green, similar to the control (0 μM α -ketobutyrate) in the standard assay (Table 1).

Table 1: Activity assay indicating non-production of α -aminocyclopropane-1-carboxylic acid deaminase by *Streptomyces acidiscabies* E13 under Fe and Cd/Ni interplay. Experiments were repeated twice and one representative data is presented.

Standard curve (μM α -ketobutyrate)	Abs (540 nm)	<i>S. acidiscabies</i> E13	
		Trt	Abs
0.0	0.128	-Fe-Cd	0.127
0.1	0.303	-Fe+Cd	0.087
0.2	0.614	+Fe-Cd	0.109
0.3	0.958	+Fe+Cd	0.109
0.4	1.305	-Fe-Ni	0.108
0.5	1.613	-Fe+Ni	0.112
0.6	1.764	+Fe-Ni	0.108
0.7	1.934	+Fe+Ni	0.106
0.8	1.992		
0.9	2.113		
1.0	2.234		

*Since absorbances (abs) are comparable to absorbance of 0.0 μM α -ketobutyrate in standard, sample absorbances did not account for those of assay reagent + ACC, and bacterial extract + assay reagent in the absence of ACC.

Based on these results, it could, therefore, be assumed that *S. acidiscabies* E13, though siderophore and auxin producers, could not directly enhance plant growth owing, probably, to high levels of stress ethylene induced by Cd (Pennasio and Reggero, 1992), which, in these cases, were not neutralized due to the absence of an ACCD. Belimov *et al.* (2005) obtained a positive correlation between ACCD activity and plant root elongation, further emphasizing the importance of this enzyme in bacterial plant growth enhancement under stress conditions (Glick *et al.*, 1998). Thus, it can be concluded that apart from the non-trivial observations that scab-producing *Streptomyces* strains (for example, *S. acidiscabies* E13) are pathogenic even to plants other than potatoes (Lambert, 1991), the non-plant

growth promoting traits of *S. acidiscabies* E13 can be due, in part, to their inability to counteract the negative effect of stress ethylene induced by metals.

CHAPTER 4

IDENTIFICATION OF SIDEROPHORE AND CADMIUM-INDUCIBLE PROTEINS IN *STREPTOMYCES TENDAE* F4**4.1. Introduction**

Though primarily produced by most microorganisms in response to environmental iron (Fe) deficiency, siderophores also bind other metals, where present. Such metals have been shown not only to interact with released siderophores, but also to stimulate siderophore production in different bacteria (see for e.g., Huyer and Page, 1988; Visca *et al.*, 1992; Hofte *et al.*, 1993; Hu and Boyer, 1996; Dao *et al.*, 1999; Sinha and Mukherjee, 2008; Wichard *et al.*, 2008; Greenwald *et al.*, 2008). *Streptomyces tendae* F4 is a cadmium (Cd)-resistant bacterium (Schmidt *et al.*, 2005) which, when inoculated in sunflower in the presence of Cd, could supply significant amounts of Fe to the plant (Dimkpa *et al.*, unpublished). It has been previously shown that this bacterium can simultaneously produce three different hydroxamate siderophores, namely desferrioxamines B and E, and coelichelin; all of these were capable of binding divalent metals such as Cd, and nickel (Ni) (Dimkpa *et al.*, 2008). In addition, the ability of desferrioxamine E to form complexes with aluminum (Al) and copper (Cu) was also demonstrated (Dimkpa *et al.*, 2009). Interestingly, all of these metals significantly stimulated siderophore production in the bacterium (Dimkpa *et al.*, 2008).

The presence of Fe represses siderophore production in bacteria; however, in *S. tendae* F4, both Cd and Ni could circumvent the repression of siderophore production caused by low Fe concentrations (Dimkpa *et al.*, 2008). Indeed, induced scarcity of Fe is a common occurrence in the overwhelming presence of other metals due, apparently, to interference by such metals in the Fe acquisition process (Baysse *et al.*, 2000). As a result, more siderophores are exuded by the affected bacteria. Nevertheless, binding of the metals by the released siderophores reduces free siderophore pool; Fe acquisition becomes a serious challenge, which bacteria try to overcome by further release of siderophores. In the case of a toxic metal such as Cd, the situation becomes even direr, as the bacterium is faced with two stress conditions: Cd toxicity and Cd-induced Fe deficiency.

Bacteria have evolved strategies to prevent or reduce heavy metal intoxication, including the elaboration of efflux pumping systems, sequestration and immobilization in cellular

compartments (Prasad, 2001). However, exclusion of metal entry into cells is also a possibility. Consistent with this, it has been shown that the uptake of Cd was reduced in growing cells of *S. tendae* F4 by siderophores produced during growth under Cd stress (Dimkpa *et al.*, unpublished), similar to the siderophore-mediated reduction in Cd and Al uptake previously reported in *Alcaligenes* and *Bacillus*, respectively (Hu and Boyer, 1996; Gilis *et al.*, 1998). Thus, by binding metals in the environment, siderophores can prevent or reduce metal uptake, and hence their toxicity, in bacteria. However, the molecular mechanisms underlying this role are not clearly understood, though it appears that the uptake of metal-bound siderophores is highly specific, depending on whether such complexes are recognized by specific bacterial uptake receptors (Moore *et al.*, 2005; Greenwald *et al.*, 2008). In the case of *S. tendae* F4, several attempts to detect Cd-bound siderophores in cell extracts using mass spectrometry have so far proved unsuccessful (Dimkpa *et al.*, unpublished observation), indicating that Cd can enter the cell through other uptake systems (Que and Helmann, 2000).

In the chrome azurol S (CAS) assay, the detection of siderophores in the presence of metals is usually underestimated since only free siderophores can be detected. However, by allowing a longer incubation time to enable affinity-dependent metal displacement from siderophores, Cd-bound siderophores could be detected. Coupling this with mass spectrometry, the stimulation of siderophore production by Cd was confirmed (Dimkpa *et al.*, 2008). In order to further investigate the observed stimulatory effects of Cd on siderophore production, as well as identify the proteins involved in the observed siderophore-mediated reduction of Cd uptake in *S. tendae* F4, a proteomics approach was applied to study whether the high siderophore contents has a genetic basis - in which case proteins responsible for the production of siderophores would be up-regulated - or whether the phenomenon is merely due to an accumulation of Cd-bound siderophores in the medium, especially given the apparent rejection, or at least, reduction, of siderophore-mediated uptake of Cd by the strain. It was envisaged that a proteomics approach combining two-dimensional gel electrophoresis and mass spectrometry can reveal the functional complement of the genetic information surrounding metal-induced siderophore production, especially in the absence of any genome sequence information on *S. tendae* F4.

4.2. Materials and Methods

4.2.1. Bacterial strain and growth conditions

Streptomyces tendae F4 was cultured under Cd (100 μ M or 300 μ M CdCl₂) stress and, where applicable, in the presence of Fe (100 μ M FeCl₃) as previously described (Dimkpa *et al.*, 2008). Three different Cd/Fe interplays were set up as follows: -Fe-Cd, -Fe+Cd and +Fe+Cd, where “-Fe” treatments represent siderophore-inducing conditions. Cells were grown for 3 days and thereafter harvested via centrifugation at 4000g.

4.2.2. Protein isolation

Following centrifugation, cell pellets were twice washed in extraction buffer containing sucrose (330 mM), Tris/HCl, pH 8.0 (50 mM), EDTA (3 mM) and DTT (1 mM). Proteins were then isolated from the different treatments by homogenization in liquid nitrogen using a ceramic mortar and pestle. Ground cells were mixed in 10 mL of the extraction buffer and centrifugated at 11, 000 rpm for 10 min. The surfactant was mixed (1:1) with a solution of 20% TCA in acetone, containing 0.0462 g DTT, and incubated for 20 min at -20°C, followed by a further incubation for 2 hours at 4°C. The samples were centrifuged at 11, 000 rpm for 10 min, pellets were washed 5 times with acetone, dried in a Speedvac (\leq 5 min), dissolved in 0.5 mL of rehydration buffer (8M Urea; 2M Thiourea; 4% CHAPS; 200 μ l IPG buffer and 120 μ l Destreak™ Reagent (GE Healthcare Europe GmbH, Munich, Germany), or 40 mM DTT, quantified using the Bradford assay (Bradford, 1976) and stored at -20°C for 2-D electrophoresis gel analysis.

4.2.3. Two-dimensional gel electrophoresis

Protein dilutions (500-600 μ g) were loaded onto IPG strips (pH 3-10, non-linear, 24 cm; GE Healthcare Europe GmbH, Munich, Germany) corresponding to the IPG buffer used for rehydration, and allowed to rehydrate overnight in a re-swelling tray covered with mineral oil (Drystrip Cover Fluid; GE Healthcare Europe GmbH, Munich, Germany) to prevent drying. The rehydrated strips were then focused in steps for 9 hrs: 45 min (300 V, 15 min; 500 V, 30 min; 1000 V, 1 hr; 3000 V, 1 hr; 8000 V, 7 hr; 50 μ A/strip; 20°C) using an Ettan™ IPGPhor II (GE Healthcare Europe GmbH, Munich, Germany). The focused IPG strips were equilibrated in two steps: buffer I (6M urea; 4% SDS; 30% glycerol; 1.5 M Tris/HCl, pH 8.8; 1% DTT) and buffer II (6M urea; 4% SDS; 30% glycerol; 50 mM of 1.5 M Tris/HCl, pH 8.8; 4% iodoacetamide) for 15 min each. The equilibrated strips were subsequently loaded onto an Ettan Dalt Six Electrophoresis Unit (GE Healthcare Europe GmbH, Munich, Germany) containing 1x and 2x electrode buffers (30 g TRIS; 10 g SDS; 144 g glycine, in 1 l water) in the lower and upper buffer chambers, respectively. SDS gel

electrophoresis was performed overnight (12.5% polyacrylamide gel; 600 V; 400 mA; 5 W/gel; 5°C).

4.2.4. Proteome analysis

The resolved gels were stained with Coomassie Brilliant Blue (5X Roti Blue™, Roth, Karlsruhe, Germany), according to the manufacturer's description; gels were scanned using GS-800 Calibrated Densitometer (Bio-Rad, Minnesota, USA) and protein spots were picked from gels and digested with trypsin, following the procedure of Giri *et al.*, 2006. Digested peptides were subjected to mass spectrometry and database search for identification.

4.3. Results and Discussion

Streptomyces tendae F4 was cultured in the presence and absence of Cd under siderophore-inducing conditions. Figure one (upper panel) shows cytoplasmic protein pattern induced by Cd in the absence of added Fe. There were obvious horizontal streaks in both gels, despite the use of a destreak reagent. Sample clean-up and repeated 2-D gel electrophoresis did not improve the gel patterns. However, approximately 15 soluble protein spots were picked from the Cd (300 µM)-treated sample. These spots were either completely absent or were of higher, and in a few cases, lower visual intensity, compared to the absence of Cd. The lower panel depicts protein pattern of a Fe-sufficient (100 µM) sample containing Cd (100 µM; A), compared to sample with similar Cd concentration, in the absence of added Fe (B). A low number of detectable spots were observed in the presence, compared to the absence of Fe, from where approximately 29 spots were randomly picked for identification. At the moment, MALDI-TOF and ESI-MS/MS analyses are ongoing, to identify the selected spots, based on which further discussion of the results is pending.

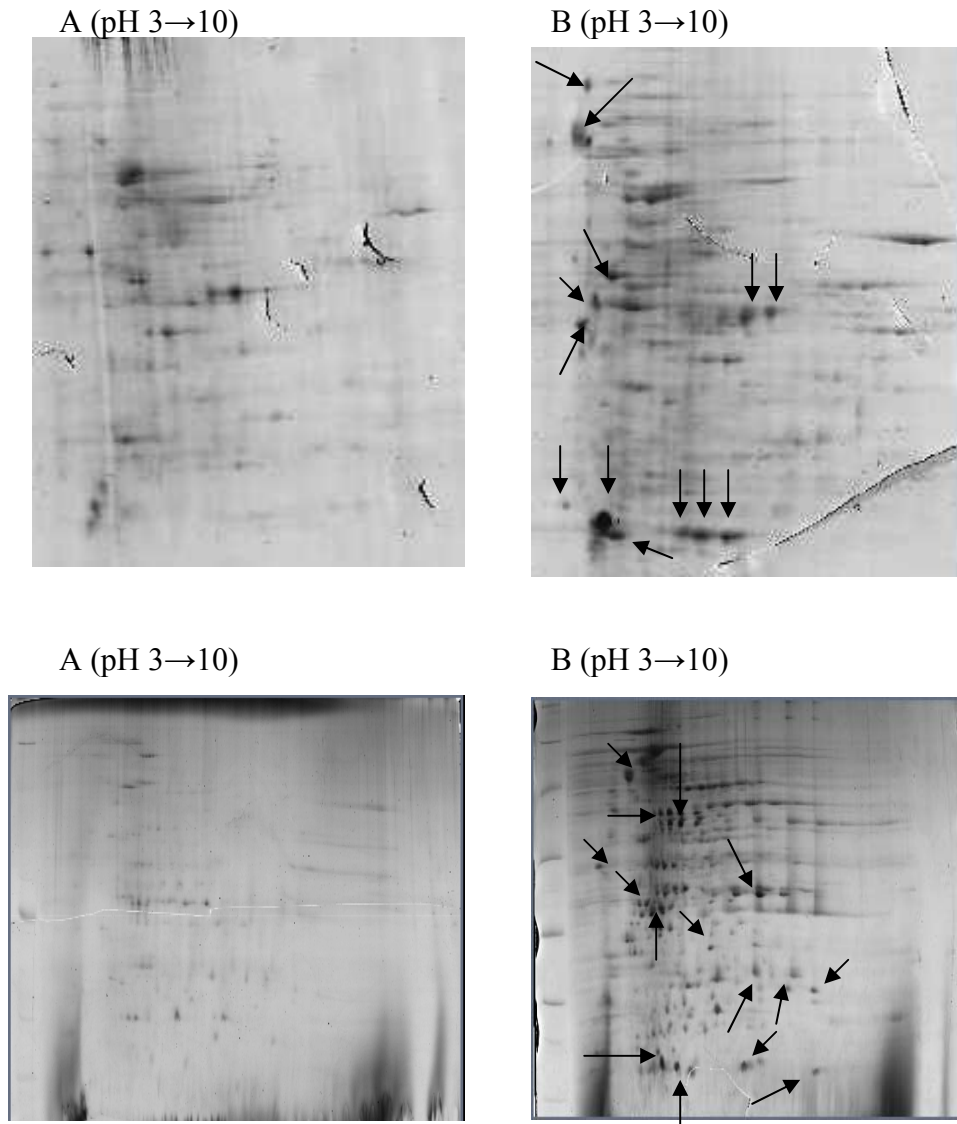


Fig. 1. Two-dimensional gel electrophoresis of *S. tendae* F4 cytoplasmic proteins. Upper Panel: gels of cells grown in the absence of Fe and Cd (-Fe-Cd; A), and in the absence of Fe with Cd (300 μ M) amendment (-Fe+Cd; B). Lower Panel: gels of cells grown in the presence of Fe and Cd (+Fe+Cd; A), and in the absence of Fe with Cd (100 μ M) amendment (-Fe+Cd; B). Arrows represent some of the spots picked for identification.

GENERAL DISCUSSION

Streptomyces acidiscabies E13 and *S. tendae* F4 produce hydroxamate siderophores which, by means of mass spectrometric methods, were confirmed to be desferrioxamines B and E, and coelichelin. All three siderophores can be produced at the same time; however, unlike coelichelin, the two desferrioxamines are expected to be encoded in the same biosynthetic operon, as was shown with *S. coelicolor* (Barona-Gomez *et al.*, 2006). Like all siderophores, the production of DFOB, DFOE and Cch were shown to be regulated by iron, and could reach a peak 72 hours after culture inoculation of *S. acidiscabies* E13 and *S. tendae* F4, which is more or less similar to the result for a different hydroxamate siderophore in another actinomycetes (Oliveira *et al.*, 2006). The presence of metals resulted in a stimulation of siderophore production (Dimkpa *et al.*, 2008b). In particular, high levels of Ni led to a slowed-down but increasing cell growth and siderophore production patterns, so that a higher final siderophore concentration was obtained after 120 hours of culture incubation (see also Hu and Boyer, 1996b). Considering that siderophores bind metals other than Fe, it was imagined that the stimulation of siderophore production by these metals resulted from the fact that free siderophore concentrations was decimated following their interaction with the metals, so that there arises the need for continuous siderophore production to acquire sufficient Fe for cell growth in the presence of toxic concentrations of other metals.

Metal contamination of soils is a critical impediment to crop and animal production, contributing to the occurrence of undesirable levels of toxic metals in the food chain. In general, the remediation of heavy metal polluted soils is conventionally often carried out by excavation and removal of soil to landfills, which are considered “secured”. Obviously, this is expensive and would, in any case, require the subsequent restoration of the excavated site. The heavy metal contaminated site Wismut, near Ronneburg in Thuringia, Eastern Germany, is an example of an anthropogenically-induced soil pollution following the mining of uranium. Remediation of the area began by placing the waste rock material in the former open pit. The water table is allowed to rise, thereby re-establishing anoxic conditions in saturated zones, and so preventing further oxidation of the pyrite-rich material and production of acid mine drainage (AMD) waters. Seepage waters resulting from leaching of the former waste rock piles by AMD have, however, infiltrated adjacent soils,

and surface waters, bringing with them large amounts of heavy metals such as nickel, cadmium, copper, chromium, and rare earth elements, among others (Merten *et al.*, 2004). In contrast to the above remediation strategy, phytoremediation - the use of plants to extract metals from contaminated soil- is a relatively recent technology employed to reduce soil metal load. In comparison with conventional technologies to remove heavy metals from soil, phytoremediation offers the benefit that it is relatively inexpensive and safe for humans and the environment (Krämer, 2005): comparative lower cost; applicability to a broad range of metals; potential for recycling the metal-rich biomass; minimal environmental disturbance, and minimization of secondary air- and water-borne wastes. However, plants in contaminated environment often experience slow growth and low biomass production due to the increased stress conditions imposed by the metals (Cakmak and Horst, 1991; Kumar *et al.*, 1995; Burd *et al.*, 2000; Dimkpa *et al.*, 2008a, 2009). Thus, phytoremediation is impeded by the fact that even the best metal-accumulating plants can be affected by high concentrations of the metals (Fischerova *et al.*, 2006). Furthermore, poor metal solubility, especially in alkaline soils, is also a major hindrance to the successful application of phytoremediation (Nowack *et al.*, 2006, and references therein).

One of the strategies applied since the last few decades to increase soil metal solubility for subsequent plant uptake is the use of a variety of synthetic metal chelators, especially EDTA, which solubilizes the metals, making them more available for plant uptake (Hale and Wallace, 1970; Liphadzi *et al.*, 2003; Wenger *et al.*, 2005; Lopez *et al.*, 2005, 2007). Whereas this has led to improved metal uptake by plants, the use of synthetic chelators in soil for the purpose of assisted phytoremediation is fraught with a number of disadvantages, the main being that they are not readily degraded from the soil, thereby constituting a new source of environmental pollution (Bucheli-Witschel and Egli, 2001; White, 2001; Nowack *et al.*, 2006). Thus, new complementary strategies become necessary to assist otherwise efficient metal accumulating plants overcome this problem. In addition to synthetic metal chelators, the use of synthetic phytohormones to facilitate plant growth in metal contaminated soil was recently demonstrated (Lopez *et al.*, 2005, 2007; Liphadzi *et al.*, 2006). The rationale behind this strategy is that phytohormones enhance root growth and consequently overall plant growth. An enhanced root growth will, in turn, augment metal uptake, since more roots will be available to take up more metals from the soil. Together with EDTA, the uptake of metals was shown to be higher when IAA, the archetypal phytohormone, was applied (Liphadzi *et al.*, 2006; Lopez *et al.*, 2007). Yet, the use of

synthetic IAA is not a sustainable option for large scale phytoremediation, due to reasons of cost, especially in resource-poor countries. Therefore, the exploitation of other cost and environmentally-friendly alternative technologies to facilitate the phytoremediation of contaminated sites such as the Wismut site becomes imperative. In this regard, the use of microorganisms readily comes to mind.

As has been shown, both *S. acidiscabies* E13 and *S. tendae* F4, as well as several other *Streptomyces* spp. can simultaneously produce siderophores and auxins (Dimkpa *et al.*, 2008b). Tryptophan-dependent biosynthesis of IAA has been described in *Streptomyces* spp. (El-Sayed *et al.*, 1987; El-Shanshoury, 1991; Manulis *et al.*, 1994); but *S. acidiscabies* E13 and *S. tendae* F4 could also produce IAA in the absence of added tryptophan (Dimkpa *et al.*, 2008a, b). Like its synthetic analogue, microbially-produced IAA can directly (Aldesuquy *et al.*, 1998; Patten and Glick, 2002), or indirectly (Dimkpa *et al.*, 2009), promote plant growth. Therefore, it was thought that microbial siderophores and auxins, respectively, can provide concurrent alternatives to the use of synthetic chelators and synthetic IAA in assisted-phytoremediation of metal pollution from soil. However, the presence of one or more metals negatively affects the production of microbial auxins (Kamnev *et al.*, 2005; Dimkpa *et al.*, 2008a), a phenomenon which was subsequently confirmed by gas chromatography-mass spectrometry (Dimkpa *et al.*, 2008b). Apparently, in addition to inhibiting auxin biosynthesis, metals also affect auxin activity, due to metal-induced oxidative stress which causes the increased production of IAA-degrading oxidases (Chaoui and El Ferjani, 2005; Johri *et al.*, 2005; Potters *et al.*, 2007; Dimkpa *et al.*, 2009). Despite this, further confirmatory studies based on the use of a siderophore-biosynthetic mutant and iron-replete conditions which precluded siderophore production, did indicate that co-produced siderophores can alleviate metal inhibition of auxin production by chelating the metals present. At the same time, the production of siderophores was upregulated in the presence of the metals, even when Fe was present; and the siderophores were shown to bind these metals on release to the environment (Dimkpa *et al.*, 2008b). At a nickel concentration of 2mM, a higher abundance of DFOE-Ni was detected at 72 and 96 hours after culture establishment of *S. acidiscabies* E13, than were DFOB-Ni and Cch-Ni. Increasing Ni concentration to 5mM, however, led to significant loss of the DFOE-Ni complex at the peak production time points of 96 and 120 hours. At this excessive concentration, Ni was able to out-compete the trace amounts of Fe present in the medium

for siderophore binding, so that a DFOE-Fe complex was not detectable. In contrast, an increase in DFOB-Ni and Cch-Ni complexes was observed (Chapter 1). Addition of low levels of Fe to Cd and Ni-containing cultures of *S. tendae* F4 led to increased detection of DFOB and Cch complexes of both Cd and Ni, which contrasted with the observed formation of low DFOB and Cch complexes with both metals under Fe deficient conditions (Dimkpa *et al.*, 2008b). At first glance, the finding that considerable abundances of all three siderophores remained metal free, despite the availability of high levels of Cd and Ni, may be surprising; yet earlier hypothesis of Davis and Byers (1971) on the formation of bio-unusable polymers between Fe and other metals may be considered a possible reason influencing metal binding, given that siderophore-metal interaction is a 1:1 relationship. Nevertheless, the differing metal binding specificities by the different hydroxamate siderophores, together with the observation that a high concentration of Ni affected the Fe scavenging ability of DFOE, led to the postulation that the biosynthesis of multiple siderophores illustrates the benefit of producing chelators with varying metal affinities and preferences that ultimately helps bacterial survival in heterogeneously polluted soils, and contributes to their usefulness in bioremediating such soils (Dimkpa *et al.*, 2008b).

Investigation of Cd uptake by *S. tendae* F4 under Fe deficiency condition confirmed that the produced siderophores can prevent, or at least, reduce metal uptake in bacteria, as previously reported for other bacteria (Hu and Boyer, 1996b; Gilis *et al.*, 1998). This contrasts with evidence of the facilitation of plant metal uptake by siderophores as reported in this work. Thus, mechanisms that reduce the bioavailability of toxic metals in the environment for rhizosphere bacteria, as well as provide plants with improved access to metals, are likely to determine ecotoxicologically-relevant metal concentrations in the soil, and their influence in microbial metabolite-assisted phytoremediation. Investigations involving the identification of proteins inducible under siderophore-producing conditions and in the presence of Cd in *S. tendae* F4 are currently underway. At present, protein analysis based on 2-D gel electrophoresis indicated the presence of candidate protein spots that have been selected for identification by MALDI-TOF and ESI-MS/MS. It is hoped that results from this might provide clues on the observed siderophore-mediated reduction of Cd uptake as well as the metal stimulation of siderophore production in *Streptomyces*.

In addition to the general unavailability of iron induced by high soil pH, competition for iron between plants and associated bacteria can cause iron-deficiency symptoms in the

environment, usually to the detriment of the plants (von Wirén *et al.*, 1995; Yang and Crowley, 2000; Winkelmann, 2007). Moreover, it was demonstrated by a number of studies that iron deficiency symptoms become aggravated in the presence of other metals (see for example, Alcántara *et al.*, 1994; Yoshihara *et al.*, 2006; Dimkpa *et al.*, 2008b), which seems to be supported by the hypothesis of Davis and Byers (1971). However, metal-induced Fe deficiency can also result due to other metals competing with Fe at root binding sites (Alcántara *et al.*, 1994). In such scenarios, both plants and associated microbes can be pictured as being equally iron-stressed. On the basis of the idea that microbial siderophores might facilitate the uptake of iron by plants, their release by associated microbes becomes advantageous to plants. Thus, based on their metal tolerance and prolific siderophore and auxin production, *S. acidiscabies* E13 and *S. tendae* F4 were deployed for testing plant growth and metal accumulation in the contaminated test field site. *S. acidiscabies* E13 was also tested in vitro. Surprisingly, however, neither of the strains resulted in any dramatic plant growth differences, compared to control maize, cowpea and sunflower (in the case of *S. tendae* F4 only) plants, nor did they positively influence plant chlorophyll contents, and uptake of Fe, Ni and Cd by maize (Chapter 2). If competition for siderophore-mediated uptake of iron between plants and microbes can be considered, a comparative study of Figure 6 (manuscript I) and Figures 2 and 4 (manuscript 4) may provide some hint as to which organism, bacteria or plant, can better maximize the presence of siderophores for iron uptake under metal stress conditions. In making this comparison, it is, however, recognized that the metal content analyses were not conducted with samples obtained during actual plant-microbe interactions.

Since both microbial auxins and siderophores are plant growth promoting substances, the co-production of auxins and siderophores by bacteria can potentially confound any attempt to evaluate the lone effect of microbial siderophores in plant growth under metal stress conditions. In the absence of an IAA biosynthetic mutant of *Streptomyces*, this drawback was circumvented by either growing the bacteria in the presence of a high concentration of nickel, which significantly reduced the presence of auxins in the siderophore-containing culture filtrates before use for plant growth, (Dimkpa *et al.*, 2008a), or by applying equivalent amounts of synthetic IAA to control the effect of microbial auxins contained in culture filtrates (Dimkpa *et al.*, 2009). In these studies, it could be shown that in the presence of inhibitory levels of one or more metals, siderophores enhanced Fe reductase

activity in the root, leading to enhanced uptake of reduced Fe and consequently higher leaf chlorophyll content. It was also demonstrated that in the absence of a metal chelator, IAA can be far less efficient in promoting plant growth in metal-contaminated soil, due to the occurrence of metal-induced oxidative stress. Elevated levels of oxidative stress induced by the metals significantly inhibited plant growth, reduced chlorophyll, RNA, protein and residual IAA contents, and caused an increase in superoxide dismutase activity, but not in carotenoid content. By means of liquid chromatography-mass spectrometry, the superior affinity of siderophores for Fe than other metals was confirmed; however, it could be demonstrated that Al can compete with Fe for siderophore binding. It was therefore concluded that not would siderophores only avail plants with solubilized metals, thus directly facilitating phytoremediation, but that by binding metals, they also reduce oxidative stress in plants through lowering the formation of metal-catalyzed free radicals that damage plant cells. Apparently, this role directly enabled plants to be healthy enough to utilize co-available microbial auxins for plant growth, with the results that, in general, more metals could be taken up by siderophore-treated plants. Fortunately, unlike auxins, the production of siderophores by bacteria is stimulated by metals other than Fe (Huyer and Page, 1988; Visca *et al.*, 1992; Hu and Boyer, 1996b; Dimkpa *et al.*, 2008b). Thus, an advantage is conferred by bacteria able to produce auxins and siderophores simultaneously, since it implies that metal stimulation dramatically increases the siderophore pool in the rhizosphere, some of which will scavenge for and chelate Fe needed for plant growth, while the rest sequester and thereby inactivate the other metals present, reducing free toxic metal concentrations that would otherwise interfere with auxin synthesis and activity (plant root development). Ultimately, a microbial auxin-enhanced root system ensures that more of the sequestered metals are taken up by the plant.

Considering that factors other than iron uptake and auxins can play a role in plant growth promotion by rhizobacteria, the ability of *S. acidiscabies* E13 and *S. tendae* F4 to produce ACCD was evaluated, and it could be demonstrated that this ability is lacking in both strains, irrespective of the presence or not of metals. It is worthy of note that ACCD is not an extra-cellular enzyme, and as such, is not expected to be present in the *Streptomyces* culture filtrates that promoted plant growth (Dimkpa *et al.*, 2008a, 2009, submitted). Interestingly, as yet, there are no reports of the existence of ACCD in *Streptomyces* spp, as was also recently indicated (Kuffner *et al.*, 2008). On the strength of these observations,

and the well-reported role of ACCD in plant growth under metal stress condition, it is concluded that *S. acidiscabies* E13 and *S. tendae* F4 lack a critical intrinsic plant growth promoting feature described in previous reports (Burd *et al.*, 1998; Penrose and Glick, 2003; Belimov *et al.*, 2005; Rajkumar *et al.*, 2006; Sheng *et al.*, 2008).

Synthesising findings from (i) production, or otherwise, of siderophores, auxins and ACCD by *S. acidiscabies* E13 and *S. tendae*; and (ii) the ability of the produced metabolites, but not live cells of the strains, to promote plant growth, the question then arises as to whether the above-mentioned plant growth promoting stimuli are simultaneously required to produce a synergistic plant growth promoting effect, or whether they act independent of one another. Using 35 bacterial strains, Sessitsch *et al.* (2004) showed that depending on individual strains, the effects of these traits can be co-dependent or stand-alone. For example, *Pantoea agglomerans* and *Clavibacter michiganensis* did not promote plant growth, yet they produced siderophores and auxins; *Pseudomonas* spp. and *Microbacterium testaceum* promoted plant growth and produced both siderophores and auxins; *Brevundimonas* spp. and *Arthrobacter* spp. promoted plant growth and produced siderophores but not auxins; *Sphingomonas aurantiaca* and *Frigoribacterium* spp. did not promote plant growth neither did they produce siderophores and auxins. Likewise, Belimov *et al.* (2005) showed that a non-plant growth promoting strain of *Pseudomonas* spp. produced high amounts of siderophores, low amounts of auxins but no ACCD. In the same study, it was also demonstrated that *Acidovorax facilis* could produce ACCD and auxins but no siderophores, and did not promote plant growth; *Variovorax paradoxus* could produce ACCD, auxins and siderophores and promoted plant growth, whereas *Flaviobacterium* spp. produced only auxins, while promoting plant growth. A recent report in which *Agromyces* spp., which did not produce any of IAA, siderophores and ACCD, promoted growth and thereby increased Cd and Zn accumulation by willow plants seems to support the argument that these molecules do not necessarily act in concert to enhance plant growth under metal stress conditions, and that other factors also play significant roles in plant-microbe interactions (Kuffner *et al.*, 2008). These results further indicate that mere testing and confirming the production of siderophores, auxins or ACCD *in vitro* may not generally potentiate plant growth promotion by specific bacteria, as the elaboration of these traits during actual plant-microbe interactions often requires specific and favorable interplay with other microbes and associated plants, as well as with prevailing abiotic

factors such as pH, and presence of toxic metals, among others. In particular, it would appear that the interaction between siderophores and plants depends largely on host specificities of the different siderophore types, since the same type of siderophore showed different responses in different plants (Klopper *et al.*, 1980; Becker *et al.*, 1985). In the light of this, assessing the specific effect of siderophores on plant growth and metal uptake might warrant the direct testing of the isolated substance on different plant species, to establish specific siderophore-mediated plant growth enhancement.

Parra *et al.* (2008) tested the metal extraction ability of a variety of metal-chelating substances, and purified DFOB showed relatively good performance for uranium extraction from soil. However, the procedures involved in siderophore isolation, purification and analytical identification are not trivial in terms of time and cost requirements (for example, as of January 2008, one mg of purified DFOE costs 200 Euros, exclusive of VAT and shipment cost). The cost of the amount of purified siderophores required to conduct pilot tests in contaminated field sites can, thus, be imagined. To reduce such costs considerably, cell-free siderophore-containing culture filtrates were used to demonstrate that, indeed, siderophores can promote plant growth and augment metal uptake by plants faced with metal stress. The culture filtrate contained three different hydroxamate siderophores co-produced by *S. acidiscabies* E13 and *S. tendae* F4, and these compounds might complement each other in terms of high affinity and redox potential, based on structural effects on their complex stability (Boukhalfa and Crumbliss, 2002). Our findings directed us to advocate for the use of microbial siderophores for assisted phytoremediation of metal pollution in the form of relatively cheap (in terms of production cost) liquid applications of culture filtrates containing these siderophores, which can be bio-fortified by the presence of microbially co-produced auxins to produce enhanced effects on plants in a sustainable and more environmentally-friendly manner, in consideration of the unwanted persistence in the environment, of synthetic chelators such as EDTA. To test out this proposal, culture filtrates of *S. tendae* F4 containing simultaneously-produced DFOB, DFOE and Cch was compared with EDTA for the uptake of cadmium by sunflower (*Helianthus annuus*). Results from this investigation indicated that siderophores can be better than EDTA for assisting Cd removal by sunflower, and that continued secretion of siderophores by a siderophore-producing bacterium can provide more Fe to plants. Nevertheless, as a dramatic siderophore concentration than was used will be required to yield significant

metal uptake by plants, we suggest that further investigations be conducted to optimize siderophore concentrations, and extensively testing the siderophores with a range of metals and plants.

Given that all the possible mechanisms surrounding the use of microbial siderophores as Fe source for strategy I plants have not yet been fully elucidated, the widespread possibility of the direct uptake of Fe-bound microbial siderophores still remains a topic of interest in the relevant scientific community. This warranted the proposal of a third mechanism for Fe uptake, strategy III, following an indication of Fe uptake strategies other than Fe reduction in strategy I plants (Bar-Ness *et al.*, 1991), its confirmation by the detection of DFOB-Fe chelates in cucumber xylem fluid (Wang *et al.*, 1993), which is also supported by a much recent study with *Arabidopsis* (Vansuyt *et al.*, 2007). However, our attempts to detect intact siderophore-Cd complexes in shoot tissues of cowpea and sunflower using mass spectrometry have, so far, not been successful; at this stage, it cannot be concluded that siderophore uptake systems of these plants, if any, do not recognize the siderophore-Cd complexes. It would, therefore, be interesting to pursue this aspect more intensely, to investigate whether metal uptake systems in plant roots discriminate between siderophore-Fe complexes and siderophores bound to metals other than Fe, as well as to elucidate molecular changes in plant root gene expression patterns engendered by the siderophores.

SUMMARY

Siderophores are primarily iron (Fe) deficiency-responsive metal chelators produced by most microbes and monocotyledonous plants for Fe acquisition; although they can bind other metals as well. In the current study, the role of siderophores from two heavy metal-resistant strains of *Streptomyces* (*S. acidiscabies* E13 and *S. tendae* F4) in rhizosphere interactions between bacteria and metal-stressed plants was evaluated with a view to harnessing siderophores for chelator-assisted phytoremediation of metal pollution. Since not all bacteria produce siderophores, the first task was to investigate whether the selected strains are able to do so. To this end, standard siderophore detection assays, coupled with mass spectrometry, was used to identify the presence of siderophores and the specific siderophore types produced by both strains, showing that these bacteria simultaneously produce two desferrioxamine siderophores and coelichelin, all of which could scavenge for scarce Fe at varying abundances, following their release into the Fe-deficient culture medium. These findings are only the third evidence of the production of coelichelin by any microbe, and the first report of siderophore production in a heavy metal resistant streptomycetes.

Following the confirmation of siderophore production in the strains, the effect of metals in the process was evaluated. Cadmium (Cd) and nickel (Ni) affected the growth of the bacteria, especially under siderophore-producing conditions (-Fe); however, by means of mass spectrometry, it was revealed that the siderophores could bind added Cd and Ni. Real-time siderophore production under metal stress conditions was then followed, indicating a peak production after 72 hours and a decline thereafter. The presence of high levels of Ni, nevertheless, altered siderophore production kinetics, shifting peak production to 120 hours, similar to its effect on bacterial growth under siderophore-producing conditions. Furthermore, it was shown that aluminum (Al), Cd, copper (Cu) and Ni stimulated siderophore production. In particular, Ni and Cd overrode low Fe repression of siderophore production, confirming the interference of metals in the Fe acquisition process.

In microbe-assisted phytoremediation, synthetic indole acetic acid (IAA auxins) is often used to facilitate plant growth, thus increasing overall metal contents of plants. On this basis, the production of auxins by the strains was tested, whereby it could be shown that,

whereas *Streptomyces* strains are able to produce indole acetic acid, the presence of metals dramatically affected this ability, in both Fe-replete conditions precluding siderophore production, and in a siderophore-biosynthetic mutant (*S. coelicolor* W13). Thus, by binding and inactivating the metals, co-produced siderophores alleviated metal-induced inhibition of auxin synthesis. These results indicate that in the presence of siderophores, the negative effect of metals on microbial auxin production is reduced and is, thus, significant, considering the role of microbial auxins in plant growth.

Using *Streptomyces* spores and siderophore-containing cell-free culture filtrates, plant growth under metal stress, as well as metal uptake capability of the plants, was assessed both *in vitro*, and in contaminated soil. Whereas metals inhibited pea and sunflower growth, addition of siderophores improved growth. There were evidences for increased oxidative stress and chlorosis induced by Ni: enhanced membrane peroxidation, increased superoxide dismutase, IAA peroxidase and Fe-reductase activities; lowered chlorophyll, RNA, protein and carotenoid contents. However, all of these metal-induced plant physiological changes were significantly improved by microbial siderophores. Moreover, Ni-induced inhibition of Fe-reductase activity was alleviated by siderophores, resulting in more Fe in siderophore-treated plants. To the best of our knowledge, this is the first evidence of the use of microbial siderophores to alleviate metal-induced oxidative stress in plants. Furthermore, siderophore competition assays between Al and Fe on the one hand, and Fe and Cu on the other, confirmed that Al can compete with Fe for siderophore binding, unlike Cu which showed relatively poor affinity towards the produced siderophores. This suggests that siderophore-mediated Fe acquisition by plants and microbes in soil contaminated with trivalent metals may be more challenging than in soil contaminated with divalent metals.

By means of inductively-coupled mass spectroscopy or inductively-coupled plasma optical electron spectroscopy, it was shown that siderophores dramatically augmented metal uptake from soil by the plants, which, for Cd in sunflower, was slightly more than did EDTA - the synthetic but toxic substance currently used for chelator-assisted phytoremediation. On the other hand, the presence of siderophores limited Cd uptake by bacteria, indicating a preventive role for siderophores in metal intoxication in bacteria. Apparently, mechanisms which reduce the bioavailability of toxic metals in the environment for rhizosphere bacteria but provide plants with improved access to metals are likely to determine ecotoxicologically-relevant metal concentrations in the soil. Studies

involving the identification of proteins inducible under siderophore-producing conditions and in the presence of Cd in *S. tendae* F4 are currently being conducted. Already, 2-D gel electrophoresis could show the presence of a number of candidate protein spots. It is hoped that results from this might shed more light on the observed siderophore-mediated reduction of Cd uptake as well as the metal stimulation of siderophore production in *Streptomyces*.

In contrast to their siderophores, both *S. acidiscabies* E13 and *S. tendae* F4 did not show clear plant growth-promoting effects, nor did they influence the uptake of Cd and Ni by plants; although *S. tendae* F4 did significantly increase shoot Fe content in sunflower in the presence of Cd. These surprising observations directed the investigation of the production of α -aminocyclopropane-1-carboxylic acid deaminase (ACCD) in both strains. Plant stress ethylene levels can be enhanced by the presence of toxic metals, resulting in plant growth inhibition; however, bacterial ACCD ameliorates ethylene-induced oxidative stress by cleaving ACC, the ethylene precursor, into less toxic products. ACCD enzyme assay revealed that both *S. acidiscabies* E13 and *S. tendae* F4 do not produce the enzyme, which, in part, could explain their inability to promote plant growth under metal stress conditions.

On the strength of these findings, it was concluded that auxins and siderophore-containing culture filtrates, especially from multi-siderophore producing bacterial strains, can be used for assisted-phytoremediation of metal pollution in soil, instead of EDTA and synthetic auxins. Use of siderophores in culture filtrate is cost-effective, compared to purified siderophores or even synthetic chelators. Moreover, siderophores are readily biodegradable, so that their use might not bring about a new type of environmental contamination, unlike synthetic chelators which are currently used for the same purpose.

Zusammenfassung

Siderophore sind in erster Linie Eisen-(Fe)-Chelatoren, die bei Fe-Mangel von den meisten Mikroben und Monokotyledonen für die Fe-Mobilisierung gebildet werden, obwohl sie auch andere Schwermetalle binden können. In der vorliegenden Arbeit wurde die Rolle der von zwei schwermetallresistenten Stämmen von *Streptomyces* (*S. acidiscabies* E13 and *S. tendae* F4) gebildeten Siderophore in der Rhizosphäreninteraktion zwischen Bakterien und Pflanzen auf metallkontaminierten Böden untersucht, um abzuschätzen, ob Siderophoren für Chelator-unterstützte Phytoremediation genutzt werden können. Da nicht alle Bakterien Siderophore bilden, wurde zunächst die Siderophorenproduktion der ausgewählten Stämme untersucht. Ein standardisierter Nachweis, gekoppelt mit Massenspektrometrie, wurde genutzt, um gebildete Siderophore zu identifizieren. Beide Stämme produzieren gleichzeitig zwei Desferrioxamin-Siderophore sowie Coelichelin. Unter Mangelbedingungen können diese Fe mit unterschiedlicher Affinität binden, nachdem sie in ein Fe-armes Kulturmedium abgegeben wurden. Diese Ergebnisse sind erst der dritte Nachweis für die Produktion von Coelichelin durch Mikroben und der erste Bericht über eine Bildung von Siderophoren durch (schwermetallresistente) Streptomyceten.

Nachdem die Bildung von Siderophoren durch beide Stämme bestätigt wurde, wurde der Effekt von anderen Metallen auf die Synthese und Fe-Bindung evaluiert. Cadmium (Cd) und Nickel (Ni) beeinträchtigten das Wachstum der Bakterien, insbesondere unter Siderophor-produzierenden Bedingungen (-Fe); allerdings konnte durch Massenspektrometrie gezeigt werden, dass die Siderophore hinzugefügtes Cd und Ni binden konnten. Anschließend wurde die Siderophorenbildung unter Schwermetallstress getestet. Die maximale Produktion trat nach 72 Stunden auf, danach nahm die Produktion ab. Die Anwesenheit von hohen Ni-Mengen änderte allerdings die Kinetik der Siderophorenproduktion, indem sich das Maximum auf 120 Stunden verschob. Dieser Effekt ähnelt dem, den Nickel auf das Bakterienwachstum unter Siderophor-bildenden Bedingungen hat. Außerdem wurde gezeigt, dass Aluminium (Al), Cd, Kupfer (Cu) und Ni die Bildung von Siderophoren stimulierten, wobei insbesondere Ni und Cd in der Lage sind, die Fe-Repression der Siderophorenbildung aufzuheben. Dies deutet auf eine Interferenz von Metallen mit dem Fe-Mobilisierungsprozess hin.

Bei der Phytoremediation mit Hilfe von Mikroben wird häufig synthetische Indolelessigsäure (IAA) genutzt, um das Pflanzenwachstum durch das Phytohormon zu stimulieren und als Folge die Menge extrahierten Metalls zu erhöhen. Auf dieser Grundlage wurde die Produktion von Auxinen durch die untersuchten Stämme analysiert, wobei gezeigt werden konnte, dass Metalle diese generell vorhandene Fähigkeit dramatisch beeinträchtigt. Dabei interferiert unter Fe-gesättigten Bedingungen, die die Siderophorenbildung beeinträchtigen, und in einer Siderophoren-Biosynthese-Mutante von *Streptomyces* die Eisenkonzentration mit anderen Metallen. Infolge der Bindung und Inaktivierung anderer Metalle hob die gleichzeitige Produktion von Siderophoren die Metall-induzierte Hemmung der Auxinsynthese auf. Diese Ergebnisse zeigen, dass die Anwesenheit von Siderophoren den negativen Effekt von Metallen auf die mikrobielle Auxinproduktion reduziert. Daraus lässt sich folgern, dass die Rolle von Auxin für Pflanzenwachstum und –entwicklung für Phytoextraktionsstrategien zu berücksichtigen ist.

Pflanzenwachstum unter Metallstress, aber auch die Aufnahmefähigkeit der Pflanzen für Metalle, wurden sowohl *in vitro* als auch in kontaminiertem Boden unter Verwendung von *Streptomyces*-Sporen und Siderophor-haltigem, zellfreiem Kulturfiltrat analysiert. Während Metalle das Wachstum von Erbse und Sonnenblume hemmte, verbesserte die Zugabe der Siderophore ihr Wachstum. Es gab Anzeichen für erhöhten oxidativen Stress und Chlorosen bei Pflanzenwachstum auf nickelhaltigen Böden: erhöhte Membranperoxidation, erhöhte Aktivitäten von Superoxiddismutase, IAA Peroxidase und Fe-Reduktase, geringere Chlorophyll-, RNA-, Protein- und Carotinoidgehalte. Diese, durch Metall –induzierten, physiologischen Veränderungen in der Pflanze wurden durch mikrobielle Siderophore signifikant verbessert. Darüber hinaus wurde die Ni-induzierte Hemmung der Fe-Reduktaseaktivität durch Siderophoren vermindert. Dies resultierte in höheren Fe-Gehalten in den mit Siderophoren behandelten Pflanzen. Nach unserem Kenntnisstand ist dies der erste Nachweis, dass die Verwendung von Siderophoren Metall-induzierten oxidativen Stress in Pflanzen lindert. Außerdem bestätigte ein Siderophoren-Konkurrenztest zwischen Al und Fe einerseits, und Fe und Cu andererseits, dass Al mit Fe um die Bindung an Siderophoren konkurrieren kann, während Cu mit einer relativ geringen Affinität gebunden werden konnte. Dies legt nahe, dass Siderophoren-vermittelte Fe-Mobilisierung durch Pflanzen und Mikroben in Böden, die mit trivalenten Metallen kontaminiert sind, eine größere Herausforderung sein kann, als Verunreinigungen mit divalenten Metallen.

Mit Hilfe von induktiv gekoppelter Massenspektrometrie und induktiv gekoppelter optischer Plasmaelektronenspektroskopie wurde gezeigt, dass Siderophore die Metallaufnahme durch Pflanzen aus dem Boden dramatisch erhöhten; für Cd war sie in Sonnenblumen etwas höher als für EDTA, eine synthetische, aber toxische Substanz, die derzeit für Chelator-unterstützte Phytoremediation verwendet wird. Andererseits begrenzte die Anwesenheit von Siderophoren die Cd Aufnahme durch Bakterien, was auf eine präventive Rolle von Siderophoren bei der Metallvergiftung von Bakterien hindeutet. Es scheint wahrscheinlich, dass Mechanismen, die die Bioverfügbarkeit von toxischen Metallen in der Umwelt reduzieren, aber den Pflanzen verbesserten Zugang zu Metallen liefern, die ökotoxikologisch relevanten Metallkonzentrationen im Boden bestimmen. Untersuchungen zur Identifizierung von Proteinen unter von *S. tendae* F4 unter Siderophorbildenden Bedingungen und in der Anwesenheit von Cd werden derzeit durchgeführt. Durch 2-D-Gelelektrophorese konnte bereits die Anwesenheit einer Reihe von Kandidaten-Proteinspots gezeigt werden. Es wird gehofft, dass die Ergebnisse dieses Experiments weitere Erkenntnisse zur beobachteten Siderophor-vermittelten Reduktion der Cd Aufnahme wie auch zur (Schwer-)Metallstimulierung der Siderophorproduktion in Streptomyceten liefern wird.

Im Gegensatz zu ihren Siderophoren, zeigten Sporen, sowohl von *S. acidiscabies* E13 als auch *S. tendae* F4, weder einen klaren wachstumsfördernden Effekt noch beeinflussten sie die Aufnahme von Cd und Ni durch die Pflanzen, obwohl *S. tendae* F4 den Fe-Gehalt im Spross von Sonnenblumen in der Anwesenheit von Cd signifikant erhöhte. Diese überraschenden Ergebnisse führten zu weiteren Untersuchungen zur Produktion von α -Aminocyclopropan-1-carbonsäure Deaminase (ACCD) in beiden Stämmen. Stress-induzierte Ethylengehalte in Pflanzen können durch die Anwesenheit von toxischen Metallen erhöht werden, was wiederum zu einer Wachstumshemmung führt; allerdings verbessert bakterielle ACCD Ethylen-induzierten oxidativen Stress, indem ACC, der Vorläufer, in weniger toxische Produkte gespalten wird. ACCD Enzymtests zeigten, dass weder *S. acidiscabies* E13 noch *S. tendae* F4 das Enzym produzierten, was ihre Unfähigkeit, das Pflanzenwachstum unter Schwermetallstressbedingungen zu fördern, zum Teil erklären könnte.

Aufgrund dieser Ergebnisse konnte gefolgert werden, dass Auxin und Siderophor-haltiges Kulturfiltrat, insbesondere von Bakterienstämmen, die mehrere Siderophore produzieren, zur Unterstützung der Phytoremediation von Metallverunreinigungen in Böden an Stelle von EDTA und synthetischen Auxinen genutzt werden können. Die Verwendung von Siderophoren in Kulturfiltraten ist kostengünstig, verglichen mit gereinigten Siderophoren oder auch synthetischen Chelatoren. Darüber hinaus sind Siderophore leicht biologisch abbaubar, so dass ihre Verwendung keine neue Art von Umweltbelastung mit sich bringt, im Gegensatz zu synthetischen Chelatoren, die derzeit für die Phytoremediation verwendet werden.

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EIGENSTÄNDIGKEITSERKLÄRUNG

Hiermit erkläre ich, dass ich die Dissertation “**Microbial Siderophores in Rhizosphere Interactions in Heavy Metal-Containing Environments**” selbständig und nur mit der darin angegebenen Hilfe verfasst habe. Die Dissertation wurde in keiner anderen Fakultät oder Universität eingereicht.

DECLARATION

I hereby declare that this dissertation “**Microbial Siderophores in Rhizosphere Interactions in Heavy Metal-Containing Environments**” is an original work. I vouch that the content of this dissertation has not, in part or whole, been accepted for the award of any Degree in any university or institution.

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CURRICULUM VITAE

MEMBERSHIP OF PROFESSIONAL ORGANISATIONS:

European Plant Science Organization (EPSO)

International Foundation for Sustainable Development in Africa and Asia (IFSDAA)

International Society of African Bioscientists and Biotechnologists (ISABB)

African-Asian Student Forum (AASF, Germany)

AWARDS AND HONORS:

Doctoral fellowship, Max Planck Society, Germany (2006-2009)

MSc scholarship, Belgian Inter-university Development Council (2003-2005)

IITA 'Outstanding Performance' award (2000, 1999, 1998)

Award for best graduating National Diploma student from Abia State, Nigeria (1992)

Award for best graduating National Diploma student in Animal Health course (1992)

SHORT COURSES ATTENDED:

Project and Time Management (Graduate Academy, FSU; 2008)

How to write a World Class Paper (Elsevier Publishers/FSU; 2008)

Course on RNA isolation, Reverse Transcription and Real-Time PCR, including hands-on training (International Leibniz Research School Jena/FSU; 2007)

Mass spectrometry for Chemical Ecology: I. Fundamentals and Basics (MPICE; 2007)

Advanced Photoshop for life sciences (MPICE; 2007)

Workshop on "Phylogenetics" (MPICE; 2006)

Workshop on "RapidPCR", including hands-on training (Hans Knoell Institute, Jena; 2006)

Proteomics Basic Training Course (MPICE/GE Healthcare Europe GmbH; 2006)

Course on Scientific Writing Skills (MPICE; 2006)

Safe Handling of Agriculture and Laboratory Chemicals (IITA; 1995)

HOBBIES: Writing, reading, traveling, and swimming.

PUBLICATIONS:

- Dimkpa, C.O., Merten, D., Svatoš, A., Büchel, G., and Kothe, E. (in press) Siderophores mediate reduced and increased uptake of cadmium by *Streptomyces tendae* F4 and sunflower (*Helianthus annuus*), respectively (Journal of Applied Microbiology).
- Haferburg, G., Schmidt, A., Schmidt, A., Schütze, E., Dimkpa, C., Kothe, E. (submitted) *Streptomyces* heavy metal resistance: extra- and intracellular mechanisms. *In: Soil Heavy Metals*. Sherameti I. and Varma A. (Eds.) Soil Biology Series, Springer.
- Dimkpa C., Gherghel F., Haferburg G., Reinicke M., Schindler F., Schlunk I., Schmidt A., Schmidt A., Schütze E., Zeggel L., Merten D., Büchel G., Kothe E. (submitted) The effect of acid mine drainage on soil microbiology. *In: Jabalpur Reviews*; Behl, R.K. (Ed.).
- Dimkpa, C.O., Merten, D., Svatoš, A., Büchel, G., and Kothe, E. (2009) Metal-induced oxidative stress impacting plant growth in contaminated soil is alleviated by microbial siderophores. *Soil Biology and Biochemistry* 41:154-162.
- Dimkpa, C.O., Svatoš, A., Dabrowska, P., Schmidt, A., Boland W., and Erika Kothe (2008) Involvement of siderophores in the reduction of metal-induced inhibition of auxin synthesis in *Streptomyces* spp. *Chemosphere* 74:19-25.
- Dimkpa, C., Merten, D., Svatoš, A., Büchel, G., and Kothe, E. (2008) Hydroxamate siderophores produced by *Streptomyces acidiscabies* E13 bind nickel and promote growth in cowpea (*Vigna unguiculata* L.) under nickel stress. *Canadian Journal of Microbiology* 54:163-172.
- Kothe E., Dimkpa C., Gherghel F., Haferburg G., Krause K., Schmidt A., Schindler F., Zellmer A., Kießig G., Merten D., Büchel G., Zeggel L., (2007) Microbial adaptation and its use for bioremediation. *In: Proceedings of the WISMUT remediation*, Gera, Germany, pp 309-316.
- Ude, G.N., Dimkpa, C.O., Aneghbeh, P.O., Shaibu, A.A., Tenkouano, A., Pillay M., and Tchoundjeu, Z. (2006) Analysis of genetic diversity in accessions of *Irvingia gabonensis* (Aubry-Lecomte ex O'Rorke) Baill. *African Journal of Biotechnology* 5: 219-223.
- Pillay, M., Hartman, J., Dimkpa, C., and Makumbi, D. (2003) Short communication: establishing the genome of *Sukali ndizi*. *African Crop Science Journal* 11:119-124.

PUBLICATIONS:

Pillay, M., Dimkpa, C., Ude, G., Makumbi, D., and Tushmereirwe, W., (2003) Genetic variation in banana cultivar *Sukali ndizi* grown in different regions of Uganda. African Crop Science Journal 11:87-95

Shaibu, A.A., Dimkpa, C., and Ogburia, M.N., (2003) Biodiversity in diploid *Musa acuminata* (AA group) and their hybrids using AFLP. African Journal of Agricultural Research 1:67-72.

CONFERENCE CONTRIBUTIONS:

Christian Dimkpa. Siderophores in metal-induced microbial and plant processes. Proceeding of the Jena School of Microbial Communication (JSMC) symposium, Jena, Germany, December 15-16, 2008.

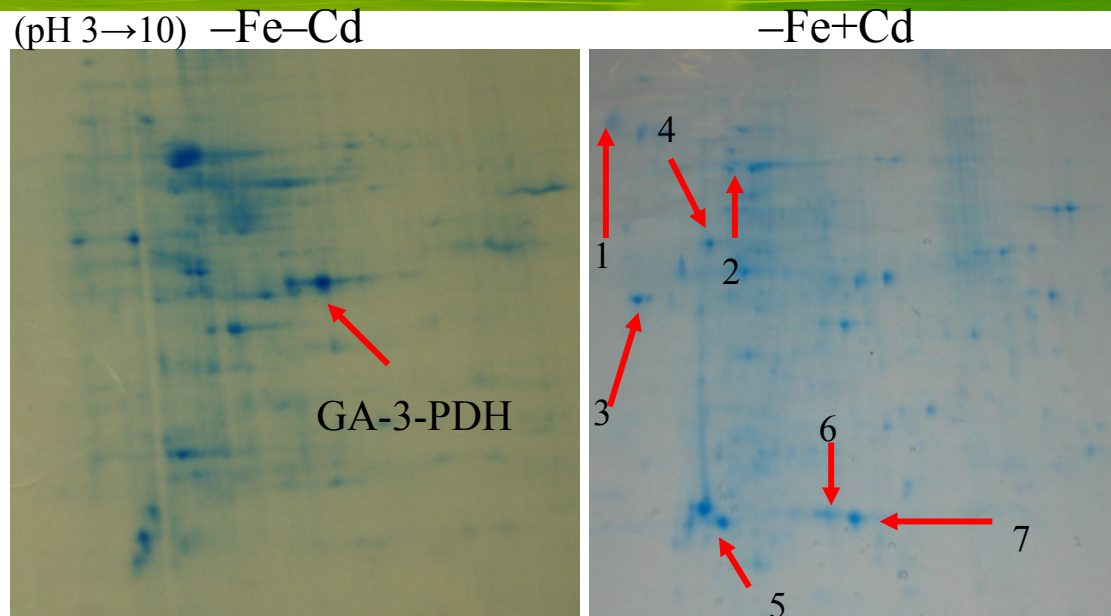
Dimkpa C., Svatoš A and Kothe E. Microbial Auxins and Siderophores: Promoting Plant Growth by Biofertilization and Bioprotection. Proceedings of the International Seminar on Food Security, Biomass Energy and Livelihood Strategies, Goettingen, Germany. 18-20 November, 2007.

Dimkpa C., Svatoš A and Kothe E. Siderophores Alleviate Heavy Metal-induced Inhibition of Auxin Synthesis in *Streptomyces* and Enhance Plant Growth in Soil Contaminated with Heavy Metals. Proceedings of the Biannual International Max Planck Research School symposium, Jena, Germany. September 25-26, 2007.

Dimkpa C., Svatoš A, Merten D, Büchel G, and Kothe E. Hydroxamate Siderophores Produced by *Streptomyces acidiscabies* E13 Bind Nickel and Promote Growth in Cowpea (*Vigna unguiculata* L.) under Nickel Stress. Proceedings of the International Symposium on Microbial Adaptation to Stress and Environment, Marburg, Germany. 12-14 April, 2007

Dimkpa C., Gherghel F., Haferburg G., Reinicke M., Schindler F., Schlunk I., Schmidt A., Schütze E., Zeggel L., Merten D., Büchel G., Kothe E., 2007. The effect of acid mine drainage on soil microbiology, Bucharest, Romania.

Dimkpa C., Svatoš A and Kothe E. Siderophores: Production by *Streptomyces* E13 and F4 under Nickel and Cadmium Stress and Plant Growth-promoting Potentials. Proceedings of the international conference on “Biology of Bacteria Producing Natural Products”. European Association for General and Applied Microbiology. Tuebingen, Germany. 20-22 October 2006.

Effect of Cd on the proteome *S. tendae* F4 under Fe deficiency condition

Spot

Corresponding protein

1 (93% id)	Dihydrolipoamide S-succinyltransferase
2 (100% id)	Dihydrolipoamide S-acetyltransferase
3 (100% id)	Dihydrolipoamide dehydrogenase
4 (85% id)	Succinyl-CoA synthetase
5 (92% id)	Tellurium resistance protein
6 (100% id)	Fe-Zn Superoxide dismutase (Fe-ZnSOD)
7 (93% id)	FeSOD

1 and 2:

-acyl transferases, respectively

-involved in succinyl/acetyl-CoA reactions

-Acetyl/Succinyl-CoA → DFOB/DFOE biosynthesis

4: Succinyl-CoA synthetase involved in DFOE biosynthesis

6 and 7: involved in metal-induced oxidative stress removal