Identification of specific interactions between bacteria and heavy metal accumulating plants

Dissertation

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CONTENTS

Summary

Various plants growing on metalliferous soils accumulate metals in their shoot tissues and have the potential to be used for the extraction of heavy metals from polluted soils. *Salix caprea* trees have been observed to accumulate high amounts of Zn and Cd and are particularly promising for phytoremediation purposes, due to their high biomass production. Previous studies on heavy metal accumulating plants gave evidence that plant-associated bacteria are involved in the heavy metal accumulation process. The mechanisms underlying these plant-microbe interactions are still unknown.

In this work more than one hundred different highly Zn and Cd resistant rhizosphere bacteria and endophytes were isolated from *Salix caprea* trees growing at a Zn/Cd/Pb contaminated site in Austria. Sequence analysis of the 16S rDNA gene revealed that rhizosphere and endosphere harboured distinct bacterial communities. Culturable endophytes were clearly dominated by methylobacteria and sphingomonads, which seem to be characteristic colonizers of heavy metal accumulating plants. The isolates were screened for the production of 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase and siderophores, because these substances have been associated with increased heavy metal uptake and tolerance in plants. Selected strains were further tested for the release of the phytohormone indole-3-acetic acid (IAA) and for the ability to produce specific Zn, Cd and Fe mobilizing compounds. IAA and ACC deaminase were produced by many rhizosphere bacteria and endophytes, whereas siderophore production was virtually limited to rhizosphere isolates. In liquid cultures most of the analysed bacteria produced metabolites that immobilized Zn, Cd and Fe, when added to soil. Four slowly growing actinobacteria mobilized high amounts of Zn, Cd and/or Fe most likely by the release of specific metal chelators. Ten rhizosphere strains were introduced into the rhizosphere of *Salix caprea* plantlets and analysed for their effect on plant growth and heavy metal uptake. One plant growth promoting *Agromyces* strain and one *Streptomyces* enhancing Zn and Cd uptake were identified. Promotion of plant growth enhanced total metal extraction more efficiently than improved metal uptake. Both of the identified beneficial strains were unable to produce siderophores, ACC deaminase or IAA. This highlights the importance of novel undescribed interaction mechanisms between bacteria and heavy metal accumulating plants.

To identify genes involved in such novel interaction mechanisms, a *Methylobacterium* strain was analysed by suppression subtractive hybridization (SSH). With SSH, sequences specific to a strain of interest are isolated by DNA-hybridization with a closely related

reference strain. A well described *M. extorquens* isolate from the Ni hyperaccumulator *Thlaspi goesingense* was selected for the SSH experiment and a closely related Ni sensitive type strain from soil was used as reference. 1088 random DNA sequences isolated by the SSH procedure were screened by microarray analysis for their distribution in *M. extorquens* isolates from various environments. The vast majority of SSH products was specific to strains associated with Ni hyperaccumulating *T. goesingense*. 109 SSH products were sequenced and 36 sequences were unique. BlastX search of the NCBI database revealed that sequences unique to the original strain which was used for the SSH procedure, were related to DNA methylases and metabolic genes. Sequences specific to all *M. extorquens* isolates from *T. goesingense* were affiliated with cell surface proteins of plant pathogens and with regulatory genes. More than half of the unique sequences did not show significant similarity to any described gene.

For further research on the role of plant-associated bacteria in heavy metal accumulation by *Salix caprea*, experimental systems for large scale plant-inoculations need to be established. Comprehensive plant experiments will reveal which of the described phenotypic characteristics and novel genetic traits are the most relevant for heavy metal accumulation in the natural system. Detailed *in planta* studies will further allow the development of techniques to stimulate beneficial plant-microbe interactions in phytoremediation systems.

Kurzfassung

Verschiedene Pflanzenarten die an Schwermetallstandorten auftreten, akkumulieren Metalle im Sproß und können zur Extraktion von Schwermetallen aus kontaminierten Böden eingesetzt werden. In Salweiden (*Salix caprea*) wurde starke Zn und Cd Akkumulation beobachtet. Aufgrund hoher Biomasseproduktion ist diese Baumart für die Phytoremediation besonders interessant. Untersuchungen an Schwermetall akkumulierenden Pflanzen zeigten, dass pflanzenassoziierte Bakterien am Akkumulationsprozess mitwirken. Die Mechanismen, die dieser Interaktion zwischen Pflanzen und Mikroorganismen zugrunde liegen, sind unbekannt.

In vorliegender Arbeit wurden an einem Zn/Cd/Pb-kontaminierten Standort in Österreich über hundert verschiedene hoch Zn- und Cd-resistente Bakterien aus der Rhizosphäre und Endosphäre von *Salix caprea* Pflanzen isoliert. Anhand von 16S rDNA Sequenzenanalysen wurde ersichtlich, dass Rhizosphäre und Endosphäre von unterschiedlichen Bakteriengemeinschaften besiedelt werden. Bei den kultivierbaren Endophyten zeichnete sich eine deutliche Dominanz von Methylobakterien und Sphingomonaden ab. Diese Genera scheinen für die Mikroflora von Schwermetall akkumulierenden Pflanzen charakteristisch zu sein. Die Isolate wurden auf Produktion von 1-aminocyclopropan-1-carboxylsäure (ACC) Deaminase und Siderophoren getestet, da diese Substanzen mit erhöhter Schwermetallaufnahme und -toleranz von Pflanzen in Verbindung gebracht werden. Ausgewählte Stämme wurden darüber hinaus auf die Synthese des Pflanzenhormons Indol-3-Essigsäure (IAA) und auf die Abgabe von Zn, Cd und Fe mobilisierenden Verbindungen untersucht. Zahlreiche Rhizosphärenbakterien und Endophyten produzierten IAA und ACC Deaminase. Die Siderophorenproduktion war hingegen fast ausschließlich auf Rhizosphärenisolate beschränkt. Die Mehrheit der analysierten Stämme erzeugte in Flüssigkultur Metabolite, die Zn, Cd und Fe immobilisierten, wenn sie Bodenproben zugesetzt wurden. Vier langsam wachsende Aktinobakterien mobilisierten große Mengen von Zn, Cd und/oder Fe, höchstwahrscheinlich durch Abgabe spezifischer Metallchelatoren. Zehn Rhizosphärenbakterien wurden in einen Inokulationsversuch mit *Salix caprea* Pflänzchen auf ihren Einfluß auf Pflanzenwachstum und Metallaufnahme überprüft. Ein wachstumsfördernder *Agromyces* Stamm und ein *Streptomyces*, der die Aufnahme von Zn und Cd erhöhte, wurden identifiziert. Für die Gesamtmenge an Zn und Cd, die pro Pflanze aus dem Boden extrahiert wurde, war die Wachstumsförderung von größerer Bedeutung als die gesteigerte Metallaufnahme. Keiner der

beiden Stämme mit positivem Effekt im Pflanzenversuch produzierte Siderophore, ACC-Deaminase oder IAA. Dies unterstreicht Bedeutung neuer noch nicht beschriebener Interaktionen zwischen Bakterien und Schwermetall akkumulierenden Pflanzen.

Zur Identifikation von Genen, die in solche neuen Interaktionsmechanismen involviert sein könnten, wurde ein *Methylobacterium* Stamm mit Suppression Subtraktive Hybridization (SSH) untersucht. SSH isoliert Gene, die für einen bestimmten Bakterienstamm spezifisch sind, durch DNA-Hybridisierung mit einem nahe verwandten Referenzstamm. Für das SSH Experiment wurde ein gut beschriebenes *M. extorquens* Isolat aus der Rhizosphäre des Ni-Hyperakkumulators *Thlaspi goesingense* ausgewählt. Ein nahe verwandter Ni-sensitiver aus dem Boden stammender Typstamm diente als Referenz. 1088 durch SSH isolierte DNA Fragmente wurden zufällig ausgewählt, auf einen Microarray aufgebracht und auf ihr Vorkommen in *M. extorquens* Isolaten aus verschiedenen Habitaten untersucht. Die überwiegende Mehrheit der SSH-Produkte erwies sich als für Isolate aus *T. goesingense* spezifisch. 109 SSH-Produkte wurden sequenziert. 36 Sequenzen waren verschieden und wurden mittels blastX mit Einträgen in der NCBI Datenbank verglichen. Sequenzen, die ausschließlich im Ausgangstamm des SSH Experimentes gefunden wurden, zeigten Homolgien mit DNA-Methylasen und metabolischen Genen. Sequenzen die für alle *M.extorquens* Isolate aus *T. goesingense* spezifisch waren, waren mit Proteinen der Zelloberfläche von Pflanzenpathogenen und mit regulatorischen Genen verwandt. Mehr als die Hälfte der 36 analysierten Sequenzen hatten keine signifikante Ähnlichkeit mit beschriebenen Genen.

Für die weitere Erforschung der Funktion von pflanzenassoziierten Bakterien bei der Schwermetallaufnahme von *Salix caprea* müssen Systeme für groß angelegte Pflanzenversuche entwickelt werden. Umfassende Inokulationsversuche werden aufklären, welche der beschriebenen phenotypischen Charakteristika die größte Bedeutung für die Schwermetallakkumulation im natürlichen System haben. Gezielte Pflanzenexperimente werden auch die Entwicklung von Strategien zur Stimulation von Interaktionen zwischen Pflanzen und Bakterien ermöglichen, die für die Schwermetallakkumulation förderlich sind.

INTRODUCTION

Introduction

1. Heavy metal toxicity and heavy metal pollution

Heavy metals are metals with a density above 5 g/cm^3. Of the 90 naturally occurring elements 53 are heavy metals. Most heavy metals are transition elements with incompletely filled d-orbitals, that allow the formation of complex compounds. Therefore certain heavy metals are cofactors in enzymatic reactions. Traces of these elements are essential for life, whereas higher concentrations of heavy metals in living tissues lead to unspecific reactions with toxic effects. Excess heavy metal ions tend to replace other cations in physiological reactions, to denature proteins by binding to -SH groups and to disturb charge balances of membranes. In addition, redox active heavy metal cations can cause the formation of radicals in cells which results in oxidative stress (Nies, 1999).

H																	He
Li	Be											B	C	N	O	F	Ne
Na	Mg											Al	Si	P	S	Cl	Ar
K	Ca	Sc	Ti	V	Cr	Mn	Fe	Co	Ni	Cu	Zn	Ga	Ge	As	Se	Br	Kr
Rb	**Sr**	Y	Zr	Nb	Mo	Tc	Ru	Rh	Pd	Ag	Cd	In	Sn	Sb	Te	I	Xe
Cs	Ba	*	Hf	Ra	W	Re	Os	Ir	Pt	Au	Hg	Tl	Pb	Bi	Po	At	Rn
Fr	Ra	**	Rf	Db	Sg	Bh	Hs	Mt									

*	La	Ce	Pr	Nd	Pm	Sm	Eu	Gd	Tb	Dy	Ho	Er	Tm	Yb	Lu
**	Ac	Th	Pa	**U**	Np	Pu	Am	Cm	Bk	Cf	Es	Fm	Md	No	Lr

Figure 1. Periodic table of the elements. Heavy metals are marked in grey, widespread heavy metal and metalloid pollutants are marked in dark grey. Soil contaminations with the metalloids As and Se and with radioactive Cs, Sr and U are sometimes included in the discussion about metal pollution, toxicity and remediation.

Figure 1 highlights heavy metals and metalloids, that have become widespread environmental pollutants. Metals chromium, copper, cobalt , nickel and zinc (Cr, Cu, Co, Ni, Zn) are essential as trace elements, but toxic at higher concentrations. Lead, cadmium and mercury

(Pb, Cd, Hg) are heavy metal pollutants with particularly high toxicity and without known biological function (Nies, 1999). The metalloids arsenic and selenium (As, Se) are often included in the discussion about metal pollution, toxicity and remediation. Soil contaminations with radioactive isotopes of the metals caesium, strontium and uranium (Cs, Sr, U) are sometimes addressed with similar remediation techniques, but have to be considered as a distinct category of environmental hazards.

In most natural environments heavy metals exist in trace concentrations. Naturally metalliferous soils can occur on metal rich mineral underground. Examples are Ni-Cr-Co rich ultramafic soils, formed by weathering of ultramafic rocks, or cadmium-lead-zinc enriched calamine ("zinc ore") soils (Reeves and Baker 2000). Global industrialisation, in particular mineral mining and processing, coal combustion, waste dumping and incineration, military activities, and applications of sewage sludge and organometallic agrochemicals, have led to a massive release of heavy metals into the environment (Nriagu 1979). Over a million polluted sites have been estimated in the EU-15 countries (European topic centre on soil, 1998). Many new member states of the European union have to deal with severe metal contaminations left behind from heavy industrial activities with antiquated technology (Rautengarten et al., 1995; Anderberg et al., 2000,). Soil sites contaminated with Pb, As, Cr, Cd and Ni are dominant issues in the National Priority List of the US environmental protection agency (Adriano et al., 2001). While the release of heavy metals to the environment started to decrease in most industrial countries at the end of the 20th century, emissions are increasing in many developing countries (Järup 2003).

Moderate heavy metal contamination of soils may not affect plant growth, but may convey heavy metals to the food chain and can make agricultural soils unsuitable for food cropping (Sridharta Chary et al., 2007; Khan et al., 2007). Heavily polluted sites such as mine tailings exhibit an altered or reduced vegetation (Álvarez et al., 2003; Li et al. 2003) and can endanger human health by leaching of heavy metals to the groundwater (Schwab et al., 2007; Zheng et al., 2007).

2. Phytoremediation of heavy metal contaminated soils

Phytoremediation is the use of green plants to remove or neutralize environmental contaminants (Van der Lelie et al. 2001). This concept offers a non-destructive alternative to

landfilling and chemical soil treatments. Phytoremediation processes are driven by solar energy and therefore sustainable and cheaper than mechanical and chemical clean-up techniques (Singh et al., 2003). Since the early 1990s, phytoremediation developed into a field of research and has gained significant commercial interest (Pilon-Smits, 2004). A variety of phytoremediation mechanisms can be employed, to remediate different types of contaminations in soil, water and air (Table 1).

Table 1. Current phytoremediation techniques (modified from Singh et al., 2003)

Phytoremediaion techniques	Mechanisms
Phytostabilization	Stabilization of the ground and control of the groundwater flow by re-vegetation; precipitation of metals by root exudates
Phytostimulation	Microbial degradation of organic contaminants in the rhizosphere
Rhizofiltration	Absorption of metals by plant roots from water
Phytodegradation	Uptake and degradation of organic contaminants by plant roots
Phytotransformation	Detoxification of organic contaminants in roots and shoots
Phytoextraction	Uptake of contaminants into harvestable parts of plants
Phytovolatilization	Uptake and evaporation of Se and volatile organic contaminants
Air filtration	Absorption of volatile organic contaminants by leaves

Unlike organic contaminants heavy metals cannot be degraded and only a few species can be detoxified by biological redox processes. Phytoextraction with plants, that accumulate metals in their harvestable parts, is a promising strategy to remove heavy metals from soil. Dried, ashed or composted plant residues, highly enriched in heavy metals can be isolated as hazardous waste (Singh et al., 2003). Certain metals can even be recycled from plant material, in a process termed phytomining (Chaney et al., 2000).

3. Heavy metal accumulating plants

3.1 Plant strategies towards heavy metals

Natural metalliferous soils and sites exposed to anthropogenic heavy metal pollution are colonized by specialized heavy metal resistant plants. Three different strategies of adaptation can be distinguished: metal excluders, indicators and accumulators. These adaptation types can be characterized by the bio-accumulation factor (BCF) which is calculated as follows:

BCF = metal concentration in shoots / metal concentration in the soil

Heavy metal excluders prevent heavy metals from entering their shoot tissues and therefore have a BCF<1 (Figure 2). Heavy metal indicators allow heavy metals to enter their shoot tissues and attain leaf concentrations similar to the concentration of metals in the soil. The result is a BCF = 1. Heavy metal accumulators, accumulate heavy metals actively in their shoot tissues to a certain saturation level and to BCF values above 1 (Baker et al., 2000).

The exclusion of heavy metals from shoot tissues is a strategy of heavy metal tolerance (Schat et al. 2000). The ecological function of heavy metal accumulation is still unclear. There is evidence that high metal concentrations in leaves protect heavy metal accumulating plants from insect grazing and fungal diseases (Reeves et al., 1981, Pollard and Baker 1997). In perennial species, metal accumulation in leaves may be a way to eliminate heavy metals via leaf fall (Vergnano Gambi et al., 1982). The evolutionary costs of heavy metal accumulation are high. Heavy metal accumulators are endemic on metalliferous sites, because they cannot compete with other plants in non-contaminated environments (Mcnair and Baker, 1994).

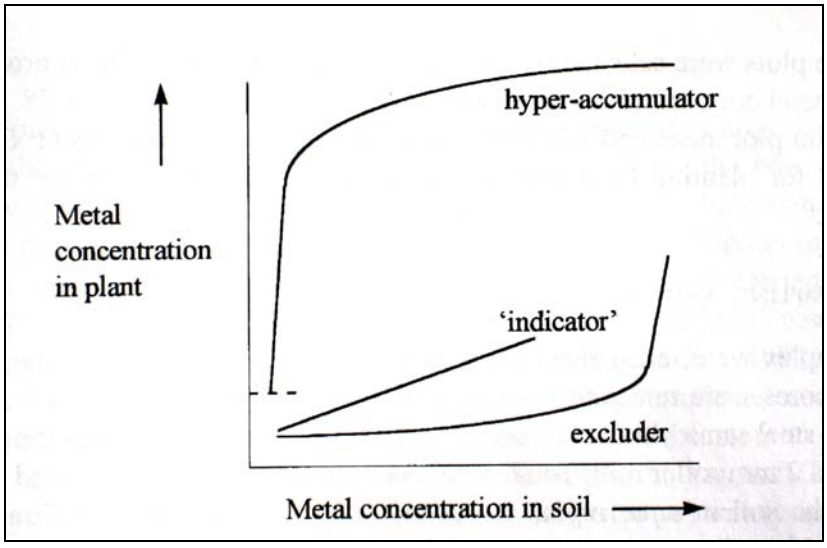

Figure 2. Response strategies of heavy metal excluders, indicators and (hyper-) accumulators to increasing heavy metal concentrations in the soil. (McGrath et al., 2000).

Plants accumulating extremely high amounts of one or more heavy metals are referred to as "hyperaccumulators". Threshold metal concentrations in leaves are used to define hyperaccumulation. These thresholds are 10.000 mg (or 1%) per kg leaf dry weight for Mn and Zn, 1.000 mg kg^{-1} (0.1%) for Co, Cu, Ni, As and Se and 100 mg kg^{-1} (0.01%) for Cd

(Baker and Brooks 1989). More than 400 heavy metal hyperaccumumating plant species have been identified, 75% of which are Ni hyperaccumulators growing on ultramafic soils (Baker and Brooks 1989). Tropical hyperaccumulators are mainly trees and shrubs of the families *Violaceae, Flacourtaceae, Buxaceae* and *Euforbiaceae*. The most impressive example of hyperaccumulation may be the New Caledonian tree *Sebertia acuminata* producing a blue green latex, with a Ni content of 26% (referring to dry weight). Most heavy metal accumulators in the northern temperate zone are herbaceous *Brassicaceae* (Baker and Brooks 1989), in particular many species of the genera *Alyssum* and *Thlaspi*. For instance, *Alyssum bertolonii*, and *Thlaspi goesingense* accumulate up to 3% of Ni in their leaves and *Thlaspi cearulescens* can reach leaf concentrations of 3.5% Zn and 0.1% Cd. Therefore these plants are well described model hyperaccumulators.

3.2 Mechanisms of heavy metal tolerance and accumulation in plants

Both, the retention of heavy metals in the roots of metal excluders, and their accumulation in the leaves of metal accumulators, require internal detoxification mechanisms. Detoxification of heavy metals in plants is based on complexation and/or sequestration (Schatt et al. 2000). Organic acids, in particular malate, citrate and the amino acid histidine have been observed to complex heavy metals in plants (Salt et al. 1999, Küpper et al., 2004). Cystein rich proteins and peptides (metallothioneins and phytochelatins), are synthesised in response to heavy metal exposure and have a high affinity to metal cations (Cobbet and Goldsbrough 2000). However their function in metal resistance is unclear, as they are produced also in metal sensitive plants (Baker et al., 2000). Sequestration, i.e. the removal of heavy metals from the cytoplasma to cell walls and vacuoles, takes place in the roots of heavy metal excluders and in the leaves of heave metal accumulators. In hyperaccumulators the highest concentrations occur in vacuoles of the leaf epidermis (Vasquéz et al., 1994).

In addition to extreme heavy metal tolerance, heavy metal accumulators are characterized by enhanced heavy metal uptake and translocation to shoots. Metal ions can passively enter plant roots and move freely in intercellular spaces. For translocation to shoots, they have to be imported actively into cells, latest at the root endodermis. The import of essential heavy metals is carried out by specific membrane transporter proteins. When non-essential metals are present in high concentrations, they probably can enter the plant cells through transporters for ions with similar properties (Clarkson and Lüttge 1989). Enhanced expression of Zn tranporter proteins has been observed in roots of the Zn hyperaccumulator *Thlaspi*

caerulescens (Assuncao et al., 2001; Lombi et al., 2001). In addition, active root proliferation towards heavy metal enriched batches of soil seems to contribute to an increased metal uptake in hyperaccumulators (Schwartz et al., 1999). In contrast, there is no direct evidence for heavy metal mobilization by root exudates (McGrath and Zhao., 2003). Little is known about the mechanisms of the massive root-to-shoot transport in hyperaccumulators, which may envolve enhanced xyleme loading, transfer to leaf cells and finally to leaf vacuoles. Assuncao et al. (2001) reported high expression of Zn transporters also in the leaves of *Thlaspi caerulescens*.

3.3 Phytoextraction with heavy metal accumulating plants

As illustrated in Figure 3, the time necessary to half the heavy metal contamination in a given soil, is determined by the annual biomass production of the extractor plants and by the bio-concentration factor (BCF). Biomass production and BCF are therefore the key factors of phytoextraction efficiency (McGrath and Zhao, 2003). Non-accumulator plants (BCF $\leq$ 1) cannot accomplish soil decontamination within any reasonable time frame, irrespective of their growth rate. Heavy metal accumulators and hyperaccumulators can be suitable for phytoextraction, depending on their adaptability to the anthropogenic pollution and on their on-site biomass production (McGrath and Zhao, 2003).

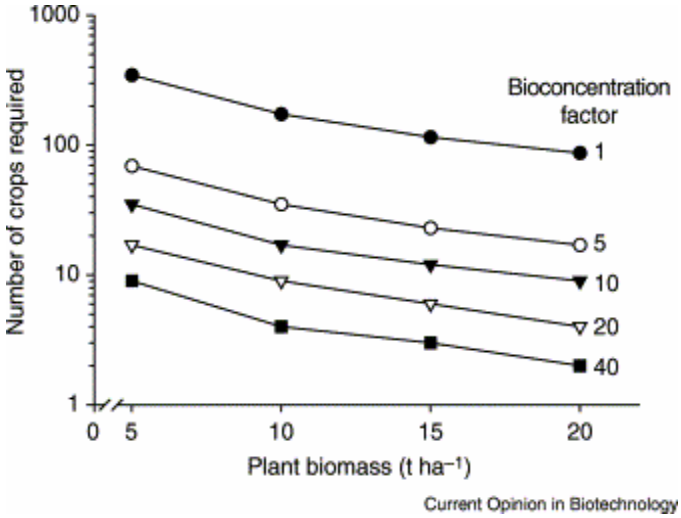

Figure 3. Model calculations of the number of crops (harvests) required to halve metal concentration in the top soil, assuming that the plants take up metals from the top 20 cm of soil. The results show that both metal hyperaccumulation and a good biomass yield are essential for efficient phytoextraction (McGrath and Zhao, 2003).

Several field surveys have been carried out, to explore the feasibility of phytoextraction with heavy metal hyperaccumulators. Ni extraction with *Alyssum sp.*, allying a Ni enrichment

potential of 3% with an annual biomass of 9t per hectare, was very successful. Ni-phytomining with *Alyssum bertolonii* has even been commercialised (McGrath and Zhao, 2003). Similarly, fast growing As-hyperaccumulating ferns performed well as As-extractor plants (Ma et al., 2001). In contrast, McGrath and Zhao (2003) state a lack of fast growing Zn, Cd, Pb, Cu and Cr hyperaccumulators. The Zn/Cd hyperaccumulator *Thlaspi caerulescens* produces only 2-5 tonnes of biomass per hectare annually. Thirteen crops were estimated necessary to decontaminate a moderately polluted soil from Zn with *Thlaspi caerulescens* (McGrath et al., 2000). Therefore high biomass crop plants, such as *Brassica juncea* (Indian mustard), *Brassica napus* (oilseed rape), which accumulate moderate amounts of metals, have been considered for phytoextraction purposes. In a short term experiment, *Brassica juncea* removed more Zn from soil than *Thlaspi caerulescens*, due to it's ten times higher biomass production (Ebbs and Kochian, 1997). Considerable heavy metal removal from soil and water has also been achieved with sunflower (*Helianthus annuus*) (van der Lelie et al, 2001; Wu et al., 2006). A recent discovery is the ability certain *Salix* (willow) and *Populus* (poplar) species to accumulate Zn and Cd (Pulford and Watson 2002). Trees are particularly interesting as extractor plants, since they access a much bigger volume of soil with their roots than herbaceous species and produce large amounts of easily harvestable biomass. Moreover willows and poplars are characterized by high transpiration rates and consequently by massive uptake of soil solutes. Wieshammer et al. (2007) observed Cd hyperaccumulation in leaves of *Salix* species up to 250 ug kg^{-1} and could reduce the Cd content in a polluted agricultural soil by 20% within three seasons. Results of field and greenhouse experiments indicate, that phytoextraction is feasible in reasonable time for moderately contaminated soils. On heavily contaminated substrates, accumulators and hyperaccumulators accumulate heavy metals to the same maximal leaf concentrations as on moderately contaminated soils (Figure 3). Accordingly, the BCF on severely polluted soils will approximate one and the time required for remediation will increase exponentially (McGrath and Zhao, 2003). In the USA certain phytoextraction concepts were successful enough to be adopted by commercial companies, such as Phytotech (www.phytotech.com) or MSE Technology Applications Inc. (www.mse-ta.com).

Phytoextraction is still an emerging technology. Its greatest challenge is the reduction of the remediation time requirement (Van der Lelie et al., 2001). The scientific effort to improve phytoremediation efficiency goes into several directions. One is the identification of novel performing extractor plants. Discoveries in this field are communicated in online databases

(www.dsa.unipr.it/phytonet; http://www.ec.gc.ca/science:PHYTOREM). Another approach is the development of agricultural techniques to improve the productivity of wild hyperaccumulator species or to increase metal accumulation in crop plants (Chaney et al., 2000, van der Lelie et al., 2001). The application of fertilizers has been found to increase metal tolerance and uptake in certain plants (Shetty et al., 1995), clipping can enhance root exudation, aeration and irrigation can stimulate rhizosphere processes. The accessibility of heavy metals can be improved mechanically by bore hole planting and by pumping up contaminated groundwater for irrigation (Pilon-Smits, 2005), and chemically by applying chelating agents such as ethylene diamine tetra acetic acid (EDTA). Soil application of EDTA can even induce metal hyperaccumulation in non-accumulating plants, but bares the risk of heavy metal mobilization to the ground water (McGrath and Zhao, 2003). Genetic engineering projects aim at increasing heavy metal tolerance and accumulation by overexpressing metal chelators, by transferring metal transporters from hyperaccumulators to high biomass plants, and by introducing bacterial resistance genes (Zhu et al., 1999; van der Lelie et al., 2001; McGrath et al., 2003, Dhanker et al., 2002). However, the introduction of genetically modified plants to ecosystems remains controversial (van der Lelie et al., 2001). A distinct area of research, is the exploration of the microflora associated with heavy metal accumulating plants (van der Lelie et al., 2000; Glick, 2003; Gadd, 2004; Khan et al., 2005).

4. Bacteria associated with heavy metal accumulating plants

4.1 Plant growth promoting rhizosphere bacteria and endophytes

The rhizosphere is a narrow zone of soil adherent to and influenced by the plant root. Root exudates, mostly sugars, organic acids, amino acids and phenols, are a nutrient source for bacteria and heterotrophic fungi. As a result, microbial density and activity is much higher in the rhizosphere than in the surrounding bulk soil, a phenomenon referred to as “rhizosphere effect” (Pinton et al., 2007). Also the intercellular spaces and vascular tissues are colonized by non pathogenic bacteria, referred to as “endophytes” (Wilson et al., 1995). Some of the endophytes are seed-borne and inhabit their plant host from the beginning of its development, others enter the plant through root hairs, stomata, lenticells and little wounds (Hallmann et al., 1997; Lamb et al., 1996; Roos and Hattingh 1983, Huang et al.; 1986).

Many of the rhizosphere bacteria and endophytes have a beneficial effect on their host plant and are therefore called plant growth promoting bacteria (PGPB). Rhizosphere bacteria can release acids, exoenzymes or siderophores, that mobilize essential nutrients like phosphorus or iron from soil (Gupta et al., 2002; Glick, 2003). Rhizosphere bacteria and endophytes can assist plant development by the synthesis of auxin and cytokinin hormones (Patten and Glick 1996, Timmusk et al., 1999, Sturz et al. 1995, Glick, 2003,). Furthermore, plant-associated bacteria can control pathogens by outcompetition, the production of antibiotics or by inducing systemic resistance responses (Dekkers et al., 2000; Compant et al., 2005).

4.2 Bacterial communities associated with heavy metal accumulating plants

The rhizosphere of heavy metal accumulating plants is a hostile environment with elevated concentrations of bio-available toxic metals compared to the surrounding bulk soil (McGrath et al., 1997; Wenzel et al., 2003). Still, DNA based community analysis revealed great bacterial diversity in the rhizosphere of heavy metal hyperaccumulating *Alyssum* and *Thlaspi*, and a high proportion of *Proteobacteria* (Mengoni et al., 2004, Idris et al., 2004). In addition many *Actinobacteria* were detected in the rhizosphere of *Thlaspi caerulescens* (Gremion et al., 2003). Gremion et al., (2003) observed further, that only a minor subset of the bacteria identified by DNA analysis, could be detected by RNA analysis and can therefore be considered active. Most of the active bacteria in the rhizosphere of *Thlaspi caerulescens* and in the surrounding contaminated bulk soil were *Actinobacteria*.

DNA based analysis of the endophyte community in *Thlaspi goesingense* (Idris et al., 2004) revealed higher endophyte diversity than in other plants (e.g. Rasche et al., 2006; Reiter and Sessitsch, 2006), a dominance of *Proteobacteria* and the presence of *Acidobacteria*, which have not been detected in plant shoots before. Moreover this study showed that within the same plant, rhizosphere and endosphere community are composed of similar taxa, but differ in intra species diversity.

Only about 1% of the bacteria occurring in the environment can be cultured on laboratory media (Amann et al., 1995). Rhizosphere isolates collected from the or *Alyssum murale* were mainly *Proteobacteria* (Mengoni et al., 2001), those from *Thlaspi goesingense* mostly alpha-*proteobacteria* and *Actinobacteria* (high-GC gram positive bacteria) (Idris et al., 2004). Endophyte collections from *Thlaspi caerulescens* and *Thlaspi goesingense* were dominated by alpha-*Proteobacteria* of the genera *Methylobacterium* and *Sphingomonas* (Lodewykx et al.,

2002, Idris et al., 2004). Analysis of culturable bacteria revealed, that on contaminated sites bacteria colonizing the rhizosphere of plants tolerate higher heavy metal concentrations than bulk soil bacteria (Kunito et al. 2001). This is particularly true for the rhizosphere of heavy metal accumulators (Schlegel et al., 1991). In Ni and Zn hyperaccumulating *Thlaspi* the heavy metal resistances of endophytes exceeded those of the rhizosphere bacteria (Lodewyckx et al., 2002; Idris et al., 2004). Most bacterial isolates from heavy metal accumulators show multiple resistances to various combinations of heavy metals (Mengoni et al., 2001; Lodewyckx et al., 2002; Abou-Shanab et al., 2005; Idris et al., 2006) suggesting an independent development of different heavy metal resistance determinants (Mengoni et al., 2001).

4.3 The role of plant associated bacteria in heavy metal accumulation and extraction

There is increasing evidence that the bacteria associated with heavy metal accumulating plants are not only adapted to, but also actively involved in the accumulation process. Soil sterilization experiments showed that heavy metal accumulators can reach their full uptake potential only in the presence of their indigenous rhizosphere microflora (deSouza et al 1999a; Abou-Shanab et al., 2003b). Inoculations of heavy metal accumulating plants demonstrated that many rhizosphere isolates can promote plant growth in the presence of heavy metals and/or enhance heavy metal uptake (Table 2). As metal extraction efficiency is given by BCF and biomass production (Figure 3), both the enhancement of metal uptake and the promotion of plant growth contribute to the metal extraction from soil. In mycorrhized plants, also mycorhizal fungi have been observed to improve heavy metal tolerance and uptake (Khan et al., 2005; Vivas et al., 2006). In contrast, there is a lack of surveys addressing the function of endophytes in heavy metal accumulating plants. As endophytes generally exhibit similar plant growth promoting properties as rhizosphere bacteria (Sturz et al., 1995), they are likely to support also heavy metal accumulators in their “way of life”.

The mechanisms by which bacteria enhance heavy metal tolerance and uptake are not yet understood. Any plant growth promoting bacterial activity mentioned above, can facilitate survival and biomass production in the contaminated environment (i.e. increase heavy metal resistance). Three mechanisms of bacterial plant growth promotion are particularly discussed in the context of heavy metal accumulation. These are the production of the auxin hormone indole-3-acetic acid (IAA), the synthesis of the enzyme 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase and the release of bacterial siderophores (Glick, 2003). Heavy metal uptake can be promoted by any bacterial activity that increases the mobility and bio-

Table 1: Effects of bacterial inocula on plants growing in the presence of heavy metals

Host plant	Metal	Effect of bacterial inocula	Reference
Brassica campestris, Lycopersicum esculentum	Ni	Increased root and shoot growth and lower ethylene levels in seedlings	Burd et al. 1998
Brassica juncea	Cd	Precipitation of Cd near roots decreasing toxic effect on roots	Salt et al., 1999
Scirpus robustus, Polypogon monspeliensis	Hg, Se	Increased uptake	deSouza et al. 1999
Brassica juncea	Se	Increased accumulation in roots and shoots; increased volatilization by roots	DeSouza et al. 1999
Brassica juncea, Brassica campestris, Lycopersicum esculentum	Ni, Pb, Zn	Increased shoot growth, increased chlorophyll concentrations	Burd et al. 2000
Brassica napus Pisum sativum	Cd	Increased root and shoot growth	Belimov et al. 2001
Thlaspi caerulescens	Zn	mobilization and increased uptake in shoots	Whiting et al. 2001
Eichhornia crassipes	Cu	Increased accumulation in roots	So et al. 2003
Alyssum murale	Ni	strain dependent mobilization/immobilization; increased uptake in shoots	Abou-Shanab et al. 2003
Elsholtzia splendens	Cu	Mobilization and increased uptake	Chen et al 2004
Brassica juncea	Cr (VI)	Increased root and shoot growth	Rajkumar et al. 2006
Brassica juncea	Zn, Pb, Cu ,Cd	Zn Mobilization; Pb immobilization, increased shoot and root growth	Wu et al. 2005
Brassica juncea	Ni	Increased shoot growth, increased uptake	Zaidi et al. 2006
Trifolium repens	Zn	Increased shoot growth, reduced uptake	Vivas et al 2006
Pityrogramma calomelanos	As	Increased shoot growth and increased uptake	Jankong et al. 2007
Brassica campestris,	Ni	Increased shoot growth	Farwell et al. 2007

availability of heavy metals. Bacterial immobilization and sequestration of heavy metals can inhibit heavy metal uptake in the rhizosphere and reduce heavy metal toxicity in the endosphere. Figure 4 schematizes bacterial activities that are thought to contribute to heavy metal tolerance and accumulation in plants

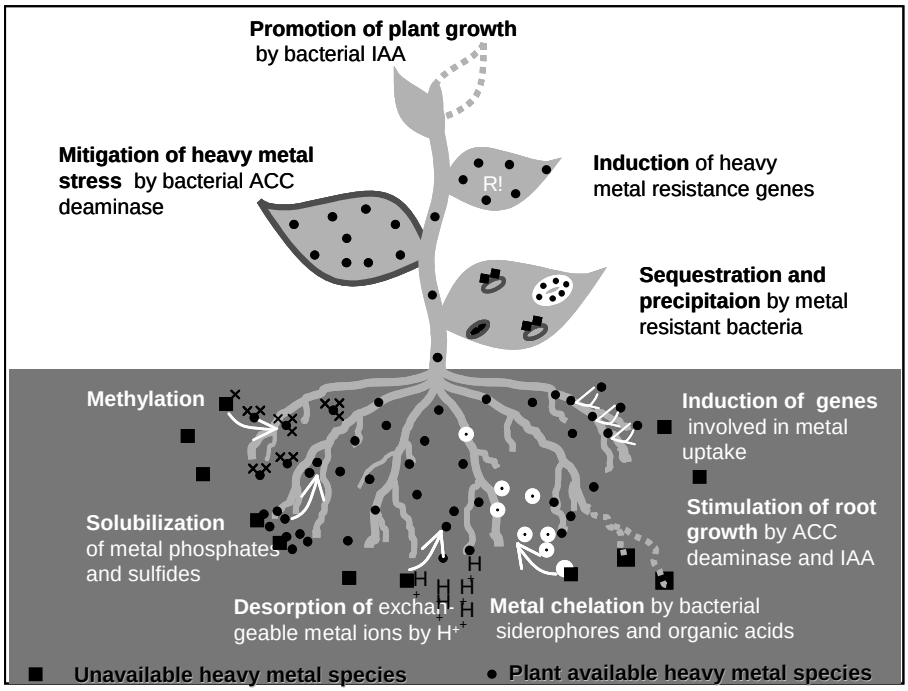

Figure 4. Putative plant-microbe interactions contributing to heavy metal accumulation

4.3.1 Bacterial production of the plant hormone IAA

Indole-3-acetic acid (IAA) is the main auxin hormone in plants, controlling many important processes, including cell enlargement and division, tissue differentiation and responses to light and gravity (Leveau and Lindow., 2005). Many plant associated bacteria, including plant growth promotors and pathogens are able to synthesize IAA (Patten and Glick 1996, 2002; Manulis and Barash, 2003) and to influence plant development by modulating IAA levels (reviewed by Spaepen et al., 2007). Depending on the amount released, rhizobacterial IAA has been observed to induce root elongation, root branching and inhibition of root growth (Davies 1995). IAA production is a frequent feature of rhizosphere bacteria on heavy metal contaminated sites (Dell'Amico et al., 2005). IAA producing bacteria have been shown to promote growth of *Brassica juncea* in the presence of Cr^{6+} and to enhance growth and Ni uptake by this plant in Ni contaminated soil (Rajkumar et al., 2006; Zaidi et al., 2006). Moreover IAA producers reduced Zn toxicity in the non-accumulator *Trifolium repens* (Vivas

et al., 2006). Increased metal accumulation may have been a result of root growth promotion, which expands the metal importing surface.

4.3.2 Bacterial production of the enzyme ACC deaminase

1-aminocyclopropane-1-carboxylic acid (ACC) is a precursor molecule in the ethylene synthesis pathway of plants. Many soil and plant borne bacteria can synthesize an enzyme that cleaves ACC into alpha-ketobutyrate and ammonia. This enzyme, called ACC deaminase, has no obvious function in free living bacteria. Plant associated ACC deaminase producers can consume plant borne ACC as a source for ammonia and at the same time inhibit ethylene synthesis in the plant (Glick et al., 2003). Ethylene is a regulator of plant development, and involved e.g. in breaking seed dormancy, in the succession of early seedling stages, in senescence processes and in stress response (Deikman, 1997). High ethylene levels however can inhibit plant growth (Abeles et al., 1992). Severe environmental stress and pathogen attack can induce accelerated ethylene synthesis (Abeles et al., 1992). This “stress” ethylene causes damage to the plant organism. Treatment with ACC deaminase producing bacteria reduced stress symptoms in plants exposed to various stress situations (Glick et al., 2003). The presence of toxic heavy metals can cause the formation of stress ethylene (Weckx et al., 1993) and heavy metal toxicity symptoms are partially due to the deleterious effects of ethylene (Glick et al., 2003). An ACC deaminase producing bacterium reduced stress ethylene in canola seedlings that were grown in presence of toxic Ni concentrations, and alleviated Ni-induced root and shoot growth inhibition (Burd et al., 1998). ACC deaminase production has been detected in an important proportion of the rhizosphere bacteria and endophytes of Ni hyperaccumulating *Thlaspi goesingense* (Idris et al., 2004).

4.3.3 Bacterial production of siderophores

Under iron limiting conditions, many organisms, including bacteria, release high affinity iron chelators, called siderophores (“iron carriers”) (Hider, 1984). Soil environments are generally characterized by low iron bioavailability, as most iron is bound in insoluble Fe(III)hydroxides. Therefore, grasses release phytosiderophores (non proteinaceous amino acids), which chelate Fe(III) and are subsequently recognized and re-imported by specific membrane receptors (Pinton et al., 2007). Dicotyledons do not produce siderophores, and acquire iron mainly via Fe(III) solubilization by proton extrusion and via reduction to Fe(II) by membrane bound Fe(III) reductases. To some extent they also chelate Fe(III) by the

exudation of organic acids. Bacteria produce various types of siderophores. Important classes are hydroxamates, catechols and the fluorescent pyoveridines of *Pseudomonas* species (Kalinowski et al., 2004). Bacterial siderophores have a low molecular weight (<1000 Da), but are more complex than the iron chelators released by plant roots and have a higher affinity to Fe(III) (Glick, 2003). Plant roots can take up certain bacterial siderophore-iron complexes (Bar-Ness et al., 1992; Cline et al., 1984). Siderophore producing rhizosphere bacteria have therefore been hypothesed to contribute to the iron nutrition of their host plant. Bacterio-siderophore assisted iron acquisition could be particularly important for plants growing in the presence of heavy metals. Heavy metals interfere with iron uptake and often cause iron deficiency symptoms such as chlorosis and reduced chlorophyll content (Mishra and Kar 1974; Imsande, 1998). Indeed Burd et al. (1998; 2000) demonstrated that a siderophore producing bacterium mitigated heavy metal stress symptoms in canola and that the beneficial effect could be increased by siderophore overexpression. Moreover many bacterial siderophores have been observed to chelate divalent heavy metal ions, including Mn, Zn, Cd, Pb and Cr(III) (Neilands, 1981; Birch and Bachofen; 1990; Diels et al., 1999;). The complexes formed with heavy metals are less stable than those formed with iron (Hernlem et al. 1996), still the affinity is significant and bacterial siderophore production can be induced by heavy metals. Extracellular complexation by siderophores may even be a bacterial mechanism of heavy metal resistance (van der Lelie et al., 2000). There are conflicting theories, whether complexation with bacterial siderophores make heavy metals more or less available for plants (van der Lelie et al., 2000; Abou-Shanab et al., 2003; Glick, 2003). Siderophore producers seem to be abundant in heavy metal hyperaccumulating plants. Most of the rhizosphere and endophyte isolates, that Idris et al. (2004) collected from Ni hyperaccumulating *Thlaspi goesingense*, were siderophore producers. It is therefore at least possible, that bacterial siderophores contribute to heavy metal accumulation. Plant roots may import bacterial siderophore-heavy metal complexes along with siderophore-iron complexes; inside the plant heavy metals may be detoxified by chelation with endophyte siderophores. This concept is supported by the results of Abou Shanab et al. (2003), who observed siderophore producing bacteria to increase Ni mobility and uptake by the hyperaccumulator *Alyssum murale*.

4.3.4 Bacterial mobilization of heavy metals

Although excess concentrations of heavy metals are available in the natural habitats of heavy metal accumulating plants, partial depletion of bio-available metal pools has been observed in their rhizosphere (Fitz et al., 2003; Puschenreiter et al., 2003, 2005; Whiting et al., 2001b). Application of living bacteria and bacterial metabolites to contaminated soil increased metal mobility, which is a measure for metal bio-availability (Whiting et al., 2001a; Abou-Shanab et al., 2003). The same metal mobilizing bacteria described by these authors were also able to promote Ni and Zn uptake in heavy metal hyperaccumulating plants. Bacteria can increase heavy metal mobility by various processes (Gadd, 2004) and Amir and Pineau (2003) stated a correlation between bacterial activity and heavy metal mobility in an ultramafic soil. One important mobilization mechanism is soil acidification by the release of protons and organic acids from bacterial cells. Protons are exported by the activity of H^+ATPases, for maintenance of charge balance and as a result of respiratory carbon dioxide accumulation. Among the various organic acids secreted from bacterial cells, citrate and oxalate have the greatest relevance to heavy metal mobility, as they form stable complexes with most metals. In the rhizosphere protons can replace exchangeable metal cations and organic acids can protect the ions resorption and precipitation. Moreover, protons and organic acid anions can dissolve metal containing minerals (Gadd, 2004). The dissolution of metal phosphates is of particular importance, as it releases simultaneously heavy metal cations and the essential nutrient phosphate (Halstead et al., 1969; Gupta et al., 2002). Inoculation with phosphate solubilizing rhizosphere bacteria increased Ni uptake in the hyperaccumulator *Alyssum murale* (Abou-Shanab et al., 2003) and both, biomass production and Ni uptake in *Brassica juncea* (Zaidi et al., 2006). Sulfur-oxidizing bacteria can liberate heavy metals from insoluble metal sulfides, and other specialists can change the solubility of heavy metals by redox transformations (Gadd, 2004). For example Mn(IV), Cr(III) and Hg(0) can be transformed into the more soluble species Mn(II), Cr(VI) and Hg(II). Soil bacteria and fungi have been further observed to transfer methyl groups enzymatically to Hg, As, Se, Te and Pb, altering the solubility, volatility and toxicity of these elements (Gadd, 2004). However there is no information about the importance of sulphur reduction, metal redox transformations and metal bio-methylation in the rhizosphere of heavy metal accumulators.

4.3.5 Bacterial heavy metal resistance and post-efflux sequestration

Bacteria can detoxify low amounts of heavy metals inside their cells by complexation and reduction. Species of toxic metals, which can be reduced by bacteria, include Hg(II), Cr(VI)

arsenate and Cu(II). Higher concentrations of heavy metals can only be managed by efflux-based resistance mechanisms (Nies, 1999). Numerous heavy metal export systems have been described and their genetic determinants have been identified (Nies, 1999, 2003; van der Lelie 2000). Detailed analysis of the Co/Zn/Cd exporting complex of *Ralstonia metallidurans* CH34 (previously *Alcaligenes eutrophus* CH34) revealed, that metal export is followed by sequestration to avoid re-entrance of heavy metals into the cells. This extremely metal resistant bacterium was able to remove high amounts of Zn and Cd from laboratory medium, and Zn, Cd, Ni, Co and Cu from polluted effluents in a bioreactor (Diels et al., 1990; 1995). The mechanisms of post-efflux sequestration are poorly understood. Exported Cd was found to be precipitated as $CdCO_3$ and Cd $(HCO_3)_2$ at the cell surface of *Ralstonia metallidurans* (Diels et al., 1995). Other bacteria can precipitate heavy metals as metal phosphates (Mcaskie et al., 1994). Moreover bacteria produce metallothioneins, phytochelatins, siderophores and extracellular polysaccharides with high metal binding capacity in response to heavy metals (Gadd, 2004). All of these compounds are thought to be involved in post-efflux immobilization and detoxification of heavy metals by highly resistant bacteria (Diels et al., 1995). Cd resistant bacteria were shown to protect *Brassica juncea* from Cd toxicity by Cd precipitation (Salt et al., 1999). In the rhizosphere of heavy metal hyperaccumulators resistance-related bioprecipitation and sequestration may counteract heavy metal accumulation. Heavy metal resistant endophytes however, may sequester and detoxify heavy metals in shoot tissues and hence increase the accumulation capacity of their host plant (Lodewyckx et al., 2001). Indeed, compared to bulk soil and rhizosphere, the highest heavy metal resistances have been found in shoot endophytes of heavy metal hyperaccumulators (Lodewyckx et al., 2002; Idris et al., 2004). This suggests that heavy metal hyperaccumulators, can benefit from metal sequestration by resistant bacteria in their shoots and therefore favour shoot colonization by particularly resistant strains.

4.3.6 Identification of novel plant-microbe interactions during heavy metal accumulation by Suppression subtractive hybridization (SSH)

The production of IAA, ACC deaminase and siderophores as well as metal mobilization and metal resistance can be assessed *invitro*, and are therefore well described. Numerous other mechanisms of adaptation to and interaction with the host plant may exist in culturable and unculturable bacteria associated with heavy metal accumulators. DeSouza et al. (1999) speculated for instance, that rhizosphere bacteria can induce the expression of heavy metal transporters in the plant. In return, heavy metal accumulating plants may induce or require the

expression of bacterial genes responsible for metal mobilization and detoxification. Novel genes encoding phenotypic features can be identified by molecular analysis. A standard approach is to generate random knock out mutants and to localise the mutation in mutants that lost the feature of interest (de Lorenzo et al. 1990). The detection of genes involved in plant-microbe interaction by random mutagenesis requires laborious *in planta* screening of several thousand mutants. Alternatively, a range of innovative molecular techniques can be used to identify genes that are related to a specific ecological function. Comparative genome analysis can identify genomic differences between organisms fulfilling or not fulfilling a certain function (Schoolnik, 2002). In Vivo expression technology (IVET), is based on promotor trapping and reveals which genes are upregulated in a certain situation (Silby and Levy, 2004). Transcriptome analysis is typically RNA-based microarray screening for genes expressed under specific conditions and can detect up- and downregulated genes (Schena et al., 1998). Proteome analysis detects proteins synthesised in a specific situation (Wilkins et al., 1996). Specifically released bacterial metabolites can be identified by metabolome analysis (Oliver et al., 1998). Each of the mentioned procedures has been successfully applied to elucidate plant-microbe interactions during plant colonization, symbiotic procedures and pathogenesis (Triplett et al., 2006; Kiely et al., 2006; Gourion et al., 2006). In chapter III of this thesis, comparative genomic analysis based on suppression subtractive hybridisation (SSH), will be introduced as an approach to identify novel genes, that characterize bacteria associated with heavy metal accumulating plants.

SSH is a technique to isolate DNA sequences that are present in a DNA sample of interest (tester) and absent from a reference DNA sample (driver). Basically, tester and driver are hybridized and tester specific sequences that do not form hybrids with driver DNA are selective amplified by suppression PCR. SSH has been developed for gene expression studies in eukaryotes, i. e. for the detection of differences between cDNA populations (Diatchenko et al. 1996; Gurskaya et al., 1996). Akopyants et al. (1998) adapted SSH to the comparison of bacterial genomes. From this protocol Clontech Laboratories Inc. (Palo Alto, CA) developed the PCR-Select^{TM} Bacterial Genome subtraction Kit, which is currently used in most SSH-based genomic studies. The PCR-Select^{TM} procedure involves seven reaction steps (Figure 5).

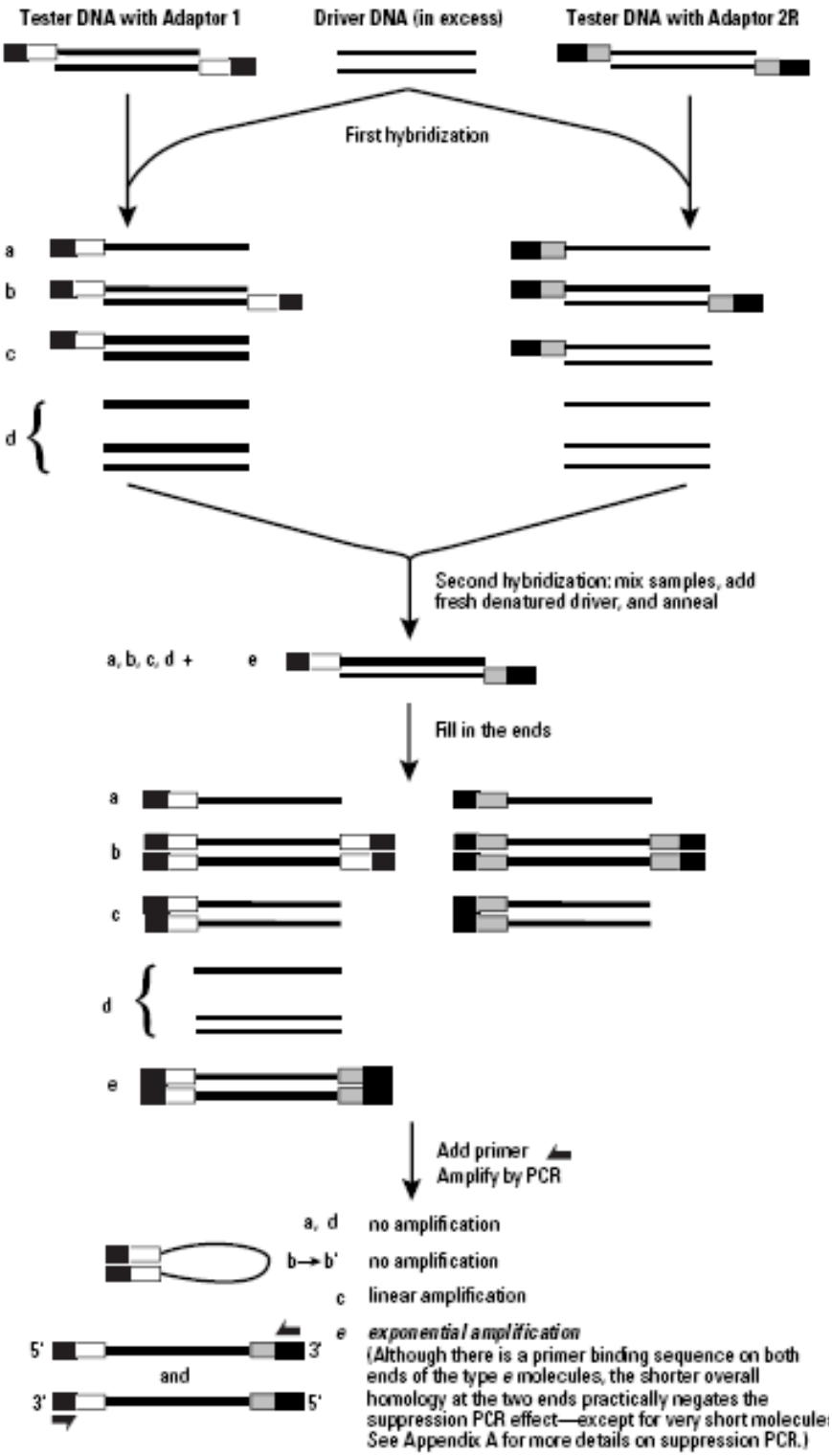

Figure 5. Molecular events during SSH using the PCR-Select^{TM} Bacterial Genome subtraction Kit .The Procedure involves seven steps: 1.Digestion of tester and driver gDNA 2. Subdivision of the tester DNA and ligation to two different adaptors 3. Separate hybridization of tester subsamples to driver DNA 4. Pooling of tester subsamples and secondary hybridization with driver DNA 5. Amplification of single stranded tester-sepcific sequences with adaptor specific primers 6. Further enrichment by nested PCR 7. Isolation of individual sequences by cloning

SSH is not a quantitative method, and fragments common to tester and driver may remain single stranded in presence of excess driver DNA and form amplifiable hybrids with complementary tester sequences. Such unsubtracted sequences are referred to as “false positives”. If the genome sequence of the driver is available, false positives can be easily identified by blast analysis. SSH libraries obtained with undescribed drivers can be screened for false positives and tester specific sequences by dot blot hybridization (Zhang et al., 2005), southern hybridization (Shen et al., 2005), PCR (Radnedge et al., 2001, 2003; Purdy et al., 2005) or microarray hybridization (Soule et al., 2005; Pelludat et al., 2005). PCR primers designed from SSH sequences and microarrays carrying entire SSH libraries can be applied further to study the distribution of tester-specific sequences in other strains (Radnedge et al., 2001; Soule et al., 2005). The blastx function of the ncbi (Altschul et al., 1997) con be used to obtain information about the functions of tester specific genes. Blastx translates DNA sequences and searches the database for proteins related to the corresponding gene products.

SSH cannot detect point mutations or interruptions of individual genes, but it can identify larger sequences in the tester genome (<20bp) that are either absent from the driver or substantially different. Therefore SSH is suitable to identify divergent gene families and large scale genome alternations such as insertions and deletions arising from horizontal gene transfer via plasmids, phages and transposons (Akopyants et al., 1998; Purdy et al., 2005). Large genome rearrangements drive bacterial evolution (Radnedge et al., 2003) and influence the environmental adaptation and distribution of individual strains (Purdy et al., 2005). SSH is mainly employed for the analysis of intra species diversity. Within a species, usually 5%-15% of the genes vary between strains (Purdy et al., 2005). Typical categories of strain specific genes are related to horizontal gene transfer, DNA restriction-modification, cell surface structures, metabolism, toxin and antibiotic resistance and virulence (Akopyants et al., 1998; Purdy et al., 2005). Cell surface formation, metabolism, resistance and virulence are processes of communication with the environment.

SSH is widely used to study the evolution of human pathogens and to identify virulence factors in and diagnostic markers for such dangerous bacteria (Akopyants et al., 1998; Radnedge et al., 2001, 2003; Purdy et al., 2005; Soule et al., 2005). Recently, SSH has been successfully applied for the detection of genes related to virulence and host specificity of plant-pathogenic bacteria (Zhang et al., 2005; Triplett et al., 2006).

4.3.7 Potential improvement of plant-microbe interactions in phytoextraction technology

Most studies about the role of bacteria in heavy metal accumulation by plants are based on plant inoculation experiments in the greenhouse. Such experiments allow the identification of individual bacteria that efficiently promote heavy metal extraction by plants (Table 2). Isolates selected in this manner could be enriched and subsequently reintroduced into their native polluted environment to enhance metal extraction by their original host plant (bioaugmentation). However, these strains cannot serve as ready-to-use inocula for every polluted site, because in non-native ecosystems introduced bacteria are easily outcompeted by the established microflora (Pilon-Smits, 2004). Particularly beneficial isolates can rather be used as model-organisms to study mechanisms of plant-microbe interactions and to identify the genes encoding these processes. The analysis of individual isolates can further help to determine the optimal conditions for the beneficial interaction processes. Agricultural measures, such as the supply of sun, air, water or specific nutrients to the extractor plants can then be adapted to support these processes in phytoremediated sites. Based on genetic information obtained from culturable bacteria, molecular tools can be developed to monitor the expression of the beneficial genes during phytoremediation and to learn more from naturally metalliferous ecosystems.

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Objectives and outline

The overall objective of this Ph.D. thesis was to contribute to the development of sustainable technologies for the remediation of heavy metal contaminated soils. This work was part of a project on the mechanisms of heavy metal accumulation in plants and on the potential of these accumulating plants and their associated microflora for soil remediation. As highlighted in the general introduction, *Salix caprea* trees are promising extractor plants, but little is known about the factors influencing the metal extraction efficiency of these trees. Surveys on herbaceous heavy metal accumulators revealed that apart from plant-internal uptake and detoxification mechanisms, microorganisms contribute to the accumulation process. Therefore, metal uptake, genetic characteristics, mycorrhizal fungi and bacterial microflora of *S. caprea* were studied by four collaborating research groups of the Austrian Research Centers GmbH and the University of Natural Resources and Applied Life Sciences Vienna. The specific aims of the presented Ph.D. work were i) the isolation and characterization of rhizosphere bacteria and endophytes associated with Zn/Cd accumulating *Salix caprea*, ii) the identification of bacterial strains promoting heavy metal accumulation, and iii) the elucidation of mechanisms enabling bacteria to enhance heavy metal accumulation by their host plant.

Chapter 1 describes the collection or heavy metal resistant rhizosphere bacteria and endophytes of *Salix caprea* trees growing at a Zn/Cd/Pb contaminated site in Arnoldstein (Austria). The isolates were phylogenetically identified by 16S rRNA gene analysis and tested *in vitro* for phenotypic characteristics that are frequently associated with the enhancement of heavy metal accumulation in plants. The analysed characteristics included the production of the plant growth hormone IAA, the release of the "anti stress agent" ACC deaminase and the secretion of iron ligands (siderophores). A subset of isolates was also tested for the ability to produce metabolites that can mobilize heavy metals from soil. Isolations and characterizations were primarily done in preparation for greenhouse experiments and molecular analysis of plant-microbe interactions. At the same time these experiments gave insight into the phylogenetic and functional diversity of the bacterial communities associated with *Salix caprea*. Strains with various combinations of phytoextraction-relevant characteristics were selected for a plant inoculation experiment. The assay failed due to inhomogeneous growth and high mortality of the experimental willow plantlets.

A parallel inoculation experiment with bacteria from a different collection of *S. caprea* rhizosphere isolates was successful and is described in chapter 2. The bacteria were tested for the same characteristics as the strains described in chapter one and were introduced into the rhizosphere of *S. caprea* plantlets. The aim of this greenhouse assay was to determine the effect of rhizosphere bacteria on biomass production and metal uptake in *Salix caprea* and to assess the relevance of *in vitro* determined characteristics to *in vivo* effects.

Apart from metal mobilization and from bacterial production of IAA, ACC deaminase and siderophores, numerous other ways of interaction between bacteria and heavy metal accumulating plants may exist. The nature of such interaction mechanisms can be elucidated by analysing the bacterial genes involved. Therefore the experiments described in the third chapter aimed at identifying genes specific to bacteria associated with heavy metal accumulators and at obtaining information about the functions of such genes. *Methylobacterium extorquens* was selected for the molecular experiments, because methylobacteria were dominant among endophyte isolates from *Salix caprea* and among isolates obtained from other heavy metal accumulators described in literature. Suppression subtractive hybridization (SSH) was carried out for the isolation of strain specific genes. In SSH, sequences specific to a bacterial strain are isolated by DNA hybridization with a closely related reference strain lacking the features of interest. The closer the two experimental strains are related, the higher is the efficiency of the SSH procedure. Therefore rather than analysing a *Methylobacterium* isolate from *Salix caprea*, an isolate from the Ni hyperaccumulator *Thlaspi goesingense* known for its close relatedness to a Ni sensitive type strain from soil was selected for SSH. The functions of genes specific to the *T. goesingnese* associated strain were predicted by sequence comparison to a public database and are discussed regarding their relevance to interactions with heavy metal accumulating plants.

The final chapter of this PhD thesis presents a synopsis of the conclusions from the three individual experiments and reflects lessons learnt from experimental success and failure. In addition an outlook is given on further research objectives in the field of plant-microbe interactions related to phytoextraction of heavy metals.

CHAPTER 1

Phenotypic characterization of bacteria associated with roots and shoots of heavy metal accumulating *Salix caprea*

Abstract

The aim of this work was to isolate and characterize bacteria associated with Zn/Cd accumulating *Salix caprea* and to select isolates for detailed analysis of plant-microbe interactions in the accumulation process. Three Zn containing isolation media of different nutrient strength allowed the isolation of 64 different rhizosphere strains and of 49 different endophytes. 16S rDNA sequencing revealed high diversity among rhizosphere isolates, particularly within the genera *Variovorax, Bradyrhizobium, Flavobacterium,* and *Flexibacter* and within the division *Actinobacteria*. Methylobacteria and Sphingomonads accounted for 65% of the endophyte diversity, which is in accordance with previous findings on heavy metal accumulators and suggests a specific function of these genera in the shoots of these plants. Bacterial 1-aminocyclopropane-1-carboxylic acid deaminase (ACCD) impairing stress ethylene formation, and bacterial siderophores, mobilizing iron and possibly also heavy metals, are thought to enhance heavy metal tolerance and uptake capacity of plants. Therefore all identified isolates were screened for ACC deaminase and siderophore production. ACC deaminase activity was found in 37% of the rhizosphere bacteria and in 8% of the endophytes. Siderophores were produced by 50% of the rhizosphere bacteria, but only by one endophyte. The isolates were extremely resistant to Zn and Cd, and certain strains also tolerated moderate concentrations of Pb. Twenty selected strains representing the dominant rhizosphere and endophyte genera were tested for release of the plant growth hormone indole-3-acetic acid (IAA), and for the production of Zn, Cd and Fe mobilizing metabolites. IAA production was detected in 40% of the analysed strains. Bacterial mobilization of metals was assessed by extracting contaminated soil with filtrates of liquid cultures. Most of the analysed strains immobilized Zn, Cd and Fe, probably by the release of non-specific metal binding agents. One siderophore producing rhizosphere bacterium and curiously also one siderophore negative endophyte mobilized iron. Four slowly growing actinobacteria mobilized Zn and/or Cd most likely by the release of specific non-siderophore metal-chelators, which were produced in the stationary phase and could be inactivated by boiling. The invitro experiments identified several isolates with multiple heavy metal relevant characteristics, which could be the basis for molecular analysis of the interactions between *Salix caprea* trees and their associated bacteria during heavy metal accumulation.

Introduction

Metal accumulation in shoots is an adaptation of various plants to metalliferous substrates (Baker et al., 1989) and can be exploited for the removal of heavy metals from polluted soils (Phytoextraction; McGrath and Zhao, 2003). Zn and Cd hyperaccumulators occurring in the northern temperate zone are typically small herbaceous *Brassicaceae*, such as *Thlaspi cearulescens*, and *Arabidopsis halleri* (Baker et al., 1989). Despite high concentrations of metals accumulated in their leaves, the metal extraction efficiency of these plants is moderate due to low biomass production (Chaney et al., 2000). Recently, trees of the genus *Salix* (willow), growing on heavy metal contaminated sites in central Europe have been discovered to accumulate high amounts of Cd and Zn in their leaves (Unterbrunner et al., 2007). Metal accumulating trees are ideal extractor plants, as they produce high amounts of easily harvestable leaf biomass and because of their massive root system (Pulford and Watson, 2002). Indeed, the Zn content and particularly the Cd content of a moderately contaminated soil could be successfully reduced by phytoextraction with *Salix* (Wieshammer et al., 2007).

Little is known about the specific requirements of *Salix* trees for optimal heavy metal uptake and about the environmental factors supporting the accumulation process. Observations on herbaceous heavy metal accumulators indicate that rhizosphere bacteria and bacteria colonizing the shoot tissues (endophytes) contribute to heavy metal uptake and tolerance (deSouza et al., 1999; Whiting et al., 2001; Lodewyckx et al., 2001; Abou-Shanab et al., 2003). The mechanisms underlying this plant-microbe interaction are not yet understood. However, bacterial production of, 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase, siderophores and indole-3-acetic acid (IAA), as well as metal detoxification and metal mobilization seem to be involved (van der Lelie et al., 2000; Glick, 2003; Gadd; 2004). ACC deaminase is an enzyme that has no function in bacteria, but antagonizes ethylene synthesis in plants, by cleaving the ethylene precursor ACC. Accelerated ethylene synthesis (“stress ethylene”) occurs in plants under environmental stress - including heavy metal stress- and damages the plant organism. ACC deaminase producing bacteria can inhibit stress-ethylene formation (Glick, 2003) and aleviate heavy metal toxicity (Burd et al., 1998). Bacterial siderophores are high affinity iron chelators that mobilize insoluble Fe(III) under iron limiting conditions and can be taken up by plant roots (Bar-Ness et al., 1992). Iron deficiency is a frequent symptome of plants under heavy metal stress and may be prevented by the import of bacterial-siderophore iron complexes (Glick, 2003). Moreover, bacterial siderophores can complex a variety of heavy metal ions (Neilands,1981) and have been speculated to mediate

their uptake by heavy metal accumulators (Abou-Shanab et al., 2003). The auxin hormone IAA controls important processes in plants, such as growth and tissue differentiation. Auxin levels in plant tissues can be modulated by IAA producing bacteria (Davies, 1995) and bacterial supply of IAA, may be important for successful growth in contaminated environments. Certain efflux-based systems of bacterial heavy metal resistance seem to involve extracellular metal precipitation and sequestration by exopolymers and chelators (Diels et al., 1995). Endophytes equipped with such resistance mechanisms may contribute to heavy metal detoxification in plants (Lodewyckx et al., 2001). Soil and rhizosphere bacteria increase metal mobility and plant-availability by various processes, potentially including redox transformations and the release of protons and organic acids (Gadd, 2004). Bacteria producing ACC deaminase, siderophores and IAA as well as highly metal resistant phenotypes and efficient metal mobilizers are frequently found in association with herbaceous heavy metal accumulators (Lodewyckx et al., 2001; Abou-Shanab et al., 2003; Idris et al., 2004, Zaidi et al., 2006). Moreover, isolates with these characteristics have been shown to promote plant growth in presence of heavy metals and/or to enhance metal accumulation (Burd et al., 1998; 2000; Salt et al., 1999; Whiting et al., 2001; Abou-Shanab et al., 2003b; Zaidi et al., 2006).

The objective of this work was to get an insight into the bacterial communities associated with roots and shoots of Zn/Cd accumulating *Salix caprea*, and to assess the functional potential in regard to support plant growth under heavy metal exposure or to promote heavy metal accumulation.

Materials and methods

Plant material and experimental soil

For the isolation of bacteria, *Salix caprea* rhizosphere and shoot samples were taken in June 2004, at a former lead mining site in Arnoldstein/Austria. The site is contaminated with Pb, Zn and Cd and has been described by Wenzel and Jockwer (1999). Fine roots and young branches with leaves were taken from four trees growing close to the contamination source, cooled to 4°C within 4h and processed latest after 48h. The Zn/Cd/Pb contaminated soil for mobilization experiments origniated from the same site. Physico-chemical characteristics of this soil have been analysed by Dos Santos Utmazian and Wenzel (2007) and are summarized in Table 1.

Table 1. Selected parameters of the experimental soil derived from Arnoldstein, Austria

Characteristic	value	unit
Texture (sand/silt/clay)	350/550/100	g kg^{-1}
Cation exchange capacity*	247	mmol kg^{-1}
Organic carbon	24.6	g kg^{-1}
pH	7.2	
Total metal contents (in aqua regia)		
Zn	1760	mg kg^{-1}
Cd	32.7	mg kg^{-1}
Pb	6560	mg kg^{-1}
Mobile fraction of metals (in 1M NH_4NO_3)		
Zn	2.56	mg kg^{-1}
Cd	0.64	mg kg^{-1}
Pb	3.81	mg kg^{-1}

* measured at soil pH

Isolation of rhizosphere bacteria and endophytes

For the isolation of rhizosphere bacteria, five grams of fine roots and adherent soil were suspended in 50 ml of 1% tryptic soy broth (TSB, Merck). After 2h of shaking at room temperature, soil particles and roots were allowed to settle for 1h. Various tenfold dilutions of the suspension were plated on 10% tryptic soy agar (TSA, Merck), 1% TSA and 0.08% diluted nutrient broth agar (DNBA, Janssen et al. 2002). All media were amended with (100 µg ml^{-1}) cycloheximidine to inhibit fugal growth and with $ZnSO_4$ (2 mM) to select for Zn resistant bacteria.

For specific isolation of endophytes, leaves (3.2-5 g) and green branches (2-3.5 g) were surface sterilized in 5% NaOCl for 5 min and rinsed with sterile water. Branches were further dipped into 70% ethanol, flamed and peeled. To check the sterility of plant surfaces, randomly selected leaves and branches were blotted onto 10% TSA plates which were subsequently incubated for 7 days at room temperature. Surface sterilized leaves and branches were cut into small pieces, mixed with 50 ml of 0.9% NaCl and ground in a stomacher (Stomacher Circulator, Seward) five times for 1min. In the intervening intervals the suspensions were cooled on ice to prevent heating and damage to living cells. Tenfold dilutions were plated on the isolation media described above. Xylem sap was extracted from lignified branches using a Scholander bomb and plated without diluting. 10% TSA plates were incubated for 1 week, 1% TSA plates for 3 weeks and 0.08% DNBA plates for up to 12 weeks to allow colony

formation of slowly growing bacteria. The number of culturable bacteria was determined on each of the media. Colonies of all distinguishable morphology types were picked an streaked on phosphate poor morpholinepropanesulfonic acid medium (MOPS; Neidhardt et al., 1974) containing 0.1% glucose and 1mM $ZnSO_4$. After 2-10 days of incubation at room temperature, the cell material was suspended in 10% tryptic soy broth (TSB) containing 15% of glycerol and stored at -80°C.

PCR-RFLP analysis of rhizosphere and endosphere isolates

In order to allow discrimination at the strain level 16S-23S intergenic spacer DNA was amplified from all isolates, using the primers p23SRO1 (5-GGCTGCTTCTAAGCCAAC-3') and pHr (5'-TGCGGCTGGATCAC-CTCCTT-3') (Massol-Deya et al., 1995). DNA was released by boiling one loop of bacterial cells for 10 min in 150 µl sterile deionised H_2O. One µl of lysis product was used as template in 50 µl PCR reactions containing 2 units Taq DNA polymerase (Invitrogen), 0.2 mM of each dNTP, 0.15 µM of each primer and 1,5mM $MgCl_2$. The thermal programme comprised an initial denaturation of 5 min at 95°C, followed by 30 cycles of 30 sec denaturation at 95°C, 1 min annealing at 55°C and 2 min extension at 72°C, and a final elongation step of 10 min at 72°C. 15 µl PCR products were digested for 4 h at 37°C with *Alu*I (Invitrogen). Restriction fragments were electrophoretically separated in 3% agarose gel. One representative of each IGS-RFLP type was selected for 16S rDNA sequence analysis.

DNA extraction and partial 16S rDNA sequencing

For the amplification and analysis of the 16SrRNA gene, bacterial DNA was isolated by bead beating and phenol-chloroform extraction (Sessitsch et al., 2001). Half a µl of DNA extract was used for 50 µl PCR reactions with the primers 8f (5'-AGAGTTTGATCCTGGCTCAG-3'; Weisburg et al., 1991) and 1520r (5'-AAGGAGGTGATCCAGCCGCA-3'; Edwards et al., 1989). Thermal programme and composition of the reaction mix were identical with those described for the IGS PCR, except for the annealing temperature, which was reduced to 53°C. After purification through sephadex G-50 (Amersham Biosciences) columns, 2 µl PCR product were added as template to 10 µl sequencing reactions using primer 518r (0.4µM; 5'-ATTACCGCGGCTGCTGG-3'; Liu et a., 1997) and the BigDye terminator cycle sequencing kit (ABI prism). After a second purification with sephadex G-50, the DNA fragments were sequenced with an ABI 373A automated DNA sequencer (Applied biosystems Inc.). Nucleotide BLAST was used to search the sequence database of the National Center for

Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/) for identified relatives of the isolates.

ACC deaminase activity

ACC deaminase activity was tested on the minimal medium described by Brown and Dilworth (1975) containing 0.7 g ACC l^{-1} as sole nitrogen source. To exclude traces of nitrogen, that might be present in conventional agar, pure agarose (Saekem) was used to solidify the medium. To avoid potential contamination with ammonium during the autoclaving, nutrient stock solutions were sterile-filtered and the agarose matrix was sterilized by 20 min of boiling. Minimal medium without nitrogen was used for negative controls, positive controls contained 0.7 g NH_4Cl l^{-1}. Plates were incubated for two weeks at room temperature.

Siderophore production

Siderophore production was analysed on chrome azurol S agar plates. Bacteria were grown for two weeks at room temperature on plates (5 mm diameter) filled half with iron free MM9 medium (6.8 g l^{-1} Na_2HPO_4, 0.3 g l^{-1} KH_2PO_4, 0.5 g l^{-1} NaCl, 1 g l^{-1} NH_4Cl, 0.4% glucose) and half with CAS-blue agar (Milagres et al., 1999). Siderophore producing organisms are able to extract iron from the blue Fe-CAS complex forming an orange depletion zone in the medium. Size and progress rate of the depletion zone were taken as estimates for siderophore production intensity.

Indole acetic acid production

The ability of the isolates to produce indole acetic acid (IAA) was initially tested according to the method of Sawar and Kremer (1995). Since many strains failed to grow in the broth described by these authors (1 g l^{-1}peptone, 2 g l^{-1}glucose, 1 mM tryptophane), the medium was solidified with 15% of agar. Bacterial cells were suspended in 0.9% saline. Fifty µl of the suspension were placed in the centre of an agar plate and incubated for 72 h at room temperature, with protection from light. Two hundred µl of Salkowsky reagent (35% perchloric acid, 3 mM $FeCl_3$) were applied to each plate on top of the bacterial cells. After 30

min of incubation in the dark, the diameter of the pink zone around the cells was used to estimate the relative amount of IAA produced.

Heavy metal tolerance

Heavy metal resistances were determined on phosphate-poor morpholinepropanesulfonic acid medium (MOPS; Neidhardt et al.,1974) with 0.1% glucose as carbon source. Bacteria were streaked on MOPS agar plates containing Zn, Cd, and Pb respectively, in concentrations of 0 mM, 2 mM, 4 mM, 6 mM, 8 mM and 10 mM. Zn resistance was further tested up to a concentration of 16 mM, Cd resistance was tested also at concentrations of 0.5, 1 and 1.5 mM. For each strain and each metal the lowest concentration that inhibited visible growth at room temperature within 7 days was determined.

Bacterial growth kinetics

One loop of bacteria was suspended in 3 ml of 10% TSB and incubated at 25°C on a rotatory shaker (200 rpm) until the cultures were tumid with cells. Three ml of this starter culture were transferred to 30 ml of fresh 10% TSB and again shaken at 25°C and 200 rpm. The optical density of the 30 ml cultures was measured at 600 nm (OD 600) with a DU® 640 Spectrophotometer (Beckman, USA). For fast growing organisms the OD 600 was determined every three hours, for slowly growing organisms every 5 hours. For each strain, two 30 ml cultures were started in the morning and two in the evening, to allow a 24 h survey of bacterial growth.

Metal mobilization by bacterial metabolites

Bacteria were grown in 10% TSB medium (Merck, pH 7.2) at room temperature and 200 rpm. Late-log cultures grown until at the moment when highest cell density was reached (Table 4). Late stationary cultures were allowed to grow twice the time needed to reach maximal density. Cells were removed from the cultures were by centrifugation (8000xg, 15min, 4°C) and filtration (0,2 μm Ministart filters; Sartorius). Aliquots of heat denatured culture filtrate were prepared by 10 min incubation in a boiling water bath. One gram of Zn/Cd/Pb contaminated soil was shaken with 5 ml of the cell free supernatants (2 h, 20 inversions perminute). The soil particles were removed again by centrifugation (7000 rpm, 5 min) and filtration (0.45 μm filters, Roth) and Zn, Cd and Fe in the filtrates were quantified by Atomic Absorption Spectroscopy (AAS, Perkin Elmer 2100). Fresh 10% TSB medium was used for

control extractions. Late logarithmic cultures, late stationary cultures and control medium were prepared in triplicates. From each replicate three aliquots of were shaken with soil and analysed, to obtain average metal concentrations from nine individual measurements.

Statistical analysis

Statistic analysis was done with STATISTICA 6 (StatSoft, Tulsa, USA). Analysis of variance (ANOVA) followed by post-hoc Fisher LSD test was carried out to identify significant effects of strains in mobilization experiments. Correlations between the resistance to different metals as well as correlations between the mobilization of different metals were determined by product moment correlation analysis.

Results

Abundance and diversity of culturable Zn resistant bacteria

For the isolation of plant associated bacteria, xyleme sap as well as extracts from leaves, branches and rhizosphere material were plated on three different media (10% TSA, 1% TSA, 0.08% DNBA) containing 2 mM of Zn.The number of colony forming units (CFUs) varied between samples from different trees. Leave and branch extracts yielded $4x10^2$ - $2x10^3$ CFUs per g fresh material. From 1 ml xyleme sap $3x10^3$ - 10^4 CFUs were obtained. All endophyte samples produced more colonies on 10% TSA than on the other media. For 1 g of rhizosphere soil 10^5 – $2x10^6$ CFUs were counted on each of the three media. Colonies of all distinguishable morphotypes were picked from each medium. The resulting collection of 180 endophytes and 180 rhizosphere isolates was screened by RFLP analysis of 16S-23S intergenic spacer DNA (IGS). Sixty-four different IGS-types were identified among the rhizophere bacteria, 49 among the endophytes. 48% of the rhizosphere-IGS types originated from 10% TSA, 17% from 1% TSA, 25% from 0.08% DNBA. Among endophytes, 10%TSA, 1%TSA and 0.08% DNBA, respectively, contributed 40%, 17% and 21% to isolate diversity. 11% of the rhizosphere IGS types and 21% of the endophyte IGS-types were found on more than one medium. One representative strain of each IGS-type was selected for further analysis.

Phylogenetic diversity of rhizosphere and endosphere isolates

For phylogenetic identification, 16S rDNA was amplified from the selected isolates. Fragments of about 500 bp were sequenced and compared to NCBI sequence database entries. The divisions *Alphaproteobacteria (Bradyrhizobium, Rhizobium, Sphingomonas, Afipia), Betaproteobacteria (Variovorax, Burkholderia, Janthinobacterium, Collimonas)* and *Actinobacteria (Streptomyces, Arthrobacter, Nocardia, Rhodococcus, Leifsonia, Microbacterium, Mycobacterium)* each accounted for about 20% of the rhizosphere isolates. (Table 2). Furthermore, *Gammaproteobacteria (Pseudomonas, Hafnia), Bacteroidetes (Flavobacterium, Chryseobacterium, Flexibacter, Cythophaga, Pedobacter,* unidentified *Bacteroidetes)* and *Firmicutes (Carnobacterium)* were represented in the rhizosphere isolate collection. 65% of the analysed endophytes were affiliated with *Alphaproteobacteria (Sphingomonas, Methylobacterium, Ochrobactrum)*, and 20% belonged to the *Actinobacteria (Microbacterium, Arthrobacter, Rhodococcus, Frigoribacterium, Leifsonia, Subtercola,* unclassified *Actinobacteria)* The remaining endophyte isolates clustered with *Betaproteobacteria (Zoogloea), Bacteroidetes (Pedobacter, Spirosoma)* and *Firmicutes (Bacillus).* Eleven rhizosphere bacteria and five endophytes had less than 97% sequence identity to described bacteria and may therefore belong to novel bacterial species. Table 2 summarizes the type of genera found among isolates.

Table 2. Putative divisions and genera detected in association with Zn/Cd accumulating *Salix caprea*

64 Zn-resistant rhizosphere isolates				49 Zn-resistant endophyte isolates	
Genera	**nb of isolates**	**Genera**	**nb of isolates**	**Genera**	**nb of isolates**
Actinobacteria		***Firmicutes***		***Actinobacteria***	
Streptomyces	2	*Carnobacterium*	1	*Microbacterium*	3
Arthrobacter	2	***α-Proteobacteria***		*Actinobacterium*	2
Nocardia	2	*Bradyrhizobium*	11	*Arthrobacter*	1
Rhodococcus	2	*Sphingomonas*	2	*Leifsonia*	1
Leifsonia	1	*Afipia*	1	*Rhodococcus*	1
Microbacterium	2	*Rhizobium*	1	*Subtercola*	1
Mycobacterium	1	***β-Proteobacteria***		*Frigoribacterium*	1
Flavobacteria		*Variovorax*	13	***Sphingobacteria***	
Flavobacterium	5	*Janthinobacterium*	2	*Pedobacter*	2
Chryseobacterium	1	*Burkholderia*	1	*Spirosoma*	1
Sphingobacteria		*Collimonas*	1	***Firmicutes***	
Flexibacter	3	***γ-Proteobacteria***		*Bacillus*	1
Pedobacter	1	*Pseudomonas*	2	***α-Proteobacteria***	
Bacteroidetes		*Hafnia*	1	*Sphingomonas*	17
undescribed	6			*Methylobacterium*	16
				Ochrobactrum	1
				β-Proteobacteria	
				Zoogloea	1

ACC deaminase activity and siderophore production

ACC deaminase activity was determined as the ability to use ACC as sole nitrogen source. Among the 64 rhizosphere strains, 17% grew moderately and 20% grew intensively on ACC amended medium (Table 3). 59% showed no ACC deaminase activity and for 4% ACC deaminase activity was not determined due to the lack of a suitable medium. Four of the 49 selected endophytes (8%) were able to use ACC as nitrogen source, 59% did not show ACC deaminase activity. For the remaining endophyte isolates ACC deaminase production could not be determined as no suitable minimal medium could be identified. Siderophore production was tested in a colour reaction with Fe-CAS agar. Half of the selected rhizosphere bacteria produced siderophores, 5% with moderate and 45% with high intensity. In contrast only one siderophore producing endophyte was identified. In Table 3 the analysed rhizosphere bacteria and endophytes are grouped according to the results of the siderophore and ACC deaminase assays. Fifteen rhizosphere isolates and 5 endophytes were selected for further analysis. Selected strains included isolates producing ACC deaminase, siderophores, both ACC deaminase and siderophores as well as isolates with none of these characteristics (Table 4).

Table 3A. Phylogenetic affiliation, ACC deaminase (ACCD) and siderophore (SID) production of rhizosphere bacteria representing different IGS types

Isolate*	Closest identified relatative ** [accession #]	% homology	*** ACCD	*** SID
Rhizosphere bacteria producing ACC deaminase and siderophores				
RX232	*Burkholderia phytofirmans* [AY962606]	100	++	++
RX26,RI27,**RD74**;RX32,RX33,RX273	*Variovorax paradoxus* [AF451851]	99-100	++	++
RX141,RX275	*Variovorax paradoxus* [AY127900]	99	++	++
RD308	*Variovorax* sp. [AB098595]	99	++	++
RX229	*Hafnia alvei* [M59155]	99	++	++
RX8; **RX228**	*Pseudomonas tolaasii* [AF320989]	99	+; ++	++
RD311	*Flavobacterium* sp. [AF541927]	97	+	++
RI3	*Nocardia brasiliensis* [AF430038]	97	+	+
RX84	*Rhodococcus erythropolis* [AY357220]	99	+	++
RI234	*Carnobacterium* sp. [AY573049]	97	++	++
Rhizosphere bacteria producing ACC deaminase, but no siderophores				
RX18	*Bradyrhizobium* sp. [AF408956]	98	++	-
RX14	*Variovorax paradoxus* [AF451851]	99	+	-
RI251	*Arthrobacter* sp. [AY572482]	98	+	-
RX22	*Microbacterium* sp. [AY017057]	100	+	-
RX138	*Rhodococcus* sp. [AY336559]	99	+	-
RX17	*Streptomyces acidiscabies* [D63865]	98	++	-
RI9	*Streptomyces* sp. [AF112159]	97	+	-
RX68	*Leifsonia xyli* [AJ717351]	99	+	ND
RD319	*Cytophagales* [AB022889]	96	+	-
RX272	Uncultured bacterium [AB117715]	92	+	ND
Rhizosphere bacteria producing siderophores but no ACC deaminase				
RI12	*Bradyrhizobium elkanii* [AY904749]	99	-	++
RD343, RD358	*Bradyrhizobium* sp. [AY547290]	99	-	+
RI158	*Bradyrhizobium* sp. [AJ558031]	97	-	+
RX265	*Collimonas* [AJ496445]	100	-	++
RX56	*Variovorax* sp. [AB196432]	98	-	++
RD335, **RD336**, RD351	*Flavobacterium* sp. [AF541927]	96	-	++
RD91, RI118, **RX233**	*Flexibacter japonensis* [AB078055]	96	-	++
RX2	*Nocardia salmonicida* [AJ298932]	94	-	++
RD341, **RD344**, RD352	*Bacteroidetes bacterium* [AY444818]	96	-	++
Rhizosphere bacteria producing neither siderophores nor ACC deaminase				
RD293	*Bradyrhizobium elkanii* [AY904749]	99	-	-
RD268	*Bradyrhizobium* sp. [AJ558025]	100	-	-
RI270, RD355; RI140	*Bradyrhizobium* sp. [AY547290]	98-99	-	-
RX29	*Bradyrhizobium* sp. [AJ558031]	97	ND	-
RI252	*Rhizobium etli* [AY460185]	99	-	-
RX101	*Sphingomonas* sp. [U63962]	97	-	-
RX30	*Sphingomonas suberifaciens* [D13737]	97	-	-
RX290	*Afipia genosp*. 9 [U87779]	99	-	-
RX243, RX256	*Janthinobacterium* sp. [AJ551147]	99	-	-
RD318	*Flavobacterium* sp. [AY336561]	96	-	-
RX139	*Chryseobacterium* sp. [AY468446]	97	ND	-
RX196	*Pedobacter* sp. [AY275498]	94	-	-
RD334	*Mycobacterium* sp. [AY215324]	97	-	-
RX225	*Arthrobacter* sp. [AY572482]	98	ND	-
RX99	*Cytophagales* str. [AB022889]	96	-	-
RI269	Rhizosphere soil bacterium [AJ252591]	91	-	-
RI121	Uncultured CFB gr. clone [AF431578]	94	-	-
RX97	*Bacteroidetes* bacterium [AY337604]	95	-	ND

*The strain nomeclature gives information about the isolation medium (X for 10% TSA, I for 1% TSA and D for diluted nutrient broth agar). The isolates selected for further analysis are highlighted. **Phylogenetic affiliations are based on sequence analysis of about 500 bp of the 16S rRNA gene. ***ND For certain strains the production ofACC deaminase production and/or siderophores could not be determined due to lack of suitable minimal media.

Table 3B. Phylogenetic affiliation, ACC deaminase (ACCD) and siderophore (SID) production of endophytes representing different IGS types

Isolate*	Clostest identified relative ** [Accession #]	% homology	*** ACCD	*** SID
Endophytes producing ACC deaminase				
EI215	*Methylobacterium* sp. [Z23158]	100	++	-
EX276	*Ochrobactrum anthropi* [AB120120]	99	++	-
ED222	*Leifsonia rubeus* [AJ438585]	95	++	-
EX150*	*Rhodococcus* sp. [AY336559]	98	+	-
Endophytes producing siderophores				
EI208	*Pedobacter* sp. [AY275498]	99	-	++
Endophytes producing neither ACC deaminase nor siderophores				
ED329	*Methylobacterium* sp. [AY369236]	99	-	-
EX107, EI172, **EI198**	*Methylobacterium* sp.[AY741724]	99	-	-
EX72	*Microbacteriaceae* bact.[AY504472]	98	-	-
EX135	*Methylobacterium* sp. [Z23158]	99	-	-
EX44	*Microbacterium* sp. [AY336540]	100	-	-
EX104	*Microbacterium* testaceum[AF474325]	99	-	-
EX129, EX131	*Sphingomonas asaccharolytica* [AJ871435]	95	-	-
ED154	*Sphingomonas* sp. [AY444826]	98	-	-
EX127; EX128, **ED306**	*Sphingomonas* sp.[AF395031]	98	-	-
EX145; ED123, EI149, EX151, EX152, ED160, EX161, ED162, ED312,ED314	*Sphingomonas* sp.[AY336556]	99	-	-
EX109	*Zoogloea* sp. [D84593]	96	-	-
EX241	*Bacillus pumilus* [AB109633]	99	-	-
EX166	Frigoribacterium sp. pfB31[AY336560]	99	-	-
Endophytes with undetermined ACC deaminase activity not producing siderophores				
EI174,EI189	*Methylobacteriaceae bact*.[AY673204]	100	ND	-
ED325	*Methylobacterium organophilum* [D32226]	97	ND	-
ED320, ED323	*Methylobacterium* sp. [AY364032]	99	ND	-
EI178, EI187, EI188, ED328	*Methylobacterium* sp. [AY741724]	99	ND	-
EI199	Methylobacterium sp. [AF395035]	100	ND	ND
EI54	Sphingomonas sp. [AY336556]	98	ND	ND
EX45	*Pedobacter* sp.[AY275498]	95	ND	-
EX36	*Spirosoma*-like sp. [X89911]	96	ND	ND
EX283	*Actinobacterium* iRIII11 [AY358003]	95	ND	-
EX48	*Actinobacterium* [AY275511]	99	ND	-
EX51	*Arthrobacter* sp. [AB017354]	95	ND	-
EX244	*Subtercola pratensis* [AJ310412]	100	ND	-

*The strain nomeclature gives information about the isolation medium (X for 10% TSA, I for 1% TSA and D for diluted nutrient broth agar). The isolates selected for further analysis are highlighted.

**Phylogenetic affiliations are based on sequence analysis of about 400 bp of the 16S rRNA gene.

***ND For certain strains the production ofACC deaminase production and/or siderophores could not be determined due to lack of suitable minimal media.

Indole acetic acid (IAA) production

Cultivation in tryptophane amended broth was not possible with 18 of the 20 selected isolates. When the tryptophane medium was solidified with agar all strains grew, and the produced IAA could be visualized with Salkowsky colour reagent. Strong IAA production leading to an intensive pink coloration of a large zone in the medium was found in 7 strains (Table 4). One additional strain released low amounts of IAA, visible as a faint pink stain in the cell material and the surrounding agar. Four rhizosphere bacteria and four of the five analysed endophytes produced IAA.

Zn Cd and Pb resistance of endophyte and rhizosphere isolates

All 64 identified rhizosphere bacteria and all 49 identified endophytes (Table 2) were tested on plates containing 5 mM and 10 mM of Zn. 90% of the rhizosphere bacteria and 70% of the endophytes were resistant to 10 mM of Zn, the remaining isolates tolerated Zn at least in 5mM concentration (data not shown). Table 4 shows precise Zn, Cd and Pb resistances of the 20 isolates that were selected for detailed analysis. For these strains, minimal inhibitory concentrations (MICs) of Zn generally ranged between 12 and 14 mM, four rhizosphere isolates grew even with 14 mM of Zn. The highest observed Cd MIC was 8 mM, for *Bradyrhizobium* RI12. Three other rhizosphere isolates and one endophyte were inhibited by 6mM of Cd. *Frigoribacterium* EX166 was inhibited by 0.5 mM, the lowest Cd dose given. The Cd MICs of the remaining strains ranged between 4 and 6 mM. Pb MICs were between 4 mM and 8 mM, except for strain RI 121 which was sensible to 2 mM of Pb. The levels of Cd and Pb resistance significantly correlated among each other ($r = 0.513$, $p<0.01$), but not with Zn resitance.

Heavy metal mobilization from soil

The ability of bacterial metabolites to mobilize metals was tested by leaching contaminated soil with filtrates of bacterial cultures and quantifying the extracted Zn, Cd and Fe (Figure 1). The pH was 7 in fresh sterile 10% TSB, 7.5 in stationary *Mycobacterium* RD334 cultures and 8-8.7 in the remaining stationary cultures (data not shown). pH differences between replicate cultures were minimal. Sterile TSB medium extracted 2.62 mg Zn, 173 µg Cd and 1.24 mg Fe per kg soil. Cd extractability was reduced by metabolites from 14 out of 15 rhizosphere bacteria and by 4 out of 5 endophytes. Ten of these rhizosphere bacteria and two of the endophytes also immobilized Zn. Four siderophore producers (RX74, RX228, RX232 and RX265) immobilized Zn at a particularly high rate to values around 1 mg kg soil^{-1}. One

rhizosphere bacterium (RD334) increased Zn and Cd mobility. Three endophyte strains (EX72, EX104 and EX166) doubled Zn extractability. EX72 also strongly enhanced Cd extraction. The effects of the bacterial metabolites on Zn and Cd mobility correlated positively ($r = 0.60$; $p<0.05$). Fe mobilization showed a clearly different pattern over the 20 isolates (Figure 1C). Fe extractability was increased 5 times by the Zn mobilizing endophyte EX104 and three times by the rhizosphere strain RI12, which had immobilized Zn and Cd.

Zn immobilizing metabolites were present in late logarithmic and late stationary cultures of RI12 (Figure 2). Similarly, both late logarithmic and late stationary cultures of EX104 contained Zn mobilizing metabolites. But the Zn mobilizing effect of the EX104 culture filtrate grew significantly from the late logarithmic to the late stationary phase. Four culture filtrates and fresh control medium were tested for heat stability in a separate assay. In this experiment the Zn extraction capacity of the control medium and of all analysed culture filtrates was higher than in the above described assay (Figure 3), and the mobilization effect of EX166 was not significant. Ten minutes of boiling did not change the pH of control medium and culture filtrates. However the heat impact significantly reduced the Zn extraction capacity of fresh 10% TSB and eliminated the positive effects of the strains EX72 and EX104. Boiled culture filtrates of these strains did not mobilize significantly more Zn than boiled control medium. The immobilizing effect of RX232 was not affected by boiling.

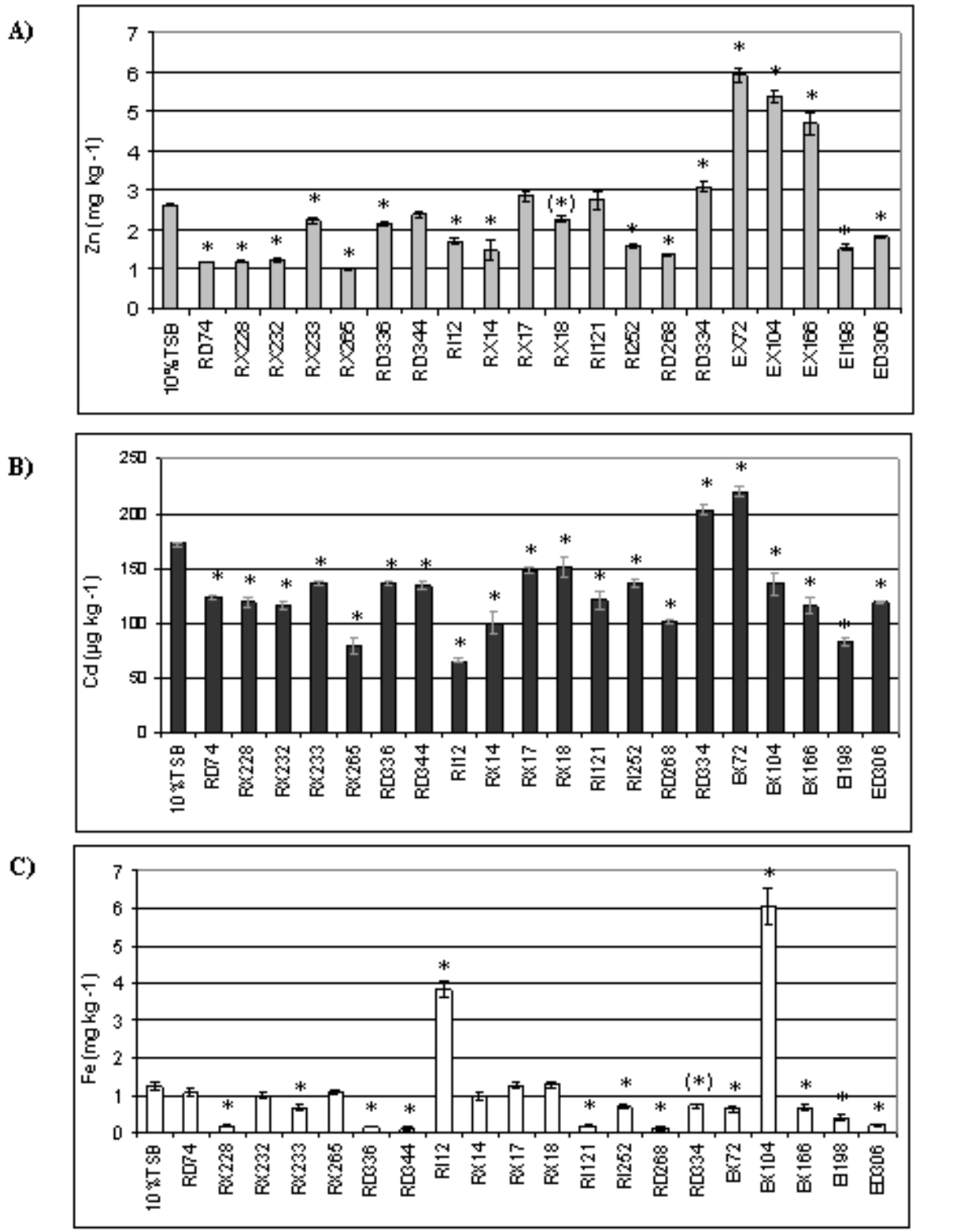
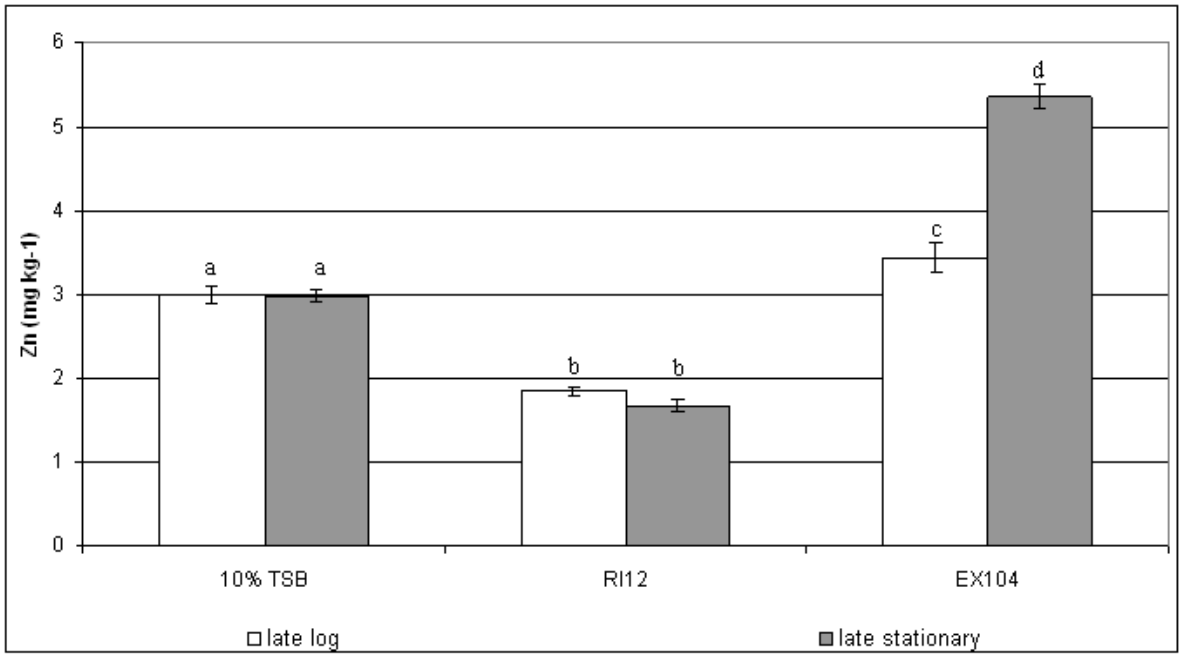


Figure 1. Mobilization of heavy metals from contaminated soil by bacterial exudates. Contaminated soil was shaken with filtrates of stationary bacterial TSB cultures. The columns show mean values obtained from three replicate cultures which were measured three times each. A) zinc B) cadmium C) iron



Different letters above columns indicate significant differences ($p<0.01$)

Figure 2. Mobilization of Zn from contaminated soil by bacterial exudates. Soil was shaken with filtrates of late logarithmic and late stationary TSB cultures. The columns show mean values obtained from three replicate cultures which were measured three times each.

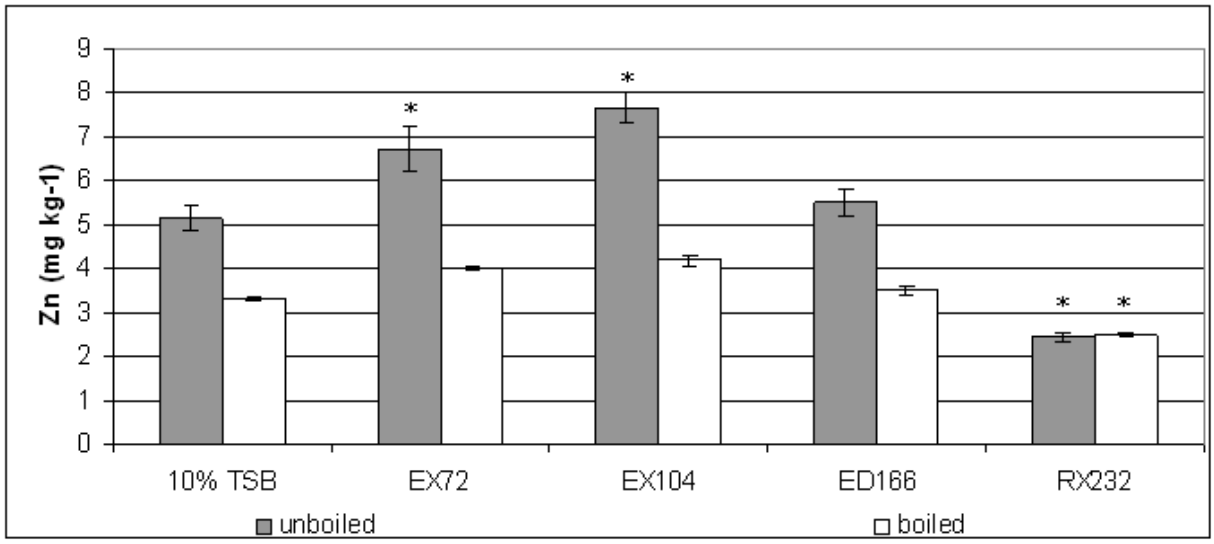


Figure 3. Mobilization of Zn from contaminated soil by bacterial boiled and unboiled exudates. Soil was shaken with filtrates of late stationary TSB cultures. The columns show mean values obtained from three replicate cultures which were measured three times each.

* significant differences of unboiled culture filtrates to unboiled control TSB medium and of boiled filtrates to boiled control TSB medium ($p<0.01$)

Table 4. Overview over phylogenetic affilitaion, culturability and heavy metal relevant phenotypic characteristics of selected rhizosphere bacteria and endophytes

		Max density in 10% TSB					MIC** (mM)			Impact on metal mobility*		
Isolate	Genus	h	OD 600	Sid	ACCD	IAA	Zn	Cd	Pb	Zn	Cd	Fe
Rhizosphere bacteria												
RX74	*Variovorax*	18	1,0	++	++	-	12	4	6	-	-	~
RX228	*Pseudomonas*	9	0,8	++	++	-	12	1,5	6	-	-	-
RX232	*Burkholderia*	9	0,9	++	++	-	16	4	6	-	-	~
RX233	*Flexibacter*	30	0,4	++	-	++	12	6	8	-	-	-
RX265	*Collimonas*	15	1,2	++	-	++	12	1	6	-	-	~
RD336	*Flavobacterium*	4	1,0	++	-	-	16	1	6	-	-	-
RD344	*Bacteroidetes bact.*	5	0,9	++	-	-	16	1	6	~	-	-
RI12	*Bradyrhizobium*	61	0,4	++	-	.-	16	8	8	-	-	++
RX14	*Variovorax*	16	0,7	-	+	++	12	4	6	-	-	~
RX17	*Streptomyces*	24	0,7	-	++	-	12	1	6	~	-	~
RX18	*Bradyrhizobium*	62	0,3	-	++	-	14	6	8	-	-	~
RI121	*CFB group bacterium*	45	0,6	-	-	-	12	4	2	~	-	-
RI252	*Rhizobium*	18	0,5	-	-	++	14	4	6	-	-	-
RD268	*Bradyrhizobium*	20	0,6	-	-	-	12	4	6	-	-	-
RD334	*Mycobacterium*	45	0,1	-	-	-	12	6	8	+	++	-
Endophytes												
EX72	*Microbacteriaceae*	28	0,4	-	-	-	12	4	6	++	++	-
EX104	*Microbact. testaceum*	15	0,5	-	-	+	12	6	6	++	-	++
EX166	*Frigoribacterium*	45	0,3	-	-	++	12	0	6	++	-	-
EI198	*Methylobacterium*	43	0,4	-	-	++	14	4	6	-	-	-
ED306	*Sphingomonas*	20	0,8	-	-	++	12	1,5	6	-	-	-

*impact of metal extractability from soil: +) mobilization, -) immobilization, ~) no effect
** minimal inhibitory concentration

Discussion

Each plant represents a unique environment for bacteria and harbours a specific microflora in its rhizosphere and endosphere (Kent and Triplett, 2002). Heavy metal accumulating plants with high metal concentrations in shoots and elevated amounts of bio-available metals in the rhizosphere provide a niche for adapted metal resistant microorganisms (Kunito et al, 2001). Several studies have addressed the microflora associated with heavy metal accumulating herbal species (Lodewyckx et al., 2002; Belimov et al., 2005; Abou-Shanab et al., 2003b; Gremion et al. , 2003; Mengoni et al., 2004; Idris et al., 2004). In this work we analysed the culturable endophytes and rhizosphere bacteria of Zn/Cd accumulating willow trees.

Colony counts of Zn resistant endophytes extracted from leaves and branches ($4x10^2$ - $2x10^3$ CFU g^{-1}) were within the range of previous observations for heavy metal accumulating plants (Lodewyckx et al., 2002; Idris et al., 2004). A decrease of endophyte density from roots to stems and further to the leaves has already been observed by Lamb et al. (1996). CFU values counted for rhizospere samples ($1x10^5$ – $2x10^6$ CFU g^{-1}) were slightly higher than those found in the rhizosphere of metal accumulating herbs (Lodewyckx et al., 2002; Idris et al., 2004). Apart from plant specific influences and suitability of the isolation media, the high yield of colonies may have partly originated from the rhizoplane which was not separated from the rhizosphere .

Hundred-eighty endophyte isolates and 180 rhizosphere bacteria were collected and screened by IGS-RFLP analysis. One representative of each IGS-type was selected and identified by 16S rDNA sequencing. Sixty-four different rhizosphere strains belonging to 22 different genera and 49 endophyte strains belonging to 14 genera were identified. Highest diversity of endophytes and rhizosphere bacteria was observed on 10% TSA. Still, 1% TSA and 0.08% DNBA each contributed approximately 20% to isolate diversity of both endophytes and rhizosphere bacteria. Only 12% of the rhizospere isolates and 22% of the endophytes could be recovered from more than one medium. Thus parallel plating on three media of different nutrient strength was a successful strategy to increase isolate diversity.

Since the majority of environmental bacteria cannot be cultured on standard laboratory medium (Amann et al., 1995), it is likely that the identified strains represent only a small subset of the bacterial community associated with heavy metal accumulating *Salix caprea*. Nevertheless, due to their functional activities the isolated strains might be involved in the heavy metal tolerance and accumulation strategy of these trees. The majority of endophyte isolates were related to the genera *Sphingomonas* and *Methylobacterium*, whereas among rhizobacterial isolates high diversity of *Variovorax-, Bradyrhizobium-, Flexibacter-* and *Flavobacterium*- like strains was observed. Plant growth promoting activity has been reported for members of these genera (Holland and Polacco, 1994; Holland, 1997; Belimov et al., 2005, Cao et al., 2004; Cattalena et al., 1999). Moreover these genera have been found previously in association with heavy metal accumulating plants (Lodewyckx et al.,2002; Idris et al.,2004; Gremion et al., 2003 Abou-Shanab et al.,2003a; Belimov et al., 2005). In particular, predominance of *Sphingomonas* and *Methylobacterium* in the endosphere of Zn and Ni accumulating *Thlaspi* has been documented by both cultivation-dependent and -

independent surveys (Lodewyckx et al., 2002; Idris et al., 2004). Strains of *Variovorax*, and *Flavobacterium* have been shown to promote the growth of heavy metal accumulating plants in contaminated soil (Belimov et al., 2005). Heavy metal accumulation in plants has been enhanced by inoculation with *Sphingomonas, Pseudomonas* and *Microbacterium* strains (Whiting et at., 2001; Abou-Shanab et al.,2003). Specific relations may also exist between Zn accumulating willows and the rhizosphere bacteria and endophytes, which were not or only distantly related to described bacteria from other environments. Based on 16S sequence information, 16 isolates might be novel species. Most of the potentially novel rhizosphere bacteria clustered with CFB group organisms.

Bacterial ACC deaminase, impairing the synthesis of stress ethylene, and bacterial siderophores, mobilizing iron and heavy metals, are thought to improve heavy metal tolerance and uptake capacity of plants (Glick, 2003). Therefore all identified isolates were screened for ACC deaminase and siderophore production. ACC deaminase activity was determined as the ability to grow on medium with ACC as sole nitrogen source. Positive results were obtained for 37% of the rhizosphere bacteria and for 8% of the endophytes (Table 3B). Thirty-seven % of ACC deamniase positive rhizosphere isolates is a high proportion in comparison to observations on heavy metal tolerant bacteria associated with *Graminaceae* (Dell'Amico et al., 2005) or Ni accumulating *Thlaspi* (Idris et al., 2004). One of the best documented effects of bacterial ACC deaminase activity on plants, is increased root elongation. *Salix caprea* may take particular advantage of bacterial ACC deaminase to develop a sound root system in heavy metal contaminated soil. In contrast, Idris et al. (2004) found a higher percentage of ACC deaminase producing bacteria in the endosphere (36%) than in the rhizosphere (20%) of Ni accumulating *Thlaspi goesingense,* and explained this with the more intimate relationship of endophytes to their host plant. The proportion of ACC deaminase producing endophytes in *Salix caprea* may have been underestimated. For 32% of the endophyte isolates ACC deaminase activity could not be determined as they failed to grow on standard agarose based minimal media, irrespective of the nitrogen source added (Table 3). Difficulties to cultivate endophyte isolates on minimal media have already been reported by Lodewyckx et al. (2002). Siderophore production was determined as depletion of iron from a blue CAS agar. Although the selected minimal medium was suitable for most of the isolates, only one siderophore producing endophyte was identified. Among rhizosphere bacteria about 50% showed siderophore production (Table 3). Active siderophore mediated iron acquisition appears to be important for certain bacteria in the root zone of *Salix caprea*. Individual siderophore

producing endophytes such as *Pedobacter* EI208, may compete with the host plant for the limited iron present in shoots. In a study about the microflora associated with *Thlaspi goesingense* all culturable endophytes and rhizosphere bacteria produced siderophores (Idris et al. 2004). This suggests that in *Salix caprea* the competition for iron is less pronounced than in these herbal metal accumulators. However, there is no information about siderophore and ACC deaminase production by the large majority of unculturable plant associated bacteria. Fifteen rhizosphere isolates and five endophytes representing dominant genera were selected for more detailed analysis and included strains which were either able to produce ACC deaminase, siderophores, both siderophores and ACC deaminase or which did not have the ability to produce these substances.

These isolates were tested for the ability to synthesize the plant growth hormone IAA, because bacterially formed IAA has been observed to promote plant growth and heavy metal accumulation (Patten and Glick, 1996; Zaidi et al., 2006; Rajkumar et al., 2006). Quantitative determination of bacterial IAA production with the method of Sawar and Kremer (1995) failed, because most of the strains did not grow in the tryptophane amended broth described by these authors. However, it was possible to determine IAA production on agar plates prepared from the same medium. Four out of 15 rhizosphere bacteria and four out of five endophytes produced IAA. The number of isolates tested for IAA synthesis was too small to allow a statement about the importance of IAA producing bacteria in association with *Salix caprea*. However it has to be noted, that the analysed representatives of both dominant endosphere genera, *Methylobacterium* and *Sphingomonas*, were IAA positive.

All 360 strains were isolated on Zn containing media and enriched on a minimal medium containing 2 mM of Zn. Hence their Zn resistance was above 1 mM, which is inhibitory for Zn sensitive bacteria (Nies, 1999). Sixty-four rhizosphere bacteria and 49 endophytes were further analysed and were resistant to 5 mM of Zn. Ninety % of the rhizosphere bacteria and 70% of the endophytes even grew with 10 mM of Zn in solid MOPS medium. These Zn resistances are much higher than those observed in bacteria associated with Zn hyperaccumulating *Thlaspi caerulescens* (Lodewyckx et al., 2002) and exceed the concentration of mobile Zn in bulk soil of the sampling site (Table 1) more than 100 times. This suggests massive Zn mobilization in the rhizosphere of *Salix caprea* and high bioavailability of Zn also in the endosphere. The highly Zn resistant bacteria may contribute to Zn detoxification in the rhizosphere and in the shoots of *Salix caprea*. Efficient efflux-

based Zn and Cd resistance systems have been observed to involve post-efflux precipitation and sequestration (Diels 1995) and to mitigate metal toxicity in plants (Salt et al., 1999). However, in liquid medium, the Zn resistance of most strains was much lower and ranged between 1 and 4 mM (data not shown). Studies on Zn and Ni hyperaccumulating *Thlaspi* (Lodewyckx et al, 2002; Idris et al., 2004) reported higher heavy metal resistances in endophytes than in rhizosphere bacteria. The endophytes isolated in this work grew more reluctantly than the rhizosphere bacteria. Suboptimal growth conditions for endophytes on laboratory medium may have increased their metal sensitivity.

For 20 selected strains the level of resistance to the dominant heavy metal pollutants in Arnoldstein soil, Zn, Cd and Pb was determined more precisely. These experiments revealed record Zn MICs of up to 16 mM. All isolates except RI121 could grow in the presence of 0.5 mM Cd, which is the minimal inhibitory concentration for *E. coli* (Nies et al., 1999). The Cd resistances of most isolates were between 1 mM and 4 mM and thus did not exceed the tolerance limit of heavy metal sensitive bacteria as dramatically as the Zn resistances did. Only four out of 20 strains tolerated more than 6 mM of Pb, and can therefore be considered Pb resistant (Nies, 1999). Water solubility of Pb salts is low, leading to low Pb bioavailability in bulk soils (Table 1), and *Salix caprea* accumulates high concentrations of Zn and Cd, but only low amounts of Pb. Apparently no or little Pb mobilization takes place in the rhizosphere of these plants and therefore there might be no pronounced selection for Pb resistant rhizosphere bacteria or endophytes. Elevated Pb tolerance was found in bacteria, which showed the highest Cd resistance levels, allowing speculations about a combined Pb/Cd resistance mechanism. Indeed, Cd-efflux systems have been observed to export also Pb ions (Nies, 1999). Nineteen of 20 isolates showed resistance to both Zn and Cd, and Co-detoxification of Cd and Zn by broad range exporters such as the czc complex, is frequent in bacteria (Nies, 1995). However the levels of Zn and Cd resistance did not correlate. Many different combinations of heavy metal resistance have been observed in bacteria colonizing heavy metal contaminated rhizospheres (Mengoni et al., 2001; Dell'Amico et al., 2005) and heavy metal hyperaccumulating plants (Idris et al., 2006). Therefore an independent evolution of different metal resistance traits has been postulated (Mengoni et al., 2001). The highest Zn tolerances (14 mM) were found in siderophore producing bacteria (RX232, RD366, RD344, RI12). This may be coincidental, as RX232, RD366, RD344 were fast growing strains, and appeared to be well adapted to laboratory media (Table 4). This adaptation may have facilitated their tolerance to heavy metals in agar plates. However, the release of siderohores

can be induced by the presence of heavy metals (van der Lelie et al., 2000). Therefore siderophores may be involved in bacterial Zn resistance, for instance in post-efflux chelation.

Bacterial mobilization of metals is the most common explanation for increased heavy metal accumulation by plants in the presence of rhizosphere bacteria (Gadd, 2004) and a positive correlation between bacterial activity and heavy metal mobility in ultramafic soils has been observed (Amir and Pineau, 2003). However, most of the isolates analysed in this work reduced the extractability Zn, Cd and Fe, when contaminated soil was shaken with filtrates of liquid cultures. Since the mobility of Zn and Cd is highest at low pH values, we suspected that the immobilization may have resulted from an increase of medium pH during bacterial growth. Indeed, the pH of the medium rose in all cultures from 7 to values between 7.5 and 8.7. NH_3 released as a waste product from the consumption of N containing compounds may have lead to this alkalization of the medium (Kalinowski et al., 2004). However, pH adjustments of fresh 10% TSB to values between 7.2 and 8.9 did not significantly alter Zn extraction (data not shown). Heavy metal immobilization has been observed previously in an experiment, where rhizosphere bacteria were directly applied to Ni contaminated soil (Abou-Shanab et al., 2003). Various products of bacterial growth, ranging from simple organic acids and alcohols to extra-cellular polymeric substances can bind metal ions (Gadd 2004). Such non-specific metal-binding compounds may have been present in the culture filtrates and may have trapped Zn, Cd and Fe on the surface of soil particles. Moreover, heavy metal resistant bacteria have been observed to remove heavy metals from liquid medium by precipitation (Diels, 1995). Precipitating agents in the culture filtrates may have scavenged metals after extraction from soil and the resulting crystals may have been removed during filtration.

Culture filtrates of individual stains strongly increased the extractability of certain metals. One rhizosphere strain (*Mycobacerium* RD334) improved Zn and Cd extractability, another strain (*Rhizobium* RI12) increased the mobility of iron. Among endophytes, *Microbactericeae bacterium* EX 72 mobilized Zn and Cd, *Microbacteirum* EX 104 mobilized Zn and Fe, and *Frigoribacterium* EX166 mobilized Zn alone. These mobilizations were again not an effect of pH, since the medium pH rose in metal mobilizing and metal immobilizing cultures at the same rate. Authors who observed bacteria to raise pH and to mobilize metals at the same time, speculated that bacterial siderophores may extract heavy metals from soil along with iron (Whiting et al., 2001; Kalinowski et al., 2004). Yet four of the five metal mobilizing strains (RD334, EX72, EX104, EX166) were unable to produce siderophores in the plate assay

(Table 4). These slowly growing *Actinobacteria* seem to synthesise specific ligands for Zn and/or Cd, which cannot be detected in Fe-based siderophore tests. Interestingly, three of the identified metal mobilizers (*Microbacterium* EX72 and EX104, and *Frigoribacterium* EX166) were endophytes. Under natural conditions, shoot associated bacteria cannot mobilize metals from soil. Many endophytes derive from the rhizosphere (Huang, 1986) and may mobilize heavy metals during root and rhizosphere colonization. Later they may be involved in metal mobilization and translocation inside the plant. Rhizosphere *Microbacterium* strains have been observed previously to mobilize heavy metals, in similar experiments with culture filtrates, and also during direct incubation with soil (Whiting et al., 2001; Abou-Shanab et al., 2003).

None of the analysed siderophore producers mobilized Zn or Cd, and the most severe immobilization of Zn was observed for siderophore positive strains (RD74, RX228, RX232 and RX265). It is of course possible, that siderophores produced by these strains scavenged Zn from the solution and were adsorbed to soil particles. However, most likely siderophores were not involved in this mobilization experiment, as it was carried out in an iron containing medium, which should not induce the release of bacterial siderophores. This theory is confirmed by the fact that seven out of eight siderophore producers did not mobilize iron. The outstandingly high Zn immobilization by siderophore producing bacteria may be related to their abundant growth in the culture medium. RD74, RX228, RX232 and RX265 reached the highest cell densities in 10% TSB (Table 4) and may therefore have produced the highest amounts of unspecific metal-binding compounds. The iron mobilization by the siderophore producer *Rhizobium* RI12 may have been mediated by siderophores. Surprisingly, also *Microbacterium* EX104, which did not mobilize iron in the siderophore plate test, multiplied iron extractability from soil. Apparently, growth conditions in liquid TSB induced the release of an iron chelator, which was not produced in solid iron free medium. The efficient iron mobilization by the rhizosphere strain RI12, may contribute to the iron nutrition of *Salix caprea*. In contrast, the endophyte EX104 appears to be a strong competitor for iron inside the plant, which may on the one hand deplete the iron resources of the host, but on the other hand prevent the proliferation of pathogens (Penyalver et al., 2001).

This mobilization experiment with metabolites produced from TSB medium can only give information about the potential of bacteria to produce metal-binding compounds. It remains to be confirmed that the same metabolites are produced from the substrates available in the

rhizosphere and endosphere habitat. Chemical analysis is necessary to reveal the nature of the metal mobilizing and immobilizing compounds and to clarify the relevance of these laboratory results to the natural situation. Our results showed that the metal mobilizing metabolites of one selected strain (EX104) were secondary growth products, released in the stationary phase (Fig 2). Heat stability of the metal mobilizing and immobilizing metabolites was assessed by boiling culture filtrates prior to soil extraction. The results of the boiling experiment have to be handled with care, because it is unclear, why in this assay the Zn mobilization of all samples was higher than in all other repetitions (data not shown) of the mobilization test. It is further questionable, whether the control medium, which had been previously autoclaved, really contained heat labile compounds. However, this simple experiment allowed to demonstrate that the Zn mobilizing substances produced by EX72 and EX104 can be denatured by heat without a change of pH, suggesting a protein-like structure or these metal-ligands. In contrast it became evident that the metal immobilizing growth products of RX232 were heat-stable.

Conclusions

The rhizosphere and the endosphere of Zn/Cd accumulating *Salix caprea* harbour distinct communities of highly Zn resistant bacteria. Our results and the results of previous studies suggest that spingomonads and methylobacteria are characteristic endophytes of heavy metal accumulators and may have a special function in these plants. *In vitro* experiments allowed to identify organisms with multiple characteristics relevant to heavy metal accumulation (Table 4): An extremely Zn/Cd/Pb resistant *Rhizobium* (RI12) produced siderophores and showed the ability to mobilize iron. Members of the numerically important rhizosphere genus *Variovorax*, were able produce siderophores and ACC deaminase (RX74), and ACC deamniase and IAA (RX14) respectively. Representatives of the well known plant-associated genera *Pseudomonas* and *Burkholderia* (RX228, RX232) synthesised high amounts of ACC deaminase and siderophores. Several slowly growing actinobacteria, affiliated with the genera *Microbacterium* (EX72, EX104), *Mycobacterium* (RD334) and *Frigoribacterium* (EX166) were able to mobilize metals by the release of unknown non-siderophore metal ligands. Members of the genera *Methylobacterium* and *Spingomonas*, as well as the described stains with several potentially heavy metal relevant traits could be promising candidates for plant inoculation experiments and for molecular analysis of heavy metal related plant-microbe interactions.

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CHAPTER 2

Rhizosphere bacteria affect growth and metal uptake of heavy metal accumulating willows

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Abstract

A variety of plants growing on metalliferous soils accumulate metals in their harvestable parts and have the potential to be used for phytoremediation of heavy metal polluted land. There is increasing evidence that rhizosphere bacteria contribute to the metal extraction process, but the mechanisms of this plant-microbe interaction are not yet understood. In this study ten rhizosphere isolates obtained from heavy metal accumulating willows affiliating with *Pseudomonas, Janthinobacterium, Serratia, Flavobacterium, Streptomyces* and *Agromyces* were analysed for their effect on plant growth, Zn and Cd uptake. In plate assays Zn, Cd and Pb resistances and the ability of the bacteria to produce IAA, ACC deaminase and siderophores were determined. The isolates showed resistance to high Zn concentrations, indicating an adaptation to high concentrations of mobile Zn in the rhizosphere of *Salix caprea*. Four siderophore producers, two IAA producers and one strain producing both siderophores and IAA were identified. None of the analysed strains produced ACC deaminase. Metal mobilization by bacterial metabolites was assessed by extracting Zn and Cd from soil with supernatants of liquid cultures. Strain *Agromyces* AR33 almost doubled Zn and Cd extractability, probably by the relase of Zn and Cd specific ligands. The remaining strains, immobilized both metals. When *Salix caprea* plantlets were grown in sterile, Zn/Cd/Pb contaminated soil and inoculated with the Zn resistant isolates, *Agromyces* AR33 promoted plant growth, whereas *Streptomyces* AR17 enhanced Zn and Cd uptake. The total amounts of extracted Zn and Cd per plant were increased only by the plant growth promoter *Agromyces* AR33 indicating that plant growth promotion was more important for heavy metal extraction than increased uptake. The IAA producing strains did not affect plant growth, and the siderophore producers did not enhance Zn and Cd accumulation. Apparently other mechanisms than the production of IAA, ACC deaminase and siderophores were involved in the observed plant-microbe interactions.

Introduction

Due to global industrialisation, in particular to mining activities, metal processing, coal combustion, intensive agriculture, traffic, incineration and dumping of waste, heavy metal pollution has become an increasing problem throughout the world (Nriagu 1979). Phytoextraction is a sustainable, cost efficient clean-up strategy using metal accumulating plants to extract heavy metals from soil (Singh et al., 2001).

The extraction potential of a plant is given by the metal concentration in the shoots and by shoot biomass production. A variety of plants growing on metalliferous soils accumulate metals in their shoot tissues to levels exceeding the metal concentration in the soil (Singh et al., 2001; McGrath and Zhao, 2003). Fast growing plants accumulating and tolerating high concentrations of heavy metals in their harvestable parts have to be selected for application in phytoextraction technology. Willows (*Salix caprea*) growing on a contaminated site in Arnoldstein (Austria) accumulate high amounts of Zn and Cd. *Salix caprea* clone BOKU 01 AT-004, a willow clone available in tree nurseries, shows a similar accumulation potential (Dos Santos Utmazian et al., 2007). High biomass production and adaptability to various environments designate willow trees as promising extractor plants.

Bioremediation systems must provide optimal conditions for metal accumulation and plant growth. There is increasing evidence that besides climatic factors and soil properties, plant-microbe interactions determine the efficiency of metal extraction. Comparisons between sterile and non sterile systems showed that heavy metal accumulators reach their full accumulation capacity only in the presence of their indigenous rhizosphere microflora (De Souza et al., 1999). Whiting et al. (2001) showed that *Thlaspi caerulescens* plants inoculated with rhizosphere bacteria produced more biomass and accumulated higher amounts of Zn. Other studies showed that certain bacteria may improve metal uptake, whereas others promote plant growth (Abou Shanab et al., 2003; de Souza et al., 1999; Belimov et al., 2001; Burd et al., 1998 and 2000; Rajkumar et al., 2006). The mechanisms enabling bacteria to promote the accumulation process are unclear. Metal uptake may be enhanced by bacterial siderophores. Siderophores are iron complexing compounds released for iron acquisition and may mobilize also other heavy metals (Hu and Boyer, 1996). Plant growth in hostile metal polluted environments may be supported by Fe supply from bacterial siderophores, by bacterial production of auxin hormones such as indole acetic acid (IAA), and by bacterial 1-amino-cyclopropane-1-carboxylic acid deaminase (ACC deaminase) activity. ACC deaminase

inhibits stress ethylene synthesis and was shown to mitigate stress caused by the presence of heavy metals in plant tissues (Burd et al., 1998; Glick et al., 1998).

Most observations on rhizosphere processes in regard to heavy metal accumulation have been made with crop plants (DeSouza et al., 1999; Burd et al. 1998) or herbaceous metal hyperaccumulators (Whiting et al., 2001; Abou-Shanab et al., 2003). The use of crop plants for phytoremediation is limited by their high nutrient requirements, and metal extraction using wild herbs is inefficient due to their low biomass production. Information on the metal accumulation strategy of fast growing trees and on their interactions with rhizosphere bacteria is needed for the development of efficient phytoextraction systems. The objective of this study was to test the potential of rhizosphere bacteria to increase heavy metal accumulation in *S. caprea* and to investigate potential mechanisms involved in this process.

Materials and Methods

Rhizosphere samples, experimental soil and willow plantlets

Rhizosphere samples for the isolation of bacteria were taken 2001 in Arnoldstein (Austria), a lead mining area, which has been described by Wenzel and Jockwer (1999). The experimental soil for the mobilizsation experiments was taken from the same site and has been previously used by Dos Santos Utmazian and Wenzel (2007). Key soil characteristics as well as the concentration of total and labile bioavailabe Zn, Cd and Pb in the experimental soil are given Table 1. The soil was air-dried and passed through a 2-mm stainless steel mesh. For sterile experiments, the soil was gamma-ray irradiated with 25 kGray for 24 h by MediScan GmbH (Seibersdorf, Austria). For pot experiments, one year old plantlets of the *S. caprea* clone BOKU 01 AT-004 (Dos Santos Utmazian and Wenzel , 2007) were used.

Table 1. Selected parameters of the experimental soil derived from Arnoldstein, Austria

Characteristic	**value**	**unit**
Texture (sand/silt/clay)	350/550/100	g kg^{-1}
Cation exchange capacity*	247	mmol kg^{-1}
Organic carbon	24.6	g kg^{-1}
pH	7.2	
Total metal contents (in aqua regia)		
Zn	1760	mg kg^{-1}
Cd	32.7	mg kg^{-1}
Pb	6560	mg kg^{-1}
Mobile fraction of metals (in 1M NH_4NO_3)		
Zn	2.56	mg kg^{-1}
Cd	0.64	mg kg^{-1}
Pb	3.81	mg kg^{-1}

* measured at soil pH

Isolation of rhizosphere bacteria

For the isolation of bacteria ten gram of rhizosphere soil were shaken with 100 ml 0.8 % NaCl solution for 30 min. Dilutions of the suspension were plated on R2A (Difco) + 1mM Cd $(NO_3)_2$ + 1mM $Pb(NO_3)_2$ + 1mM $Zn(NO_3)_2$ and incubated at room temperature for 1-2 weeks. Morphologically distinct bacteria were further analysed.

DNA extraction and partial 16S rDNA sequencing

DNA was isolated from bacterial strains by bead beating (Sessitsch et al. 2001) and the 16S rDNA gene was amplified with the primers 8f (5'-AGAGTTTGATCCTGGCTCAG-3'; Weisburg et al., 1991) and 1520r (5'-AAGGAGGTGATCCAGCCGCA-3'; Edwards et al., 1989). A volume of 0.5 µl DNA extract was used for 50 µl PCR reactions containing 2 units Taq DNA polymerase (Invitrogen), 0.2 mM of each dNTP, 0.15 µM of each primer, and 1.5 mM $MgCl_2$. The thermal programme comprised an initial denaturation step of 5 min at 95°C, followed by 29 cycles of 30 sec denaturation at 95°C, 1 min annealing at 53°C and 2 min extension at 72°C, and a final elongation step of 10 min at 72°C. The amplified 16S rDNA was digested with *Hin6I* (Invitrogen) according to the instructions of the manufacturer for RFLP-fingerprinting. Isolates with different RFLP profiles were identified by sequencing of 600 bp – 1500 bp of the 16S rDNA gene (Table 2). Partial sequences of strains AR16, AR35 and AR36 were obtained with the primer 8f. For the remaining strains three separate reactions were prepared with the primers 8f, 926r (5' CCGTCAATTCCTTTRAGTTT 3',Weisburg et al., 1991) and 1520r respectively, to sequence the whole 16S rDNA gene. Two microliters of sephadex G-50 (Amersham Biosciences) purified PCR product were added as template to 10 µl sequencing reactions containing the BigDye terminator cycle sequencing kit (ABI prism) and 0.4 µM of primer. After a second purification with sephadex G-50 columns , DNA fragments were sequenced with an ABI 373A automated DNA sequencer (Applied Biosystems Inc., Foster City, CA). Sequencher 4.2 software (Gene Codes Corporation, Ann Arbor Michigan, USA) was used to create sequence contigs from the fragments obtained with the three individual primers. The sequences were subjected to BLAST analysis with the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/) in order to identify closest described relatives. Ten isolates were selected for this study (Table 2).

Nucelotide sequence accession numbers

The 16S rDNA sequences of the bacteria analysed in this study are accessible in the National Center for Biotechnology Information (NCBI) database. Strains PR01, PR02 and PR04 have the accession numbers DQ640006-8, whereas strains PR13, AR16, AR17, AR35, AR36 and BR780 have been deposited under accession numbers EF672646-52.

Heavy metal tolerance

Heavy metal resistances were determined on phosphate-poor morpholinepropanesulfonic acid medium (MOPS; Neidhardt et al.,1974) with 0.1% glucose as carbon source. Bacteria were streaked on MOPS agar plates containing Zn, Cd, Pb and an equimolar mixture of Zn/Cd/Pb respectively, in concentrations of 0 mM, 2 mM, 4 mM, 6 mM, 8 mM and 10 mM. Zn resistance was further tested up to a concentration of 16 mM, Cd resistance was tested also at concentrations of 0.5, 1 and 1.5 mM. For each strain and each metal the highest concentration that allowed visible growth at room temperature within 7 days was determined.

Indole acetic acid production

The ability of the isolates to produce indole acetic acid (IAA) was initially tested according to the method of Sawar and Kremer (1995). Since many strains failed to grow in the broth described by these authors (1g l^{-1}peptone, 2g l^{-1}glucose, 1mM tryptophane), the medium was solidified with 15% of agar. Bacterial cells were suspended in 0.9% saline. Fifty µl of the suspension were placed in the centre of an agar plate and incubated for 72 h at room temperature, with protection from light. Two hundred µl of Salkowsky reagent (35% Perchloric acid, 3mM $FeCl_3$) were applied to each plate on top of the bacterial cells. After 30 min of incubation in the dark, the diameter of the pink zone around the cells was used to estimate the relative amount of IAA produced.

ACC deaminase activity

ACC deaminase activity was tested on the minimal medium described by Brown and Dilworth (1975) containing 0.7 g ACC l^{-1} as sole nitrogen source. To exclude traces of nitrogen, that might be present in conventional agar, pure agarose (Saekem) was used to solidify the medium. To avoid potential contamination with ammonium during the autoclaving, nutrient stock solutions were sterile-filtered and the agarose matrix was sterilized by 20 min of boiling. Minimal medium without nitrogen was used for negative controls, positive controls contained 0.7 g NH_4Cl l^{-1}. Plates were incubated for two weeks at room temperature.

Siderophore production

Siderophore production was analysed on chrome azurol S agar plates. Bacteria were grown for two weeks at room temperature on plates (5 mm diameter) filled half with iron free MM9 medium (6.8 g l^{-1} Na_2HPO_4, 0.3 g l^{-1} KH_2PO_4, 0.5 g l^{-1} NaCl, 1 g l^{-1} NH_4Cl, 0.4% glucose) and half with CAS-blue agar (Milagres et al., 1999). Siderophore producing organisms are able to extract iron from the blue Fe-CAS complex forming an orange depletion zone in the medium. Size and progress rate of the depletion zone were taken as estimates for siderophore production intensity.

Zink and cadmium mobilization by bacterial metabolites

Bacteria were grown in 30 ml of 10% TSB at 25°C and 200 rpm until the late stationary phase (i.e. twice the time needed to reach the end of the log phase). Then cells were removed from the medium by centrifugation (8000xg, 15min, 4°C) and filtration through 0.2 μm Ministart filters (Sartorius) . The filtrates were stored at -20°C and the pH of each filtrate was measured prior to mobilization analysis. Five ml of the filtrates were shaken with 1 g of γ-sterilized Zn/Cd/Pb contaminated soil from Arnoldstein, for two hours at room temperature. As controls, 1 g aliquots of the same soil were shaken with 5 ml of fresh 10% TSB. Soil particles were removed by centrifugation (7000 rpm, 5 min), and filtering (0.45 μm filters) and the concentrations of Zn and Cd in the filtrates were quantified by Atomic Absorption Spectroscopy (AAS, Perkin Elmer 2100). For each strain three late stationary cultures were prepared and from each culture three 5 ml aliquots were analysed.

Inoculations of *Salix caprea* Mauerbach

Bacteria were grown for 3 days in 10% TSB containing 1 mM of $ZnSO_4$ and harvested by centrifugation (2420 x g, 10', 4C). Cell pellets were resuspended in 50 mM potassium phosphate buffer (pH 7) resulting in an optical density of 0.1 at 600 nm. Actual cell densities in the OD 0.1 suspensions were determined by plating tenfold dilutions on 10% TSA containing 2 mM $ZnSO_4$. Roots of one year old *S. caprea* clone BOKU 01 AT-004 plantlets were washed with sterile water and soaked in the OD 0.1 bacterial suspensions for 30 minutes. Then the plantlets were potted in 1.5 kg aliquots of γ-sterilized Arnoldstein soil, watered with ion-free water and with 12.5 ml bacterial suspensions. Four replicate plants were inoculated with each bacterial strain. After 12 weeks of incubation at 25°C in a growth

chamber leaves were harvested. Leaves were dried at 80°C and their dry biomass was recorded. Dried leaves were ground in a metal-free mill and digested in a mixture of HNO_3 and $HClO_4$ (5:1, v/v). Zn and Cd concentrations in the digested leaf material were determined by AAS (Perkin Elmer, 2100).

Statistical analysis

Statistic analysis was done with STATISTICA 6 (StatSoft, Tulsa, USA). Analysis of variance (ANOVA) followed by post-hoc Fisher LSD test was carried out to identify significant effects of strains in mobilization and plant inoculation experiments. Differences to control groups with $p<0.01$ in mobilization experiments and with $p<0.1$ in plant inoculation experiments were considered significant. Correlations between the resistance to different metals as well as correlations between the mobilization of different metals were determined by product moment correlation analysis.

Results

Phylogenetic affiliation of the analysed bacteria

The analysed organisms were phylogenetically affiliated by sequencing of the 16S rRNA gene. Blast analysis of the complete 16S rDNA showed that strains PR04, PR02, PR13, BR780, PR01, AR17 and AR33 were affiliated with the genera *Pseudomonas, Janthinobacterium, Serratia, Flavobacterium, Streptomyces* and *Agromyces*, respectively (Table 2), with sequence identities of 98-100%. Blast analysis of the first 600 bp indicated that strains AR16, AR35 and AR36 were affiliated with *Streptomyces* again with identities of 99%-100%.

Heavy metal resistances

Extremely high Zn resistance (11 mM of Zn in solid medium) was observed for *Streptomyces* AR16 (Table 2), whereas strain *Pseudomonas* PR04 showed relatively low tolerance to Zn (2 mM). For the remaining strains Zn resistances ranged between 6 mM and 8 mM. *Serratia* BR780 grew with 4 mM of Cd in the medium, *Flavobacterium* PR01 with 2 mM. The Cd

resistance of *Agromyces* strain AR33 was below 0.5 mM, the lowest dose tested. The remaining strains tolerated Cd in concentrations between 0.5 and 1 mM. Pb resistance levels were between 2 and 4 mM. Zn, Cd and Pb resistances did not correlate with each other. *Serratia* BR780, *Streptomyces* AR36 and *Flavobacterium* PR01 tolerated 2 mM of Zn, Cd and Pb in combination. For the remaining bacteria a combination of these three metals was inhibitory in 2 mM concentration.

Table 2 . Analysed bacteria and their characteristics with potential relevance to the phytoextraction process.

	Closest described relative [a]	MIC (mM) [b]				Production of		
Strain	[Accession nb.] (bp, identity)	Zn	Cd	Pb	Zn,Cd,Pb	siderophores	IAA	ACCD[c]
PR04	*Pseudomonas* sp. BE3dil [AY263472] (1389bp,100%)	2	0.5	2	<2	+	-	-
PR02	*Janthinobacterium lividum* [AF174648] (1375bp,99%)	6	0.5	4	<2	-	+	-
PR13	*Janthinobacterium lividum* [AF174648] (1471bp, 99%)	6	1	4	<2	-	+	-
BR780	*Serratia marcescens* [AB061685] (1503bp,99%)	8	4	2	2	+	+	-
PR01	*Flavobacterium frigidimaris* [AB183888] (1338bp,99%)	6	2	4	2	-	-	-
AR16	*Streptomyces* sp. 10-6 [AB222069] (624bp,99%)	11	1	4	<2	+	-	-
AR17	*Streptomyces* sp. VTT E-042639 [EF564804] (1483bp,100%)	8	0.5	2	<2	-	-	-
AR35	*Streptomyces* sp. VTT E-042639 [EF564808] (588bp,100%)	6	0.5	2	<2	-	-	-
AR36	*Streptomyces* sp. VTT E-042677 [EF564805] (647bp,99%)	6	1	2	2	+	-	-
AR33	*Agromyces terreus* DS-10 UMS-101 [EF363711] (1443bp,98%)	6	<0.5	2	<2	-	-	-

[a] determined by sequencing of the 16S rDNA gene
[b] Minimal Inhibitory Concentration
[c] ACC deaminase

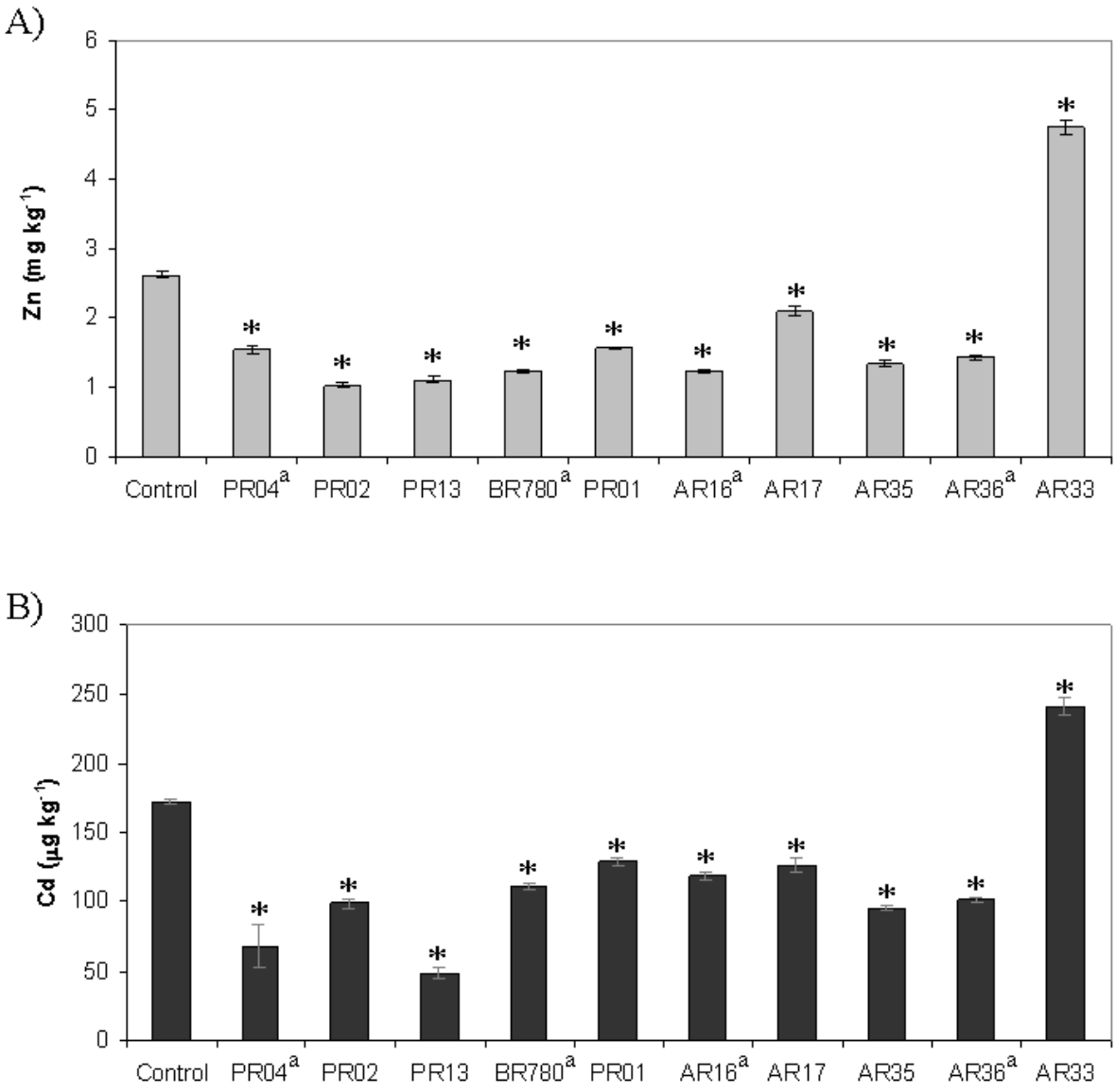
Siderophores, IAA and ACC deaminase

P. borealis PR04, *S. marcescens* BR780 and two of the *Streptomyces* strains (AR16 and AR36) produced siderophores (Table 2). IAA production was found in *Serratia* BR780 and at a lower level in the *Janthinobacterium* strains PR01 and PR13. ACC deaminase activity was not observed in any of the tested strains.

Quantitative determination of bacterial IAA production with the method of Sawar and Kremer (1995) failed, because most of the strains did not grow in the tryptophane amended broth described by these authors. The plate test developed in this study enabled a differentiation between IAA negative organisms, moderate and strong IAA producers .

Zn, Cd and Fe mobilization by bacterial metabolites

The ability of bacterial metabolites to mobilize metals was tested by extracting Zn and Cd from contaminated soil with filtrates of bacterial cultures and quantifying the extracted metals. Sterile 10% TSB medium had a pH of 7 and mobilized 2.5-2.8 mg of Zn and 170 µg of Cd from 1 kg of Arnoldstein soil. The pH of 10% TSB that had supported bacterial growth until the late stationary phase was between 8 and 8.7 (data not shown) . Figure 1 shows the amounts of Zn and Cd mobilized by the growth products of the analysed rhizospere bacteria. *Agromyces* AR33 almost doubled Zn and Cd extraction to values of 5 mg kg^{-1} and 250 µg kg^{-1}, respectively. The exudates of the remaining strains decreased both Zn and Cd mobility in comparison to the control. The effects of the analysed strains on Zn and Cd mobility strongly correlated ($p<0.001$). The ability of the analysed bacteria to produce siderophores did not affect Zn or Cd mobilization.



* significant difference to control ($p<0.01$) [a] strains producing siderophores

Figure 1. Mobilization of heavy metals from contaminated soil by bacterial exudates. Contaminated soil was shaken with filtrates of stationary bacterial TSB cultures. Mean values obtained from three replicate cultures which were measured three times each. A) Zinc B) Cadmium

Inoculations of *Salix caprea* plantlets

Suspensions of bacteria were introduced into the rhizosphere of *Salix caprea* Mauerbach plantlets grown in sterilized, metal contaminated Arnoldstein soil. Exact cell densities of the inoculant suspensions were determined by colony counts, and varied between 10^4 and 10^7 cells applied to 1 g soil. Twelve weeks after inoculation leaf biomass, Zn and Cd content were determined (Figure 2). *Agromyces* AR33 was the only inoculant with a positive impact on leaf growth (p=0.06). Inoculation with *Streptomyces* AR17 increased the uptake of both Zn (p=0.09) and Cd (p=0.05) in *Salix caprea* Mauerbach. In contrast, *Pseudomonas* PR04, and *Streptomyces* AR36 significantly reduced the uptake of Zn (p=0.01 and 0.01) and also tended to decrease Cd accumulation (p=0.06 and 0.08 respectively). A tendency to reduced Zn uptake was also observed in plants treated with *Janthinobacterium* PR13 (p=0.09). Leaf biomass and Zn/Cd contents were calculated to show the total amount of metal extracted by each plant (Figures 2D, 2E). The resulting values showed, that the growth promoting strain AR33 tendentiously improved Zn extraction per plant in comparison to the sterile control. PR04, and AR36 reduced the effective extraction of Zn (p=0.07 and 0.07) and with lower significance also that of Cd. Inoculations with the other strains, including the uptake-promoter AR17, had no effect on the total amount of heavy metal extracted from soil.

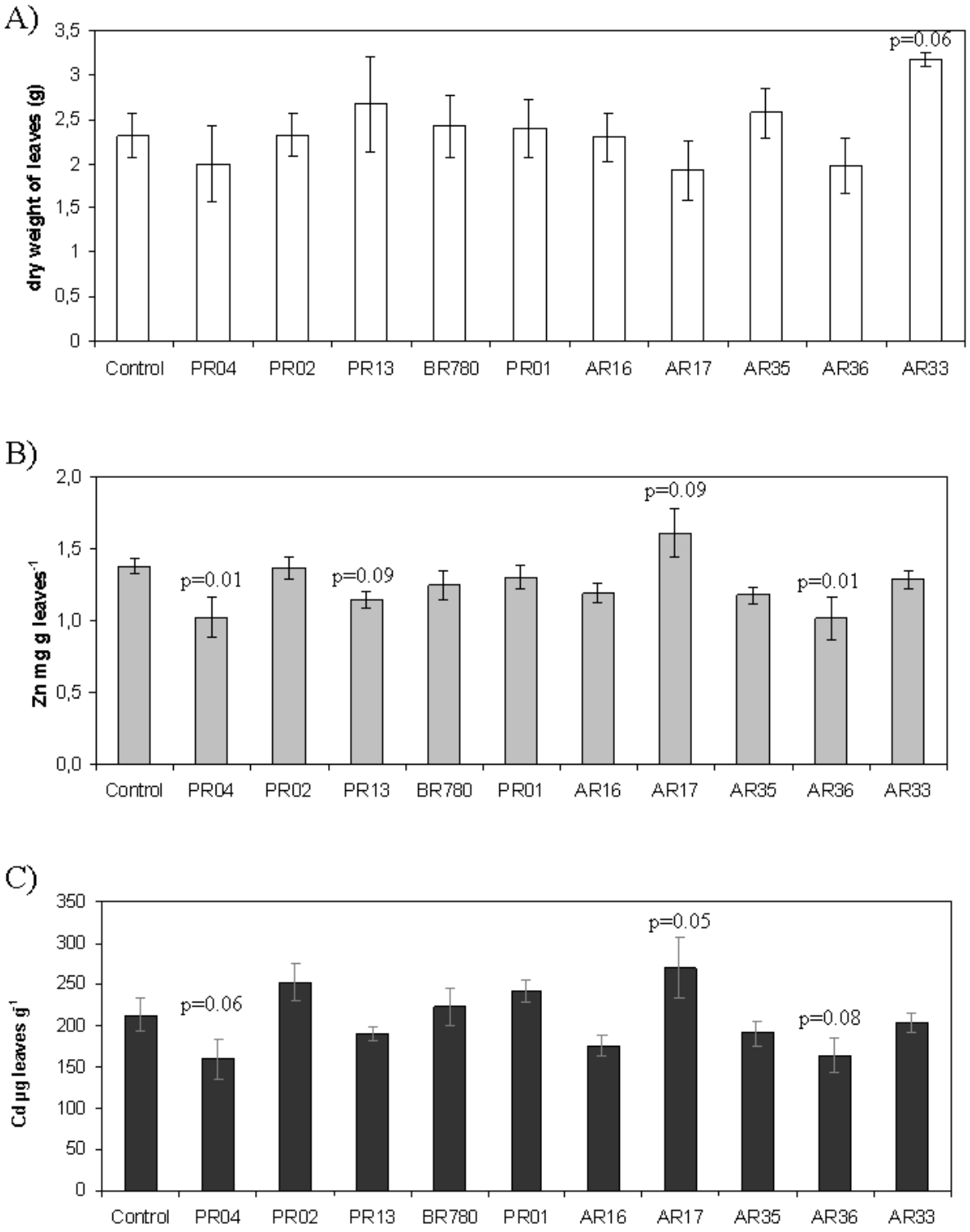


Figure 2. Leaf biomass and heavy metal content of *Salix caprea* Mauerbach 3 months after inoculation with 10 rhizosphere isolates from heavy metal accumulating willows. A) Leaf dry weight B) zinc concentration in leaves C) cadmium concentration in leaves D) total zinc accumulated in the leaf biomass E) total cadmium accumulated in the leaf biomass

For significant differences to the control ($p<0.1$), p-Values are indicated in the graphic.

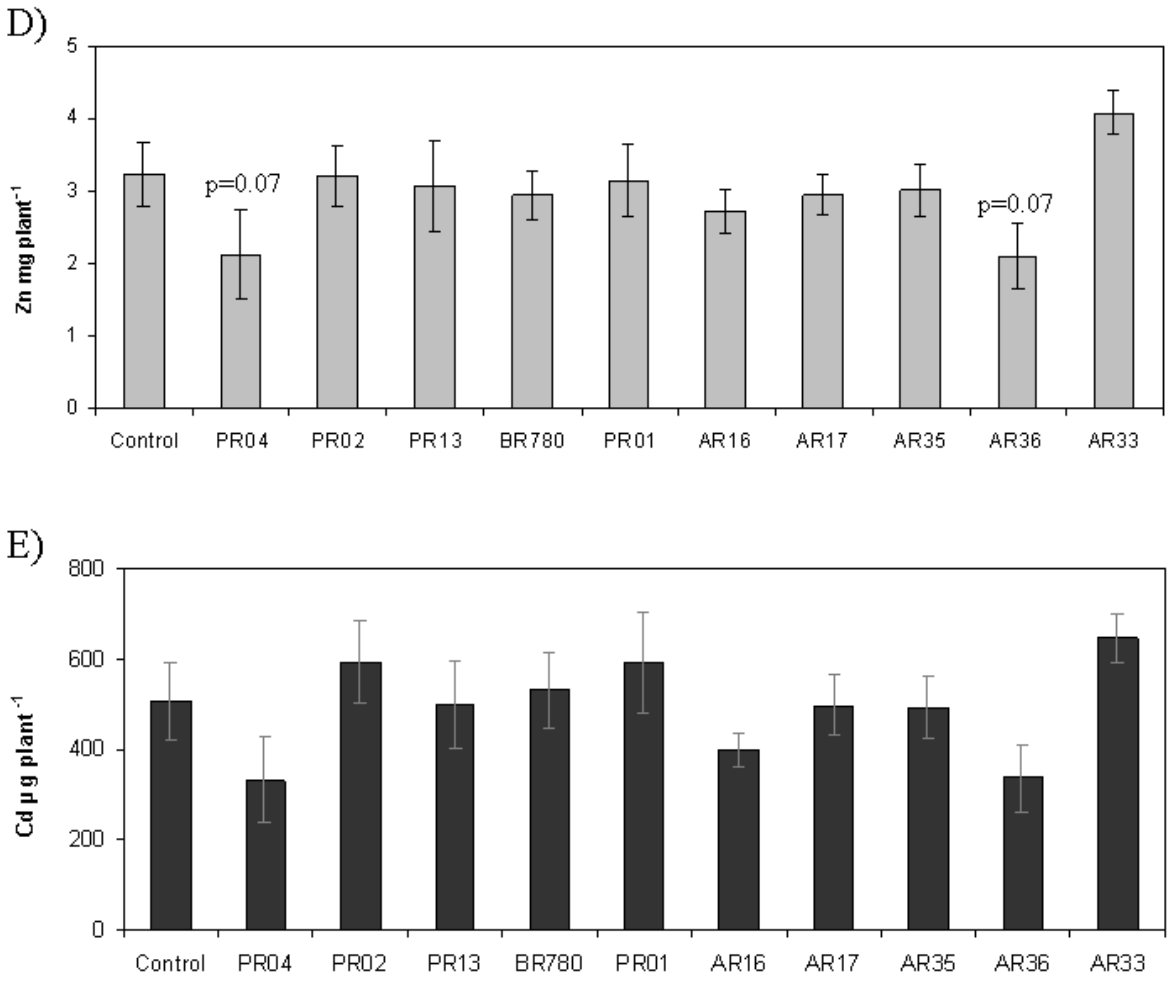


Figure 2 (continued). Leaf biomass and heavy metal content of *Salix caprea* Mauerbach 3 months after inoculation with 10 rhizosphere isolates from heavy metal accumulating willows. A) Leaf dry weight B) zinc concentration in leaves C) cadmium concentration in leaves D) total zinc accumulated in the leaf biomass E) total cadmium accumulated in the leaf biomass.

For significant differences to the control ($p<0.1$), p-Values are indicated in the graphic.

Discussion

The rhizosphere of heavy metal accumulating plants provides a niche for adapted metal resistant microorganisms (Lodewyckx et al., 2002; Idris et al., 2004) and the mobility of heavy metals is higher in the rhizosphere of metal accumulators than in bulk soil, due to active mobilization by roots and microorganisms (Lasat et al., 1996; McGrath et al., 1997). Zn resistances of the *Salix caprea* rhizosphere bacteria ranged between 2 mM and 11 mM and were much higher than those of bacteria associated with Zn hyperaccumulating *Thlaspi* (Lodewyckx et al., 2002). This suggests a high bioavailability of Zn in the rhizosphere of *Salix caprea* and a specific adaptation of the associated bacteria. Strains *Serratia* BR780 and *Flavobacterium* PR01 showed exceptionally high Cd tolerance (4 mM and 2 mM, respectively). In contrast strain AR33 was sensitive to 0.5 mM of Cd. This strain may originate from a low-Cd niche within the *Salix caprea* rhizosphere. The remaining strains were again clearly adapted to Cd stress, as they tolerated Cd in concentrations between 0.5 mM and 1 mM. Pb resistances ranged between 2 mM and 4 mM, which is below the minimal inhibitory concentration of Pb-sensitive *E.coli* (Nies et al., 1999). *Salix caprea* trees growing at the contaminated site in Arnoldstein accumulate high amounts of Zn and Cd, but less Pb. This may indicate low availabiltiy of Pb in the rhizosphere for both, plants and microorganisms, and explain the relatively low Pb tolerance of the bacteria. The simultaneous resistance to equimolar concentrations of Zn, Cd and Pb was determined by the most inhibitory metal, Cd or Pb respectively. Synergistic effects were not observed. Zn, Cd and Pb resistance levels did not correlate, suggesting that different detoxification mechanisms were responsible for the resistance to different metals.

Bacterial IAA, ACC deaminase and siderophores have been associated with enhanced growth and accumulation under heavy metal exposure (Whiting et al. 2001; Abou-Shanab et al., 2003; Patten and Glick, 1995; Burd et al., 1998; Glick et al., 2003). Therefore the rhizosphere isolates were tested for their ability to synthetize IAA, ACC deaminase and siderophores. The *in vitro* tests identified four siderophore producers (*Pseudomonas* PR04, *Serratia* BR780 and *Streptomyces* strains AR16 and AR36), two IAA producers *(Janthinobacterium* strains PR02 and PR13), one strain producing siderophores and IAA (*Serratia* BR780), but none of the strains tested was able to produce ACC deaminase. Similarly, siderophore and IAA production have been detected more frequently than ACC deaminase activity in rhizosphere isolates of metallicolous *Graminaceae* and of the Ni hyperaccumulator *Thlaspi goesingense* (Dell Amico et al., 2005, Idris et al., 2004).

The ability of the bacterial isolates to produce metal mobilizing metabolites was assessed by extracting contaminated soil with TSB culture filtrates and quantifying the extracted Zn and Cd (Figure 1). Nine out of ten strains produced culture filtrates that reduced Zn and Cd mobilization as compared to the control. This somewhat contradicts the general observation that bacterial activity correlates with heavy metal mobility (Whiting et al., 2001; Amir and Pineau, 2003). The immobilization was not a result of the rise of medium pH from 7.0 to 8.0-8.7, which occurred during the growth of all analysed strains. In pure TSB medium adjustments of the pH to values between 7.2 and 8.7 did not reduce Zn extractability (data not shown). The Zn/Cd immobilizing strains included all four siderophore producers analysed in this study. Bacterial siderophores have been suspected to mobilize heavy metals along with iron (Whiting et al., 2001; Kalinowski et al., 2004). Most likely, siderophores were not involved in this TSB based mobilization assay. TSB medium contains sufficient iron for bacterial growth and hence should not induce siderophore production. *Agromyces* AR33 was the only isolate, which was able to mobilize Zn and Cd. Similarly, Whiting et al. (2001) observed that bacterial metabolites increased the Zn extraction capacity of TSB medium. Again, a pH effect can be excluded, as the pH of the AR33 culture filtrate was within the range of the other samples. The observed Zn and Cd mobilization by this siderophore negative strain (Table 2) suggests that certain bacteria are able to synthesize substances, which increase Zn and Cd mobility. Those may include organic acids or specific ligands that cannot be detected in Fe-based siderophore tests. Chemical analysis of the culture filtrate is necessary in order to reveal the nature of the metal mobilizing compounds released by strain AR33. The correlation between Zn and Cd mobilization suggests that the released bacterial metabolites affected Zn and Cd in a similar way. Mobilization experiments with metabolites derived from TSB medium can only give an indication about the ability of an organism to produce metal chelating compounds from a rich substrate. The obtained results do not allow to conclude that the same compounds are produced from the substrates available in the rhizosphere. However, in parallel mobilization experiments using TSB and RSM, a medium designed to resemble the rhizosphere habitat, congruent results were obtained (Whiting et al. 2001). Altogether our results indicate that different organisms have different effects on heavy metal mobility. A larger and more representative selection of isolates has to be analysed before general conclusions about mobilization and immobilization processes in the rhizosphere of *Salix caprea* can be drawn.

When *Salix caprea* clone BOKU 01 AT-004 plantlets were grown in sterilized contaminated soil and inoculated with bacteria, *Agromyces* AR33 increased leaf biomass (Figure 2A). This

strain was not able to produce the plant growth hormone IAA or the stress reducing enzyme ACC deaminase, nor did it produce siderophores (Table 2). *Agromyces* RA33 seems to synthetize different plant growth promoting agents, mobilize other essential nutrients than iron or directly affect gene expression in the plant.

The ability of *Agromyces* AR33 to produce Zn and Cd mobilizing metabolites (Figure 1) did not lead to an increased accumulation of these metals in the plant. The only strain improving Zn and Cd uptake was *Streptomyces* AR17, which slightly decreased Zn and Cd extractability in the culture supernatant experiment (Figure 1). This shows clearly that the production of metal mobilizing (or immobilizing) compounds in TSB does not necessarily indicate metal mobilization (immobilization) in the rhizosphere. However strains PR04 and AR36, which reduced Zn and Cd mobilization in the supernatant experiment (Figure 1) also impaired Zn and Cd uptake into the plant (Figures 2B and 2C).

Among the siderophore producers, two strains slightly decreased Zn and Cd uptake (PR04, AR36) and two had no effect (AR16, BR780). These results suggest that bacterial siderophores had no or even a negative effect on Zn and Cd uptake by *Salix caprea*. Indeed, siderophores might scavenge free Zn and Cd ions from the soil solution and make their uptake more difficult. On the other hand it is not clear whether siderophores were actually produced in the rhizosphere. The fact that the siderophore negative strain *Streptomyces* AR17 enhanced Zn and Cd uptake, highlights the importance of other interaction mechanisms for heavy metal accumulation by *Salix caprea*. The specific properties enabling *Streptomyces* AR17 to enhance heavy metal uptake in plants, remain to be identified. Apart from mobilizing metals this strain may directly influence gene expression in the roots of *Salix caprea* to stimulate metal uptake and translocation.

The extraction capacity of a plant is given by the concentration of metal accumulated in the leaf tissues and leaf biomass production (McGrath and Zhao, 2003). To obtain total Zn and Cd amounts extracted from soil and accumulated in harvestable plant material, leaf biomass was multiplied with leaf Zn and Cd content. The resulting values did not differ with statistical significance. However, the plant growth promoting effect of *Agromyces* AR33 tended to improve total Zn and Cd extraction per willow plant. *Streptomyces* AR17, which enhanced metal uptake, did not increase total Zn and Cd extraction due to the relatively low biomass production of the inoculated plants. The inhibition of metal uptake by *Pseudomonas* PR04 and *Streptomyces* AR36 were not compensated by increased biomass production and lead to slightly reduced Zn and Cd extraction per plant.

In conclusion, the tested rhizosphere strains had independent effects on metal uptake and growth of *Salix caprea*. For the total amounts of extracted metal our results indicated that plant growth promotion might be more important than the enhancement of metal uptake. There was no correlation between the production of metal mobilizing compounds and heavy metal uptake in the plant. The commonly discussed interaction mechanisms of bacterial IAA, ACC deaminase and siderophore production were not involved in the observed promotion of metal accumulation and plant growth. Further research is required to better understand the interactions between microorganisms and heavy metal accumulating willows and to elucidate the mechanisms how bacteria can promote heavy metal accumulation in plants.

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CHAPTER 3

Identification of genes specific to *Methylobacterium extorquens* strains associated with the Ni hyperaccumulator *Thlaspi goesingense* by suppression subtractive hybridization

Abstract

Methylobacteria are characteristic and numerically important colonizers of heavy metal accumulating plants. Their specific functions in these host plants are largely unknown. Suppression subtractive hybridization (SSH) was carried out to identify genes specific to methylobacteria associated with heavy metal accumulators. A Ni resistant *Methylobacterium extorquens* isolate (iRIII1) from the rhizosphere of the Ni-hyperaccumulator *Thlaspi goesingense* was used as tester and a Ni sensitive *M. extorquens* strain from soil (DSM1337) was used as driver. 1088 randomly selected SSH products were spotted on a microarray. Probing with tester and driver DNA confirmed 688 SSH products to be intact and tester-specific. The SSH microarray was further used to study the distribution of the SSH products in 15 *M. extorquens* isolates from *T. goesingense*, from non-accumulating plants, from soil and air. The hybridization signal patterns partially reflected phylogenetic relatedness and habitat. 18% of the SSH products were detected exclusively in the tester, and 80% were detected only in isolates from *T. goesingense*. 109 SSH products were sequenced and 36 of these sequences were unique. Sequences detected only in the tester or only in the tester and another isolate from *T. goesingense* were affiliated with DNA methylases and with metabolic genes. Sequences characteristic to all three *T. goesingense*-associated strains were related to cell surface proteins of plant-pathogens and to regulatory sequences. One of these regulators was a family member of the ros/MucR proteins, which are involved in the communication of *Rhizobium meliloti* and *Agrobacterium tumefaciens* with their host plants. None of the 1088 SSH products was detected in all Ni resistant strains and none of the identified sequences had similarities with described Ni-resistance genes. Sixteen SSH sequences did not match any described protein. RNA-based experiments are planned to assess, which of the characterized sequences are expressed during plant colonization and to identify Ni-responsive SSH products**.**

Introduction

Bacteria belonging to the genus *Methylobacterium* are rod shaped, strictly aerobic alpha-proteobacteria. They are characterized by the capability to utilize C1 compounds such as methanol via the serine pathway (Green et al. 1992). Methylobacteria are also referred to as pink pigmented facultative methylotrophs (PPFMs), since most strains synthesize pink or red carotenoids (Hirashi et al., 1995). Methylobacteria have been isolated from soil, water, air, lake sediments and anthropogenic environments (Green 2001, Corpe 1985), but seem to have

a particularly intimate relationship with plants. Methylobacteria have been detected in the phyllosphere of more than 70 different plant species, and have been hypothesed to be the dominant phyllosphere genus (Corpe and Rheem, 1989). Apart from the phyllosphere, methylobacteria may colonize the endosphere (Araujo et al., 2002; Lacava et al. 2004) and the rhizosphere (Idris et al., 2004; Madhaiyan et al., 2006) of plants. The closest plant-*Methylobacterium* associations have been found in legumes, where N-fixing methylobacteria formed root nodules similar to rhizobia (Jourand et al., 2004), and in pine trees, where they lived as endosymbionts in meristematic bud cells (Pirttilä et al., 2000).

Methylobacteria consume methanol released by plants as a by-product of cell wall synthesis (Sy et al., 2005). Many plant-borne *Methylobacterium* isolates show characteristics, that suggest beneficial effects on the host plant such as the production of vitamin B12 (Kalyaeva et al., 2001), of auxin (Ivanovna et al., 2001) and cytokinin homones (Ivanovna et al., 2000). Other studies describe the synthesis of urease, which may be involved in the host's nitrogen metabolism (Holland and Polacco 1994), of 1-aminoclopropane-1-carboxylic acid deaminase (Madhaiyan et al., 2006), which may increase plant stress tolerance, and of siderophores potentially improving iron supply to the plant (Idris et al., 2004). *Methylobacterium* isolates from poplar have been found to degrade toxic organic compounds (van Aken et al., 2004). Inoculation experiments gave evidence that plant associated methylobacteria can favour seedling growth (Abanda-Nkpwatt, 2006) and root elongation (Madhaiyan et al, 2006), and interact with plant pathogens (Araùjo et al., 2002).

Particularly high abundance and diversity of methylobacteria has been observed in association with heavy metal accumulating plants. Methylobacteria dominated the culturable shoot endophytes of Zn/Cd accumulating *Salix caprea* (Chapter 1 of this thesis), and the culturable root endophytes of the Zn hyperaccumulator *Thlaspi cearulescens* (Lodewyckx et al., 2002). In Ni hyperaccumulating *Thlaspi goesingense,* methyloabacteria were dominant among endophyte and rhizosphere isolates and their numerical importance was confirmed by culture independent analysis (Idris et al., 2004). These observations suggest, that in addition common plant-beneficial activites, methylobacteria are highly adapted to heavy metal accumulating plants and may have specific functions in relation to the ability of these plants to grow in heavy metal contaminated soils.

Information about genetic specificities of methylobacteria associated with heavy metal accumulators can help to understand the mechanisms underlying these plant-microbe interactions. In this study suppression subtractive hybridization (SSH) was carried out in order to identify DNA sequences specific to methylobacteria colonizing the Ni-hyperaccumulator *T. goesingense*. With SSH, sequences unique to a genome of interest (tester) are isolated by hybridization with the genome of a reference strain (driver). Tester specific fragments that cannot hybridize with the driver DNA are selectively amplified and isolated by cloning (Diatchenko et al., 1996; Gurskaya et al., 1996). For efficient isolation of specific genes, it is important to use two closely related strains as tester and driver, which differ only in the features of interest (Radnedge et al., 2003). Here *Methylobacterium extorquens* iRIII1 (Idris et al., 2004) was used as tester, and the type strain *M.extorquens* DSM 1337 was used as driver. Idris et al. (2006) reported that iRIII1 and DSM1337 have 100% identical 16S rDNA sequences, 80% relatedness in DNA-DNA hybridization, nearly identical C-source utilization patterns, common key enzyme activities, and high similarity in fatty acid composition. However, isolates iRIII1 and DSM1337 differ in habitat and in heavy metal resistance. iRIII1 was isolated from the rhizosphere of the Ni hyperaccumulator *T. goesingense* and is resistant to Ni, Zn and Cr (Idris et al., 2004, 2006). DSM1337 is a soil isolate (Bassalik et al., 1960) resistant to Zn, but sensitive to Ni and Cr (Idris et al., 2006). SSH was therefore expected to identify genes involved in plant-colonization, in specific communication with heavy metal accumulators and in heavy metal resistance. Heavy metal resistance determinants of iRIII1 were of interest in so far, as PCR-tests did not detect any described Ni resistance gene in this strain (Idris et al., 2006). A library of SSH products was arrayed on a microarray and probed with genomic DNA of additional *M. extorquens* isolates obtained from *T. goesingense*, non-accumulating plants, soil and air. The microarray hybridization patterns were compared to study the intra-species distribution of the isolated fragments in relation to phylogeny and habitat, and to select SSH products for sequence analysis.

Materials and Methods

Bacterial strains and media

The *Methylobacterium extorquens* strains analysed in this work are listed in Table 1. Strains iRIII1 (the tester), iRII2 and iRIV1 have been isolated from the rhizosphere of *Thlaspi goesingense* by Idris et al. (2004). Strains DSM1337 (the driver), DSM1340 and DSM13060 were purchased from the German collection of microorganisms and cell cultures (www.dsmz.de). The isolates from *Arabidopsis thaliana* and *Medicago truncatula* as well as the airborne strain AM1 were kindly provided by Dr. C. Knief and Prof J. Vorholt, Swiss Federal Institute of Technology, Zurich. Ni resistant strains were grown in minimal salts medium (Krema and Lidstrom, 1990) containing 0.5% methanol as carbon source and 1 mM Ni to prevent loss of potential plasmid borne Ni resistances. Ni sensitive stains were grown in DSMZ medium #1 (5 g l^{-1} tryptone, 3 g l^{-1}meat extract) amended with 1% methanol.

Table 1. *Methylobacterium extorquens* strains analysed in this work

strain	sampling site /environment	Host plant / habitat	Compart-ment	MIC of Ni (mM)
iRIII1 (tester)	Ni rich ultramafic soil site	*Thlaspi goesingense*	rhizosphere	5
iRII2	Ni rich ultramafic soil site	*Thlaspi goesingense*	rhizosphere	10
iRIV1	Ni rich ultramafic soil site	*Thlaspi goesingense*	rhizosphere	10
156	Urban vegetation	*Arabidopsis thaliana*	leaf	<0.5
157	Urban vegetation	*Arabidopsis thaliana*	leaf	<0.5
160	Urban vegetation	*Arabidopsis thaliana*	leaf	<0.5
229	Urban vegetation	*Arabidopsis thaliana*	leaf	<0.5
304	Mediterranean pine forest	*Arabidopsis thaliana*	leaf	<0.5
396	Mediterranean scrubland	*Quercus coccinea*	leaf	2
554	Mediterranean wine yard	*Medicago truncatula*	leaf	<0.5
603	Mediterranean wine yard	*Medicago truncatula*	parent fruit*	<0.5
849	Mediterranean wine yard	*Medicago truncatula*	parent fruit*	<0.5
F46	Greenhouse	*Arabidopsis thaliana*	leaf	2
DSM13060	Natural pine stand, Finland	*Pinus sylvestris*	bud	2
AM1	/	air	/	5
DSM1340	/	soil	/	<0.5
DSM1337(driver)	/	soil	/	<0.5

*Parent fruit refers to the under ground residuals of the fruits from which *M. truncatula* plants developed

Determination of Ni resistances

Ni resistances were determined on phosphate-poor morpholinepropanesulfonic acid medium (MOPS; Neidhardt et al.,1974) with 1% methanol as carbon source. Bacteria were streaked on MOPS agar plates containing 0 mM, 0.5 mM, 1 mM, 2 mM, 5 mM and 10 mM $NiCl_2$. For each strain the highest concentration that allowed visible growth at room temperature within 7 days was determined.

Isolation of genomic DNA

Cells were harvested from 4 ml over night cultures by centrifugation (1 min, 13000 rpm, room temperature). Genomic DNA was isolated from the cell pellets by bead beating and phenol chlorophorm extraction as described by Sessitsch et al. (2001).

Amplification and sequencing of 16S-23S intergenic spacer (IGS) DNA

IGS DNA was amplified with the primers p23SRO1 (5-GGCTGCTTCTAAGCCAAC-3') and pHr (5'-TGCGGCTGGATCAC-CTCCTT-3') (Massol-Deya et al., 1995). 0.5 µl of DNA were used as template in 50µl PCR reactions containing 2 units or FIREPol DNA polymerase (Solis Biodyne), 0.2mM of each dNTP, 0.15 µM of each primer and 1.5mM $MgCl_2$. The thermal programme comprised an initial denaturation of 5 min at 95°C, followed by 30 cycles of 30 sec denaturation at 95°C, 1 min annealing at 55°C and 2 min extension at 72°C, and a final elongation step of 10 min at 72°C.

Suppression Subtractive Hybridization (SSH)

The genome of *Methylobacterium extorquens* DSM1337 was used as driver and subtracted from that of the tester *Methylobacterium extorquens* iRIII1. The PCR-select TM Bacterial Genome Subtraction Kit (CLONTECH) was used for the SSH procedure, according to the instructions of the manufacturer and with the following modifictaions. Four µg of genomic DNA from tester and driver were digested with *RsaI* and purified with phenol, chlorophorm and isoamylalcohol. For precipitation of the purified digests, NaOAc (pH 5.2, final concentration 0.3M) was used instead of NH_4OAc and glycogen (final concentration 0.07 g l^{-1}) was added as DNA carrier to increase precipitation efficiency. Adaptor ligation of tester DNA, hybridizations with driver DNA and PCR amplification of specific DNAs were carried out following the CLONTECH user manual.

Cloning of the SSH products

The final PCR product obtained from the SSH procedure was purified and inserted into the pGEM-T ® vector system I (Promega). The vector was introduced into *E.coli* DH5α by electroporation with a Gene Pulser®II (Biorad) following the instructions of the manufacturer. 1152 colonies were transferred to freezing medium (10 g l^{-1}tryptone, 5 g l^{-1} yeast extract, 5 g l^{-1}NaCl, 44 ml glycerol, 8.2 g $l^{-1}K_2HPO_4$, 1.8 g $l^{-1}KH_2PO_4$, 0.5 g l^{-1} Na_3-citrate, 1 g $l^{-1}MgSO_4$, 0.9 g l^{-1} $(NH_4)_2SO_4$, 100 mg l^{-1} ampicillin), grown over night at 37°C and stored at -80°C for further analysis.

Amplification of the SSH products and microarray fabrication

1088 randomly chosen inserts from the SSH library were amplified from the pGEM-T® vector with the primers M13f (5' GTAAAACGACGGCCAG 3') and M13r (5'CAGGAAACAGCTATGAC 3'). 1.5 µl of freezing-culture were directly used as template for 100 µl PCR reactions. A 100 µl reaction mixture contained 2 units of FIREPol (Solis Biodyne), 0.15 µM of each primer, 200 mM dNTPs, 2,5 mM $MgCl_2$ and BD PCR buffer in 1-fold concentration. After an initial denaturation of 5 min at 95°C, 35 cycles of 30 sec denaturation at 95°C 1 min annealing at 50°C and 2 min elongation at 72°C were performed. The thermal program was terminated by an additional elongation step of 10 min at 72°C. Two µl of PCR product were loaded onto 1% agarose gels to assess the performance of the PCR reaction and the fragment length. The remaining volume of the PCR products was cleaned by centrifugation through Sphadex G-50 (Amersham Biosciences) and dried in an SC250 EXP SpeedVac concentrator (Thermo) at 45°C and 16.2 mbar of vacuum pressure. Twenty four µl of spotting buffer (3xSSC, 1.5M betaine, 0.1% SDS) were added to the dry pellets and the PCR products were allowed to resolve under constant shaking for two hours at room temperature. For spotting, 10 µl of each sample were transferred to a 384 well plate. The difference products were spotted onto CSA-100 silanated amine slides (Cel Associates Inc. Pearland, USA) with an OmniGrid spotter (1 Tele-Chem SMP3 pin) at 55% relative humidity and 21 °C, using 16 pins. All samples were spotted in duplicates and 16S rDNA samples from tester and driver were included as internal controls for hybridization.

Labelling of genomic DNA

Per hybridization experiment, about 1.5 µg of genomic DNA were labelled with Cy5-dCTP (Amersham Biosciences), using the Bioprime Array CGH genomic labelling system (Invitrogen, Carlsbad, CA) following the protocol of Kostić et al. (2005). The labelled DNA

was purified using the purification module of the labelling kit. UV-vis spectra of the labelled DNA were analysed in a NanoDrop apparatus (NanoDrop Technologies, Wilmington), to calculate the labelling efficiency. Labelling rates between 20 and 30 nucleotides per dye molecule were considered suitable for hybridization experiments.

CSA slide processing and hybridization

Prior to hybridization, printed CSA slides were UV- crosslinked with 650 mJ, treated with sodium borohydride to prevent autofluorescence (Martinez et al., 2003) and subsequently blocked by acylation with succinic anhydride (Eisen and Brown, 1999). The purified labelled target (30 µl) was added to 180 µl of hybridization buffer and denatured for 3' at 95°C. The final composition of the hybridization reaction was 50% formamide, 5xSSC, 0.1% SDS and 100 µg ml^{-1} salmon sperm DNA. The total of 210 µl was applied to the blocked CSA slides and hybridized over night at 42°C as described by Stralis-Pavese et al. (2004). Genomic DNA from each *M. extorquens* strain was hybridized with two replicate slides. The slides were washed in 4 steps of 5 min: first at 50°C in 1xSSC containing 0.2% SDS, then at room temperature in 0.1xSSC containing 0.2% SDS, and finally twice at room temperature in 0.1xSSC (Kostić et al., 2005). Washed slides were dried with an oil free air gun and immediately scanned.

Scans and data analysis

The hybridized microarrays were scanned at 635 nm with a GenePix 4000A laser scanner (Axon Instruments, Foster City, CA). Scans were carried out at three lines to average and with a resolution of 10 µm. Maximal laser power and PMT gain were set to the highest level possible without signal saturation. Multilayer tiff images of the scans were analysed with the GenePix Pro 6.0 software (Axon Instruments). Based on the median feature – background signal intensities, data analysis was carried out in microsoft excell. Within each slide the signals of the individual spots were normalized to the median signal of 32 internal positive controls (16S rDNA). In a next step means were calculated, comprising four values per spot (obtained from two replicate hybridizations with slides carrying duplicated spots). Finally, the hybridization profiles of each strain were normalized to the hybridization profile of the tester (iRIII1). SSH products (spots) were considered present in a strain, if the corresponding hybridization signal was at least 25% of the signal obtained with the tester DNA. Cluster analysis of the presence-absence patters was done with STATISTICA 6 (StatSoft, Tulsa, USA).

Sequence analysis and annotation

For sequencing PCR products were purified through Sephadex G-50 (Amersham Biosciences) and 2 µl product were added as template to 10 µl sequencing reactions containing 2 µl of the BigDye terminator cycle sequencing kit (ABI prism) and 0.4 µM primer. In order to sequence the 16S-23S rRNA intergenic sequence (IGS DNA) sequencing was performed with the primer pHr (Massol-Deya et al., 1995). The primer M13f was used to sequence clones of the SSH library. After a second purification with Sephadex G-50, DNA fragments were sequenced with an ABI 373A automated DNA sequencer (Applied Biosystems Inc.). The ARB software package (Ludwig et al., 2004) was used to create a dendrogram from the IGS sequence data. The sequences obtained from SSH clones were cleaned from vector and adaptor residuals and assembled to contigs using online EGassembler service of the University of Tokyo (Masoudi-Nejad et al., 2006). BlastX (Altschul et al., 1997) was used to search the NCBI database for proteins related to the hypothetical gene products of the SSH sequences.

Results

Phylogenetic relatedness of the M. extorquens isolates

The phylogenetic relatedness of the *M. extorquens* isolates analysed in this study was assessed by sequence comparison of the 16S-23S intergenic spacer (IGS).The isolates from *T. goesingense* (iRIII1, iRII2 and iRIV1) and isolate 229 from the leaves of an urban *A. thaliana* could not be distinguished based on the IGS sequence and were extremely similar to the type strain DSM 1337. The neighbour joining tree constructed from the IGS sequences (Fig.1) showed, that the remaining M. extorquens isolates clustered in three distinct branches with a maximal evolutionary distance of about 0.2 to the *T. goesingense* isolates.

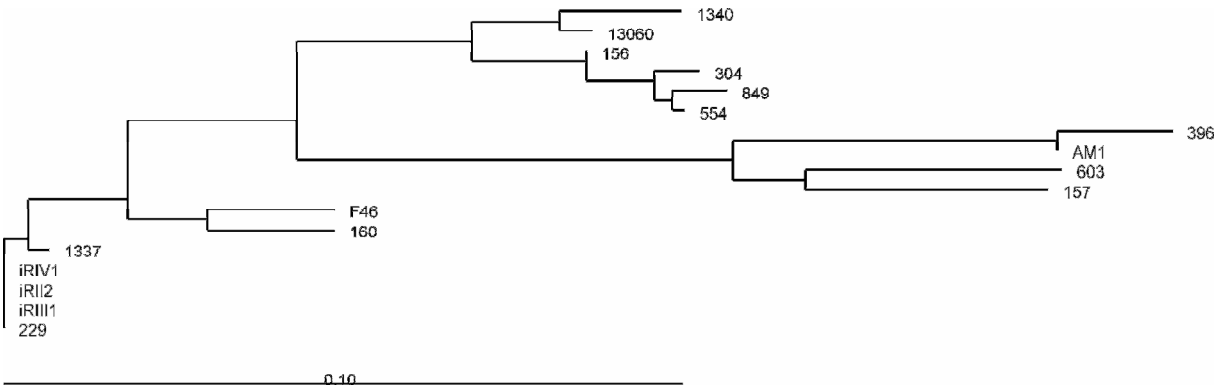

Figure 1. Neighbour tree of the of the *M. extorquens* strains based on 16S-23S intergenic spacer DNA sequences. The scale bar indicates the evolutionary distance.

Nickel resistance

As described previously (Idris et al., 2006) the minimal inhibitory concentrations (MIC) of Ni for the *T. goesingense*-associated strains iRIII1, iRII2 and iRIV1 were 5 mM, 10 mM and 10 mM respectively. Moreover the airborne strain AM1 was resistant to 5 mM Ni. Three isolates from non-accumulating plants (DSM13060, 396, F46) were resistant to 2 mM of Ni (Table1). The MIC of Ni-sensitive *E. coli* is 1 mM (Nies 1999). Therefore the remaining strains, which were inhibited by 0.5 mM of Ni, were considered Ni sensitive.

SSH and microarray hybridization

SSH was carried out with iRIII1, a Ni resistant rhizosphere isolate from *T. goesingense* as tester and DSM1337, a Ni sensitive soil isolate as driver. 1088 randomly selected SSH products were amplified from clones and spotted in duplicates onto a microarray. Microarray slides were hybridized with gDNA of tester, driver and 15 additional *M. extorquens* isolates. Image data of four strains (iRIII1, DSM1337, 160 and DSM13060) were used to compare the signal intensities obtained from replicate spots. Signals obtained from replicate spots correlated strongly (correlation coefficients (r) : 0.96, 0.98, 0.99, 0.97). Therefore mean values of replicate spots were used for all further calculations. Within each slide, signals were normalized to internal control spots (16S rDNA of *M. extorquens*) and were expressed as percentage of the control signal. gDNA from each strain was labelled twice in two separate reactions and hybridized with two replicate slides. Signal patterns obtained from replicate hybridizations with gDNA from isolates iRIII1, DSM1337, 160 and DSM13060 correlated with r values of 0.79, 0.85, 0.87 and 0.86, respectively. Stringent detection criteria were defined to account for this moderate hybridization reproducibility. An SSH product was considered present in a gDNA sample, if the normalized hybridization signal was at least 25% as strong as the corresponding signal obtained with the tester (iRIII1). Spots with normalized signal intensities of less than 5 during hybridization with the tester, were excluded from the analysis. To evaluate these detection criteria, the data of replicate hybridizations with strains DSM1337, 160 and DSM13060 were analysed separately. In each case the detection of more than 98% of the spots was reproducible. Average signal intensities were calculated from replicate hybridizations and transformed into presence-absence patterns using a 25% threshold.

Distribution of the tester Specific SSH products in M. extorquens isolates

Hybridization of 1088 SSH products with 17 *M. extorquens* strains yielded 71 different hybridization patterns (Figure 2). 317 SSH products (29%) had normalized hybridization signals below 5 with the tester and were therefore excluded from the analysis. 103 SSH products hybridized with both tester and driver and were considered as unsubtracted residuals of the SSH procedure (false positives). 668 SSH products were tester specific, i.e. detected in the tester and absent in the driver. The tester-specific SSH products showed 27 different distribution profiles which were grouped into 7 distribution types (Table 2). The distribution types were defined to distinguish between sequences detected only in the tester, only in the tester and another *T. goesingense* isolate, in all *T. goesingense* isolates, only in Ni resistant isolates, only in plant-isolates and scattered sequences. More than 80% of the tester specific sequences were detected only in isolates from *T. goesingense*: 18% were detected exclusively in the tester iRIII1, 28% were also detected in iRII2, 9% were also detected in iRIV1 and 37% were detected in all three *T. goesingnese* isolates. Three tester specific SSH products (0.4%) were detected only in Ni resistant strains and 52 (8%) only in plant-associated strains. However there was no tester-specific SSH product detected in all Ni resistant or in all plant associated isolates and absent in all other strains. 109 tester specific SSH products including sequences unique to iRIII1, to iRIII1 and iRII2, to all *T. goesingense* isolates, to Ni resistant isolates and to plant isolates were selected for sequencing (Table 2).

Presence-absence patterns of the SSH products in *M. extorquens* strains were also compared by cluster analysis. The resulting dendrogram had two deep branches. The first comprised the closely related *T.goesingense* isolates, and the second assembled all other strains (Figure 3). At the basis of the second branch, certain pairs of most similar presence-absence patterns, corresponded to pairs of most closely related IGS-sequences in figure 1 (e.g. AM1 and 396, 1340 and 13060, 157 and 603). Most of these consistencies with phylogenetic relatedness were lost, when false positives were excluded from the cluster analysis (data not shown). Apart from the separation of *T. goesingense*-associated strains from other isolates, clustering of presence-absence patterns did not reflect habitat, host plant or Ni resistance.

Sequence analysis of SSH products

109 SSH products were one-way sequenced and had, after removal of vector residuals and adaptors, lengths of 125-780 bp and GC contents of 40-60%. Sequence assembly resulted in

36 different sequences, 14 of which were contigs formed from identical and overlapping fragments. With a few exceptions (<5%, see footnotes in Table 3) fragments with different distribution profiles had different nucleotide sequences. Identical and overlapping sequences were found among SSH products with identical profiles, i.e. within distribution types A, B and C (Table 2). In each of these distribution types about 30% of the analysed sequences were unique. Three contigs were composed of more than 10 individual sequences. However rarefaction analysis showed that the potential SSH library was not exhausted by sequencing 109 inserts (Figure 4). BlastX search was carried out with the 36 different sequences and matches with E scores below 0.01 were considered significant (Table 3). 16 sequences, including individuals of each distribution type did not have any significant match with the NCBI protein database. In contrast, a large contig of 1490 bp (Ct1) contained two entire open reading frames, that matched two different proteins. Three sequences were related to hypothetical proteins with unknown functions. Among the 18 SSH products with significant hits to annotated database entries, four matched sequences from *Methylobacterium* species. Sequences specific to the tester, or to the tester and iRII2 (distribution types A and B) were mostly related to DNA methylases and to proteins involved in transport and metabolism. Sequences specific to all three *T. goesingense* isolates (distribution type C) were related to cell surface proteins and to proteins from *M. chloromethanicum* CM4 with regulatory functions. One of the sequences that were detected only in Ni-resistant strains (distribution type E), matched a transposase from *M. populi* BJ001. The other two sequences of this distribution type did not have significant matches. Two SSH products present only in plant-associated strains (distribution type F) matched proteins from *M. chloromethanicum* CM4. Two sequences of distribution type F were identical with sequences of types A and B respectively and were therefore integrated into the corresponding contigs.

		Ni resistant							Ni sensitive										
distr type	profile #	*Thlaspi* iRIII1 tester	*Thlaspi* iRII2	*Thlaspi* iRIV1	*Pinus* 13060	Air AM1	*Quercus* 396	F46	*Arabidopsis* 229	*Arabidopsis* 304	*Arabidopsis* 156	*Arabidopsis* 157	*Arabidopsis* 160	*Medicago* 554	*Medicago* 849	*Medicago* 603	Soil 1340	Soil 1337 driver	nb of sequences
A	1	■																	121
B	2	■	■																184
C	3	■		■															61
D	4	■	■	■															244
E	5	■	■	■		■													1
	6	■	■	■			■												1
	7	■	■	■				■											1
F	8	■	■	■					■										16
	9	■	■	■						■									4
	10	■	■	■							■								1
	11	■	■	■				■	■										9
	12	■	■	■					■	■									1
	13	■	■	■						■	■								6
	14	■	■	■				■	■		■								1
	15	■	■	■					■	■	■		■						1
	16	■	■	■						■	■	■	■						1
	17	■	■	■					■	■	■	■	■						2
	18	■	■	■	■					■						■			1
	19	■	■	■					■					■					2
	20	■	■	■					■	■				■	■				1
	21	■	■	■				■	■	■	■			■	■				3
	22	■	■	■				■	■	■	■	■		■					1
	23	■	■	■				■	■	■	■	■			■	■			1
	24	■	■	■				■	■	■	■	■	■	■	■	■			1
G	25	■				■													1
	26	■	■	■		■		■		■		■		■	■				1
	27	■	■	■	■					■	■						■		1
H	28	■	■															■	1
	29	■	■		■	■		■							■		■	■	1
	30	■	■		■			■		■	■	■		■		■	■	■	1
	31	■	■	■														■	4
	32	■	■	■						■								■	1
	33	■	■	■		■								■				■	1
	34	■	■	■						■	■							■	1
	35	■	■	■	■					■	■						■	■	6
	36	■	■	■				■	■		■							■	2
	37	■	■	■					■									■	27
	38	■	■	■				■	■									■	2
	39	■	■	■	■				■									■	1
	40	■	■	■		■			■									■	1
	41	■	■	■					■							■		■	1
	42	■	■	■					■		■						■	■	1
	43	■	■	■					■		■				■	■		■	1
	44	■	■	■					■	■								■	1
	45	■	■	■				■	■	■	■			■	■			■	8
	46	■	■	■					■	■	■		■		■		■	■	1
	47	■	■	■	■				■	■						■		■	1
	48	■	■	■	■				■	■	■						■	■	1
	49	■	■	■	■	■			■	■						■	■	■	1
	50	■	■	■	■	■			■	■					■	■	■	■	1
	51	■	■	■	■	■			■	■			■	■	■		■	■	1
	52	■	■	■	■	■			■	■		■		■		■	■	■	1
	53	■	■	■	■			■	■	■	■			■		■	■	■	1
	54	■	■	■	■				■	■	■		■	■	■		■	■	1
	55	■	■	■	■	■		■	■	■		■			■	■	■	■	1
	56	■	■	■	■	■		■	■			■		■	■	■	■	■	1
	57	■	■	■	■			■	■	■	■			■	■	■	■	■	1
	58	■	■	■				■	■	■	■	■	■	■	■	■		■	1
	59	■	■	■	■	■			■	■	■		■	■	■	■	■	■	2
	60	■	■	■	■	■		■	■	■	■			■	■	■	■	■	1
	61	■	■	■	■			■	■	■	■		■	■	■	■	■	■	1
	62	■	■	■	■			■	■	■	■	■	■	■	■	■	■	■	1
	63	■	■	■	■			■	■	■	■	■		■	■	■	■	■	2
	64	■	■	■	■	■	■	■	■	■			■	■	■	■	■	■	1
	65	■	■	■	■	■		■	■	■	■	■		■	■	■	■	■	1
	66	■	■	■	■	■		■	■	■		■	■	■	■	■	■	■	2
	67	■	■	■	■			■	■	■	■	■	■	■	■	■	■	■	4
	68	■	■	■	■	■		■	■	■	■	■	■	■	■	■	■	■	2
	69	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		■	3
	70	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	9
I	71																		317
	total	771	558	461	51	33	14	64	125	84	71	36	34	56	54	44	47	103	1088

Figure 2 (previous page). Distribution profiles of 1088 random SSH sequences in *M extorquens* strains. Filled cells indicate, that an SSH product was detected in the genomic DNA of a strain by microarray hybridization. SSH products were considered present, if the microarray hybridization signal was at least 25% as strong as the signal obtained with DNA from the tester. For each distribution profile, the number of SSH products showing this profile is indicated in the right column. Distribution profiles were grouped into distribution types, which are indicated in the first column. Distribution type H, assembles SSH products also detected in the driver (false positives). Distribution type I are defective SSH products. Distribution types A-G comprise tester specific SSH products and are explained in table 2.

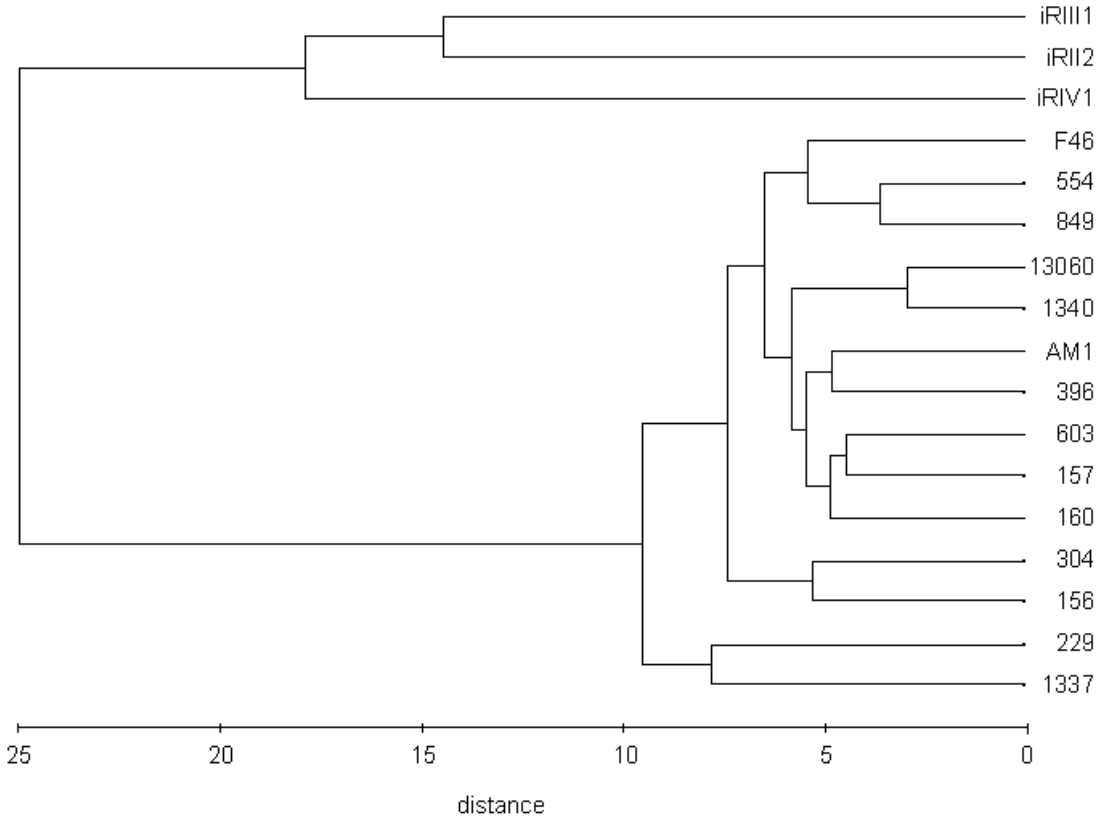

Figure 3. Relatedness of the *M. extorquens* strains based on hybridization patterns with the SSH-library microarray. Presence-absence patterns were clustered with STATISTICA software package using Euclidean distance and average linkage.

Table 2 . Grouping of tester-specific SSH products with respect to their distribution in *M. extorquens* strains and sequence analysis

Distribution type	Distribution profile # (Fig.2)	detected in	SSH products (%)		sequenced	unique sequences
A	1	iRIII1	121	(18)	26	8
B	2	iRIII1,iRII2	184	(28)	32	9
C	3	iRIII1, iRIV1	61	(9)	/	/
D	4	iRIII1, iRII2, iRIV1	244	(37)	43	13
E	5-7	Ni-resistant strains	3	(0.4)	3	3
F	8-24	Plant-associated strains	52	(8)	5	3
G	25-27	Scattered in *M. ext*. strains	3	(0.4)	/	/
total			668	(100)	109	36

Table 2. BlastX affiliations of 109 SSH sequeneces (14 contigs and 22 singletons)

distr type	Contig*/ singulet	Sequences (nb)	bp	BlastX E value	Accession#	Organism	Putative function
DNA Restriction-Modification							
A	Ct14	8	414	1e-43	ZP_01039292	*Erythrobacter sp. NAP1*	DNA methylase, type II
A	Ct2	2	434	7e-33	ZP_01037573	*Roseovarius sp. 217*	DNA methylase type II
A	6g10	1	522	1e-63	YP_001350665	*Pseudomonas aeruginosa PA7*	DNA methylase type II
A	2a6	1	528	8e-32	ZP_01037573	*Roseovarius sp. 217*	DNA methylase, type II
B	Ct8	11	1198	3e-67	YP_001047007	*Methanoculleus marisnigri* JR1	DNA methylase, type I
B	4g9	2	603	6e-19	NP_842486	*Nitrosomonas europaea ATCC 19718*	DNA methylase, type I
B	Ct4	3[c]	603	2e-25	NP_869965	*Rhodopirellula baltica SH 1*	DNA restriction modification
Transport and metabolism							
A	Ct7	8[d]	993	0.010	YP_996317	*Verminephrobacter eiseniae EF01-2*	TRAP dicarboxylate transporter
B	Ct1[a]	12[e]	1490	4e-23	NP_925215	*Gloeobacter violaceus PCC 7421*	distant relative of
B	1e11	1	608	2e-04	NP_925215	*Gloeobacter violaceus PCC 7421*	cell wall-associated hydrolases
F	2e1	1	453	2e-61	ZP_02058415	*Methylobacterium chloromethanicum CM4*	kojibiose phosphorylase
Cell surface							
D	Ct6	19	1078	3e-14	ZP_00946544	*Ralstonia solanacearum UW551*	Rhs family protein (outer membr)
D	8e4	1	501	5e-29	ZP_01998770	*Beggiatoa sp. PS*	WxcM-like protein (lipopol.synth.)
D	7f9	2	505	2e-16	ZP_01347765	*Rickettsia canadensis str. McKiel*	glycosyl transferase
F	6c7	1	416	2e-47	ZP_02056832	*Methylobacterium chloromethanicum CM4*	mannosyltransferase
Gene regulation and signal transduction							
D	Ct5	8	531	6e-23	ZP_02057732	*Methylobacterium chloromethanicum CM4*	MucR-like transcriptional regulator
D	2h9	1	307	0.003	ZP_02055863	*Methylobacterium chloromethanicum CM4*	response regulator receiver
Mobile elements							
E	10c4	1	421	9e-15	EDO72273	*Methylobacterium populi BJ001*	transposase
Unknown function							
B	Ct1[b]	12[e]	1490	2e-15	YP_001515075	*Acaryochloris marina MBIC11017*	hypothetical protein
B	7c7	1	626	7e-14	YP_001515075	*Acaryochloris marina MBIC11017*	hypothetical protein
D	Ct11	3[f]	463	1e-14	YP_779791	*Rhodopseudomonas palustris BisA53*	hypothetical protein
No significant BLASTx match							
A	Ct10	5	433	*****	**********	**********	**********
A	5e8	1	265	*****	**********	**********	**********
A	8b3	1	171	*****	**********	**********	**********
B	Ct3	2	125	*****	**********	**********	**********
B	2d9	1	250	*****	**********	**********	**********
B	8a4	1	395	*****	**********	**********	**********
D	Ct13	2	426	*****	**********	**********	**********
D	3e9	1	327	*****	**********	**********	**********
D	Ct9	2[g]	256	*****	**********	**********	**********
D	Ct12	2	682	*****	**********	**********	**********
D	2g4	1	375	*****	**********	**********	**********
D	7b3	1	426	*****	**********	**********	**********
D	8c8	1	297	*****	**********	**********	**********
E	11c7	1	288	*****	**********	**********	**********
E	2h8	1	247	*****	**********	**********	**********
F	8d3	1	321	*****	**********	**********	**********

*Contigs are named Ct1- Ct14. Singulets, have names starting with numbers. [**a** contig1 bases 342-941] [**b** contig 1 bases 987-1415] [**c** contig4 assembled 2 sequences of distribution type B and 1 sequence of distribution type D] [**d** contig7 assembled 7 sequences of distribution type A and 1 sequence of distribution type F] [**e** Contig1 assembled 10 sequences of distribution type B and 2 sequences of distribution type D] [**f** contig11 assembled 2 of distribution type D and 1 sequence of distribution type B] [**g** contig9 assembled 1 sequence of distribution type D and 1 sequence of distribution type F]

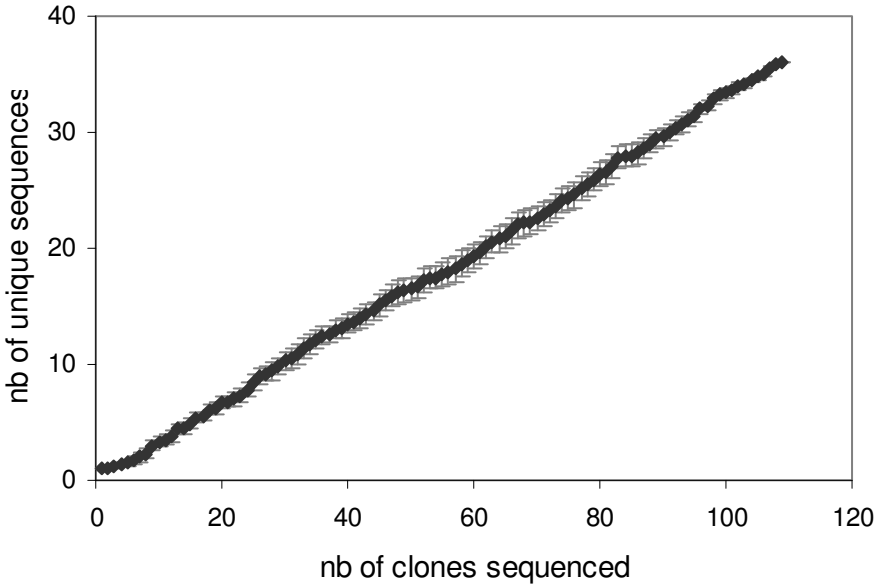

Figure 4. Rarefaction curve showing the diversity of the identified SSH sequences. The data points are mean values of 10 rarefaction curves. From a library of 1088 SSH clones, 109 clones were sequenced and assembled to 14 contigs and 22 singletons.

Discussion

Bacteria of the genus *Methylobacterium* are characteristic and numerically important colonizers of heavy metal accumulating plants (Lodewyckx et al., 2001, Idris et al., 2004, Chapter 1 of this thesis). However, little is known about their specific adaptations to and functions in heavy metal accumulating host plants. The aim of this work was to identify genes specific to methylobacteria associated with the Ni hyperaccumulator *Thlaspi goesingense* and to get information about their function.

SSH was carried out to isolate genes that are specific to *M. extorquens* iRIII1, a rhizosphere strain from *T. goesingense* (tester), and absent in the soil isolate *M. extorquens* DSM1337 (driver). Sequence analysis of 16S-23S intergenic spacer DNA gave new evidence for the close relatedness of iRIII1 and DSM1337, which has already been observed by Idris et al. (2006). High similarity between tester and driver is favourable for the detection of specific genomic differences by SSH (Radnedge et al., 2003).

Random SSH products (1088 clones) were amplified and spotted on a microarray. Upon hybridization with genomic DNA from the tester strain, 29% of the spots did not give a positive hybridization signal. These spots included clones that contained no insert or failed to be amplified from freezing cultures. Partial screening on agarose gel showed, that about 10% of the SSH clones gave no PCR product (data not shown). Additional spots may have been lost due to punctual problems in the spotting procedure. Certain tester-negative spots may have been complementary to fragments that failed to hybridize under the applied conditions, due to exceptional GC contents or to the formation of secondary structures. Finally, SSH products that actually hybridized with tester DNA, may have been assigned as "absent", because the hybridization signal was less than 5% of the 16S rDNA signal. The 5% threshold was very stringent, because the 16S rDNA was longer than the SSH products and therefore gave particularly strong signals. Stringent thresholds were applied, because the microarray hybridization signals were not perfectly reproducible. However it was possible to define reliable detection criteria, and the microarray allowed rapid and extensive screening of the SSH library.

A high number (771) of SSH products were confirmed to be present in the tester iRIII1. 13% of these products were also detected in the driver (DSM1337). SSH is not quantitative and false positives arise from fragments common to tester and driver, that by chance do not meet to form heterohybrids during SSH (Agron et al., 2002). Moreover the SSH procedure does not enrich only sequences that are absent from the driver genome, but also those that are substantially different (Akopyants et al., 1998). Such fragments may contain patches of sequence able to bind driver DNA during microarray hybridization. According to the informations provided by CLONTECH TM, 13% is a very low proportion of false positives, signalising high subtraction efficiency. In total, 668 SSH products were tester specific.

The microarray was further used to study the distribution of the SSH products in *M. extorquens* isolates from *T. goesingense*, various non-accumulating plants, soil and air. According to IGS sequence similarity, the two other rhizosphere isolates from *T. goesingense* (iRII2, iRIV1) as well as one Ni sensitive strain from *Arabidopsis thaliana* (229) were closely related to tester and driver. The remaining strains were phylogenetically more distant and grouped in three distinct clusters (Figure 1). Hybridization patterns obtained with the SSH microarray partially reflected phylogenetic relationship and host plant. In particular the three closely related isolates from *T. goesingense* shared the majority of positive signals and 80%

of the tester specific SSH products were detected only in these three strains. Precisely, 18% were detected only in the tester (iRIII1), 28% were detected only in iRIII1 and iRII2, 9% only in iRIII1 and iRIV1, and 37% were characteristic to all three *T. goesingense* associated strains. Strain 229, which also closely related to the tester, but originated from a different host plant, hybridized only with about 6% of the SSH products. This suggests, that apart from phylogenetic markers the SSH library may also contain genes related to the specific colonization of *T. goesingense*. The isolates from habitats other than *T. goesingense* hybridized only with 0.1% - 6% of the tester specific SSH products, further indicating an enrichment of genes involved in host specificity. Moreover, about 50% of the putative false positives were detected only in the isolates from *T. goesingense*, in the driver and in few other strains. The signals of these spots shaped the dendrogram of hybridization patterns in a way to reflect phylogenetic relationships among strains from other sources than *T. goesingense*. When "false positives" were excluded from cluster analysis, the consistencies between IGS-based relatedness and hybridization pattern similarity were lost. In conclusion also the pool of "false positives" was enriched in tester specific sequences, and only those hybridizing with all *M. extorquens* strains were actually unsubtracted DNAs. Further evidence for the high specificity of the SSH library was given by the fact that none of the spots produced a hybridization signal with DNA from the species *M. goesingense* (data not shown). This narrow specificity appears to be a result of the high similarity between tester and driver. The hybridization patterns of strains from other isolation sources than *T. goesingense* were not related to habitat, and there was no SSH product detected in all plant-associated strains. However, only three of the 668 tester specific SSH products were detected in strains from non-plant habitats.

Selected SSH products (109) were one-way sequenced, including sequences detected only in the tester iRIII1, only in iRIII1 and iRII2, only in the *T. goesingese* associated strains (iRIII1, iRII2, iRIV1), only in Ni resistant strains and only in plant-associated strains. Thirty-six sequences were unique. Contigs were formed form identical and overlapping sequences. Most of them showed identical distribution among isolates. Only in five cases, sequences with different hybridization profiles were identical.

Most of the sequences detected exclusively in the tester iRIII1 and many of those detected only in iRIII1 and iRII2 were related to N6-adenine specific DNA methylases. DNA methylases are primarily part of DNA restriction-modification systems, that protect the cell

against phage attack. Methylases transfer methyl groups to specific sites in the bacterial chromosome and cognate restriction enzymes cleave foreign DNA that lacks site specific methylation (Gingeras et al., 1991). Methylation in the N6 position of adenine can also alter interactions of the DNA with regulatory proteins, such as activators and repressors, either by steric effects or by affecting the secondary DNA structure. In this way, additional (epigenetic) information is imparted in the DNA (Low et al., 2001). Several methylases without cognate restriction enzymes have been identified and were observed to regulate multiple cellular processes. Such processes include DNA replication, adjustment of metabolic pathways to environmental conditions and the expression of virulence genes (Kahng and Shapiro, 2001, Oshima et al., 2002). Similarly, it is conceivable that in *M. extorquens* DNA methylation regulates adaptations to the Ni-containing rhizosphere environment. On the other hand, DNA restriction modification genes are typical to vary between closely related strains and have been detected in many SSH surveys on human and animal pathogens (Akopyants et al., 1998; Purdy et al., 2005; Soule et al., 2005; Hepworth et al., 2007). In *Helicobacter pylory* the diversity of DNA methylases is so high, that they have been proposed as phylogenetic markers for strain typing (Vale and Vitor 2007). In this work, DNA methylases allowed a differentiation of the extremely similar strains iRIII1, iRII2, iRIV1 and 229. iRIII1 and iRII2 contained type I methylases that were absent in iRIV1 and 229. iRIII1 contained additional type II methylases that were absent in iRII2.

The remaining blastX hits of sequences specific to iRIII1 or both, iRIII1 and iRII2, were metabolic genes and transporters, possibly involved in the utilization of specific substrates provided by the plant root. A 1 kb contig of sequences uniquely detected in iRIII1 showed moderate similarity (E=0.01) to a tripartite ATP-independent periplasmic transporter (TRAP) for dicarboxylates. Only one member of this transporter family has been characterized and mediates the import of malate, fumarate and succinate into *Rhodobacter capsulatus* (Forward et al., 1997; Rabus et al., 1999). In the rhiozosphere this transporter may mediate the uptake of malate, which is an important constituent of plant root exudates (Pinton et al., 2007). However the relatively low similarity allows only speculations about the function of this protein. One segment of the largest contig (Ct1) matched a distant relative of cell wall associated hydrolases from *Gloeobacter violaceus*. A singulet sequence detected in several plant associated strains was highly similar to a specific disaccharide hydrolase from *M. chloromethanicum* CM4 (kojibiose phosphorylase).

The majority of sequences characteristic to all three isolates from the rhizosphere of *T. goesingense* were affiliated with cell surface proteins. In plant-associated bacteria the cell surface is the scene of interaction with the host plant. Cell surface proteins have been observed to affect host-specificty and virulence of plant pathogenic bacteria (Zhang et al., 2005; Triplett et al., 2006). Two sequences were related to glycosyl transferases, one of which was specified as outer membrane mannosyl transferase involved in cell envelope biogenesis. One sequence matched a WxcM-like protein. WxcM is a virulence determinant of the crucifer pathogen *Xanthomonas campestris* required for lipopolysaccharide antigen synthesis (Qian et al., 2005). A contig composed of 19 individual sequences matched a RhS family protein, which has been detected in a comparative genomic study on two plant pathogenic *Ralstonia solanacearum* strains with different temperature optima and host range (Gabriel et al., 2006). The function of Rhs elements is still unclear and has only been predicted from the structure of the hypothetic protein products. Rhs elements show features characteristic for binding proteins, that are either secreted or exhibited at the cell surface, such as toxins and antigens (Hill et al., 1994). In Vivo Expression Technology (IVET) based experiments showed that Rhs genes were induced in *Erwinia amylovora* during infection of pears and in *Pseudomonas syringiae* pv. tomato during infection of *Arabidopsis thaliana* (Zhao et al., 2005; Boch et al., 2002).These observations suggest that certain mechanisms involved in colonization of and communication with the host plant are similar in plant pathogens and not pathogenic *Methylobacteria*.

The remaining sequences that were detected only in *T. goesingense*-associated strains and had significant matches in the NCBI protein database, were related to regulatory proteins. A contig of eight sequences was affiliated with a transcriptional regulator of the ros/MucR family. MucR regulates in *Rhizobium meliloti* the production of exopolysaccharides, that are necessary for symbiosis with plants and may promote growth of the infection thread (Martin et al., 2000). Ros regulates exopolysaccharide production and virulence operons in octopine and nopaline strains of *Agrobacterium tumefaciens* (Cooley et al., 1999). A genome project on *Methylobacterium chloromethanicum* CM4 revealed that MucR regulators are also present in methylobacteria and the sequence identified in this work was closely related to MucR of *M. chloromethanicum* CM4. Open reading frames (ORFs) related to plant association-relevant genes of *Rhizobium* and *Agrobacterium* have also been detected in the genome of *M. extorquens* AM1 and underscore the intimate relation of methylobacteria to plants (Lidstrom and Christoserdova, 2002). Our results suggest that MucR regulators in *M. extorquens* vary

between strains with different habitats or host plants. A unique sequence showed moderate similarity to the receiver domain of a two component response regulator. In such response systems, a histidine kinase sensor phosphorylates the receiver domain of a response regulator, which activates the effector domain to trigger cellular response. Two component sytems are widespread in bacteria and allow adaptations to changing conditions and colonization of different ecological niches (Stock et al., 2000). Two component systems can receive biotic and abiotic signals and include heavy metal response regulators as well as systems related to plant-association. For istance, the czcR/czcS system in *Pseudomonas aerugniosa* can induce Zn resistance in presence of Zn (Perron et al., 2004). The GacS/GacA system controls the production of secondary metabolites and extracellular enzymes related to virulence of plant pathogens. In plant beneficial bacteria GacS/GacA regulates the synthesis of biocontrol agents (Heeb and Haas, 2001). Proteomic analysis of an epiphytic *M. extorquens* strain revealed a novel two component system PhyR, which is essential for plant colonization (Gourion et al., 2006).

SSH between the Ni resistant tester iRIII1 the Ni sensitive driver DSM1337 was expected to identify the hitherto undescribed gene conferring Ni resistance to *M. extorquens* strains like iRIII1 (Idris et al., 2006). Therefore all strains were tested for Ni resistance in plate experiments and SSH products common to Ni resistant strains were searched among the hybridization profiles. Apart from the highly Ni resistant isolates from *T. goesingense*, four strains showed phenotypic resistance to lower Ni concentrations (DSM13060, AM1, 369 and F46). Not a single SSH product was characteristic to all Ni resistant strains. This suggests, that different Ni detoxification systems existed in the highly and the moderately resistant strains. Three SSH products were detected in the three isolates from *T. goesingense* and in one additional Ni resistant strain and absent in all Ni sensitive strains. Two of these sequences had no match to any known gene and the third was a transposase. Mobile genetic elements such as transposases are frequently detected in SSH between closely related strains (Purdy et al., 2005), but are unrelated to heavy metal resistance. None of the 109 sequenced SSH products was related to the Ni resistance genes *ncc* or *nre*B, homologues of which have been detected in the genome of *M. extorquens* AM1 (operonDB, http://www.cbcb.umd.edu) or to any other known heavy metal resistance determinant. As the SSH products were not exhaustively sequenced, it is possible that orthologs of known Ni resistence genes were present in iRIII1 and were contained in unsequenced clones. Moreover, heavy metal resistances are often located on plasmids (Mergeay et al., 2003). Although the applied DNA isolation protocol is

supposed to retrieve plasmid DNA, loss of plasmids cannot be excluded. It is also possible that Ni resistance genes were located far from *Rsa*I restriction sites and failed to be enriched by SSH. Agron et al. (2002) demonstrated that the use of different restriction enzymes in SSH, yieldes slightly different sets of sequences. On the other hand iRIII1 may contain a novel Ni resistant gene, because Idris et al (2006) did not obtain PCR products from this strain with primers for *ncc*B, *ncc*N, *nre*B and *cnr*Y. iRIII1 was resistant to high Ni concentrations (5 mM) and efficient heavy metal detoxification in bacteria is efflux-based (Nies 1999). Even novel resistance systems may have homologies with elements of described transport complexes. The only transport protein identified in this work was related to a TRAP dicarboxylate transporter, as described above. Although the described representative of this transporter type is an importer for organic compounds, we may speculate that the related protein in iRIII1 could be a metal exporter. Alternatively, the novel Ni resistance gene may be among the sequences that did not match described proteins. Three SSH sequences were affiliated with unidentified hypothetical proteins. Sixteen SSH products (44%) including sequences detected only in iRIII1, in iRIII1 and iRII2, in all *T. goesingense* isolates, in Ni resistant isolates and in plant associated isolates, had no significant similarity to any NCBI database entry. Most of these fragments contained open reading frames of considerable length. Primers could be designed to asses in real time PCR experiments whether the TRAP-like transporter or the genes without described relative are Ni-inducible. The SSH microarray could be hybridized with cDNA from Ni-stressed and Ni-free cultures of iRIII1, to detect Ni-responsive genes within the SSH library.

Conclusions

SSH and microarray screening identified DNA restriction modification systems, metabolic genes, cell surface proteins and regulatory proteins specific to *T. goesingense* colonizers and unknown proteins potentially including a novel Ni resistance gene. The detection of DNA restriction modification genes, which vary between closely related strains, indicates high specificity and efficiency of the SSH procedure. Metabolic and cell surface proteins specific to *T. goesingense* associated strains are likely to be involved in plant-microbe interactions. Their relevance to plant-colonization could be confirmed by comparing cDNA from *in vivo* and *in vitro* grown iRIII1 on the SSH microarray. The function of the regulatory genes could be assessed by the creation of deletion mutants, which could be compared to wildtypes in plant-inoculation experiments. The SSH microarray could be screened for genes that depend on these regulators by probing with cDNA from wild-types and mutants.

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Final conclusions and outlook

Three strategies were applied to study the role of bacteria in heavy metal accumulation by *Salix caprea* and to elucidate the mechanisms of plant-microbe interactions during the accumulation process. At first, rhizosphere bacteria and endophytes were screened *in vitro* for characteristics commonly associated with enhanced heavy metal accumulation in plants. In a second step the effect of individual strains on growth and metal uptake by *S. caprea* plantlets was assessed in greenhouse experiments. Finally, SSH-based molecular analysis was carried out to identify novel genes involved in interactions between bacteria and heavy metal accumulating plants.

Isolation and phenotypic characterization of rhizosphere bacteria and endophytes of *Salix caprea* revealed high phylogenetic and functional diversity. Plant-inoculation experiments gave evidence that bacterial activity contributes to heavy metal accumulation by *Salix caprea*. It became apparent that bacteria can enhance heavy metal extraction either by facilitating metal uptake or by promoting growth of the accumulating biomass. Two interesting rhizsophere isolates promoting Zn/Cd uptake and respectively growth of *Salix caprea* were identified.

The most complex objective of this work was the analysis of mechanisms enabling bacteria to promote heavy metal accumulation in plants. Characteristics commonly associated with the promotion of heavy metal accumulation, i.e. the production of IAA, ACC deaminase and siderophores, were detected in many *Salix caprea*-colonizing strains. Such observations support the theory that these bacterial products affect heavy metal uptake and tolerance of plants. However plant inoculation experiments did not confirm positive effects of IAA- and siderophore producing strains on growth and heavy metal uptake of *Salix caprea*. Similarly, a bacterium producing Zn and Cd mobilizing metabolites *in vitro*, did not facilitate the uptake of these metals *in vivo*. The subset of isolates analysed in plant experiments was too small to allow general conclusions about the importance of bacterial IAA, siderophores and metal ligands for heavy metal accumulation in *Salix caprea*. The observation that bacteria unable of producing IAA, ACC deaminase, siderophores or Zn/Cd ligands, promoted growth and heavy metal uptake, indicated that other beneficial interaction mechanisms exist. Genetic analysis of a selected rhizosphere strain revealed that strain specific metabolism, cell surface structures and many unknown proteins may be involved in such interaction mechanisms.

Phenotypic analysis, plant experiments and molecular analysis provided complementary information on the functions of plant-associated bacteria in heavy metal accumulation. Polyphasic experimental strategies appear to be imperative for studying this complex system of plant-microbe-metal interactions. The molecular approach allowed the rapid identification of numerous novel genes potentially involved in specific interactions between bacteria and heavy metal accumulators. Culture dependent laboratory experiments will be required to analyse the phenotypic properties conferred by these novel genes. In the presented work, culture dependent analysis enabled reliable screening of bacterial isolates for phenotypic characteristics relevant to heavy metal availability and plant growth. Finally, plant experiments are necessary to assess the function of interesting bacterial phenotypes in the natural system and to identify particularly beneficial strains.

This PhD thesis presents a first trial of introducing bacterial inocula into the rhizosphere of heavy metal accumulating trees. Unlike herbaceous heavy metal accumulators, *Salix caprea* trees cannot be efficiently proliferated from surface sterilized seeds. This complicates the set-up of gnotobiotic inoculation systems. One of our inoculation experiments was successful. In several other trials, root washing and transplantation to sterilized soil reduced the vitality of *Salix caprea* plantlets and lead to inhomogeneous growth. Consequently information about the *in vivo* effects of many interesting bacteria was lost. One of the primary challenges for future research on the functions of bacteria in heavy metal accumulating *S. caprea* will be the development of gentle inoculation systems. It will be important to avoid transplantation and to select homogeneous individuals from robust well pre-grown *S. caprea* cuttings. Once reliable inoculation protocols are established, large scale screenings will allow to relate *in vitro* determined phenotypic characteristics to effects on plants. Moreover it will be possible to asses the *in planta* function of individual bacterial genes, e.g. by inoculation experiments with knock out mutants. Further plant experiments are particularly required for the identification of conditions that favour the beneficial plant-microbe interactions.

Our collaborators at the University of Natural Resources and Applied Life Sciences have demonstrated that *Salix caprea* trees can efficiently remove Cd from moderately contaminated soils. Bioaugmentation of beneficial plant-associated bacteria or agricultural measures supporting beneficial interactions are expected to further increase this efficiency. Severely contaminated sites can be stabilized by re-vegetation with *Salix caprea* and stimulation of

beneficial bacterial activities may facilitate tree growth in these hostile environments. Despite such measures, sites like the former mining area of Arnoldstein, where the samples for this study have been taken, will remain contaminated for the life time of many generations. Therefore, apart from developing clean-up technology, it is our responsibility to avoid and oppose further emissions of heavy metals to the environment.

Curriculum Vitae

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1985-1997	Rudolf Steiner School Pötzleinsdorf, Wien 18
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1998-2003	Studies of Ecology at the University of Vienna, graduation with honours. MSc at the Institute of Ecology and Conservation Biology under supervision of Doz. Dr. Alexander Haslberger. Thesis title: "Culture Independent Techniques for Monitoring the Impact of Agricultural Practices on Soil Microbial Communities."
2003	Research at the I.N.R.A. (Institut National de la Recherche Agronomique) in Dijon, France. Research Project: "Impact of Genetically Modified Plants and their Specific Cultivation Practices on Soil and Rhizosphere Microflora."
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