RESEARCH ARTICLE

Identification of dominant bacterial phylotypes in a cadmium-treated forest soil

Anna Lazzaro[1], Franco Widmer[2], Christoph Sperisen[1] & Beat Frey[1]

[1]Soil Sciences, Swiss Federal Institute for Forest, Snow and Landscape Research WSL, Birmensdorf, Switzerland; and [2]Molecular Ecology, Agroscope Reckenholz-Tänikon Research Station ART, Zurich, Switzerland

Correspondence: Beat Frey, Swiss Federal Institute for Forest, Snow and Landscape Research WSL, Zuercherstrasse 111, CH-8903 Birmensdorf, Switzerland. Tel.: +41 0 447392541; fax: +41 0 447392215; e-mail: beat.frey@wsl.ch

Received 9 July 2007; revised 19 October 2007; accepted 22 October 2007.
First published online January 2008.

DOI:10.1111/j.1574-6941.2007.00417.x

Editor: Max Häggblom

Keywords
Burkholderia; *Actinobacteria*; heavy metals; 16S rRNA gene; quantitative real-time PCR; terminal restriction fragment length polymorphism (T-RFLP).

Abstract

The presence of heavy metals in soils can lead to changes in microbial community structure, characterized by the dominance of groups that are able to tolerate contamination. Such groups may provide good microbial indicators of heavy-metal pollution in soil. Through terminal restriction fragment length polymorphism (T-RFLP) profiling, changes in the bacterial community structure of an acidic forest soil that had been incubated with cadmium (Cd) for 30 days were investigated. T-RFLP revealed, in particular, three operational taxonomic units (OTUs) strongly dominating in relative abundance in the contaminated soil. By cloning of the amplified 16S rRNA genes and partial sequencing of 25 clones, these three dominant OTUs were phylogenetically characterized. One dominant OTU in the cadmium-contaminated soil was derived from *Betaproteobacteria*, genus *Burkholderia*, and the other two were from uncultured members of the class *Actinobacteria*, closely related to the genus *Streptomyces*. To confirm T-RFLP data, four primers were designed on the basis of this study's dominant sequences, targeting the OTUs corresponding to *Burkholderia* or *Actinobacteria*. Real-time PCR showed that *Burkholderia* target sequences were more abundant in cadmium-treated soil ($7.8 \times 10^7 \pm 3.0 \times 10^7$ targets g^{-1} soil) than in untreated soil ($4.0 \times 10^6 \pm 8.9 \times 10^5$ targets g^{-1} soil). It was concluded that the genus *Burkholderia* includes species that may be particularly dominant under cadmium contamination.

Introduction

In recent decades, the continuous accumulation of heavy metals (HMs) in terrestrial ecosystems has reached levels of concern, and is a risk for human and environmental health, owing to their toxicity and persistence in soil (Vig *et al.*, 2003; Sharma & Agrawal, 2005). The toxicity of HMs is strongly related to their chemical forms in the soil, which determine their bioavailability to the different receptor organisms (Peijnenburg *et al.*, 2007).

Investigation of the adverse effects of HMs on the soil microbiota is of particular interest, given the well-furnished array of tools for the analysis of both microbial activity (Winding *et al.*, 2005) and microbial community structure (Smalla *et al.*, 2007). Moreover, soil microorganisms are sensitive to HM contamination. For example, HMs in soils reduced microbial biomass (Hartmann *et al.*, 2005) and carbon mineralization (Lazzaro *et al.*, 2006b), and led to disturbances of enzymatic activities (Frey *et al.*, 2006).

Microbial communities undergoing a HM stress also tend to change structure and diversity (Pennanen, 2001; Hartmann *et al.*, 2005; Frey *et al.*, 2006; Lazzaro *et al.*, 2006b). Several studies report a low bacterial diversity in HM-impacted environments, as noted for example in the denaturing gel electrophoresis profiles of mine soil (Hu *et al.*, 2007), or in the microbial composition near a copper smelter (Wang *et al.*, 2007). Decreased diversity due to the effect of HMs was also observed within a single taxa, such as with culturable members of the genus *Pseudomonas* (Brandt *et al.*, 2006). In general, it appears that HM contamination leads to a rearrangement of the microbial communities, as the HM-sensitive populations are reduced or disappear, and are gradually replaced by other indigenous populations, which can better tolerate and adapt to the disturbed environmental conditions. This effect can be clearly assessed in laboratory-based experiments, where soils are incubated under exactly the same environmental conditions. The influence of external factors on the microbial community

structure can therefore be distinguished from the effects of HMs, through comparison of the contaminated samples with an uncontaminated control. Replacement of sensitive bacterial phylotypes, and an increase in numbers of *Actinomycetales* were shown, for example, in a microcosm experiment with HM-treated soils (Nakatsu *et al.*, 2005).

Adaptation mechanisms differ with the time of exposure to the disturbance. In the short term, the intrinsically tolerant populations survive the pollution. In the long term, the surviving organisms may adapt to the disturbed environment using phenotypic or genetically based adaptation mechanisms (Dìaz-Raviña & Bååth, 1996; Oger *et al.*, 2001). Although individual genes coding for metal resistances have been assessed (Naz *et al.*, 2005), a detailed understanding of the key indigenous organisms able to tolerate HM pollution is still lacking.

In general, HM-polluted soils have been reported to be enriched in Gram-positive bacteria such as *Bacillus* and *Arthrobacter* and of Gram-negative bacteria such as *Pseudomonas* and *Burkholderia* (Kozdroj & van Elsas, 2001; Ellis *et al.*, 2003; Hèry *et al.*, 2003). *Proteobacteria* seem to represent a very important group in terms of resistance to HM, as found, for example, in a cadmium (Cd)-contaminated activated sludge (Tsai *et al.*, 2005). Among the *Proteobacteria, Betaproteobacteria* appear to include HM-sensitive groups (Tsai *et al.*, 2005), although they are also dominant in HM-impacted roadside soils (Park *et al.*, 2006) and in HM- and hydrocarbon-contaminated soil (Joynt *et al.*, 2006).

The capacity of certain bacterial groups to survive and grow in extreme or disturbed environments, such as under high levels of HM contamination, may be a useful feature for risk assessment and bioremediation of polluted sites. Molecular-based techniques have proven to be particularly accurate and useful for the characterization of microbial communities, including the analysis of unculturable populations. Terminal restriction fragment length polymorphism (T-RFLP) of the 16S rRNA gene, for example, has successfully described complex bacterial communities in disturbed soils at high resolution (Frey *et al.*, 2006; Widmer *et al.*, 2006), allowing the identification of significant T-RFs, which were particularly abundant under HM contamination (Turpeinen *et al.*, 2004; Frey *et al.*, 2006). However, direct assignment of single T-RFs to individual taxa appears to be difficult and imprecise, as different bacterial groups may often produce T-RFs of the same length (Marsh *et al.*, 2000).

The main objective of the present study was to investigate groups of the indigenous bacterial community of a forest soil responding to a cadmium treatment in a short-term microcosm experiment (Lazzaro *et al.*, 2006a) as analysed by T-RFLP. Cadmium is one of the most important toxic HMs involved in atmospheric deposition and contamination of forest ecosystems (Reinds *et al.*, 2001; Starr *et al.*, 2003). T-RFLP profiling of the 16S rRNA gene was used to obtain a wider overview of the general effects of cadmium on bacterial community structure, including HM-sensitive and HM-tolerant populations. The study was focused on the identification and characterization of bacterial groups surviving the cadmium stress, which could be important in monitoring and bioremediation of HM-contaminated sites. In the cadmium-contaminated samples, through molecular cloning and sequencing, three dominant operational taxonomic units (OTUs), representing possible candidate bioindicators of cadmium contamination were observed and identified. This study further attempted to design primer sets for these key groups to verify the results of the T-RFLP profiling and to analyse the abundance in the cadmium-treated soil. The results contribute to the characterization of adaptive responses of bacteria to HM stress.

Materials and methods

Soil sampling and microcosm experiment

The soil selected for this study derives from a natural forest site (Lausanne), with a content of total (HNO_3-extractable) cadmium, which is below 0.01 mg cadmium kg^{-1} soil dry weight. The soil is a dystric cambisol with a pH of 4.5, and with a composition of 62% sand, 25% silt and 13% clay. The soil sampling and microcosm set-up is described in detail in Lazzaro *et al.* (2006a). Briefly, the microcosms were treated with $CdCl_2$ solution (Sigma-Aldrich, St Louis, MO), to obtain 1900 mg cadmium kg^{-1} soil dry weight. This level of cadmium was the highest in a series of cadmium additions of the previous study (Lazzaro *et al.*, 2006a), and was necessary to obtain an effective water-soluble cadmium concentration. Similar cadmium treatments have been used in previous studies (Renella *et al.*, 2003, 2005). Control (untreated) microcosms received 10 mL of MQ water (Millipore) without cadmium. Untreated controls and cadmium-treated microcosms were prepared in quadruplicate, and incubated for 30 days at 20 °C under 50% external humidity. This incubation length was optimal to visualize the first bacterial responses to the cadmium treatment avoiding the effects of carbon starvation in bulk soils.

Cadmium bioavailability

Bioavailability of cadmium was measured through water (Blaser *et al.*, 2000) and Lakanen (Lakanen & Erviö, 1971) extractions, obtained through filtration of the soil samples to remove particulate matter (Lazzaro *et al.*, 2006a). Cadmium concentrations were measured by atomic adsorption spectroscopy using a spectrAA 300/400 (Varian, Palo Alto, CA).

Extraction of DNA, PCR amplification and T-RFLP of the bacterial 16S rRNA gene

DNA extraction from each of the replicate microcosms was performed using a modification of the bead-beating method described by Frey *et al*. (2006) and Lazzaro *et al*. (2006a). DNA was quantified using Pico Green (Molecular Probes, Basel, Switzerland), and herring sperm DNA standards (Invitrogen, San Diego, CA) as described by Hartmann *et al*. (2005).

Before PCR, 5 ng μL^{-1} DNA was pretreated with 3 $\mu g\,\mu L^{-1}$ bovine serum albumin (BSA) for 2 min at 95 °C (Lazzaro *et al*., 2006a). This step was necessary to reduce the effects of inhibitors on the subsequent PCR reaction. Ten to fifty nanograms of pretreated DNA was then added to the PCR reaction mix [1.5 mM $MgCl_2$-containing PCR buffer (Qiagen, Hilden, Germany), 0.5 mM additional $MgCl_2$, 400 μM of dNTP mixture (Catalys, Wallisellen, Switzerland), 0.3 $\mu g\,\mu L^{-1}$ BSA, 0.2 μM of each primer (Microsynth, Balgach, Switzerland), 2 U of HotStarTaq polymerase (Qiagen)] in a volume of 50 μL. The bacterial 16S rRNA gene was amplified with forward primer 27F [5′ labelled with 6-FAM (6-carboxyfluorescein), Table 1] and reverse primer 1378R (Heuer *et al*., 1999, Table 1). PCR was carried out in a PTC-100 thermocycler (MJ Research, Waltham, MA), with 35 cycles under the conditions described in Table 1.

The PCR amplicon was digested for 3 h at 37 °C with an equal volume of digestion mix [10 U (each) HhaI and HaeIII, 1% (v/v) Y^+ Tango buffer (Fermentas, Burlington, ON)]. Desalting of the digestions was performed with Montage single-sample microspin columns (Millipore, Billerica, MA), according to the manufacturer's instructions.

Capillary electrophoresis was performed on 2 μL of the digestion products in 11.9 μL of HIDI formamide (Applied Biosystems, Foster City, CA) and 0.1 μL of ROX500 DNA fragment length standard (Applied Biosystems). The samples were first denatured for 2 min at 95 °C and immediately chilled on ice. Electrophoresis was performed for 30 min at 60 °C with an ABI Genetic Analyzer 310 (Applied Biosystems).

Analysis of T-RFLP data

The sizes and relative abundances of T-RFs were quantified with the GENESCAN version 3.1 and GENOTYPER Version 2.5 software (Applied Biosystems). OTUs were defined as peaks with a size of $x \pm 0.5$ relative migration units (rmu) and a height of at least 150 fluorescence units in all the four replicate profiles of water control or cadmium treatment. Relative abundance was assessed by converting the peak height of each OTU into the percentage value of the total peak heights in the profile. The average ($n = 4$) relative abundances of each OTU in each cadmium treatment were then compared for significant differences with the controls by one-way ANOVA corrected with the Tukey *post hoc* test (SYSTAT 10, Statsoft inc., Tulsa, OK).

Cloning of PCR-amplified products

Two pooled PCR products were ligated in the p-GEM T-easy plasmid vector (Catalys, Wallisellen, Switzerland) according to the manufacturer's instructions. Libraries were screened with colony PCR in 15 μL of a PCR mix as described above using 0.2 μM of each vector-specific primer M13F and M13R (Catalys) and 25 amplification cycles. The PCR conditions are described in Table 1. The quality of the PCR products was assessed by electrophoresis of 1.5 μL of the

Table 1. Sequences, probe match assignments and PCR conditions of the primers used in this study and of the specific primers designed

Primer combinations		Target OTU	Primer description, Probe Match no. of hits* (affiliations)	Primer sequence (5′–3′)	PCR† annealing temperature	Reference
Forward	M13F		Vector-specific	CGCCAGGGTTTTCCCAGTCACGAC	48 °C	
Reverse	M13R		Vector-specific	TCACACAGGAAACAGCTATGAC	48 °C	
Forward	1369F		Real-time PCR	CGGTGAATACGTTCYCGG	55 °C	Smith *et al*. (2006)
Reverse	1492R		Real-time PCR	GGWTACCTTGTTACGGACTT		Smith *et al*. (2006)
Forward	27F-FAM		Universal primer	AGAGTTTGATCMTGGCTCAG		Heuer *et al*. (1997)
Reverse 1	1378R		Universal primer	CGGTGTGTACAAGGCCCGGGAACG	48 °C	Heuer *et al*. (1999)
Reverse 2	522R		Universal primer	TACCGCGGCKGCTGGCA		Widmer *et al*. (2006)
Reverse 3	Burk_484_Rev	214	15 (*Burkholderiaceae*)	AACCCAGAGGTTTTCTT	51 °C	This study
Reverse 4	Burk_225_Rev	214	82 (*Burkholderiaceae*)	CCTGTAGCGGGAGGTCC	56 °C	This study
Reverse 5	Acta_278_Rev	222/223	4406 (*Firmicutes, Actinobacteria*)	GTCGCCTTGGTAGGCCG	56 °C	This study
Reverse 6	Actb_279_Rev	222/223	287 (*Actinobacteria/ Actinomycetales*)	CAAAGCCTTGGTAGGCCAT	56 °C	This study

*Probe match was performed over 205 165 published sequences of the RDP database.

†PCR conditions were as follows: 15 min at 95 °C, 45 s at 94 °C, 45 s at *X* °C and 2 min 72 °C, where *X* is the annealing temperature specific for each primer pair, and reported in the Table.

PCR products on a 2% agarose gel and ethidium bromide staining.

In order to identify clones yielding the fragments of interest, a second PCR was performed. The PCR products obtained with the M13F and M13R primers were first purified with PCR clean-up microspin columns (Millipore). One microlitre of the purified PCR products was reamplified with 15 cycles in 15 μL PCR mix as described in Table 1 using the fluorescently labelled universal eubacterial primers 27F (5′ labelled with HEX) and 1378R. Amplicons were digested with HhaI and HaeIII, and prepared for T-RFLP as described previously. In addition, before performing T-RFLP, 1 μL of digestions of the whole bacterial community amplified previously with the FAM-labelled 27F and 1378R primers were added to the samples. Capillary electrophoresis and T-RF size can influence migration, and assignment of a fragment to a precise size can be problematic. By overlapping the whole community profile with the clone profile, the clone could be assigned to a precise fragment size category.

Sequencing reactions and analysis

Sequencing of the clones yielding the selected fragments of interest was performed on both strands using two separate sequencing reactions with the unlabelled universal eubacterial primer 27F or internal reverse primer 522R (Widmer *et al.*, 2006, Table 1). Fifty to 100 ng of DNA, quantified previously on a 2% agarose gel, was sequenced using the BigDye Version 1.1 Terminator Sequencing Kit (Applied Biosystems), containing 1.6 pmol of the primer, and HPLC water to an end volume of 10 μL. Reactions were purified through Sephadex G-50 (Sigma-Aldrich) columns according to the manufacturer's instructions and sequenced with an ABI 310 automatic sequencer using POP6 as the running polymer (Applied Biosystems) and running the samples for 45 min at 50 °C.

Sequences were assembled with AUTOASSEMBLER (Version 1.4; Applied Biosystems). The resulting consensus sequences were analysed with Chimera_Check of the Ribosomal Database Project (Cole *et al.*, 2003) in order to detect chimeric sequences in the authors's data. Sequences were matched with the sequences in GenBank using the BLASTN program of the National Centre for Biotechnology Information (Benson *et al.*, 2005). The program MULTALIN (Corpet, 1988) was used to align the sequences obtained with similar sequences retrieved from the database. Sequences of different length were cut to obtain alignments of the same length. For the phylogenetic analysis, sequences of *c.* 450 bp were finally aligned. Neighbour-joining phylogenetic trees with Jukes–Cantor distances were constructed with the TREECON package, version 1.3. (Van de Peer & De Wachter, 1994). One hundred bootstrap replicate resampling datasets were generated. The sequences were deposited in the Genbank database under accession numbers DQ646664–DQ646675.

Primer design and assessment

Specific PCR primers for the clone sequences of interest were designed on the aligned sequences of each T-RF group with the aid of BIOEDIT version 7.0.4.1. software (Hall, 1999). PCR conditions were chosen with the aid of Biomath calculator (http://www.promega.com/biomath/calc11.htm). The reverse oligonucleotide primers designed were first evaluated over the whole of the published ribosomal sequences with the Probe Match facility of the RDP database.

PCR was then performed on the replicate control and cadmium-treated DNA samples and on the amplicons of selected clones. Reactions were carried out by adding 5 ng of DNA template or 1 μL of diluted (1 : 10 with HPLC water) clone amplicon to a PCR reaction mix as described before, to give an end volume of 25 μL, using the designed primers in combination with the FAM-labelled 27F forward primer (Table 1). PCR was performed with 35 cycles (Table 1). Annealing temperatures were experimentally optimized to maximize the specificity of PCR amplification. Amplicons were digested with HhaI and HaeIII restriction enzymes, and analysed by T-RFLP as described previously. The specificity of both the reverse primers was further validated by cloning the PCR products. The HhaI/HaeIII restriction patterns of the transformed clones were examined first through RFLP, by electrophoresis on a 2% agarose gel and ethidium bromide staining, and subsequently through T-RFLP. Selected colonies containing the T-RF of interest were then sequenced as described above using the vector-specific primers T7 and SP6.

Real-time PCR assay of *Burkholderia* target sequences

Real-time quantitative (SYBR Green I) PCR was performed in MicroAmp optical 96-well plates using the automated ABI Prism 7700 sequence detector (Applied Biosystems). Each 25-μL reaction contained the following: 0.5 μM of each primer (27F/Burk484rev, see Table 1), 12.5 μL of SYBR Green PCR master mix, including HotStar Taq DNA polymerase, Quanti Tec SYBR Green PCR Buffer, dNTP mix, SYBR Green I, ROX and 5 mM $MgCl_2$ (QuantiTect SYBR Green PCR Kit, Qiagen), 0.2 mg mL^{-1} BSA, 11 μL of diluted DNA corresponding to 2.5 ng of total soil DNA and RNase-free water to complete the 25-μL volume. PCR conditions were 15 min at 95 °C, followed by 40 cycles of 95 °C for 15 s, 51 °C for 30 s (annealing) and 72 °C for 45 s (extension), followed by a final data acquisition step at 80 °C. The 16S rRNA gene was targeted for amplification using the following primers described previously by Smith *et al.* (2006): Bact

1369F and Prok 1492R (see Table 1). The conditions for 16S rRNA gene real-time PCR were the same as before, with an annealing step at 55 °C for 30 s. Each plate included triplicate reactions per DNA sample and the appropriate set of standards. After the DNA amplification cycles, melting curve analysis was performed to confirm that the obtained signals were caused by the specific amplicon. The C_t values for each PCR reaction were automatically calculated and analysed by the ABI prism sequence detection systems software (version 1.9). A plasmid standard containing the target region was generated from sequenced clones (Cd_Burk484_001 and Cd_Burk484_002). Plasmids were isolated using the Wizard Plus SV Minipreps DNA Purification System (Catalys) with DNA concentrations determined by the PicoGreen assay (Molecular Probes). A standard curve was obtained by plotting C_t values as a function of log-transformed copy numbers. Tenfold serial dilutions of the plasmid ranging from 10^1to10^8 copies were used as the template, in triplicate, to determine the calibration curve. There was a linear relationship between the log of the plasmid DNA copy number and the C_t values across the specified concentration range ($r^2 = 0.997$) and a slope of -3.47 (data not shown), indicating a high amplification efficiency of 94% (Smith *et al.*, 2006). Data were presented as the average copy number of targets per gram of soil (dry weight).

Results

Bioavailable cadmium concentrations

After 30 days of incubation, the amount of cadmium retrieved in the Lakanen extracts was 2060 ± 177 mg cadmium kg^{-1} soil dry weight, and was not significantly different from that added at the beginning of the experiment (1900 mg cadmium kg^{-1} soil dry weight). In the water controls, water-extractable cadmium was 0.01 ± 0.00 mg cadmium kg^{-1} soil dry weight. In the cadmium-treated samples, water extracts retrieved 562 ± 41 mg cadmium kg^{-1} soil dry weight, which corresponds to $29.5 \pm 2.1\%$ of the total cadmium added.

T-RFLP analysis

As all the microcosms had been incubated under exactly the same conditions, and as all the replicates within one treatment had the same T-RFLP profile, the control microcosms were used as a measure of the effects of time and nutrient availability on the bacterial communities. In this way, the community changes observed could be assigned to cadmium contamination and not to other external factors. The T-RFLP profiles of the cadmium treatments appeared to be profoundly modified and were different when compared with the untreated controls (Fig. 1a and b). Overall, 76

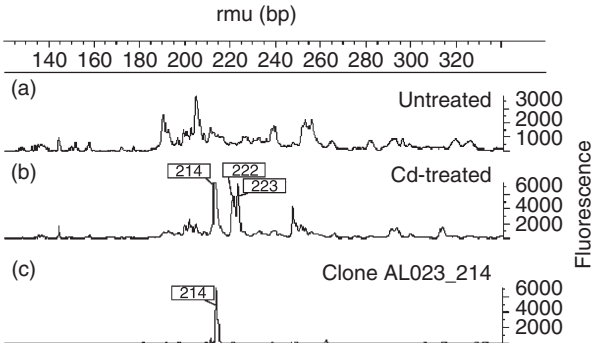

Fig. 1. T-RFLP profiles of one representative replicate of the untreated (a) and cadmium-treated (b) microcosm soil samples, overlapped with a profile of a selected clone (CdL023_214) yielding T-RF 214 (c). OTUs selected for analysis are labelled with the corresponding rmu values. Ordinates represent fluorescence.

OTUs were detected in the control samples, whereas in the cadmium-treated soils the number of OTUs detected was reduced to 64. Moreover, 33 OTUs significantly ($P < 0.05$) decreased, and 12 OTUs significantly ($P < 0.05$) increased in relative abundance in comparison with the control (data not shown). In particular, a region of the fingerprint, ranging from *c.* 190 to 240 rmu was identified, where a large number of changing OTUs were observed. In this region, the dominance of three specific OTUs of 214, 222 and 223 rmu in the cadmium treatment was noticed (Fig. 1b). They all had a relative abundance of $> 7\%$ of the total profile.

16S rRNA gene clone designation and phylogenetic profiles

As the replicate T-RFLP profiles were highly homogeneous, and to maximize throughput, two different PCR amplification products of replicate cadmium-treated samples were pooled before ligation. The profiles of the clones screened showed the presence of a dominant T-RF (peak), which had a fragment length predictable from the T-RFLP profile of the total bacterial community (Fig. 1c). More than 10% of the screened clones corresponded to OTU 214. Eighty per cent of the 25 partial sequences (400–500 nucleotides) analysed showed at least 96% identity to known sequences in the NCBI database (Table 2). The sequences corresponding to OTU 214 appeared to be affiliated with members of the *Betaproteobacteria*. *Actinobacteria* were represented by the sequences corresponding to OTUs 222 and 223 (Table 2). GENESCAN-predicted T-RF sizes differed from T-RF sizes retrieved after sequencing from 3 to 6 bp for T-RF category 214, and from 0 to 3 bp for T-RF categories 222 and 223.

Phylogenetic analyses

Phylogenetic analysis of the *Betaproteobacteria* sequences (Table 2, Fig. 2) revealed that OTU 214 was strongly

Table 2. Phylogenetic assignment of clones to the GenBank database entries

Phylogenetic affiliation	Clone	Size* predicted T-RF (bp) GENESCAN	Size† observed T-RF (bp) sequencs	Restriction site (HaeIII/HhaI)	Closest relative	Sequence identity (%)	Accession no.	Closest cultured relative	Sequence identity (%)	Accession no.
Actinobacteria										
	CdL035_222	222	222	HaeIII	*Kitasatospora putterlickiae*	95	AY189976			
	CdL036_222	222	222	HaeIII	*Streptomyces pancagri*	95	AB245388			
	CdL037_222	222	222	HhaI	*Streptomyces lavendulae*	91	DQ459019			
	CdL038_222 (2)‡	222	222	HaeIII	Uncultured bacterium	97	DQ451517	*Actinoplanes multisporangius*	93	AB037007
	CdL040_222	222	222	HaeIII	Uncultured bacterium	99	DQ451517	*Micromonosporaceae bacterium Ellin7233*	94	AY673399
	CdL041_222	222	223	HaeIII	*Actinobacterium* Aac-30	96	AB180773			
	CdL042_223 (2)	223	226	HaeIII	*Actinobacterium* Aac-30	98	AB180773			
	CdL043_223	223	225	HaeIII	Bacterium Ellin5116	98	AY234533			
	CdL044_223	223	224	HaeIII	*Streptacidiphilus carbonis*	93	AF074412			
	CdL046_223	223	222	HaeIII	Bacterium 12202	98	AY639903			
Betaproteobacteria										
	CdL031_214 (11)	214	219	HaeIII	Uncultured eubacterium	99	AJ292644	*Burkholderia* sp. WBF5	99	DQ777734
	CdL028_214	214	220	HaeIII	Uncultured soil bacterium	97	DQ378200	*Burkholderia* sp.	96	AB025790
	CdL029_214	214	217	HaeIII	Uncultured eubacterium	95	AJ233511	*Burkholderiaceae* bacterium	95	DQ490295

*Predicted T-RF sizes from GENESCAN analysis.

†Size observed T-RF (bp) sequences.

‡In the phylogenetic analysis, when more than one clone gave the same BLAST results, only one representative clone is shown. Numbers in parentheses indicate the number of clones producing the same result.

associated with unculturable *Betaproteobacteria* (AJ292644), related to members of the genera *Burkholderiaceae* (e.g. DQ490295) and *Burkholderia* (e.g. AM086244). The phylogenetic tree derived from the *Actinobacteria* sequences (Fig. 3) showed that OTU 222, except for clone AL041_222, formed two clusters. A first cluster (cluster 1, Fig. 3) included clones belonging to the genera *Streptomyces* (AY785161, AB245388) and *Kitasatospora* (AY189976), and close to the genus *Streptacidiphilus* (e.g. AF074412). A second cluster (cluster 2, Fig. 3) contained clones related to uncultured soil bacteria (e.g. AY913402). Except for clone CdL044_223, OTU 223 clustered (cluster 3, Fig. 3) close to uncultured *Actinobacteria* (e.g. AB180773). Clones CdL041_222 and CdL044_223 were not clearly associated with defined genera. Moreover, although clone CdL041_222 was classified as a member of OTU category 222, it appeared to be more closely related to members of OTU category 223.

Assessment of group-specific primers and real-time PCR

To verify and confirm the results obtained by T-RFLP analysis, four reverse primers for the specific detection of OTUs 214, 222 and 223 were designed (Table 1). Probe Match search in RDP-II identified 15 of 205 165 published sequences that matched the primer sequence Burk_484_Rev. Thirteen of the 15 sequences belonged to the genus *Burkholderia* and two to the genus *Pandoraea*, a member of the family *Burkholderiaceae*. Ten sequences, all belonging to the genus *Burkholderia*, gave theoretical T-RFs between 217 and 219 bp, all with a HaeIII restriction site. Among the hits targeted, groups were observed to also appear in the BLAST search, such as an uncultured eubacterium (AJ292644), forest soil bacterium (AY043580) and *Burkholderia* species (AJ704380, AJ704384). The second primer designed, Burk_225_Rev, gave 82 hits, all belonging to the genus *Burkholderia* and none to the genus *Pandoraea*, including sequences with both HaeIII and HhaI restriction sites.

For PCR-based assays, each reverse primer with the FAM-labelled 27F primer was used to obtain T-RF sizes comparable with those observed in the authors' T-RFLP profiles. PCR amplification of DNA from control and cadmium-treated soil samples, together with PCR of clones containing T-RF 214 (clones CdL023_214 and CdL025_214), yielded results consistent with the results of the T-RFLP analysis. In the T-RFLP analysis from the amplicon derived from the primer combination 27F/Burk_484_Rev, a major peak of size 214 rmu was visible in both untreated and cadmium-treated samples. For the primer combination 27F/Burk_225_Rev, a main peak with size 211 rmu appeared instead in the T-RFLP profiling (data not shown).

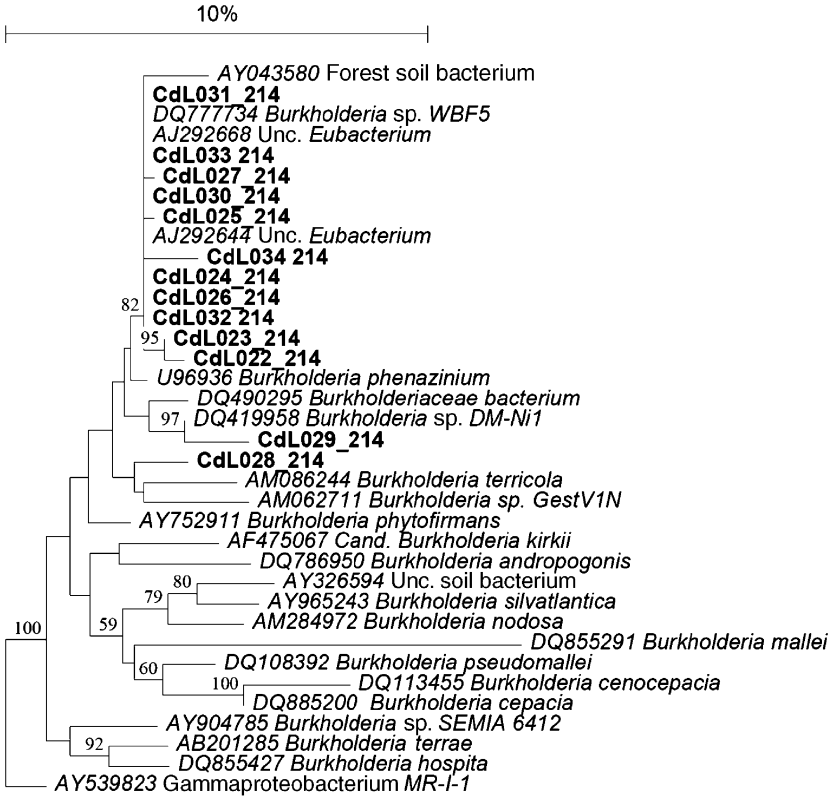

Fig. 2. Neighbour-joining phylogenetic tree showing the relationship of clones belonging to OTU category 214 with reference members of *Burkholderia* and uncultured *Betaproteobacteria* species. The horizontal scale represents 10% sequence divergence. Bootstrap values are shown for values that had > 50% support in 100 resamplings.

Primers Acta_278_Rev and Actb_279_Rev designed to target T-RFs 222 and 223 appeared to be much less accurate, and, on the basis of the clone sequences, the authors were not able to find a region that would produce fewer hits. The primer Acta_278_Rev gave 4406 hits, belonging to different phyla, in particular, *Firmicutes* (class *Clostridia*) and *Actinobacteria*. It was therefore considered to be insufficiently specific to isolate the T-RFs of interest. The primer Actb_279_Rev gave 287 hits in the RDP database, belonging mainly to *Actinobacteria*, *Chlamidiae* or unclassified bacteria. This primer was designed observing a region distinguishing five of the clones (CdL041_223, CdL043_223, CdL044_223, CdL045_223, CdL046_223) from other similar published sequences. The primer combination 27F/Actb_279_Rev, although producing different T-RFs, showed one particularly dominant peak, corresponding to T-RF 224, in the cadmium-contaminated samples (not shown). No peak at 223 rmu was detected.

Cloning and sequencing of the PCR products derived from the two primer combinations 27F/Burk_484_Rev and 27F/Actb_279_Rev revealed their efficiency in tracking the organisms of interest. For primer Burk_484_Rev, RFLP revealed an identical restriction pattern for all colonies. It was therefore sufficient to analyse 24 out of 48 starting colonies with T-RFLP profiling to identify the T-RF of interest. All clones appeared to produce a T-RF of 214 ± 0.5 rmu, which overlapped the HEX-labelled 214-rmu peak of the cadmium-treated environmental sample (data not shown). Two clones were then selected for sequencing. They revealed sequences, differing for two mismatches, with 98% or 99% sequence similarity to unculturable *Betaproteobacteria* (AJ292644, AJ292668), and to *Burkholderia* sp. WBF5 [DQ777734; (Table 3)]. Given the high level of efficiency in tracking OTU 214 with the Burk_484_Rev primer, the abundance of the corresponding *Burkholderia* target sequences in the cadmium-treated and control soils was analysed using real-time PCR. An average of $7.8 \times 10^7 \pm 3.0 \times 10^7$ targets g^{-1} dry soil were detected in cadmium-treated soils, which was significantly ($P < 0.05$, *t*-test) higher than the $4.0 \times 10^6 \pm 8.9 \times 10^5$ targets g^{-1} dry soil in untreated control soils (Table 4).

For primer Actb_279_Rev, the restriction patterns visualized through RFLP were more variable, and screening of a larger number of clones through T-RFLP profiling to detect the T-RF of interest was required. Although a T-RF of 222 or of 223 rmu was not detected, eight out of 96 clones showed a T-RF of 224 ± 0.5 rmu overlapping the corresponding HEX-labelled T-RF of 223 rmu of the cadmium-treated environmental sample (data not shown). Sequencing of four of these clones revealed (Table 3) that three had high similarities with a soil *Actinomycete* defined as *Streptacidiphilus carbonis* (AF074412), while one showed 99% sequence similarity to bacterium Ellin5516 (AY234533). The clones similar to *Streptacidiphilus carbonis* (AF074412) differed from clone CdL044_223 by

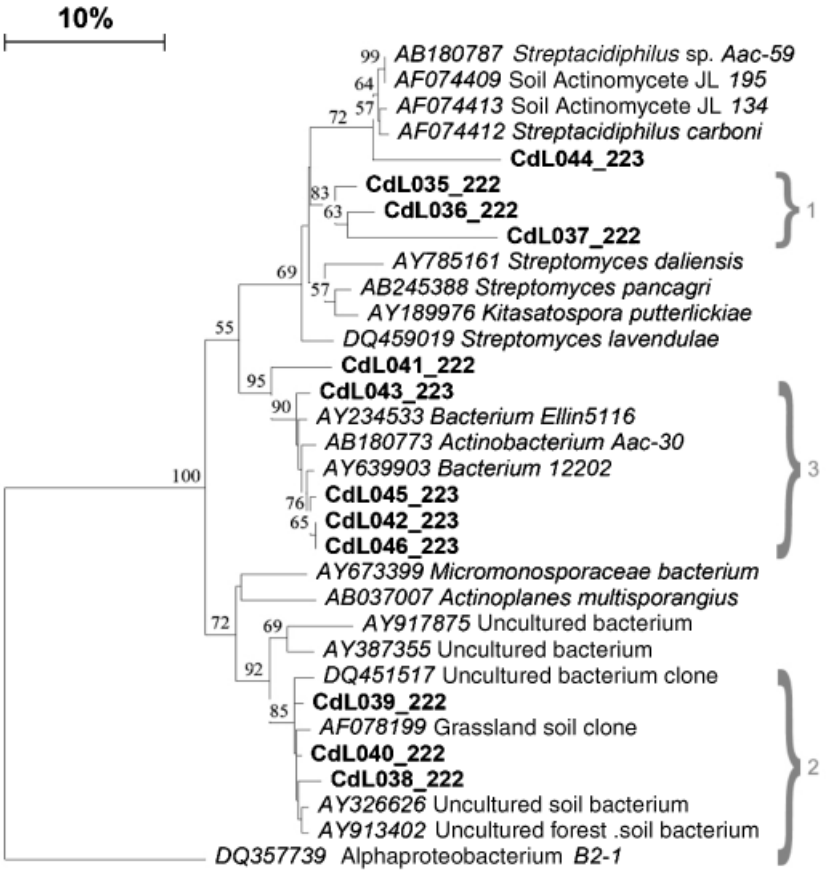

Fig. 3. Neighbour-joining phylogenetic tree showing the relationship of clones belonging to OTU categories 222 and 223 (dominant T-RFs in the cadmium-treatment) with reference members of *Actinobacteria* species. The horizontal scale represents 10% sequence divergence. Bootstrap values are shown for values that had > 50% support in 100 resamplings. Numbers near brackets indicate the main clusters formed by the analysed clones. The number following each clone name indicates the predicted T-RF size for that clone.

Table 3. Phylogenetic assignment of clones deriving from cadmium-treated samples to the GenBank database entries

Clone*	Closest relative	Sequence Identity (%)	Accession no.	Closest cultured relative	Sequence Identity (%)	Accession no.
Cd_Burk484_001	Uncultured eubacterium	98	AJ292644	*Burkholderia* sp. WBF5	98	DQ777734
Cd_Burk484_002	Uncultured eubacterium	99	AJ292668	*Burkholderia* sp. WBF5	99	DQ777734
Cd_Actb279_001	*Streptacidiphilus carbonis*	97	AF074412			
Cd_Actb279_002	*Streptacidiphilus carbonis*	99	AF074412			
Cd_Actb279_003	*Streptacidiphilus carbonis*	99	AF074412			
Cd_Actb279_004	Bacterium Ellin5116	99	AY234533			

*Clones derive from the amplification of cadmium-treated samples with a combination of universal primer 27F and group-specific primers Burk_484_Rev or Actb_279_Rev.

5–8 bp. The clone similar to bacterium Ellin5516 (AY234533) differed from clone CdL043_223 by 2 bp.

Discussion

Cadmium is a toxic HM, which exerted in the short term a strong stress on the bacterial communities of the forest soil (Lazzaro *et al.*, 2006a). The effects were evident at water-extractable concentrations above 562 mg cadmium kg^{-1} soil dry weight, which correspond to a relatively high level of cadmium pollution. Water extracts may be considered as providing a good estimation of bioavailable cadmium for soil bacteria, as they have been shown to represent a good approximation to the soil solution (Blaser *et al.*, 2000). Cadmium was also found to induce microbial community changes at lower total cadmium concentrations (40 mg cadmium kg^{-1} soil) in a nonforest soil analysed by ribosomal intergenic spacer analysis (Ranjard *et al.*, 2006), and at water-extractable concentrations up to 196 mg cadmium kg^{-1} humus in forest humus analysed by phospholipid fatty acid profiling (Fritze *et al.*, 2000).

The comparison between the T-RFLP profiles of water controls and cadmium-treated microcosms showed that after 30 days of incubation, certain OTUs dominated in the

Table 4. Abundance of *Burkholderia* target sequences (gene copy number g^{-1} soil dry weight $\pm$ SD; $n = 4$) in cadmium-contaminated and control soils analysed by real-time PCR targeting 16S rRNA gene fragments using the 27F and Burk_484_Rev primer set

Treatment	*Burkholderia* (gene copy number g^{-1} soil dry weight)	Bacterial 16S rRNA gene (gene copy number g^{-1} soil dry weight)
Untreated control	$4.0 \times 10^6 \pm 8.9 \times 10^5$	$1.6 \times 10^9 \pm 7.8 \times 10^8$
Cd-treated	$7.8 \times 10^7 \pm 3.0 \times 10^{7*}$	$4.8 \times 10^8 \pm 1.5 \times 10^8$

*Significantly different values ($P < 0.05$; *t*-test) between the cadmium treatment and the untreated control.

cadmium-treated microcosms, and not in the water controls. Such an observation suggests that only certain bacterial groups able to tolerate cadmium contamination were surviving in the soil. Bacteria possessing intrinsic tolerance to cadmium contamination have been reported in previous studies. For example, Piotrowska-Seget *et al.* (2005) found that 50% of the bacteria in a sandy loam were tolerant to cadmium. Similar results were found by Duponnois *et al.* (2006) in the bacterial community of a termite mound artificially treated with $CdCl_2$. Further investigation of specific cadmium-resistance genes such as *cadA* (Silver & Phung, 1996; Brim *et al.*, 1999) could help to elucidate the cadmium-tolerant bacterial groups.

T-RFLP is a powerful tool for the investigation of bacterial community profiles, and for characterizing how environmental factors such as HMs drive community changes (Osborn *et al.*, 2000). As a monitoring tool, however, it can be affected by experimental and analytical difficulties. In the T-RFLP profiles of some of this study's clones, for example, the appearance of peaks additional to that of the expected size was noted (data not shown). It has been reported that partial digestion can affect the homogeneity of replicate profiles and produce pseudo-T-RFs (Egert & Friedrich, 2003). The occurrence of additional T-RFs due to single-stranded DNA amplicons has also been observed (Egert & Friedrich, 2003).

The selected OTUs revealed sizes that differed between 1 and 6 bp from the actual sequence in the clones, as observed in previous studies (Kaplan & Kitts, 2003). It has been hypothesized that this effect is sequence-dependent, and can result in different discrepancies for organisms with similar predicted T-RF lengths (Kitts, 2001). This was also observed in the present results, as a higher discrepancy for T-RF 214 in comparison with the discrepancies for T-RFs 222 and 223 was noted. This effect, in addition, may have been responsible for the incorrect assignment of clone CdL041_222 to OTU group 222, instead of to OTU group 223, as would be more likely based on the corresponding phylogenetic tree (Fig. 3). Therefore, the assignment of T-RFs of similar sizes must be handled with care.

The most dominant OTU (214) in the present cadmium-contaminated T-RFLP profiles derived from the class *Betaproteobacteria* closely related in particular to the genus *Burkholderia*. The genus *Burkholderia* contains a variety of acidophilic species, which are ubiquitous in all environments. They range from plant pathogens (Burkholder, 1950) to nitrogen-fixing bacteria (Chen *et al.*, 2003), and have been shown to be plant growth-promoting bacteria (Bevivino *et al.*, 2000). Generally, *Burkholderia* was found to be more abundant in rhizosphere soil than in bulk soil (Felske *et al.*, 1998). The present results are among the few (Macnaughton *et al.*, 1999; Nogales *et al.*, 2001) demonstrating the presence of *Burkholderia* also in bulk soil. Moreover, *Burkholderia* was found to be abundant under disturbed conditions. Members of this genus have been detected, for example, in agricultural or industrial soils contaminated with various HMs such as nickel (Héry *et al.*, 2003) and cadmium (Macnaughton *et al.*, 1999; Sandrin *et al.*, 2000). Furthermore, *Burkholderia cepacia* biofilms have been shown to possess mechanisms of cellular sequestration of lead (Templeton *et al.*, 2001). *Burkholderia* species also have the ability to utilize various different carbon sources and thus have been considered in programmes of degradation of organic pollutants (Friedrich *et al.*, 2000; Sandrin *et al.*, 2000). The sequences found in the present study were indeed highly similar to sequences in the database related to *Betaproteobacteria* characterizing oil- (DQ378200) or polychlorinated biphenyl-polluted soils (AJ292644), as well as *Burkholderia* species involved in fenitrothion degradation (AB025790) or characterizing extreme environments such as volcanic deposits (DQ490295). The present laboratory-based results are also in accordance with the findings of *Burkholderia* species isolated directly in HM-impacted industrial areas (Goris *et al.*, 2001) and represent further evidence that this genus is of great relevance under HM stress.

The results from the quantitative PCR showed that the *Burkholderia* species increased in abundance after contamination with cadmium, indicating that a possible physical separation of the bacteria in the soil from the soluble HM can be excluded. In addition to resistance or tolerance mechanisms of the genus *Burkholderia*, the loss of cadmium-sensitive populations in the soils may have also provided a higher availability of resources favouring the growth of the surviving *Burkholderia* species.

OTUs 222 and 223 were assigned instead to the class *Actinobacteria*. *Actinobacteria* diversity is broad, and different groups may respond in different ways to HMs (Babich & Stotzky, 1977; Kelly *et al.*, 2003). In the present study, the family *Streptomycetaceae* appeared to be related most closely to some of the clones corresponding to the dominant OTUs 222 and 223. Members of this family are known to possess HM-resistance mechanisms (Schmidt *et al.*, 2005), such as

mercury resistance (Ravel *et al.*, 2000) or nickel resistance (Mengoni *et al.*, 2001) genes.

The results of this study indicate that some species of the genus *Burkholderia* and of the family *Streptomycetaceae* are a significant component of soils under cadmium contamination, and may therefore constitute promising potential bioindicator organisms for cadmium, or possibly of general HM, contamination in soils. The undefined nature of the corresponding OTUs, however, is a limitation in the accurate definition of potential bioindicators of cadmium contamination, because it does not allow the identification of such key organisms at a species level.

The conventional PCR approach using specific primers that was adopted in this study was effective in detecting the important OTUs focused on and in confirming the results of T-RFLP analysis. For the primer combination 27F/Burk_484_Rev, all clone sequences were similar, which allowed efficient tracking of the OTU 214 corresponding to the *Burkholderia* sp. and quantitative analysis of their abundance in the soils. Real-time PCR clearly showed that OTU 214 was an order of magnitude more abundant in cadmium-treated soils than in untreated control soils (Table 4).

With the primer Actb_279_Rev, it was possible to detect the dominant T-RF of 224 rmu, which this study was able to assign to the OTU category 223, although it appeared that this OTU may be produced by different *Actinobacteria* species and was therefore not further analysed by quantitative real-time PCR.

Considering that forest soils harbour a large bacterial diversity, it was further investigated whether the indigenous groups dominating in the short term under cadmium contamination were novel or unexpected taxa typical of forest soils. The results showed, however, that the dominant key OTU (214) identified was most probably the same uncultured betaproteobacterium described by Nogales *et al.* (2001) in a biphenyl-polluted industrial soil, although sequence identity to bacteria from a forest soil subjected to compaction (Axelrood *et al.*, 2002) was also found. While the clones corresponding to OTU 223 did not appear specifically to be associated with forest soil bacteria, the clones corresponding to OTU 222 were highly related to unculturable forest soil bacteria (e.g. DQ451517, AY913402). Moreover, the clones that clustered separately in the phylogenetic tree (Fig. 3), such as clones CdL035_222, CdL036_222 and CdL037_222, may indicate novel lineages typical of forest soils close to the genera *Streptomyces* and *Kitasatospora* (family *Streptomycetaceae*).

Conclusions

T-RFLP-based analysis revealed indigenous eubacterial populations able to tolerate in the short term high levels of cadmium. Although in some cases it appeared that single OTUs corresponded to different species, this study was able to identify the major bacterial groups of a moderately acidic cadmium-contaminated forest soil as *Betaproteobacteria* (genus *Burkholderia*) and *Actinobacteria* (family *Streptomycetaceae*). Given that primer Burk_484_Rev allowed for efficient detection of OTU 214 as corresponding to the genus *Burkholderia*, this may be validated in future studies on various cadmium-polluted soils in a quantitative manner, and further analysis is needed to clarify the ecological role of these organisms in metal-polluted soils. In addition, cultivation of members of key bacterial groups and a more specific definition of their phylogeny remains an important objective to their characterization and potential application in bioremediation strategies.

Acknowledgements

A.L. was financed by the Swiss Federal Office for the Environment (FOEN), grant no. 810.3189.004. A part of the study was supported by COST short-term scientific mission 631, and was performed with the kind collaboration of Dr Angela Sessitsch at the Austrian Research Institute, Seibersdorf, Austria. The authors would also like to acknowledge Martin Hartmann at ART, Reckenholz, Switzerland, for helpful and constructive discussions and Andreas Rüdt for excellent assistance with the real-time PCR.

References

Axelrood PE, Chow ML, Radomski CC, McDermott JM & Davies J (2002) Molecular characterization of bacterial diversity from British Columbia forest soils subjected to disturbance. *Can J Microbiol* **48**: 655–674.

Babich H & Stotzky G (1977) Sensitivity of various bacteria, *Actinomycetes* and fungi to cadmium and influence of pH on sensitivity. *Appl Environ Microbiol* **33**: 681–695.

Benson DA, Karsch-Mizrachi I, Lipman DJ, Ostell J & Wheeler DL (2005) Genbank. *Nucleic Acids Res* **33**: 34–38.

Bevivino A, Dalmastri C, Tabacchioni S & Chiarini L (2000) Efficacy of *Burkholderia cepacia* MCI7 in disease suppression and growth promotion of maize. *Biol Fertil Soils* **31**: 225–231.

Blaser P, Zimmermann S, Luster J & Shotyk W (2000) Critical examination of trace element enrichments and depletions in soils: As, Cr, Cu, Ni, Pb, and Zn in Swiss forest soils. *Sci Tot Environ* **249**: 257–280.

Brandt KK, Petersen A, Holm PE & Nybroe O (2006) Decreased abundance and diversity of culturable *Pseudomonas* spp. populations with increasing copper exposure in the sugar beet rhizosphere. *FEMS Microbiol Ecol* **56**: 281–291.

Brim H, Heyndrickx M, de Vos P, Wilmotte A, Springael D, Schlegel HG & Mergeay M (1999) Amplified rDNA restriction analysis and further genotypic characterisation of metal-

resistant soil bacteria and related facultative hydrogenotrophs. *Syst Appl Microbiol* **22**: 258–268.

Burkholder WH (1950) Sour skin, a bacterial rot of onion bulbs. *Phytopathology* **40**: 115–117.

Chen WM, Moulin L, Bontemps C, Vandamme P, Béna G & Boivin-Masson C (2003) Legume symbiotic nitrogen fixation by β-*Proteobacteria* is widespread in nature. *J Bacteriol* **185**: 7266–7272.

Cole JR, Chai B, Marsh TL *et al.* (2003) The Ribosomal Database Project (RDP-II): previewing a new autoaligner that allows regular updates and the new prokaryotic taxonomy. *Nucleic Acids Res* **31**: 442–443.

Corpet F (1988) Multiple sequence alignment with hierarchical clustering. *Nucleic Acids Res* **16**: 10881–10890.

Dìaz-Raviña M & Bååth E (1996) Development of metal tolerance in soil bacterial communities exposed to experimentally increased metal levels. *Appl Environ Microbiol* **62**: 2970–2977.

Duponnois R, Kisa M, Assigbetse K, Prin Y, Thioulouse J, Issartel M, Moulin P & Lepage M (2006) Fluorescent pseudomonads occurring in *Macrotermes subhyalinus* mound structures decrease Cd toxicity and improve its accumulation in sorghum plants. *Sci Tot Environ* **370**: 391–400.

Egert M & Friedrich MW (2003) Formation of pseudo-terminal restriction fragments, a PCR-related bias affecting terminal restriction fragment length polymorphism analysis of microbial community structure. *Appl Environ Microbiol* **69**: 2555–2562.

Ellis RJ, Morgan P, Weightman AJ & Fry JC (2003) Cultivation-dependent approaches for determining bacterial diversity in heavy-metal contaminated soil. *Appl Environ Microbiol* **69**: 3223–3230.

Felske A, Wolterink A, van Lis R & Akkermans ADL (1998) Phylogeny of the main bacterial 16S rRNA sequences in Drentse A grasslands soils (The Netherlands). *Appl Environ Microbiol* **64**: 871–879.

Frey B, Stemmer M, Widmer F, Luster J & Sperisen C (2006) Microbial activity and community structure of a soil after heavy metal contamination in a model forest ecosystem. *Soil Biol Biochem* **38**: 1745–1756.

Friedrich M, Grosser RJ, Kern EA, Inskeep WP & Ward DM (2000) Effect of model sorptive phases on phenantrene biodegradation: molecular analysis of enrichments and isolates suggests selection based on bioavailability. *Appl Environ Microbiol* **66**: 2703–2710.

Fritze H, Perkiömäki J, Saarela U, Katainen R, Tikka P, Yrjälä K, Karp M, Haimi J & Romantschuk M (2000) Effect of Cd-containing wood ash on the microflora of coniferous forest humus. *FEMS Microbiol Ecol* **32**: 43–51.

Goris J, De Vos P, Coenye T, Hoste B, Janssens D, Brim H, Diels L, Mergeay M, Kersters K & Vandamme P (2001) Classification of metal-resistant bacteria from industrial biotopes as *Ralstonia campinensis* sp. nov., *Ralstonia metallidurans* sp. nov. and *Ralstonia basilensis* Steinle *et al.* 1998 emend. *Int J Syst Evol Microbiol* **51**: 1773–1782.

Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for windows 95/98/NT. *Nucleic Acids Symp Ser* **41**: 95–98.

Hartmann M, Frey B, Kölliker R & Widmer F (2005) Semi-automated genetic analyses of soil microbial communities: comparison of T-RFLP and RISA based on descriptive and discriminative statistical approaches. *J Microbiol Methods* **61**: 349–360.

Hèry M, Nazaret S, Jaffre T, Normand P & Navarro E (2003) Adaptation to nickel spiking of bacterial communities in neocaledonian soils. *Environ Microbiol* **5**: 3–12.

Heuer H, Krsek M, Baker P, Smalla K & Wellington EMH (1997) Analysis of *Actinomycete* communities by specific amplification of genes encoding 16S rRNA and gel-electrophoretic separation in denaturing gradients. *Appl Environ Microbiol* **63**: 3233–3241.

Heuer H, Hartung K, Wieland G, Kramer I & Smalla K (1999) Polynucleotide probes that target a hypervariable region of 16S rRNA genes to identify bacterial isolates corresponding to bands of community fingerprints. *Appl Environ Microbiol* **65**: 1045–1049.

Hu Q, Qi HY, Zeng JH & Zhang HX (2007) Bacterial diversity in soils around a lead and zinc mine. *J Environ Sci* **19**: 74–79.

Joynt J, Bischoff M, Turco R, Konopka A & Nakatsu CH (2006) Microbial community analysis of soils contaminaed with lead, chromium and petroleum hydrocarbons. *Microb Ecol* **51**: 209–219.

Kaplan CW & Kitts CL (2003) Variation between observed and true terminal restriction fragment length is dependent on true TRF length and purine content. *J Microbiol Methods* **54**: 121–125.

Kelly J, Häggblom M & Tate RL (2003) Effects of heavy metal contamination and remediation on soil microbial communities in the vicinity of a zinc smelter as indicated by analysis of microbial community phospholipids fatty acid profiles. *Biol Fertil Soils* **38**: 65–71.

Kitts CL (2001) Terminal restriction fragment patterns: a tool for comparing microbial communities and assessing community dynamics. *Curr Issues Intest Microbiol* **2**: 17–25.

Kozdroj J & van Elsas JD (2001) Structural diversity of microorganisms in chemically perturbed soil assessed by molecular and cytochemical approaches. *J Microbiol Methods* **43**: 197–212.

Lakanen E & Erviö R (1971) A comparison of eight extractants for the determination of plant available micronutrients in soils. *Acta Agral Fenn* **123**: 223–232.

Lazzaro A, Hartmann M, Blaser P, Widmer F, Schulin R & Frey B (2006a) Bacterial community structure and activity in different Cd-treated forest soils. *FEMS Microbiol Ecol* **58**: 278–292.

Lazzaro A, Schulin R, Widmer F & Frey B (2006b) Changes in lead availability affect bacterial community structure but not basal respiration. *Sci Total Environ* **371**: 110–124.

Macnaughton S, Stephen JR, Chang YJ, Peacock A, Flemming CA, Leung KT & White DC (1999) Characterization of metal-

resistant soil eubacteria by polymerase chain reaction–denaturing gradient gel electrophoresis with isolation of resistant strains. *Can J Microbiol* **45**: 116–124.

Marsh TL, Saxman P, Cole J & Tiedje J (2000) Terminal restriction fragment length polymorphism analysis program, a web-based research tool for microbial community analysis. *Appl Environ Microbiol* **66**: 3616–3620.

Mengoni A, Barzanti R, Gonnelli C, Gabbrielli R & Bazzicalupo M (2001) Characterization of nickel-resistant bacteria isolated from serpentine soil. *Environ Microbiol* **3**: 691–698.

Nakatsu CH, Carmosini N, Baldwin B, Beasley F, Kourtev P & Konopka A (2005) Soil microbial community responses to additions of organic carbon substrates and heavy metals (Pb and Cr). *Appl Environ Microbiol* **71**: 7679–7689.

Naz N, Young HK, Ahmed N & Gadd GM (2005) Cadmium accumulation and DNA homology with metal resistance genes in sulfate-reducing bacteria. *Appl Environ Microbiol* **71**: 4610–4618.

Nogales B, Moore EB, Llobet-Brossa E, Rossello-Mora R, Amann R & Timmis KN (2001) Combined use of 16S ribosomal DNA and 16S rRNA to study the bacterial community of polychlorinated biphenyl-polluted soil. *Appl Environ Microbiol* **67**: 1874–1884.

Oger C, Berthe T, Quillet L, Barray S, Chiffoleau JF & Petit F (2001) Abundance of the cadmium resistance gene cadA in microbial communities in polluted estuary water. *Res Microbiol* **152**: 671–678.

Osborn AM, Moore ERB & Timmis KN (2000) An evaluation of terminal-restriction fragment length polymorphism (T-RFLP) analysis for the study of microbial community structure and dynamics. *Environ Microbiol* **2**: 39–51.

Park GS, Kim DH, Lim JG & Ohga S (2006) Heavy metal concentration and identification of microorganisms in soil under roadside trees of Daejeon city, Korea. *J Fac Agric Kyushu Univ* **51**: 53–56.

Peijnenburg JGM, Zablotskaja M & Vijver MG (2007) Monitoring metals in terrestrial environments within a bioavailability framework and a focus on soil extraction. *Ecotoxicol Environ Saf* **67**: 163–179.

Pennanen T (2001) Microbial communities in boreal coniferous forest humus exposed to heavy metals and changes in soil pH-a summary of the use of phospholipids fatty acids, Biolog® and H-3-thymidine incorporation methods in field studies. *Geoderma* **100**: 91–126.

Piotrowska-Seget Z, Cycon M & Kozdroj J (2005) Metal-tolerant bacteria occurring in heavily polluted soil and mine spoil. *Appl Soil Ecol* **28**: 237–246.

Ranjard L, Lignier L & Chaussod R (2006) Cumulative effects of short-term polymetal contamination on soil bacterial community structure. *Appl Environ Microbiol* **72**: 1684–1687.

Ravel J, Di Ruggiero J, Robb FT & Hill RT (2000) Cloning and sequence analysis of the mercury resistance operon of *Streptomyces* sp. Strain CHR28 reveals a novel putative second regulatory gene. *J Bacteriol* **182**: 2345–2349.

Reinds GJ, De Vries W & Groenenberg BJ (2001) Critical loads of lead and cadmium for European forest soils. *Modelling and Mapping of Critical Thresholds in Europe, CCE Status Report* (Posch M, De Smet PAM, Hettelingh J & Downing RJ, eds), pp. 81–92. Coordination Center for Effects, National Institute for Public Health and the Environment (RIVM), Bilthoven, the Netherlands.

Renella G, Ortigoza ALR, Landi L & Nannipieri P (2003) Additive effects of copper and zinc on cadmium toxicity on phosphatase activities and ATP content of soil as estimated by the ecological dose (ED50). *Soil Biol Biochem* **35**: 1203–1210.

Renella G, Mench M, Gelsomino A, Landi L & Nannipieri P (2005) Functional activity and microbial community structure in soils amended with bimetallic sludges. *Soil Biol Biochem* **37**: 1498–1506.

Sandrin TR, Chech AM & Maier RM (2000) A rhamnolipid biosurfactant reduces cadmium toxicity during naphthalene biodegradation. *Appl Environ Microbiol* **66**: 4585–4588.

Schmidt A, Hafeburg G, Sineriz M, Merten D, Büchel G & Kothe E (2005) Heavy metal resistance mechanisms in actinobacteria for survival in AMD contaminated soils. *Geochemistry* **65**: 131–144.

Sharma RK & Agrawal M (2005) Biological effects of heavy metals: an overview. *J Environ Biol* **26**: 301–313.

Silver S & Phung LT (1996) Bacterial heavy resistance: new surprises. *Ann Rev Microbiol* **50**: 753–789.

Smalla K, Oros-Sichler M, Milling A, Heuer H, Baumgarte S, Becker R, Neuber G, Kropf S, Ulrich A & Tebbe CC (2007) Bacterial diversity of soils assessed by DGGE, T-RFLP and SSCP fingerprints of PCR-amplified 16S rRNA gene fragments: do the different methods provide similar results? *J Microbiol Methods* **69**: 470–479.

Smith CJ, Nedwell DB, Dong LF & Osborn AM (2006) Evaluation of quantitative polymerase chain reaction-based approaches for determining gene copy and gene transcript numbers in environmental samples. *Environ Microbiol* **8**: 804–815.

Starr M, Lindroos AJ, Ukonmaanaho L, Tarvainen T & Tanskanen H (2003) Weathering release of heavy metals from soil in comparison to deposition, litterfall and leaching fluxes in a remote, boreal coniferous soil. *Appl Geochem* **18**: 607–613.

Templeton AS, Trainor TP, Traina SJ, Spormann AM & Brown GEJ (2001) Pb(II) distributions at biofilm-metal oxide interfaces. *Proc Natl Acad Sci USA* **98**: 11897–11901.

Tsai YP, You SJ, Pai TY & Chen KW (2005) Effect of cadmium on composition and diversity of bacterial communities in activated sludges. *Int Biodet Biodegr* **55**: 285–291.

Turpeinen R, Kairesalo T & Häggblom M (2004) Microbial community structure and activity in arsenic-, chromium- and copper-contaminated soils. *FEMS Microbiol Ecol* **47**: 39–50.

Van de Peer Y & De Wachter R (1994) TREECON for windows: a software package for the construction and drawing of evolutionary trees for the Microsoft windows environment. *Comput Applic Biosci* **10**: 569–570.

Vig K, Megharaj M, Sethunathan N & Naidu R (2003) Bioavailability and toxicity of cadmium to microorganisms and their activities in soil: a review. *Adv Environ Research* **8**: 121–135.

Wang YP, Shi JY, Wang H, Lin Q, Chen XC & Chen YX (2007) The influence of soil heavy metals pollution on soil microbial biomass, enzyme activity, and community composition near a copper smelter. *Ecotoxicol Environ Saf* **67**: 75–81.

Widmer F, Hartmann M, Frey B & Kölliker R (2006) A novel strategy to extract specific phylogenetic sequence information from community T-RFLP. *J Microbiol Methods* **66**: 512–520.

Winding A, Hund-Rinke K & Rutgers M (2005) The use of microorganisms in ecological soil classification and assessment concepts. *Ecotoxicol Environ Saf* **62**: 230–248.