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REGULAR ARTICLE

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 indole-3-acetic acid (IAA), 1-amino-cyclopropane-1-carboxylic acid deaminase (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 γ-sterilized, Zn/Cd/Pb contaminated soil and inoculated with the Zn resistant isolates, *Streptomyces* AR17 enhanced Zn and Cd uptake. *Agromyces* AR33 tendentiously promoted plant growth and thereby increased the total amount of Zn and Cd extracted from soil. 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.

Keywords Metal mobilization · Plant growth promotion · Phytoextraction · Rhizosphere bacteria · Siderophores · ACC deaminase · IAA

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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-3-acetic acid (IAA) in the rhizosphere, 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 (de Souza 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 1 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[a]	247	mmol kg^{-1}
Organic carbon	24.6	g kg^{-1}
pH	7.2	
Total metal contents (in aqua regia)		
Zn	1,760	mg kg^{-1}
Cd	32.7	mg kg^{-1}
Pb	6,560	mg kg^{-1}
Mobile fraction of metals (in 1 M NH_4NO_3)		
Zn	2.56	mg kg^{-1}
Cd	0.64	mg kg^{-1}
Pb	3.81	mg kg^{-1}

[a] Measured at soil pH

Isolation of rhizosphere bacteria

For the isolation of bacteria 10 g of rhizosphere soil were shaken with 100 ml 0.8% NaCl solution for 30 min. Dilutions of the suspension were plated on R2A (Difco) + 1 mM Cd $(NO_3)_2$ + 1 mM $Pb(NO_3)_2$ + 1 mM $Zn(NO_3)_2$ 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′-AGAGTTTGATCC TGGCTCAG-3′; Weisburg et al. 1991) and 1520 r (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 s 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 restriction fragment length polymorphism (RFLP)-fingerprinting. Isolates with different RFLP profiles were identified by sequencing of 600 bp–1,500 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 1520 r 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

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

Strain	Closest described relative[a] [Accession nb.] (bp, identity)	MIC (mM)				Production of		
		Zn	Cd	Pb	Zn,Cd,Pb	siderophores	IAA	ACCD
PR04	*Pseudomonas* sp. BE3dil [AY263472] (1,389 bp, 100%)	2	0.5	2	<2	+	−	−
PR02	*Janthinobacterium lividum* [AF174648] (1,375 bp, 99%)	6	0.5	4	<2	−	+	−
PR13	*Janthinobacterium lividum* [AF174648] (1,471 bp, 99%)	6	1	4	<2	−	+	−
BR780	*Serratia marcescens* [AB061685] (1,503 bp, 99%)	8	4	2	2	+	+	−
PR01	*Flavobacterium frigidimaris* [AB183888] (1,338 bp, 99%)	6	2	4	2	−	−	−
AR16	*Streptomyces* sp. 10–6 [AB222069] (624 bp, 99%)	11	1	4	<2	+	−	−
AR17	*Streptomyces* sp. VTT E-042639 [EF564804] (1,483 bp, 100%)	8	0.5	2	<2	−	−	−
AR35	*Streptomyces* sp. VTT E-042639 [EF564808] (588 bp,100%)	6	0.5	2	<2	−	−	−
AR36	*Streptomyces* sp. VTT E-042677 [EF564805] (647 bp, 99%)	6	1	2	2	+	−	−
AR33	*Agromyces terreus* DS-10 UMS-101 [EF363711] (1,443 bp, 98%)	6	<0.5	2	<2	−	−	−

MIC = Minimal inhibitory concentration, ACCD = ACC deaminase

[a] Determined by sequence analysis of the 16S rDNA gene

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, 2, 4, 6, 8 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.

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 microliters 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 microliters 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.

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 2 weeks at room temperature.

Siderophore production

Siderophore production was analysed on chrome azurol S agar plates. Bacteria were grown for 2 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% trypticase soy broth (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 (8,000×*g*, 15 min, 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 2 h 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 (7,000 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 (2,420×*g*, 10′, 4°C). 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 1 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 min. 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 and rhizosphere soil (of treatments inoculated with strain PR04, AR16 and BR780) were sampled. 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 $HClO_4$ (4:1, v/v). Zn and Cd concentrations in the digested leaf material were determined by AAS (Perkin Elmer, 2100). Bacterial strains were extracted from rhizosphere samples as described above and plated on KB medium (PR04), GYM (10 g l^{-1} malt extract, 4 g l^{-1} yeast extract, 4 g l^{-1} glucose, 15 g l^{-1} agar) (AR16) and on 10% TSA (BR780). In the case of PR04, 20 randomly selected colonies were chosen and their identity with the inoculant strain was determined by PCR-RFLP analysis of the 16S-23S rRNA intergenic spacer (IGS) as described previously (Sessitsch et al. 1997).

Statistical analysis

Statistical 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 (the MIC was 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 MICs ranged between 6 and 8 mM. *Serratia* BR780 and *Flavobacterium* PR01 had Cd MICs of 4 mM and 2 mM respectively. The Cd MIC of *Agromyces* strain AR33 was below 0.5 mM, the lowest dose tested. The remaining strains were inhibited by 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.

Siderophores, IAA and ACC deaminase

Pseudomonas PR04, *Serratia* 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 and Cd 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.

Inoculations of *Salix caprea* plantlets

Suspensions of bacteria were introduced into the rhizosphere of *Salix caprea* 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 (Fig. 2). *Agromyces* AR33 was the only inoculant with a tendentious positive effect on leaf growth, however the effect was not significant. 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 ($p=0.09$) (Fig. 2). Leaf biomass and Zn/Cd contents were calculated to show the total amount of metal extracted by each plant (Fig. 2). The resulting values showed, that the growth promoting strain AR33 tendentiously improved Zn extraction per plant in comparison to the non-inoculated control.

The persistence of three inoculant strains, PR04, AR16 and BR780, in the rhizosphere was determined. Strain PR04 showed 6×10^5 cells g^{-1} dried rhizosphere soil and all 20 colonies tested showed the same IGS RFLP pattern as the inoculant strain (data not shown). Strains AR16 and BR780 showed densities

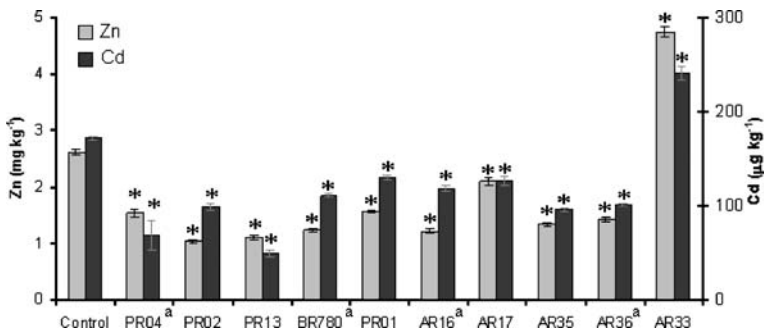

Fig. 1 Mobilization of Zn and Cd from contaminated soil by bacterial exudates. Contaminated soil was shaken with filtrates of stationary bacterial TSB cultures. Mean values of metals extracted per kg soil from three replicate cultures which were measued three times each are indicated. ANOVA for the influence of bacterial metabolites on Zn and Cd extraction capacity of TSB revealed highly significant effects: $p=0.0000$ for both Zn and Cd. Significant differences of culture filtrates to controls ($p<0.01$ identified by post hoc Fisher LSD tests) are labelled with an *asterisk* (*)

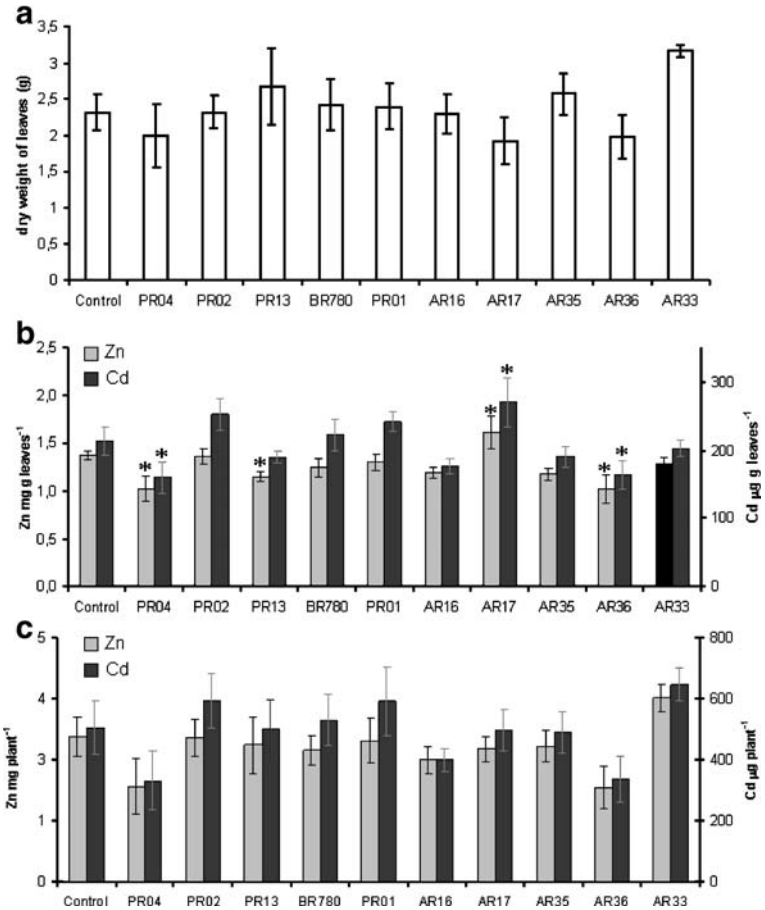

Fig. 2 Leaf biomass and heavy metal content of *Salix caprea* 3 months after inoculation with 10 rhizosphere isolates. **a** Leaf dry weight **b** Zn and Cd concentration in leaves **c** total Zn and Cd accumulated in leaf biomass (= leaf biomass × metal concentration in leaves). ANOVA for the influence of bacterial inoculations revealed significant effects on Zn and Cd concentrations in leaves ($p=0.008$ and $p=0.008$, respectively). Values differing significantly from the control ($p<0.1$, according to post hoc Fischer LSD tests) are labelled with an *asterisk* (*). Bacterial effects on leaf biomass and total accumulated Zn and Cd were statistically not significant ($p=0.321$, $p=0.179$ and $p=0.174$, respectively)

of 3×10^4 and 2×10^3 cells g^{-1} dried rhizosphere soil, respectively.

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 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 (MICs of 4 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 showed Cd MICs between 0.5 and 1 mM. Pb MICs ranged between 2 and 4 mM, which is below the minimal inhibitory concentration of Pb-sensitive *E. coli* (Nies 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 availability 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; Pattern and Glick 1996; Burd et al. 1998; Glick 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 (Fig. 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. It cannot be concluded 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 g-sterilized contaminated soil and inoculated with bacteria, *Agromyces* AR33 tendentiously increased leaf biomass (Fig. 2). 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). The ability of *Agromyces* AR33 to produce Zn and Cd mobilizing metabolites (Fig. 1) did not lead to an increased accumulation of these metals in the plant. The only strain significantly improving Zn and Cd uptake was *Streptomyces* AR17, which slightly

decreased Zn and Cd extractability in the culture supernatant experiment (Fig. 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 (Fig. 1) also impaired Zn and Cd uptake into the plant (Fig. 2b,c).

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, *Agromyces* AR33 tendentiously improved 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 tendentiously reduced Zn and Cd extraction per plant.

In conclusion, the tested rhizosphere strains had effects on metal mobilization and uptake of *Salix caprea*. For the total amounts of extracted metal our results indicated that plant growth promotion might be an important parameter besides 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 studies including e.g. detailed chemical (metabolite) and genetic analysis are 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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