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ORIGINAL ARTICLE

Culturable bacteria from Zn- and Cd-accumulating *Salix caprea* with differential effects on plant growth and heavy metal availability

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Abstract

Aims: To characterize bacteria associated with Zn/Cd-accumulating *Salix caprea* regarding their potential to support heavy metal phytoextraction.
Methods and Results: Three different media allowed the isolation of 44 rhizosphere strains and 44 endophytes, resistant to Zn/Cd and mostly affiliated with *Proteobacteria*, *Actinobacteria* and *Bacteroidetes/Chlorobi*. 1-Aminocyclopropane-1-carboxylic acid deaminase (ACCD), indole acetic acid and siderophore production were detected in 41, 23 and 50% of the rhizosphere isolates and in 9, 55 and 2% of the endophytes, respectively. Fifteen rhizosphere bacteria and five endophytes were further tested for the production of metal-mobilizing metabolites by extracting contaminated soil with filtrates from liquid cultures. Four *Actinobacteria* mobilized Zn and/or Cd. The other strains immobilized Cd or both metals. An ACCD- and siderophore-producing, Zn/Cd-immobilizing rhizosphere isolate (*Burkholderia* sp.) and a Zn/Cd-mobilizing *Actinobacterium* endophyte were inoculated onto *S. caprea*. The rhizosphere isolate reduced metal uptake in roots, whereas the endophyte enhanced metal accumulation in leaves. Plant growth was not promoted.
Conclusions: Metal mobilization experiments predicted bacterial effects on *S. caprea* more reliably than standard tests for plant growth-promoting activities.
Significance and Impact of the Study: Bacteria, particularly *Actinobacteria*, associated with heavy metal-accumulating *Salix* have the potential to increase metal uptake, which can be predicted by mobilization experiments and may be applicable in phytoremediation.

Introduction

Metal accumulation in shoots is an adaptation of various plants to metalliferous substrates (Baker *et al.* 2000) and can be exploited for the removal of heavy metals from polluted soils. This sustainable remediation strategy is referred to as phytoextraction (McGrath and Zhao 2003). Harvested shoots of certain extractor plants can be used for recovery of metals (Chaney *et al.* 2007) or for energy production (Keller *et al.* 2005; van Ginneken *et al.* 2007).

Zn and Cd hyperaccumulators occurring in the northern temperate zone are typically small herbaceous *Brassicaceae*, such as *Thlaspi cearulescens* and *Arabidopsis halleri* (Baker and Brooks 1989). Despite high concentrations of metals accumulated in their leaves, the metal extraction efficiency of these plants is limited because of low biomass production (Chaney *et al.* 2000). Recently, *Salix caprea* (goat willow) trees, growing on heavy metal-contaminated sites in central Europe, have been found to accumulate up to 116 mg Cd kg^{-1} and 4680 mg Zn kg^{-1} in their leaves (Unterbrunner *et al.* 2007). Metal-accumulating *Salix*

species are ideal extractor plants, as they produce as much as 10 dry t per ha per year of easily harvestable leaf biomass and develop a massive root system in the topsoil (Pulford and Watson 2003). Indeed, the Zn content and particularly the Cd content of a moderately contaminated soil (13·4 mg kg^{-1} Cd, 955 mg kg^{-1} Zn) 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 interior of plants (endophytes) contribute to heavy metal uptake and tolerance (de Souza *et al.* 1999; Lodewyckx *et al.* 2001; Whiting *et al.* 2001; Abou-Shanab *et al.* 2003a). The underlying mechanisms are not yet fully understood. Bacterial production of 1-aminocyclopropane-1-carboxylic acid deaminase (ACCD), siderophores and indole-3-acetic acid (IAA), as well as metal detoxification and metal mobilization, might be involved (van der Lelie *et al.* 2000; Glick 2003; Gadd 2004; Sessitsch and Puschenreiter 2008).

ACCD is an enzyme that has no known function in bacteria but antagonizes ethylene synthesis in plants, by cleaving the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC). Accelerated ethylene synthesis ('stress ethylene') occurs in plants under environmental stress – including heavy metal stress – and causes damage to the plant organism (Glick 2003). ACCD-producing bacteria can inhibit stress-ethylene formation (Glick 2003) and have been reported to alleviate heavy metal toxicity (Burd *et al.* 1998). Bacterial siderophores are high-affinity Fe(III) chelators for the acquisition of iron under iron-limiting conditions. Under certain conditions, bacterial siderophores can be taken up by plant roots (Bar-Ness *et al.* 1992). Iron deficiency is a frequent symptom 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 it has been speculated that they are involved in heavy metal mobilization (Abou-Shanab *et al.* 2003a; Kuffner *et al.* 2008). 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 postefflux sequestration of metals, i.e. the prevention of extruded metal ions from re-entering the cell by precipitation, chelation or by binding to exopolymers (Diels *et al.* 1995; Salt *et al.* 1999). Endophytes equipped with postefflux sequestration systems may contribute to heavy metal detoxification in plants (Lodewyckx *et al.* 2001). Bacteria producing ACCD, siderophores and IAA as well as metal-resistant phenotypes are commonly found in association with heavy metal-accumulating plants (Lodewyckx *et al.* 2001; Abou-Shanab *et al.* 2003b; Idris *et al.* 2004; Zaidi *et al.* 2006). Moreover, isolates with these characteristics have been shown to promote plant growth in the presence of heavy metals (Salt *et al.* 1999; Burd *et al.* 2000; Dell'Amico *et al.* 2008) and/or to enhance metal accumulation (Zaidi *et al.* 2006; Jiang *et al.* 2008; Braud *et al.* 2009). Presumably, the most direct way for bacteria to support heavy metal accumulation in plants is metal mobilization. Soil and rhizosphere bacteria can increase metal mobility and plant availability by various processes, potentially including redox transformations and the release of protons and organic acids (Gadd 2004). Although heavy metal mobilization has been assessed less frequently than IAA, ACCD, siderophores and metal resistance, individual bacteria have been demonstrated to promote heavy metal solubility and metal uptake in plants simultaneously (Whiting *et al.* 2001; Abou-Shanab *et al.* 2003a; Rajkumar and Freitas 2008).

The aim of this work was to characterize culturable bacteria associated with Zn/Cd-accumulating *S. caprea* trees regarding their potential to promote heavy metal phytoextraction. We compared rhizosphere and endophytic bacteria to gain insight into the characteristics of these subpopulations and into their role in increasing growth and heavy metal accumulation.

Materials and methods

Sampling

For the isolation of bacteria, *S. caprea* trees growing on a former zinc/lead mining and processing site in Arnoldstein, Austria (Table 1) were sampled in June 2004. The site, which has been described in detail by Unterbrunner *et al.* (2007), is contaminated with Pb, Zn and Cd. Branches with leaves and fine roots with adherent rhizosphere soil from a depth of 0–25 cm were taken from four trees growing close to the contamination source, cooled to 4°C within 4 h and processed within 48 h.

Isolation of rhizosphere bacteria and endophytes

For the isolation of rhizosphere bacteria, 5 g of fine roots and adherent soil was shaken in 50 ml of 1% tryptic soy broth (0·3 g l^{-1} TSB; Merck, Darmstadt, Germany) for 2 h at room temperature. Soil particles and roots were allowed to settle for 1 h, and tenfold dilutions were plated

Table 1 Selected parameters of the experimental soils

	Mobilization experiment	Plant experiment
Site	Arnoldstein (Austria)	Celje (Slovenia)
Sand/silt/clay (g kg^{-1})	350/550/100	450/340/210
CAC (mmol kg^{-1})	247	273
Organic carbon (g kg^{-1})	24·6	38·5
pH (H_2O)	7·21	7·54
Total metal contents (in aqua regia)		
Zn (mg kg^{-1})	1760	608·2
Cd (mg kg^{-1})	32·7	4·9
Pb (mg kg^{-1})	6560	98·5
Mobile fraction of metals (in 1 mol l^{-1} NH_4NO_3)		
Zn (mg kg^{-1})	2·56	0·268
Cd (mg kg^{-1})	0·64	0·01
Pb (mg kg^{-1})	3·81	<0·001

CAC, cation exchange capacity, measured at soil pH.

on three Zn-containing isolation media of different nutrient strength as different laboratory media select for different bacteria. Ten per cent tryptic soy agar (TSA) (3 g l^{-1} TSB, 15 g l^{-1} agar), 1% TSA and 0·08% diluted nutrient broth agar (0·08% DNBA) (Janssen *et al.* 2002) were used. All media were amended with cycloheximidine (100 μg ml^{-1}) to inhibit fungal growth and with $ZnSO_4$ (2 mmol l^{-1}) to select for Zn-resistant bacteria.

For specific isolation of endophytes, 15 leaves (3·2–5·0 g) and five green branch segments of 0·5 cm diameter (2·0–3·5 g) were randomly picked from each tree, surface-sterilized in 5% (w/v) sodium hypochlorite for 5 min and rinsed with sterile water. Branches were further dipped into 70% ethanol, flamed and peeled. Surface-sterilized leaves and branches were cut into small pieces and ground with 50 ml of 0·9% NaCl in a stomacher (Stomacher Circulator; Seward, W. Sussex, UK) five times for 1 min. In the intervening intervals, the suspensions were cooled on ice. Tenfold dilutions were plated on 10% TSA, 1%TSA and 0·08% DNBA. Furthermore, xylem sap was extracted from lignified branches using a Scholander bomb and plated without diluting. Ten per cent 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. Colonies of all distinguishable morphology types were isolated on phosphate-poor morpholinepropanesulfonic acid medium (MOPS) (Neidhardt *et al.* 1974) containing 0·1% glucose and 1 mmol l^{-1} $ZnSO_4$.

PCR-RFLP analysis of rhizosphere and endosphere isolates

To allow discrimination at the strain level, 16S–23S intergenic spacer (IGS) DNA was amplified from all isolates, using the primers p23SRO1 (5′-GGCTGCTTCTAAGCCAAC-3′) and pHr (5′-TGCGGCTGGATCACCTCCTT-3′) (Massol-Deya *et al.* 1995). DNA was released by boiling one loop of bacterial cells for 10 min in 150 μl sterile deionized H_2O. One microlitre of lysis product was used as template in 50 μl PCR containing two units Taq DNA polymerase (Invitrogen, Carlsbad, CA), 0·2 mmol l^{-1} of each dNTP, 0·15 μmol l^{-1} of each primer and 1·5 mmol l^{-1} $MgCl_2$. The thermal programme included an initial denaturation of 5 min at 95°C, 30 cycles of 1 min denaturation at 95°C, 1 min annealing at 55°C and 2 min elongation at 72°C and a final elongation of 10 min at 72°C. PCR products (15 μl) were digested for 4 h at 37°C with *Alu*I (Invitrogen). Restriction fragments were electrophoretically separated in 3% agarose gels.

DNA extraction and partial 16S rDNA sequencing

For the amplification and analysis of the 16S rRNA gene, bacterial DNA was isolated by bead beating and phenol–chloroform extraction (Sessitsch *et al.* 2001). About 100 ng of DNA was used in 50 μl PCR with the primers 8f (5′-AGAGTTTGATCCTGGCTCAG-3′) (Weisburg *et al.* 1991) and 1520r (5′-AAGGAGGTGATCCAGCCGCA-3′) (Edwards *et al.* 1989). The composition of the reaction mix was identical with that described for the IGS PCR, the annealing temperature was 53°C. PCR products were purified through Sephadex G-50 (Amersham Biosciences, Buckinghamshire, UK) columns, and 2 μl was used as template in 10 μl sequencing reactions using 0·4 μmol l^{-1} of the primer 518r (5′-ATTACCGCGGCTGCTGG-3′) (Liu *et al.* 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., Foster City, CA). Nucleotide Blast (Zhang *et al.* 2000) was used to search the sequence database of the National Center for Biotechnology Information (NCBI) for identified relatives of the isolates.

Nucleotide sequence accession numbers

The 16S rRNA gene sequences of rhizosphere and endophyte isolates described in this study have been entered to the database of the NCBI under the accession numbers GQ342532 to GQ342602. When identical sequences were obtained from several strains, one representative sequence was deposited. Sequences of the following strains are also representative for the strains indicated between parentheses: RD343 (RD358), RD344 (RD336), EI149 (ED123, EX145), ED162 (EX151, EX161), EX128 (ED306), RD74 (RX14, RD308), EI198 (EX107), EI187 (EI178, ED320, ED328), EI189 (EI174), RX196 (EX45), RI3 (RX2).

Heavy metal resistance, ACCD, IAA and siderophore production

Minimal inhibitory concentrations (MICs) of Zn and Cd, ACCD activity and siderophore release were analysed in agar plate assays as described previously (Kuffner *et al.* 2008). Briefly, heavy metal MICs were determined as the lowest concentrations inhibiting growth on phosphate-poor minimal medium. ACCD production was determined as the ability to grow with ACC as sole nitrogen source. Siderophore production was determined as the ability to scavenge iron from chrome azurol-S agar. IAA was determined in bacteria grown in the dark (on 5 g l^{-1} glucose, 0.025 g l^{-1} yeast extract and 0.204 g l^{-1} L-tryptophane, 15 g l^{-1} agar) by covering colonies with Salkowsky reagent (35% perchloric acid, 3 $mmol^{-1}$ $FeCl_3$) and dark incubation for 30 min.

Metal mobilization by bacterial metabolites

Bacteria were grown in 10% TSB medium (pH 7·2) at room temperature and 200 rev min^{-1}. Late-log cultures were grown until the highest cell density was reached. Stationary phase cultures were allowed to grow twice as long. The optical density of the cultures was measured at 600 nm (OD_{600}) with a DU® 640 Spectrophotometer (Beckman, Brea, CA). Cells were removed from the cultures by centrifugation (8000 ***g***, 15 min, 4°C) and filtration (0·2 μm Ministart filters; Sartorius AG, Göttingen, Germany). The filtrates containing bacterial metabolites were stored at −20°C, and filtrate pH was measured prior to mobilization analysis. Air-dried and sieved (2 mm) soil from Arnoldstein (Kuffner *et al.* 2008) contaminated with Zn, Cd and Pb (Table 1) was used for the mobilization experiment. One gram of soil was shaken with 5 ml of culture filtrate (2 h, 20 inversions per min). The soil particles were removed by centrifugation (7000 rev min^{-1}, 5 min) and filtration (0·45 μm filters, Roth), and Zn and Cd in the filtrates were quantified by Atomic Absorption Spectroscopy (AAS; Perkin-Elmer 2100). Fresh 10% TSB medium was used for control extractions. Bacterial cultures and control medium were prepared in triplicates. From each replicate, three aliquots were shaken with soil and analysed three times ($n = 9$). In a pre-experiment with one mobilizing and three immobilizing strains, the mobilization capacity of filtrates from late-log and stationary cultures was compared. In the stationary phase, immobilization effects were increased by 20–40%, and mobilization effects were more than doubled (data not shown). Therefore, only stationary culture filtrates were analysed from the remaining isolates.

Inoculation of *Salix caprea* plantlets

Cuttings of *S. caprea*, clone Boku 04 CZ-024 (derived from Kutna Hora, Czech Republic) (Unterbrunner *et al.* 2007), were pre-grown for 1 year in a sand–soil mixture under nonsterile conditions in a climate chamber (14/20°C day/night temperature; 80% air moisture, 16 h light per day). The experimental soil originated from Celje, Slovenia (Table 1). This moderately contaminated soil was used, because phytoextraction with trees will be principally applied to remediate intermediate heavy metal pollutions (Dickinson and Pulford 2005). The soil was air-dried, sieved (2 mm) and gamma ray irradiated with 25 kGray for 24 h by Mediscan GmbH (Seibersdorf, Austria). Bacteria were grown in 10% TSB until the late logarithmic phase, harvested by centrifugation (2420 ***g***, 10 min, 4°C) and resuspended in 50 mmol l^{-1} potassium phosphate buffer (pH 7) to an OD_{600} of 0·4 (about 10^8 cells per ml). Willow plantlets were transplanted into pots containing 800 g of Celje soil, and 10 ml of bacterial suspension were applied with a pipet into the soil surface surrounding the plantlets. Thus, about 10^9 cells were applied to each plantlet. Pots were positioned in a greenhouse (16/22°C day/night temperature; 60% relative humidity, 16 h light per day) following a randomized design, and each treatment was replicated four times. After 12 weeks, roots, shoots and leaves were harvested and washed with tap water. To remove metals from the apparent free space of the root tissues, roots were sonicated in 0·05 mol l^{-1} $CaCl_2$ for 10 min and rinsed with deionized water. Plant material was dried at 80°C for 24 h and weighed to assess the dry matter weight (dw). Subsamples of 0·5 g were digested in 4 ml HNO_3 (Puriss. p.a.; Sigma–Aldrich Handels GmbH, Vienna, Austria) and 1 ml $HClO_4$ (Puriss. p.a., Sigma–Aldrich Handels GmbH) at 225°C using an automated heating block (Digester DK 42/26; Velp Scientifica, Milano, Italy). Cd and Zn concentrations were determined by inductively coupled plasma mass spectrometry (ICP-MS, Elan 9000 DRCe; Perkin-Elmer, Waltham, MA).

Statistical analysis

Statistic analysis was carried out in STATISTICA 6 (StatSoft, Tulsa, OK, USA). Analysis of variance (ANOVA) followed by *post hoc* Dunnet test was carried out to identify significant effects of bacterial strains in mobilization assays and plant inoculation 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 on different isolation media

For the isolation of plant-associated bacteria, xylem 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 mmol l^{-1} of Zn. The number of colony-forming units (CFUs) varied between samples from different trees. One gram of fresh leaves or branches yielded $8 \times 10^2 \pm 2 \times 10^2$ CFUs, 1 ml xylem sap $6 \times 10^3 \pm 2 \times 10^3$ CFUs, 1 g of rhizosphere soil $8 \times 10^5 \pm 2 \times 10^5$ CFUs. One hundred and eighty endophytes and 180 rhizosphere isolates were screened by RFLP analysis of 16S–23S IGS DNA. Forty-four different IGS-types were identified among the rhizophere bacteria, and 44 among the endophytes. Strains with identical IGS types were grouped into operational taxonomic units (OTUs). Forty-three per cent of the rhizosphere OTUs originated from 10% TSA, 11% from 1% TSA and 21% from 0·08% DNBA (Fig. 1a). Among endophytes, 10% TSA, 1% TSA and 0·08% DNBA, respectively, contributed 43, 18 and 23% to isolate diversity (Fig. 1b). Twenty-five per cent of the rhizosphere OTUs and 16% of the endophyte OTUs were found on more than one medium. Of the endophyte OTUs, 55% originated from xylem sap, 14% from leaves, 11% from twigs, and 20% were found in more than one plant organ (Fig. 1c).

Phylogenetic affiliation of rhizosphere and endosphere isolates

For phylogenetic identification, 16S rDNA was amplified from one representative strain of each OTU. Fragments of about 450 bp were sequenced and compared to NCBI sequence database entries (Table 2). *Alphaproteobacteria*, *Betaproteobacteria*, *Actinobacteria* and *Bacteroidetes/Chlorobi* accounted for 30, 16, 23 and 25% of rhizosphere isolate diversity, respectively. Furthermore, *Gammaproteobacteria* and *Firmicutes* were represented in the rhizosphere isolate collection. Culturable endophytes were phylogenetically distinct from rhizosphere bacteria. Although rhizosphere and endophyte isolate collections included both 44 different species, endophyte diversity was much lower at the genus level. More than 60% of the analysed endophytes clustered with alphaproteobacterial genera *Sphingomonas* and *Methylobacterium.* Twenty-three per cent of the endophytes were affiliated with the division *Actinobacteria.* Moreover, the divisions *Betaproteobacteria*, *Firmicutes* and *Bacteroidetes/Chlorobi* were represented by individual endophyte isolates. Eight rhizosphere bacteria and seven endophytes had <97% 16S rRNA gene sequence identity to described bacteria and may therefore represent novel bacterial lineages. Ten of the potentially novel species clustered with *Bacteroidetes/Chlorobi* group organisms.

Production of ACCD, IAA and siderophores

ACCD activity was determined as the ability to use ACC as sole nitrogen source. Of the 44 rhizosphere strains, 41% were able to metabolize ACC (Table 2). Among endophytes, 9% were ACCD positive, and 60% were negative. For the remaining endophyte isolates, ACCD production could not be determined because of lack of a suitable minimal medium. IAA formation was found in 23% of the rhizosphere bacteria and in 55% of the analysed endophytes (Table 2). Forty-one per cent of the selected rhizosphere bacteria produced siderophores. In contrast, only one siderophore-producing endophyte was identified (Table 2). The distribution of IAA production, ACCD activity and siderophore release was not correlated. Within the genera *Bradyrhizobium*, *Variovorax*, *Methylobacterium* and *Frigoribacterium,* isolates with very similar or identical 16S rDNA sequences differed in siderophore or IAA production. Fifteen rhizosphere isolates and five endophytes, representing dominant genera with and without IAA, ACCD and siderophore production were selected for further analysis. In general, the ability to produce IAA was frequently found among endophytes, whereas siderophore and ACCD production was more common among rhizosphere bacteria.

Zn and Cd resistance of endophyte and rhizosphere isolates

Table 3 shows Zn and Cd resistance of the 20 selected isolates. MICs of Zn ranged between 12 and 16 mmol l^{-1}. The highest observed Cd MIC was 8 mmol l^{-1} for *Bradyrhizobium* RI12. *Frigoribacterium* EX166 was inhibited by 0·5 mmol l^{-1}, the lowest Cd dose given. The Cd MICs of the remaining strains ranged between 1 and 6 mmol l^{-1}. The levels of Cd and Zn resistance did not correlate with each other.

Heavy metal mobilization from soil

The ability of bacterial metabolites to mobilize metals was tested by leaching contaminated soil with filtrates of stationary bacterial cultures and quantifying the extracted Zn and Cd (Fig. 2). Sterile TSB medium extracted 2·62 mg Zn (equivalent to *c.* 0·1% of the soil concentration) and 173 μg Cd per kg soil (equivalent to *c.* 0·5% of the soil concentration). Cd extractability was reduced by metabolites from 14 of 15 rhizosphere bacteria and by four of five endophytes. Eight of these rhizosphere

(a)

Division	putative genus	OTUs and representative isolates
Alphaproteobacteria		
	Bosea	RX290
	Sphingomonas	RX30 RX101
	Bradyrhizobium	RX18 RX29 RI12 RI158 RD268 RD293 RD343 RD358 RI270
	Rhizobium	RI252
Betaproteobacteria		
	Burkholderia	RX232
	Collimonas	RX265
	Janthinobacterium	RX243
	Variovorax	RX56 RX14 RD308 RD74
Gammaproteobacteria		
	Hafnia	RX229
	Pseudomonas	RX228
Actinobacteria		
	Leifsonia	RX68
	Microbacterium	RX22
	Nocardia	RX2 RI3
	Streptomyces	RX17 RI9
	Rhodococcus	RX84 RX138
	Arthrobacter	RI251
	Mycobacterium	RD334
Firmicutes		
	Carnobacterium	RI234
Bacteroidetes/Chlorobi group		
	Chryseobacterium	RD139
	Flavobacterium	RD336 RD318 RD344
	Luteifibra	RX233
	Pedobacter	RX196 RX99
	Mucilaginibacter	RX97
	Sphingoterrabacterium	RI121
	Bacteroidetes	RD319 RI269

(b)

Division	putative genus	OTUs and representative isolates
Alphaproteobacteria		
	Methylobacterium	EX135 EI174 EI178 EI187 EI189 EI198 EI199 ED320 ED323 ED325 ED328 ED329 EI215 EX107
	Sphingomonas	EX127 EX128 EX129 EX131 EX151 EX161 EI54 ED123 ED154 ED162 ED306 ED314 EI149 EX145
	Ochrobactrum	EX276
Betaproteobacteria		
	Massilia	EX109
Actinobacteria		
	Leifsonia	EX283
	Kocuria	EX51
	Rhodococcus	EX150
	Subtercola	EX244
	Plantibacter	EX44
	Frigoribacterium	EX166 EX48
	Microbacterium	EX104 EX72
	Frondihabitans	ED222
Firmicutes		
	Bacillus	EX241
Bacteroidetes/Chlorobi group		
	Pedobacter	EX45 EI208
	Spirosoma	EX36

(c)

Division	putative genus	OTUs and representative isolates
Alpha-Proteobacteria		
	Methylobacteria	EI174 EI189 ED325 EI199 ED329 ED320 ED323 EX135 EI178 EI198 ED328 EI215 EI187 EX107
	Sphingomonas	EX127 ED306 ED314 EI54 ED123 EX145 EX131 ED154 EX151 EX161 ED162 EX128 EX129 EI149
	Ochrobactrum	EX276
Beta-Proteobacteria		
	Zoogloea	EX109
Actinobacteria		
	Leifsonia	EX283
	Subtercola	EX244
	Kocuria	EX51
	Rhodococcus	EX150
	Frigoribacterium	EX166 EX48
	Microbacterium	EX72 EX104
	Plantibacter	EX44
	Frondihabitans	ED222
Firmicutes		
	Bacillus	EX241
Bacteroidetes/Chlorobi group		
	Pedobacter	EI208 EX45
	Spirosoma	EX36

Table 2 Phylogenetic affiliation, 1-aminocyclopropane-1-carboxylic acid deaminase (ACCD), indole-3-acetic acid (IAA) and siderophore (SID) production of (A) rhizosphere bacteria and (B) endophytes

Isolate*	Closest identified relative† [accession number]	Identity (%)	ACCD‡	IAA‡	SID‡
(*A*) *Rhizosphere bacteria*					
Alphaproteobacteria					
RI270	*Bradyrhizobium* sp. [AY547290]	99	–	++	–
RD343, RD358	*Bradyrhizobium* sp. [AY547290]	99	–	++	+
RX18	*Bradyrhizobium* sp. [D84604]	98	++	–	–
RI158	*Bradyrhizobium* sp. [D84604]	98	–	–	+
RD268	*Bradyrhizobium* sp. [FJ390940]	100	–	–	–
RI12	*Bradyrhizobium* sp. [FJ390909]	99	–	–	++
RD293	*Bradyrhizobium* sp. [FJ390909]	99	–	–	–
RX29	*Bradyrhizobium* sp. [D84604]	98	ND	–	–
RI252	*Rhizobium* sp. [EU184089]	99	–	++	–
RX101	*Sphingomonas* sp. [U63962]	97	–	++	–
RX30	*Sphingomonas* sp. [EU337119]	98	–	–	–
RX290	*Bosea* sp. [FM174104]	99	–	–	–
Betaproteobacteria					
RD74, RD308	*Variovorax* sp. [CP001635]	100	++	–	++
RX14	*Variovorax* sp. [AF451851]	99	+	–	–
RX56	*Variovorax* sp. [EU934231]	99	–	–	++
RX232	*Burkholderia* sp. [U37344]	98	++	–	++
RX243	*Janthinobacterium* sp. [AM989104]	100	–	+	–
RX265	*Collimonas* [AJ496445]	99	–	–	++
Gammaproteobacteria					
RX228	*Pseudomonas* sp. [AM989273]	99	++	–	++
RX229	*Hafnia alvei* [M59155]	98	++	–	++
Actinobacteria					
RX17	*Streptomyces* sp. [EF063466]	99	++	–	–
RI9	*Streptomyces* sp. [EU594469]	99	+	–	–
RI251	*Arthrobacter* sp. [FJ517624]	100	+	+	–
RI3	*Nocardia* sp. [FJ529720]	99	+	–	+
RX2	*Nocardia* sp. [FJ529720]	99	–	–	++
RX84	*Rhodococcus* sp. [AY168597]	99	+	–	++
RX138	*Rhodococcus* sp. [EU016150]	99	+	+	–
RX68	*Leifsonia* sp. [FJ189782]	98	+	ND	ND
RX22	*Microbacterium* sp. [AM403723]	100	+	–	–
RD334	*Mycobacterium* sp. [AY215324]	97	–	–	–
Firmicutes					
RI234	*Carnobacterium* sp. [AB213026]	97	++		++
Bacteroidetes/Chlorobi group					
RD336, RD344	*Flavobacterium* sp. [AM988921]	99	–	–	++
RD318	*Flavobacterium* sp. [AM110997]	95	–	–	–
RX196	*Pedobacter* sp. [AM988946]	95	–	–	–
RX99	*Pedobacter* sp. [AM988949]	96	–	–	–
RX233	*Luteifibra* sp. [AM237312]	94	–	++	++
RX139	*Chryseobacterium* sp. [EU336939]	97	ND	ND	–
RX97	*Mucilaginibacter* sp. [EU747841]	96	–	–	ND
RD319	*Sphingoterrabacterium* sp. [AB267718]	96	+	–	–

Figure 1 Origin of bacterial isolates. Each cell represents one operational taxonomic unit as determined by RFLP analysis of 16S–23S intergenic spacer DNA. Representative isolates, which were selected for further analysis, are indicated inside the cells. The strain nomenclature gives information about the isolation medium [X for 10% TSA, I for 1% TSA and D for diluted nutrient broth agar (DNBA)]. (a) Isolation media of rhizosphere isolates. (■) 10% TSA; (▩) 1% TSA; (□) 0·08% DNBA and (■) more than one medium. (b) Isolation media of endophytic isolates. (■) 10% TSA; (▩) 1% TSA; (□) 0·08% DNBA and (■) more than one medium. (c) Plant organ habitats of endophytic isolates. (□) Xylem sap; (▩) leaves; (■) twings and (■) in more than one component.

Table 2 (*Continued*)

Isolate*	Closest identified relative† [accession number]	Identity (%)	ACCD‡	IAA‡	SID‡
RI269	*Filimonas* sp. [AB362776]	92	+	+	ND
RI121	Uncult. *Bacteroidetes* bact. [EF018676]	94	–	–	–
(*B*) *Endophytes*					
Alphaproteobacteria					
ED154	*Sphingomonas* sp. [AY444826]	98	–	+	–
EI149, EX145, ED123, ED162, EX151, EX161	*Sphingomonas* sp. [AY336556]	100	–	++	–
EI54	*Sphingomonas* sp. [AY336556]	98	ND	ND	ND
EX127, EX128, **ED306**	*Sphingomonas* sp. [AY336550]	98	–	++	–
ED314	*Sphingomonas* sp. [AM988654]	99	–	++	–
EX129, EX131	*Sphingomonas* sp. [AM900781]	94	–	–	–
EI178, EI187, ED328	*Methylobacterium* sp. [AY741724]	99	ND	++	–
EX107	*Methylobacterium* sp. [AY741724]	99	–	–	–
EI198	*Methylobacterium* sp. [AY741724]	99	–	++	–
EI215	*Methylobacterium* sp. [AM403498]	100	++	–	–
EX135	*Methylobacterium* sp. [AM403498]	100	–	++	–
ED320, ED323	*Methylobacterium* sp. [AY364034]	99	ND	++	–
ED329	*Methylobacterium* sp. [AM989028]	99	–	–	–
ED325	*Methylobacterium* sp. [DQ872157]	96	ND	–	–
EI199	*Methylobacterium* sp. [CP001002]	99	ND	–	ND
EI189, EI174	*Methylobacteriaceae bact*. [AM989029]	100	ND	–	–
EX276	*Ochrobactrum* sp. [AB120120]	100	++	+	–
Betaproteobacteria					
EX109	*Massilia* sp. [FM955855]	97	–	+	–
Actinobacteria					
EX72	*Microbacterium* sp. [AB271048]	98	–	–	–
EX104	*Microbacterium* sp. [EU373326]	100	–	–	–
EX166	*Frigoribacterium* sp. [EU584512]	99	–	–	–
EX48	*Frigoribacterium* [EU584512]	99	ND	++	–
EX150	*Rhodococcus* sp. [EU016150]	99	+	–	–
EX283	*Leifsonia* sp. [AM931710]	95	ND	+	–
EX44	*Plantibacter* sp. [AM396918]	100	–	–	–
EX51	*Kocuria* sp. [DQ448783]	95	ND	+	–
ED222	*Frondihabitans* sp. [DQ525859]	95	++	–	–
EX244	*Subtercola* sp. [AJ310412]	100	ND	+	–
Firmicutes					
EX241	*Bacillus* sp. [FJ937943]	99	–	ND	–
Bacteroidetes/Chlorobi group					
EX45	*Pedobacter* sp. [AM988946]	95	ND	ND	–
EI208	*Pedobacter* sp. [AY275498]	99	–	–	++
EX36	*Spirosoma*-like sp. [X89911]	96	ND	–	ND

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

†Phylogenetic affiliations are based on sequence analysis of about 450 bp of the 16S rRNA gene.

‡ND For certain strains, the production of ACCD, IAA or siderophores could not be determined because of lack of suitable minimal media.

bacteria and two of the endophytes also immobilized Zn. The Zn- and Cd-immobilizing rhizosphere bacteria included five of the eight analysed siderophore producers. None of the siderophore producers had a positive effect on Zn or Cd extractability. One rhizosphere bacterium (RD334) increased Cd mobility. Three endophyte strains (EX72, EX104 and EX166) doubled Zn mobility. EX72 also strongly enhanced Cd extraction. The effects of the bacterial metabolites on Zn and Cd mobility correlated positively with each other ($r = 0{\cdot}60$; $P < 0{\cdot}05$) but did not correlate with culture filtrate pH. The pH of fresh sterile 10% TSB was 7·0 and rose during the growth of all analysed strains to values between 7·5 and 8·7 in the stationary cultures (data not shown).

Table 3 Zn and Cd resistance of selected rhizosphere bacteria and endophytes

	MIC (mmol l^{-1})	
Isolate	Zn	Cd
RX74	12	4
RX228	12	1·5
RX232	16	4
RX233	12	6
RX265	12	1
RD336	16	1
RD344	16	1
RI12	16	8
RX14	12	4
RX17	12	1
RX18	14	6
RI121	12	4
RI252	14	4
RD268	12	4
RD334	12	6
EX72	12	4
EX104	12	6
EX166	12	0·5
EI198	14	4
ED306	12	1·5

MIC, minimal inhibitory concentration.

Inoculation of *Salix caprea* plantlets

The rhizosphere isolate RX232 and the endophyte EX72 were selected for an inoculation experiment with *S. caprea*. RX232 was a strong ACCD producer, siderophore producer and Zn/Cd immobilizer. In contrast, EX72 did not produce ACCD, IAA and siderophores but had the ability to mobilize Zn and Cd. About 10^9 cells were applied to 1-year-old nonsterile plantlets, which were grown in gamma-sterilized soil moderately contaminated with Zn and Cd (Table 1). Three months later, dry weight, Zn and Cd concentration of leaves and roots were determined. No visible symptoms of heavy metal toxicity (i.e. chlorosis and/or necrosis of leaves) were observed. Neither the rhizosphere strain nor the endophyte significantly influenced biomass production of roots or leaves. However, strain RX232 promoted root growth tendentiously (Fig. 3a). In all plants, Zn and Cd concentrations in leaves were significantly higher than in roots (Fig. 3b,c). With respect to noninoculated control plants, plants inoculated with RX232 showed significantly reduced concentrations of Zn and Cd in roots ($P < 0·05$), and plants inoculated with EX72 showed significantly increased Zn and Cd concentrations in leaves ($P < 0·05$).

Discussion

The majority of environmental bacteria are unable to grow on laboratory media. However, culturable rhizosphere bacteria and endophytes may serve as model organisms for studying plant–microbe interactions. Moreover, culturable bacteria can be enriched and used for various applications such as for bioaugmentation (Pilon-Smits 2005). Highest diversity of heavy metal–tolerant rhizosphere bacteria and endophytes was found on 10% TSA, suggesting that many plant-associated taxa require higher nutrient concentrations for *in vitro* growth. In contrast to that, bulk soil bacteria tend to prefer diluted media (Janssen *et al.* 2002). Still, 1% TSA and 0·08% DNBA favoured slowly growing heavy metal-tolerating species, and each medium contributed 10–20% to endophyte and rhizosphere isolate diversity. In heavy metal accumulators, leaves are the site of metal detoxification and storage (Vazquez *et al.* 1994; Schat *et al.* 2000), and remarkably, several endophyte strains have been obtained from this environment. Most endophytes were isolated from the xylem, for which we have no information on the prevailing heavy metal concentration.

In accordance with observations on other plants (Berg *et al.* 2005), the rhizosphere and endosphere of *S. caprea* were colonized by different communities. Two of our observations on community composition corresponded well with data from culture-independent surveys. First, culturable endophytes were dominated by *Sphingomonas* and *Methylobacterium*, which also prevailed in endophytic 16S rDNA sequence libraries from Zn- and Cd-accumulating *Thlaspi* (Idris *et al.* 2004). These genera seem to have a competitive advantage in metal-enriched shoots independent of the plant species and may also have plant growth-promoting effects. Individual methylobacteria have been shown to promote the growth of heavy metal–accumulating plants in contaminated soil (Madhaiyan *et al.* 2007), and inoculation with *Sphingomonas* has improved heavy metal uptake (Abou-Shanab *et al.* 2003a). Second, rhizosphere and endosphere isolates included many different organisms of the division *Actinobacteria*. rRNA-based community analysis showed that *Actinobacteria* might be a dominant part in the metabolically active rhizosphere population of heavy metal accumulators (Gremion *et al.* 2003). Our data suggest that *Actinobacteria* were similarly important in rhizosphere and endosphere of *S. caprea*. In addition, culturable methylobacteria, sphingomonads and members of the division *Actinobacteria* have been isolated previously from *Thlaspi* and *Alyssum* species accumulating Zn/Cd and Ni, respectively (Lodewyckx *et al.* 2002; Idris *et al.* 2004; Barzanti *et al.* 2007).

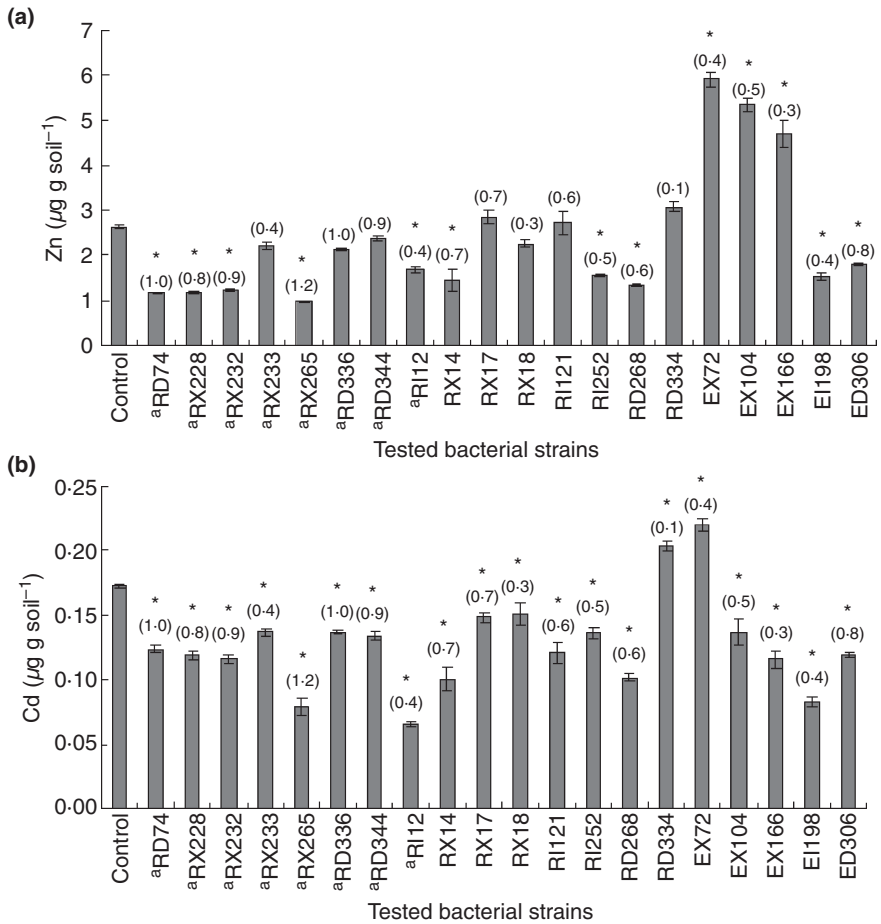

Figure 2 Effect of bacterial growth products (10% tryptic soy broth medium) on the capacity to extract (a) Zn and (b) Cd from soil. Error bars show standard errors of the mean ($n = 9$). Samples with extraction values differing significantly from the control ($P < 0{\cdot}05$) are labelled with asterisks (*). Cell densities (optical densities at 600 nm) in cultures prior to filtration are indicated between brackets above diagram bars. [a]Siderophore producers.

Rhizosphere bacteria and endophytes were isolated on Zn-containing media and showed Zn and Cd resistances much higher than those reported for isolates from Zn-hyperaccumulating *Thlaspi caerulescens* plants (Lodewyckx *et al.* 2002). Moreover, the observed tolerance levels exceeded the concentration of mobile Zn in bulk soil of the sampling site (Wenzel and Jockwer 1999), suggesting Zn and Cd mobilization in the rhizosphere of *S. caprea* and high bioavailability in the endosphere. The resistant bacteria may contribute to metal detoxification *via* postefflux sequestration (Diels *et al.* 1995; Salt *et al.* 1999).

Plate assays for IAA, ACCD and siderophore production were carried out with all 44 rhizosphere bacteria and 44 endophytes to asses their potential for plant growth promotion. Important proportions of the rhizosphere isolates produced IAA (23%), ACCD (41%) and siderophores (41%). Among endophytes, IAA synthesis was more frequent (55%) than in the rhizosphere, whereas ACCD activity and siderophore release were only detected in few individuals. The number of ACCD-producing endophytes may have been underestimated in this study. For about 30% of the endophyte isolates, ACCD activity could not be determined as they failed to grow on the test medium. Idris *et al.* (2004) found a higher percentage of ACCD-producing bacteria in the endosphere than in the rhizosphere of Ni-accumulating *Thlaspi goesingense* and explained this with the more intimate relationship of endophytes to their host plant. These authors also observed siderophore production in all culturable endophytes and rhizosphere bacteria. In *S. caprea*, the competition for iron might be less pronounced. The highest Zn resistance was found in siderophore-producing bacteria. Siderophore release can be induced by the presence of

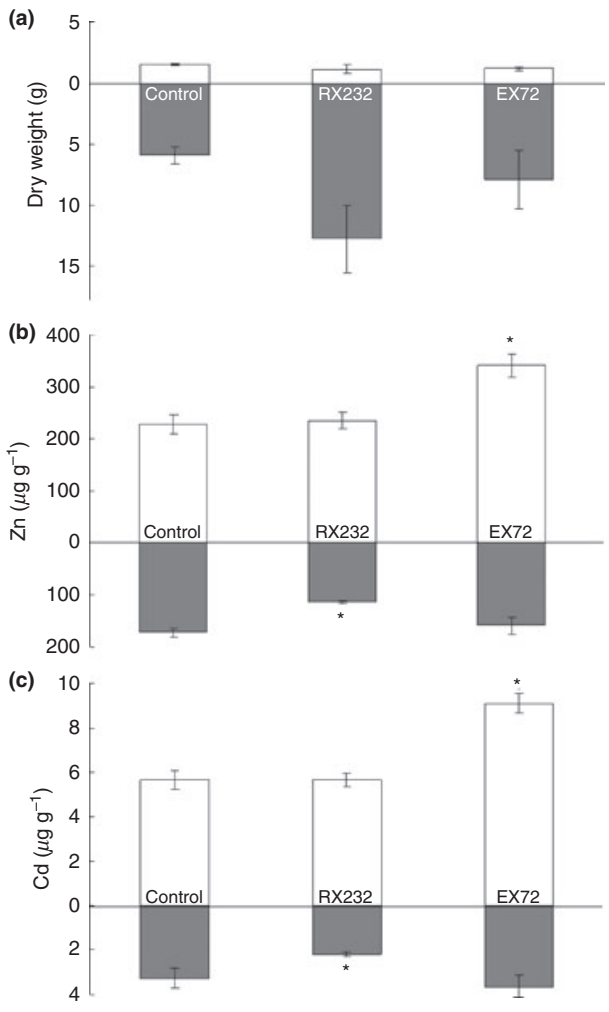

Figure 3 Biomass and concentration of Zn and Cd in *Salix caprea* roots and leaves 12 weeks after inoculation with the rhizosphere isolate RX232 and the endophyte EX72. (a) Biomass, (b) Zn content, (c) Cd content. Mean values obtained from four replicate plants are shown. Error bars are standard errors of the mean ($n = 4$). Values differing significantly from the control ($P < 0{\cdot}05$) are labelled with an asterisk (*). (□) roots and (▨) leaves.

heavy metals, and siderophores may be involved in bacterial Zn resistance, for instance in postefflux chelation (van der Lelie *et al.* 2000).

Apart from supporting growth of the accumulating biomass, rhizosphere bacteria may mobilize heavy metals for enhanced uptake by plant roots (Gadd 2004). Twenty isolates representing dominant rhizosphere and endophyte taxa with and without the capacity to produce IAA, ACCD and siderophores were tested for their Zn and Cd mobilization potential. Most of these strains reduced the extractability of Zn and Cd when contaminated soil was shaken with filtrates of stationary liquid cultures. The medium pH rose during bacterial growth in all cultures. However, the immobilization was not affected by pH, because pH adjustments of fresh 10% TSB (pH 7·2–8·9) did not alter Zn extraction (data not shown). Heavy metal immobilization has been observed previously, when rhizosphere bacteria were directly applied to contaminated soil (Abou-Shanab *et al.* 2003a; Madhaiyan *et al.* 2007). Various products of bacterial growth, ranging from organic acids and alcohols to exopolymers, can bind metal ions and may trap them to soil particles (Gadd 2004). Moreover, heavy metal–resistant bacteria have been observed to remove heavy metals from liquid medium by precipitation (Diels *et al.* 1995). Culture filtrates of four slowly growing strains affiliated with the division *Actinobaceria* strongly increased the extractability of Cd and/or Zn. This was not an effect of acidification, because 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). Additionally, in none of the studies on changes in rhizosphere pH, a significant pH decrease was found (Wenzel *et al.* 2004). This suggests that pH is generally not involved in metal mobilization by plants and their associated rhizosphere bacteria. None of the analysed siderophore producers mobilized Zn or Cd, and six of eight siderophore producers even immobilized both metals. However, the role of siderophores in the observed Zn/Cd mobilizations and immobilizations remains unclear because the experiment was carried out in an iron-containing medium, which may not have induced the release of bacterial siderophores. The four metal-mobilizing strains were unable to produce siderophores showing that other mechanisms or metabolites play an important role in mobilization. They may either synthesize-specific ligands for Zn and/or Cd or form nonspecific metal-binding compounds during growth in TSB, which were not formed in the siderophore test minimal medium. Chemical analysis is necessary to reveal the nature of the metal-mobilizing and metal-immobilizing compounds, and it remains to be confirmed whether they can be produced from the substrates available in the rhizosphere and endosphere. Interestingly, three of the identified metal-mobilizing *Actinobacteria* were endophytes. Many endophytes derive from the rhizosphere (Huang 1986) and may mobilize heavy metals during rhizosphere colonization. Inside the plant, they may be involved in metal translocation. Rhizosphere and endosphere *Actinobacteria*, particularly *Microbacterium* strains, have been observed previously to mobilize metals and to enhance their accumulation in several plants (Whiting *et al.* 2001; Abou-Shanab *et al.* 2003a; Kuffner *et al.* 2008; Sheng *et al.* 2008).

The strains RX232 and EX72 were selected for a bioaugmentation experiment with *S. caprea*, based on their contrasting characteristics. RX232 was a rhizosphere isolate, closely related to a powerful plant growth promoter, *Burkholderia phytofirmans* PsJN (Sessitsch *et al.* 2005). It showed particularly intense ACCD activity, siderophore production and Zn/Cd-immobilizing effects. EX72 was an endophyte affiliated with the family of *Microbacteriaceae* (*Actinobacteria*), unable of IAA-, ACCD- and siderophore production, but strongly mobilizing Zn and Cd in the mobilization assay. In a previous study, we demonstrated that growth of heavy metal-accumulating *S. caprea* can be promoted by bacterial inoculation (Kuffner *et al.* 2008). The ACCD- and siderophore-producing rhizosphere strain RX232 tendentiously promoted root growth. However, neither RX232 nor EX72 had a significant effect on root or shoot biomass production. *Salix caprea* tolerates higher concentrations of Zn and Cd than present in the experimental soil (Unterbrunner *et al.* 2007). Most likely, the metal stress given in our experimental system did not exceed plant's internal detoxification capacities and did not limit root or leaf growth. The presence of the rhizosphere strain RX232 reduced Zn and Cd import into the roots of *S. caprea* but did not affect the amount of heavy metal translocated to shoots. RX232 may have produced the same metal-immobilizing compounds as in the *in vitro* assay and thus may have reduced Zn/Cd availability to *S. caprea*. The endophyte EX72-enhanced Zn/Cd accumulation in leaves but did not change the amount of metals retained in roots. EX72 may have colonized the rhizosphere and may have mobilized Zn/Cd by release of those metabolites that had been detected in the *in vitro* assay. The surplus of available Zn/Cd may have been taken up by *S. caprea* roots and may have stimulated root-to-leaf translocation activity. The endophyte EX72 may also have entered the plant interior and may have induced the expression of heavy metal transporters. In all treatments, the accumulation of Zn and Cd in *S. caprea* was affected in a similar manner, suggesting that both metals were influenced by the inoculated strains likewise and no competition for uptake occurred.

Screening for IAA, ACCD and siderophore production revealed a high plant growth-promoting potential among culturable rhizosphere bacteria and endophytes from *S. caprea*. But as these traits were frequent and their distribution was not correlated, plate tests failed to identify a group of 'most promising growth promoters' producing ACCD, IAA and siderophores. Metal mobilization capacity was more laborious to test than IAA, ACCD and siderophore production and identified only few mobilizers. However, results of mobilization assays predicted the effect of individual bacteria on heavy metal accumulation in *S. caprea* more reliably. In the greenhouse experiment, the Zn/Cd mobilizer increased, and the Zn/Cd immobilizer reduced the uptake of these metals. More isolates have to be tested to confirm this correlation. Furthermore, analysis of metal mobilization drew our attention to the division *Actinobacteria*. Compiling our observations and the results of other studies, *Actinobacteria* constitute diverse and active populations in heavy metal accumulators and may fulfil key functions in metal uptake and translocation. The Zn/Cd-mobilizing *Microbaterium* EX72 could be employed for improving phytoextraction efficiency of *S. caprea*. Future studies should address the specific requirements of *Actinobacteria*, and techniques to favour their growth and activity in phytoremediation environments should be developed.

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