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, Al^{3+}, Cd^{2+}, Cu^{2+}, Fe^{3+} and Ni^{2+}, or a combination of Fe^{3+} and Cd^{2+}, and Fe^{3+} and Ni^{2+} 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 Cd^{2+} and Ni^{2+}, and thus, showing their auxin-stimulating effects. We conclude that siderophores promote auxin synthesis in the presence of Al^{3+}, Cd^{2+}, Cu^{2+} and Ni^{2+} 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 (Al^{3+}), cadmium (Cd^{2+}), copper (Cu^{2+}), nickel (Ni^{2+}), zinc (Zn^{2+}) and lead (Pb^{2+}), 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 Fe^{3+} with a high affinity (Boukhalfa and Crumbliss, 2002; Fernández and Winkelmann, 2005), but which can also interact with metals other than Fe^{3+}, 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 (Doumbou 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, Fe^{3+}-deficient siderophore-inducing medium (Alexander and Zuberer, 1991) was supplemented with L-tryptophan ($20 \mu g\,mL^{-1}$). 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 (10^6 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 Fe^{3+} (as $FeCl_3$), Al^{3+} (as $AlCl_3$), Cd^{2+} (as $CdCl_2$), Cu^{2+} (as $CuSO_4 \cdot 5H_2O$) and Ni^{2+} (as $NiCl_2$), spores were collected from *S. acidiscabies* E13, *S. tendae* F4, *S. coelicolor* A3(2), and *S. coelicolor* W13, as described above, and cultured in Cu^{2+}-deficient, siderophore-inducing medium supplemented or not supplemented with the respective metals (100 μM each), and with or without tryptophan ($20 \mu g\,mL^{-1}$). 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 Fe^{3+} and Cd^{2+} on the one hand, and Fe^{3+} and Ni^{2+}, 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 Cd^{2+} and Ni^{2+} interactions with siderophores released by *S. tendae* F4, the medium was respectively amended with 100 μM Cd^{2+} and 1 mM Ni^{2+}, under a starting Fe^{3+} concentration of 100 μM in each case, and also in the absence of added Fe^{3+}. For this, four types of Fe^{3+} and Cd^{2+}/Ni^{2+} 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 10^6 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, $[^{13}C_6]$-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 preconditioned (methanol, 5 mL; ethyl acetate, 5 mL) Chromabond NH_2 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 $15\,^{\circ}C\,min^{-1}$ to 140 °C, $5\,^{\circ}C\,min^{-1}$ to 210 °C and $15\,^{\circ}C\,min^{-1}$ 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 $m/z = 195$ ($[^{13}C_6]$-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: Fe^{3+} Cd^{2+} and Ni^{2+} (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 Schywn 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 Fe^{3+}, Al^{3+}, Cd^{2+}, Cu^{2+} and Ni^{2+}. 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 $[M - 2H^+ + Fe^{3+}]^+$ for siderophore–Fe^{3+} complexes, $[M - H^+ + Cd^{2+}]^+$ for siderophore–Cd^{2+} complexes, and $[M - H^+ + Ni^{2+}]^+$ for siderophore–Ni^{2+} 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 ($20 \mu g\, mL^{-1}$). 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]
S. acidiscabies E13	+++	+++
S. griseus	++	+++
S. avermitilis	++++	+++
S. tendae F4	+++	+++
S. tanashiensis	++++	+++
S. coelicolor A3(2)	++++	++++
S. mirabilis K7A-1	+	++++
S. chromofuscus P4B-1	+	++++
S. mirabilis P10A-3	+	++++
S. chromofuscus P10A-4	+	++++
S. prunicolor P6A-1	+	++++
S. naganishii P9A-1	+	++++
S. coelicolor W13	++++	n.d[c]

[a] +: weak reaction; ++: intermediate reaction; +++: strong reaction; ++++: strongest reaction.
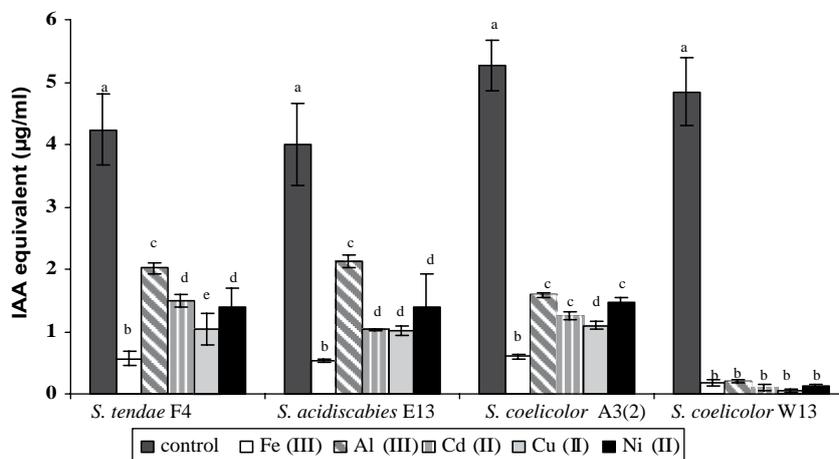[b] +++: strong reaction; ++++: stronger reaction.
[c] N.d, not detected.

**Fig. 1.** Influence of $100 \mu M$ 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 ($20 \mu g\, mL^{-1}$). Bars denote standard deviation and different letters show significantly different results ($P = 0.05$) within each strain.
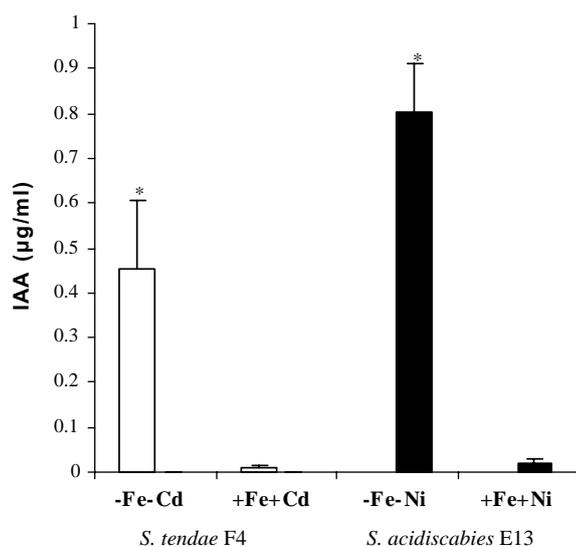
Fig. 2. Gas chromatography–mass spectrometry analysis of culture filtrates of *S. tendae* F4 and *S. acidiscabies* E13 validating IAA production in the absence of tryptophan, and its inhibition by metals. Fe^{3+}, Cd^{2+} and Ni^{2+} were each added at 100 μM. Asterisks indicate significant differences 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 72 h of growth, the presence of siderophores was assayed by incubating the samples together with the CAS assay solution for 12 h, 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 Fe^{3+} 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-
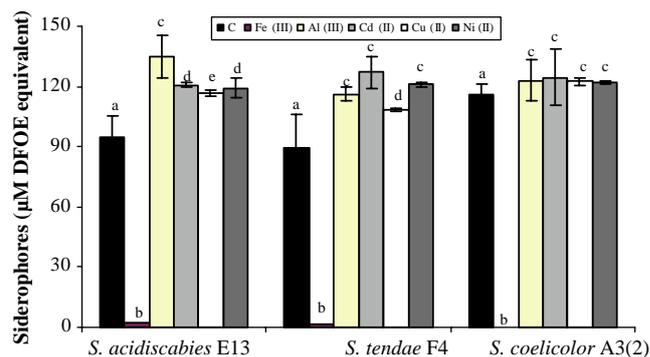

Fig. 3. CAS assay demonstrating the influence of 100 μM of Fe(III), Al(III), Cd(II), Cu(II) and Ni(II) on siderophore production by *Streptomyces acidiscabies* E13, *S. tendae* F4, and *S. coelicolor* A3(2) grown for 72 h in siderophore-inducing medium amended with (20 μg mL^{-1}) L-tryptophan. Bars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

tion. *S. tendae* F4 was treated with Cd^{2+} (100 μM), in the presence of high (100 μM), low (35 μM) or no added Fe^{3+}. 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 Fe^{3+} (100 μM) irrespective of their Cd^{2+} 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, Cd^{2+} was confirmed to stimulate siderophore production in the absence of Fe^{3+}. At the same time, Cd^{2+} was shown to override the repression of siderophore production by low Fe^{3+} (35 μM) concentrations. Without addition of Fe^{3+}, irrespective of Cd^{2+} status, desferrioxamine siderophores (DFOE and DFOB) were mainly produced. When Cd^{2+} was absent, the presence of low concentrations of Fe^{3+} resulted in low abundance of all three siderophores. In contrast, all three siderophores were significantly up-regulated when Cd^{2+} was added. Overall, in addition to chelating Fe^{3+}, where present, all three hydroxamates bound Cd^{2+} at varying abundances (Fig. 4).

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

As with Cd^{2+}, ESI-MS confirmed that Ni^{2+} stimulates siderophore production in *S. tendae* F4. In addition, the presence of a low level of Fe^{3+} (35 μM) decreased siderophore production; however,
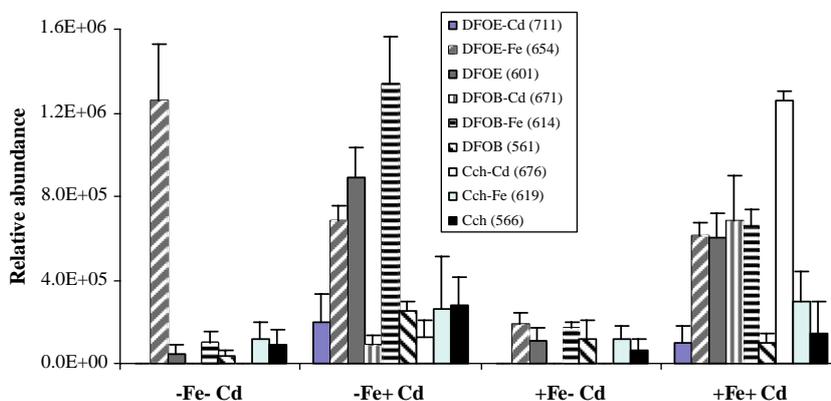
Fig. 4. Identification of DFOE, DFOB and Cch, and the detection of their metal chelate species in culture supernatants of *Streptomyes tendae* F4 grown under Fe^{3+} and Cd^{2+} set-ups. The relative abundances of the released siderophores and their respective metal chelate species are indicated. Bracketed legend numbers are measured *m/z* values used for siderophore identification (ESI-MS; $n=3$).
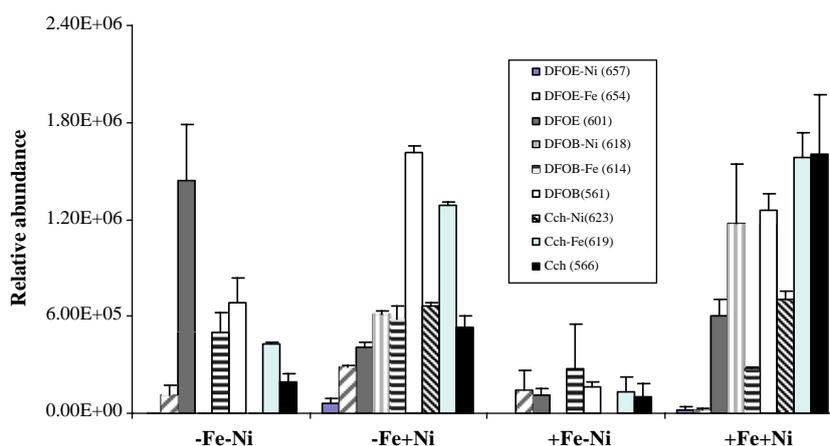
Fig. 5. Stimulation of siderophore production in *S. tendae* F4 by high concentration of Ni^{2+} after 120 h growth. The relative abundances of the released siderophores and their respective metal chelate species are indicated (ESI–MS; $n = 3$).

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 ($20\,\mu g\,mL^{-1}$), 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 (Schywn 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 Cd^{2+} and Ni^{2+}, 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 Ni^{2+}, 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 Al^{3+} for siderophore binding was experimentally demonstrated recently, in which pyoverdine produced by *Pseudomonas aeruginosa* was shown to bind Al^{3+}, 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 Thüringia, Germany, will shed light on these possibilities.

Acknowledgments

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