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Assessment of bacterial community structure in a long-term copper-polluted ex-vineyard soil

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Soil bacteria;
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Summary

The influence of long-term copper contamination on the diversity of bacterial communities was investigated in an ex-vineyard soil. Two sites of the same area but exhibiting different 3-fold exchangeable copper (Ex-Cu) concentrations were analysed. Culturable bacterial community structure was assessed using a variety of approaches: determination of culturable bacteria number, analyses of 132 isolates, and denaturing gradient gel electrophoresis (DGGE) patterns of bacterial biomass grown on agar plates and of soil DNA. There was no significant difference in the number of total heterotrophs at the two sites, whereas the percentage of fast-growing bacteria growing in 1 day, was lower at the site with the higher Ex-Cu content. A high percentage of Cu-tolerant bacteria was found in both sites (63–70%) and it was relatively independent of the Cu content. Shifts in species composition of the culturable bacterial community were detected by analysing isolates from the two soils, Gram-positive bacteria prevailed in the less-polluted soil while Gram-negative bacteria in the more-polluted soil. Each sample site had a community with a different metal resistance pattern. Our study seems to indicate that in this soil ecosystem, copper influenced the culturable bacterial communities, affecting the structural diversity and altering some of the metal resistance of the microorganisms. The Sorensen similarity index calculated on DGGE profiles of 16S rDNA of total and culturable bacterial communities indicated a different species composition at the two sites, although both sites had the same biodiversity degree and different dominance.

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Introduction

The pollution of agricultural soil by copper, as well as by various other heavy metals, commonly occurs through the addition of contaminated waste matter and mineral fertilisers, and the use of some pesticides. Copper-containing sprays of various formulations have been used to control fungal diseases as mildew, leaf spots, blights, and apple scab in orchards, vineyards and vegetable crops for well over 100 years.

Copper is an essential element for life, but high concentrations of its ionic form are toxic to soil microorganisms (Cervantes and Gutierrez-Corona, 1994). Due to its role in the production of free radicals, it has been implicated in the induction of viable but non-culturable conditions (Alexander et al., 1999). Free Cu levels within a cell must be limited, and ion transportation needs strict regulation. Once inside the cells, sequestration and cellular energy-dependent efflux systems are required to remove excess levels of cations and to protect the intracellular targets (Nies, 1999; Rensing and Grass, 2003). Genetic determinants for Cu resistance systems, such as *pco*, *cop*, and *cue* operons, are described in *Pseudomonas*, *Xantomonas*, and *Escherichia coli* (Silver and Phung, 1996). In the plant pathogen *Pseudomonas syringae*, resistance to copper, via accumulation and compartmentalisation in the cell's periplasm and outer membrane, is due to four proteins encoded on the plasmid-borne *cop* operon (Cooksey, 1994). In *E. coli*, the resistance is based on an efflux mechanism by which copper is removed from the cell. The efflux proteins are expressed by plasmid *pco* genes (Cooksey, 1993). The CopA together with CueO, a multi-copper oxidase, constitute the Cue system and are both regulated by CueR. CueO protects periplasmic enzymes from copper-induced damage, loading of copper into CueO in the cytoplasm; the subsequent transport into the periplasm could contribute to cytoplasmic copper detoxification (Rensing and Grass, 2003).

Cooksey (1993) also states that most bacterial species in the environment have acquired at least one of the aforementioned copper management systems.

Studies concerning the effect of metals on soil bacteria describe, in the case of short-term contact with the metal, the selection of resistant bacteria within a few weeks. In the event of high metal levels, the selection of resistant mutants took place slowly. Thus long-term contact leads to adaptation of the part of the microbial community which then survives well in metal-polluted soil (Giller et al., 1998; Kunito et al., 1999).

All aspects of microbial presence can, potentially, be affected by the introduction of a heavy metal: bacterial numbers, biomass, activity and diversity, and a number of different methods have been employed to evaluate such effects (Giller et al., 1998). Some groups within the bacterial community show high sensitivity to toxicants, and among the most sensitive are autotrophic nitrifiers that have been proposed as toxicity biosensors (Tanaka et al., 2002). The response of this bacterial group to Cu presence has been evaluated in both pure cultures and in a soil environment. In pure cultures, Cu toxicity was already evident at 0.05 mg/kg (Sato et al., 1986) while in soil the toxic Cu level was much higher (10 to more than 1000 mg/kg), depending on the experimental conditions (Maliszewska et al., 1985).

In the case of adaptation being already reached, contrasting results have been recorded. On analysing soils polluted by different copper amounts, Ellis et al. (2003) found comparable bacterial diversity, whereas other authors agree that the presence of Cu determines a decrease in bacterial diversity and a change in community structure (Huysman et al., 1994; Smit et al., 1997; Tom-Petersen et al., 2003; Turpeinen et al., 2004). In particular, Tom-Petersen et al. (2003) demonstrated an impact of Cu on *Rhizobium–Agrobacterium* group in field experiments. Discrepancies occur also when analysing the effect of the level of Cu contamination on the number of culturable bacteria: Hiroki (1992) and Ellis et al. (2003) found a negative correlation between the Cu level and the CFU number, whereas Huysman et al. (1994), Smit et al. (1997), and Turpeinen et al. (2004) found that the number of culturable bacteria was not affected by metal contamination. Huysman et al. (1994) and Smit et al. (1997) noted that the percentage of Cu-resistant bacteria increased with rising soil Cu content.

Within this context, the aim of this work was to compare the soil bacterial diversity of two sites belonging to the same agricultural soil but contaminated with different copper concentrations, and to reveal any differences in the communities. Modifications in bacterial community structure were assessed by (i) enumeration of culturable bacteria; (ii) phylogenetic analysis of the 16S rRNA genes from the isolates; (iii) denaturing gradient gel electrophoresis (DGGE) profile of total and culturable bacterial biomass from plates of the same sample (plate washes, PW). Moreover, as investigations of metal resistance commonly involve the study of phenotypic traits, genetic determinants for putative metal resistance were checked by PCR in copper-resistant isolates.

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Materials and methods

Soil characteristics, sampling, and bacterial counts

An agricultural area located in the Piedmont region (northern Italy) was considered. It had been a vineyard for over 30 years until the 1980s, and had thus been repeatedly subjected to copper sulphate treatments (4 per year). In the absence of an unpolluted control two sites of this area, one characterised by the highest (A) and the other by the lowest Cu content (B), were chosen to investigate a possible direct/indirect influence of long-term Cu exposure on the structure of bacterial populations. In April 2003, eight samples were collected randomly from each site, from marginal transects with no vegetation cover. The eight samples were randomly put together to form two composite samples for each site. These composite samples were put into plastic bags, sealed and transported in an ice-box to the laboratory where they were sieved (2 mm), and stored moist in the dark at 4 °C in polyethylene bags, 0.025 mm thickness, closed without head space for 2 weeks until the analyses (Wollum, 1994).

Organic carbon (Walkley and Black, 1934), total nitrogen (Kjeldahl method), and pH(H_2O) (1:2.5 soil:water ratio) were determined on the air-dried samples.

Because heavy metals are present in various forms in soil, besides to determine the total copper content we quantified the exchangeable Copper content (Ex-Cu). Ex-Cu, the most important bioavailable fraction, is considered to be toxic to soil bacteria by Kunito et al. (1998). Total copper concentration was determined in 3:1 HCl: HNO_3 (Aqua Regia) extract, the exchangeable Cu was extracted for 16 h with 0.5 M KNO_3 according to Alva et al. (2000). The concentration of total and exchangeable Cu was measured using inductively coupled plasma spectrometry (ICPAES, Plasma 40, Perkin-Elmer Corp, Norwalk, CT).

Serial 10-fold dilutions in Winogradsky 1:20 saline solution (Jensen, 1962) were prepared starting from a 5 g+45 ml suspension from each soil sample in a 1 g/l sodium pyrophosphate/water solution (Pochon and Tardieux, 1962), which was treated in a homogeniser with rotating blades (Lindahl and Bakken, 1995). Aliquots (1 ml) of the various dilutions were then inoculated into agar media in double sets of pour plates, or in a quintuple set of tubes with liquid medium. Incubation was at 28 °C for an appropriate number of days.

For the total bacterial counts, 10 times diluted tryptic soy agar (TSA/10) (Smit et al., 1997) was used. Cycloheximide at 100 mg/l was added to the media to inhibit eukaryotic growth. To analyse the metal tolerance level of bacterial community in the soil (Kunito et al., 1999), the number of Cu-tolerant bacteria was estimated in TSA/10 supplemented with 50 mg/l of $Cu(NO_3)_2$ (Sigma-Aldrich). The copper tolerance of culturable bacteria was expressed as percentage of growth on TSA/10 without metal addition. For autothrophic nitrifier (ammonium-oxidisers) counts, an ammonium liquid mineral medium was inoculated (Pochon and Tardieux, 1962). After 55 d incubation (Belser, 1979), diphenylamine reagent was used to detect the presence of nitrite and/or nitrate.

Resistance of isolates to heavy metals and growth curve determinations of a model strain

Fifty strains are normally necessary to obtain a sufficient picture of species richness and to analyse the diversity of a bacterial community (Bianchi and Bianchi, 1982). Sixty-seven isolates from site A and 65 isolates from site B grown on TSA/10 plates were randomly isolated. Single colonies were streaked to purity on the same medium. Strains were maintained in glycerol stocks at −70 °C. Prior to use, the strains were grown to mid-exponential phase in 1/10 Tryptic Soy Broth at 30 °C with shaking. To analyse the resistance level of the isolates to Cu, Cd, Zn, and Ni, Tris mineral medium with 0.6% (w/v) gluconate (TMMG) at low phosphate content (0.12 g/l of Na_2HPO_4) to avoid heavy metal precipitation was used (Sadouk and Mergeay, 1993). Exactly 1 ml of each isolate suspension was inoculated in two flasks containing TMMG supplemented with appropriate amounts of soluble metal ions and checking for growth after 5 d incubation at 30 °C by $OD_{600\,nm}$ measurements (Table 1).

With *Ralstonia* sp. 13–100, chosen as a model strain for its high Cu-resistance (4 mM), growth curves in the presence of Cu and other metals were determined. Duplicate Erlenmeyer flasks containing 200 ml of TMMG medium supplemented with $Cu(NO_3)_2$ to a final concentration of 0, 1 and 3 mM and with 0.5 mM of $CdCl_2$, $NiCl_2$, $Pb(NO_3)_2$ and $ZnSO_4$ were inoculated with 5 ml of an overnight culture ($OD_{600\,nm}$ of 1.2) of the strain grown without copper. Incubation was performed at 30 °C with shaking. At given times, aliquots were taken and used for the OD_{600} determinations.

Molecular methods

DNA was extracted twice from 1 g of soil sample by a bead-beating method according to

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Table 1. Characteristics of the soil sites

Soil site	pH (H_2O)	Organic C (%)	Total N (%)	CEC[a] ($cmol^{(+)}/kg$)	Texture[b]	Total Cu (mg/kg)	Ex-Cu (mg/kg)
A	4.8b	2.0a	0.16a	9.8a	74–20–6	372a	30a
B	5.4a	2.1a	0.16a	9.8a	74–20–6	215b	9b

Each value is the mean of four determinations and is expressed on dry matter basis. Mean separation within columns was carried out by analysis of variance at the 5% level.
[a]Cation exchange capacity.
[b]Sand/silt/clay.

Table 2. Oligonucleotide primers used for PCR amplification of genes involved in metal resistance

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)	Size of PCR product (bp)	Reference
merRTΔP	GAGATCTAAAGCACGCTAAGGC	GGAATCTTGACTGTGATCGGG	1011	Trajanovska et al. (1997)
pcoR	CAGGTCGTTACCTGCAGCAG	CTCTGATCTCCAGGACATATC	640	Trajanovska et al. (1997)
pcoB	CAMCSGCAMGMCGCGAMGAA	MYGACWKGGTAGGMCTCCKACA	540	This work
cop	GCASGGCATGGACCACAGCMA	AYRCCGATRTAMGGCGCAAA	701	This work
czcC	ACATACCTTGGTGCAATTCGA	ATGTTTTATGAATCCCGTCTTACC	1292	Dell'Amico (2003)
cadA	GACAAGACYGGMACYMTCAC	GCRTGGTTRATSCCGTC	600	Dell'Amico (2003)

manufacturer's instructions (MOBIO, USA) and from bacterial biomass of PW by G-NOME kit according to manufacturer's instructions of BIO101, UK. Proteinase K (10 mg/ml) digestion was used to extract DNA from pure strains (Cavalca et al., 2004).

PCR amplification of the 16S rDNA was performed on the extracted DNA, using the eubacterial universal primers P27f and P1495r based on the nucleotide sequence of the *E. coli* 16S rRNA gene. Nested PCR reaction for V3 amplification was carried out (Muyzer et al., 1993). Internal transcribed spacers were amplified by PCR using primers for universal small 16S and bacterial-specific large 23S ribosomal subunits (Ranjard et al., 2000).

DGGE analysis of the PCR products was performed in a DCODE Universal Mutation Detection System (Biorad, USA) apparatus. The linear denaturing gradient of urea and formamide ranged from 40% to 60%. Gels were run at a constant voltage of 70 V for 16 h at 60 °C. After completion of electrophoresis, the gels were stained in an ethidium bromide solution (0.5 μg/ml) and documented with GelDoc System (Biorad, USA). To check the electrophoresis run and to compare fragment migration between gels, markers were applied twice to each DGGE gel. Markers were obtained by mixing the same volume of each identified isolate DGGE-PCR product. The running position of each isolate fragment in the marker was estimated by a further DGGE gel loaded with markers and DGGE-PCR product of each single identified isolate.

The nucleotide sequence of 16S rDNA of isolates was determined according to the DYEnamic ET Terminator Cycle Sequencing Kit (Amersham Biosciences, UK). Primers used in the PCR reaction for sequencing products were the same as those in normal 16S rDNA PCR reactions. The forward and reverse samples were run on a 310 A sequence analyser (Applied Biosystems, USA). Thirty-one isolates, representing 46% of the Operational Taxonomic Unit (OTUs), were identified.

Genetic systems known to specifically encode for copper (*pco* and *cop*), mercury (*mer*), cadmium (*cad*), and multiple heavy metal resistances (*czc*) in isolates from contaminated environments were screened by PCR amplification. All the primers used are reported in Table 2. *CzcC* and *cadA* were amplified according to Dell'Amico (2003), *merRT ΔP* and *pcoR* according to Trajanovska et al. (1997). Primer for *copAB* were designed on the consensus region of *copAB* sequence of *P. putida* PNL-MK25 (AY321492) and of *Cupriavidus metallidurans* CH34 (AJ278983). Primer for *pco*B were designed on the consensus region of *pcoB* sequence of *C. metallidurans* CH34 (AJ278983). The PCR mix consisted of deoxynucleotide triphosphates at 200 μM each, 0.30 μM each primer, 1.75 mM $MgCl_2$, 1 × PCR buffer, and 1 U of Taq DNA polymerase (Invitrogen, UK) in a total volume of 50 μl. Annealing temperatures were 50 °C.

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Digestion of 16S rDNA amplicons from each isolate was performed by *Hha*I endonuclease (Promega, USA) according to manufacturer's instructions. Fragments were separated on a 3% agarose gel run at 70 V for 3 h and stained by ethidium bromide solution (0.5 μg/ml).

Data analysis

All bacterial counts were $\log_{10}$ transformed to normalise before analysis of variance at the 5% level; in the case of significance, Duncan's multiple range test was carried out.

RISA and ARDRA profiles were used to characterise the isolates. Fragment size was estimated using a linear regression equation between the molecular mass of the DNA ladder and the log of the distance covered by fragments within the same gel run. A distance matrix was built and the UPGA method was used to build a similarity tree by the Jaccard coefficient, using the NTSYS software package.

The indices of general diversity (H') and of dominance (D) of the two culturable bacterial communities were calculated according to Shannon (1948) and Simpson (Begon et al., 1990), respectively. The equations are the following: $H' = -\Sigma p_i \ln p_i$, and $D = \Sigma {p_i}^2$, where p_i is the proportion of members of each genus or OTU. The Sorensen similarity index (S) (Southwood, 1978) between communities was calculated on the basis of DGGE profiles and on OTUs as follows: $S = 2c/(a+b)$ where c is the number of common OTUs or common DGGE bands and a and b are the total OTUs or the total DGGE bands at sites A and B, respectively.

The Diversity Database software package (Bio-rad) was used to analyse the DGGE profiles, after assigning bands to the gel tracks.

Accession numbers

The *merRT ΔP* sequences obtained in the present study are under the following accession numbers: from AJ878863–AJ878867 for *Ralstonia* sp. strains 12–100, 13–100, 14–100, 15–100, 16–100, and AJ878862 for *Ralstonia taiwanensis* 181.

Results

Chemical and microbial characteristics of soil

Two sites of an ex-vineyard soil, contaminated by a Cu content higher than the EU safety limits (Commission of the European Community), were chosen for their different Ex-Cu content to investigate the influence of the metal on the microbial community structure and diversity. Table 1 shows the main physicochemical characteristics of the two sites. Both sites showed high Ex-Cu concentrations, but at site A the concentration was 3-fold higher than at B (Table 1). In accordance with Ellis et al. (2001) we made direct comparisons of samples from the same area, and interpreting the results, site-specific parameters, that may have affected the response of bacterial community, were considered. The only other factor that varied along with Cu concentration at the two sites was pH. Both Ex-Cu and pH might have influenced microbial community.

The culturable heterotrophs of the two sites significantly differed in the number of bacteria culturable in 1 day (1.5×10^6 and 7.8×10^6 CFU/g dry soil at sites A and B, respectively), i.e., "fast-growing bacteria". Site B contained nearly five times more *r*-strategist bacteria than the more Cu-polluted site A. For the longer incubation times (from 2 to 21 days when the maximum colony number was reached) no significant differences in bacterial number between sites were observed (1.6×10^8 and 1.3×10^8 CFU/g dry soil at sites A and B, respectively), in agreement with the result of Smit et al. (1997) and Huysman et al. (1994). The proportion of Cu-tolerant bacteria, 63% at site A and 70% at site B, was not correlated with the amount of Ex-Cu in the soils. The high ratio of Cu-tolerant bacteria at both sites suggests that the long-term exposure to copper had probably selected a Cu-tolerant community composed of two types of bacteria: the one originally resistant to Cu and the other with an acquired resistance.

The ammonia-oxidising nitrifier counts were 2.8×10^4 and 1.9×10^4 MPN/g dry soil at sites A and B, respectively. The statistical analysis showed no difference between the different Cu-polluted sites.

Diversity of culturable bacteria

The isolates of the two sites that allowed subculturing were divided into OTUs by ARDRA and RISA analysis with the objective of recognising shifts in the bacterial communities related to environmental perturbation (Fig. 1). The culturable bacterial communities at the two sites were different ($S = 0.14$), there being differences in the distribution and relative abundance of the OTUs. Based on a 1500-nucleotide segments, the BLASTIN search results of the 16S rRNA gene, reported in Table 3 evidenced the presence of 16 genera. Firmicutes was the dominant group at both

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sites, representing the 19% and the 61% of the heterotrophic community of sites A and B, respectively.

The Shannon index of the two communities did not evidence any difference in biodiversity between the two sites ($H'_A = 2.3$, $H'_B = 2.1$). However, dominance was greater in the less Cu-polluted site B ($D_B = 0.08$) where one dominant OTU of *Bacillus megaterium* (24% of the community) and 31 rare OTUs were found. In the more-polluted site A ($D_A = 0.04$), two dominant OTUs (*Ralstonia* sp. and *Stenotrophomonas acidaminiphila*, representing 7% and 8% of the community, respectively) and 23 rare OTUs were found.

Metal-resistance characteristics of isolates

Of the 132 isolates, 42% grew on TMMG medium added with 0.1 mM Cu (Fig. 1). The dominant Cu-resistant isolates belonged to common soil genera, including *Sphingomonas*, *Pseudomonas*, *Ralstonia*, *Alcaligenes*, *Bacillus*, *Paenibacillus* and *Arthrobacter*. Among the Gram-positive isolates, Cu-resistant strains were higher at site A than at site B, indicating adaptation of this class to higher Ex-Cu content. At site A the Cu-resistant heterotrophs were 48% and multiple metal-resistant strains prevailed, only a few resisting Cu alone (13%) (Figs. 1 and 2). Instead at site B with the lower Cu content, the copper-resistant strains were 38% and bacteria resistant solely to Cu were 45% (Figs. 1 and 2). Seven *Ralstonia* isolates were highly resistant to Cu: *Ralstonia* sp. E-50 grew up to 2 mM, *R. taiwanensis* 181 up to 3 mM and *Ralstonia* sp. 12–100, 13–100, 14–100, 15–100 and 16–100 up to 4 mM. All these *Ralstonia* grew in the presence of 0.5 mM Zn, Ni and Pb, but only *R. taiwanensis* 181 resisted 0.5 mM Cd.

Molecular determinants for resistance to a variety of metal ions were screened for by PCR

Table 3. Closest relative to strains identified by sequencing 16S rRNA gene

Strain	Closest relative	Accession number	% Homology
5	*Brevundimonas* sp.	DQ108394	99.4
4	*Ochrobactrum* sp.	AY331579	99.3
6	*Alcaligenes faecalis*	AJ509012	97.9
10	*Brevundimonas* sp.	BSJ227798	99.2
11	*Sphingopyxis* sp.	AB161684	100
12	*Alcaligenes* sp.	AY283260	97.3
14	*Stenotrophomonas acidaminiphila*	AF273079	99.7
16	*Sphingobacterium multivorum*	AB100738	98.5
30	*Paenibacillus lautus*	AB073188	97.6
31	*Bacillus megaterium*	AF142677	99.4
55	*Sphingomonas* sp.	AB018439	98.3
63	*Oerskovia turbata*	X79454	98.4
71	*Arthrobacter* sp.	AY371243	97.9
81	*Bacillus megaterium*	AY030338	98.3
128	*Frateuria* sp.	AF406662	98.1
134	*Bacillus* sp.	AF286480	97.8
146	*Streptomyces coelicolor*	AL939108	97.2
147	*Bacillus cereus*	AE016999	99
162	*Bacillus sp.*	AM286802	100
173	*Bacillus licheniformis*	AY017347	98.0
174	*Bacillus pumilus*	AB098578	100.0
176	*Arthrobacter* sp.	AF441731	98.9
181	*Ralstonia taiwanensis*	AJ555134	100
188	*Arthrobacter* sp.	U85895	98
195	*Paenibacillus* sp.	AF324200	97.4
206	*Sphingomonas* sp.	AB018439	98.7
215	*Sphingomonas* sp.	AF131295	98.5
233	*Sphingomonas* sp.	AB033949	98.9
13–100	*Ralstonia* sp.	AB232327	100
Es 17	*Burkholderia cepacia*	AB114607	99.1
ES-2	*Arthrobacter histidinolovorans*	AF501356	99.8
ES-8	*Pseudomonas putida*	AB109776	98.8

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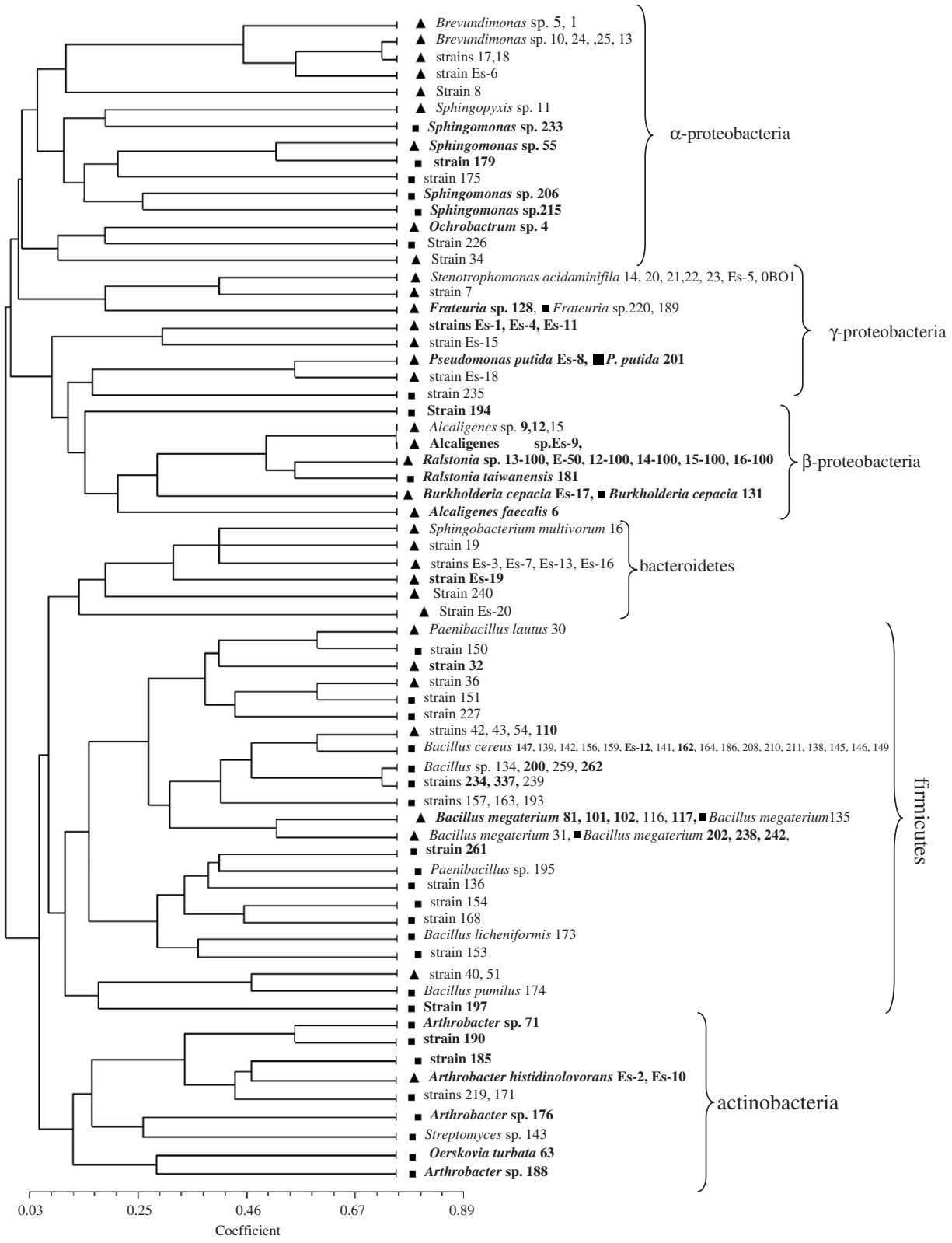

Figure 1. Distribution of isolates among 68 OTUs generated on ribosomal intergenic spacer analysis (RISA) and amplified ribosomal DNA restriction analysis (ARDRA). ■, isolates from site B; ▲, isolates from site A; bold type: 0.1 mM Cu-resistant isolates. Phylogenetic tree was constructed by NTSYS software package with Jaccard coefficient.

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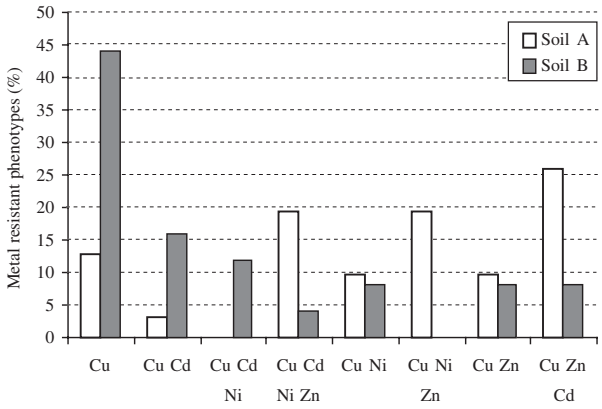

Figure 2. Heavy-metal-resistant phenotypes. The isolates were grown in minimal medium TMMG separately supplemented with either 0.1 mM Cu or 0.5 mM Cd, Ni, Zn.

amplification of gene fragments with primers for *pcoR*, *pcoB*, *copAB*, *czcC*, *cadA*, and *merRT ΔP*. None of the isolates yielded amplicons related to *pcoR*, *copAB*, *czcC*, or *cadA*, suggesting the presence of highly divergent genes or the absence of specific genetic traits for metal resistance in at least some strains.

Primers for *merRT ΔP* successfully amplified an expected fragment of 1.0 kpb in *R. taiwanensis* 181 and in *Ralstonia* sp. strains 12–100, 13–100, 14–100, 15–100, and 16–100. The restriction map of the amplicons produced two different patterns: one for strain 181 and the other clustering together strains 12–100, 13–100, 14–100, 15–100, and 16–100, evidencing the presence of two distinct alleles. The *merRT ΔP* nucleotide sequence of *R. taiwanensis* 181 and *Ralstonia* sp. strains 12–100 revealed homologies of 98% and 100%, respectively with *merRT ΔP* genes of *Ralstonia eutropha* JMP134 (Trefault et al., 2004).

Diversity of 16S rDNA gene amplicons from DGGE analysis

Direct PCR-DGGE of the total soil community and the bacterial biomass from PW allowed a description of the structure of microbial communities of the two sites. In our analysis, the number of DGGE bands was taken as an indication of the number of species present in each sample (Table 4), even though one band could possibly represent more than one species (Heuer and Smalla, 1997) and some bacteria produce more than one band on DGGE gel (Muyzer et al., 1993). Soil and PW bands were correlated to the bands of pure strains on the basis of electrophoretic mobility. Table 4 lists, by order of migration distance, the species present in the markers, prepared as described above.

Two replicates were initially produced for each DGGE sample, generating similarities between replicates of >95% (data not shown). However, to have all the samples on the same gel, only a single sample was run on the final gel.

The Sorensen index calculated by DGGE profiles of total bacterial community evidenced clear differences ($S = 0.26$) between the more (A) and the less (B) Cu-polluted site (lanes A and B, Fig. 3). Only a few bands were common to both sites (Fig. 3, Table 4): band 3 corresponding to *Sphingopyxis* sp. 11, *Stenotrophomonas*-like strain 7, *Paenibacillus*-like strain 36, *Bacillus licheniformis* 173, and band 28 corresponding to *Oerskovia turbata* 63. Bands 39 and 40 were not identified.

The bands of PW profiles were less numerous than those of the corresponding soil DNA profiles, indicating that most of the soil bacteria were not culturable under the conditions used, and that culturable bacteria grown on TSA/10 were not representative of the dominant groups in soils (Fig. 3). DGGE profiles of heterotrophs (PW_A, PW_B) and Cu-tolerant bacteria (PW_{ACu}, PW_{BCu}) confirmed differences in species composition between the two sites: S for PW_A vs. PW_B was equal to 0.25; the *S* value for PW_{ACu} vs. PW_{BCu}, 0.42, indicated that Cu had exerted a selective pressure on the bacteria. Band 26, corresponding to Cu-resistant *Alcaligenes faecalis* 6 and to strain 34, was common to A, PW_A and PW_{ACu} (Fig. 3, Table 4). Band 2 corresponding to *S. acidaminiphila* 14, *Paenibacillus*-like strain 32, *B. cepacea* Es17, *Bacillus*-like strain 157 and band 31 corresponding to *Brevundimonas* sp. strains 10 and 1, *Alcaligenes* sp. 9, were common to PW_A and PW_{ACu} (Fig. 3, Table 4). In PW_{Acu}, the intense bands 2, 26, and 27 indeed corresponded to bands of Cu-resistant strains able to grow in TMMG with 0.1 mM Cu (Fig. 3); while in PW_{BCu} the two intense bands 10 and 11 corresponded instead to those of *Bacillus* strains able to grow only in TSA/10 supplemented with 50 mg/l of $Cu(NO_3)_2$. Band 5, corresponding to *Sphingomonas* sp. 215, strain 226, *Bacillus*/*Paenibacillus*-like strain 136, *Paenibacillus*-like strain 151, and band 11 were common to PW_B and PW_{BCu} (Fig. 3, Table 4). Different intense bands in PW_B and PW_{BCu} not corresponding to any isolate were excised and sequenced. Unfortunately, many single bands from the original DGGE gel resulted in multiple bands after reamplification and therefore could not be sequenced. Only two bands named 89 and 99 were identified and showed 95% and 93% homology with *Pedobacter* sp.

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Table 4. Composition of DGGE reference markers M1, M2, M3, M4, and M5 obtained by mixing V3-PCR products from various bacterial isolates

Marker[a]	Band[a]	Strain identification
M1	7	*Sphingobacterium* – like strain Es19
	29	*Sphingobacterium multivorum* 16
	30	*Sphingobacterium* – like strain Es3
	24	*Paenibacillus lautus* 30
	2	*Stenotrophomonas acidaminiphila* 14, ***Paenibacillus*** – **like strain 32**
	3	*Sphingopyxis* sp. 11, *Stenotrophomonas* – like strain 7, *Paenibacillus* – like strain 36
	31	*Brevundimonas* sp. strains 10 and 1, ***Alcaligenes*** **sp. 9**
	32	*Ralstonia* sp. E50
	33	*Brevundimonas* – like strain 18
M2	7	*Pseudomonas putida* Es8
	22	*Pseudomonas* – like strain Es1
	23	*Bacillus megaterium* 31
	24	*Pseudomonas* – like strain Es18, *Bacillus* – like strain Es12
	24+25	*Bacillus megaterium* 117
	2	***Burkholderia cepacia*** **Es17**
	25	*Frateuria* sp. 128, *Sphingomonas* sp. 55, *Bacillus* – like strain 51, strain Es9
	26	***Alcaligenes faecalis*** **6, strain 34**
	27	***Arthrobacter histidinolovorans*** **Es2**
	28	*Oerskovia turbata* 63
M3	16	*Pseudomonas putida* 201, strain 240
	9	*Paenibacillus* – like strain 227
	5+9	*Sphingomonas* sp. 233
	11	*Frateuria* sp. 220, *Bacillus pumilus* 174, *B. megaterium* strains 135 and **202**, ***Bacillus*** – **like strain 147**
	17	*Sphingomonas* – like strain 179
	18	*Frateuria* sp. 189, *Sphingomonas* sp. 206
	19	*Ralstonia taiwanensis* 181
	20	*Arthrobacter* – like strain 185, *Arthrobacter* sp. 71, strain 176
	21	*Arthrobacter* – like strain 190, strain 197
M4	7+8	*Pseudomonas* – like strain 235
	9	*Bacillus* sp. 200
	10	*Bacillus* sp. 134
	11	*Bacillus* – like strain 141
	12	*Burkholderia cepacia* 131
	5	***Sphingomonas*** **sp. 215**, strain 226
	13	*Arthrobacter* sp. 188
	14	*Streptomyces* sp. 143
	15	strain 194
M5	1	*Bacillus/Paenibacillus* – like strain 168
	2	*Bacillus* – like strain 157
	3	*Bacillus licheniformis* 173
	4+5	*Bacillus/Paenibacillus* – like strain 153
	5	*Bacillus/Paenibacillus* – like strain 136, *Paenibacillus* – like strain 151
	6	strain 175, *Arthrobacter* – like strain 171

Some species are identified by more than one band. Cu-resistant strains (0.1 mM) are in bold character.
[a]Marker and band numbers refer to Fig. 3.

Discussion

In this study, we combined methodologies commonly used to assess changes in microbial communities of experimentally polluted soil to investigate a real-field condition by analysing two sites with high Ex-Cu-content. The effect of copper on the number of culturable bacteria is uncertain as the findings differ between studies. In the present investigation, total heterotroph numbers as well as those of ammonia-oxidising nitrifiers were comparable at the two sites, within the range found in

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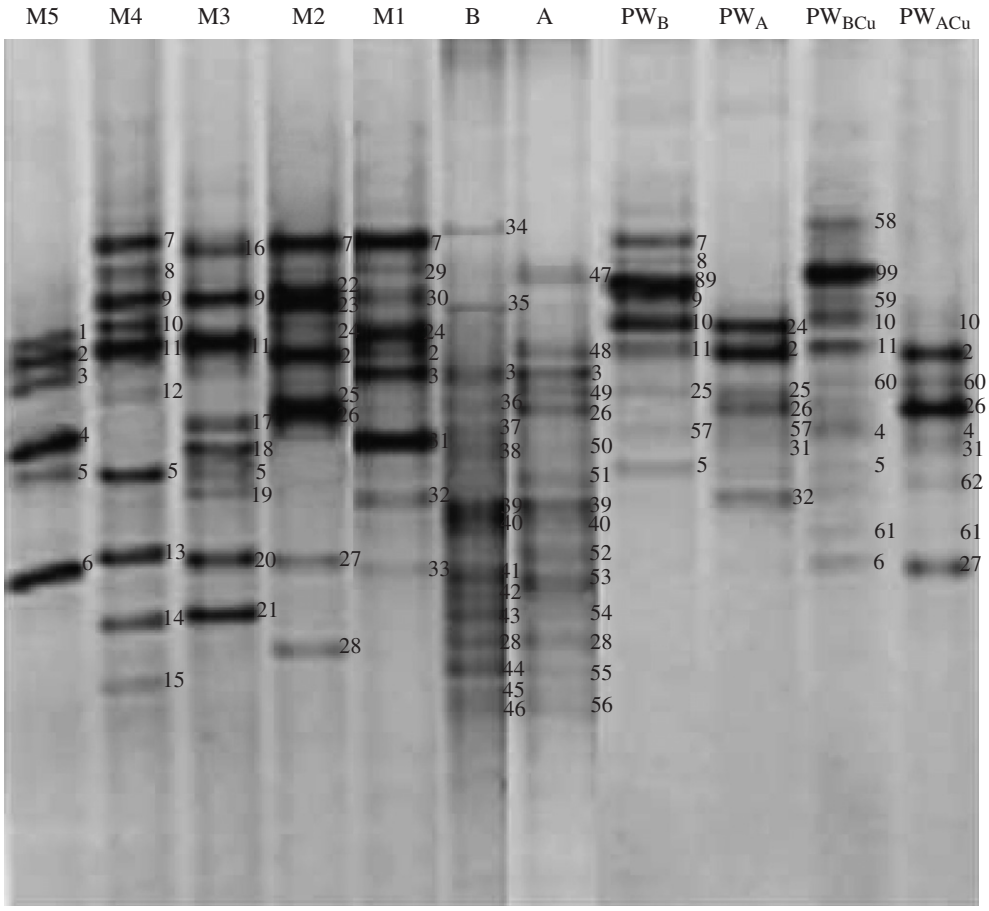

Figure 3. Linking of bacterial strain identities to DGGE profiles of total community at site A (A) and site B (B) and of biomass accumulated on TSA/10 agar plates not supplemented (PW_A, PW_B) and supplemented with 50 mg/l Cu $(No_3)_2$ (PW_{Acu}, PW_{BCu}). M1, M2, M3, M4, and M5 were marker lanes consisting of DGGE fragment mixtures of different bacterial species, according to Table 4. Sorensen's similarity index of site A vs. site B is equal to 0.26, that of PW_A vs. PW_B is equal to 0.25 and that of PW_{Acu} vs. PW_{Bcu} is equal to 0.42. An index value of 0 indicates that two samples are completely different, whereas an *S* value of 1 indicates that they are identical.

agricultural soil (Bruns et al., 1999), indicating an adaptation also of the latter group, retained sensitive to Cu. However, the presence of copper influenced the fraction of fast-growing bacteria. The greater percentage of fast-growing bacteria (20%) in the less Cu-polluted site B confirms that r-strategist bacteria are more sensitive to toxic substances than K-strategist (De Leij et al., 1994), but it contradicts the prediction that disturbed ecosystems contain a higher proportion of opportunists (Odum, 1985). The metal tolerance of soil bacteria communities was high at the two sites and relatively independent of the level of total and Ex-Cu contamination, suggesting that neither total- nor Ex-Cu content could be used to evaluate the Cu tolerance of soil bacteria communities. On the contrary Kunito et al. (1999) found a significant correlation with the amount of Ex-Cu in soils. In a long-term contaminated real-field evaluation without a non-contaminated control sample, a reliable estimation of the impact is not necessarily provided by counts of tolerant bacteria.

The different DGGE profiles at the two sites could indicate that the structural diversity changed, or at least, the community species composition was sufficiently altered to originate a different pattern in agreement with other studies (Sandaa et al., 1999; Müller et al., 2001). Shifts in the culturable bacterial community were detected also analysing the phylogenetic diversity of isolates. It was apparent that the abundance of individual groups differed in the two sites. Gram-positive bacteria (*Bacillus, Paenibacillus* belonging to Firmicutes and *Arthrobacter*) prevailed at site B with the lower Ex-Cu content, while Gram-negative bacteria prevailed at site A with the higher Ex-Cu content (Pennanen et al., 1996; Trajanovska et al., 1997), and distinctive genera as *Alcaligenes*, *Sphingobacterium*, *Brevundimonas*, and *Stenotrophomonas* were found. Roane and Kellogg (1996) and Ellis

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et al. (2003) found that the group of Firmicutes had a high relative abundance in metal-contaminated soils. In this study, *Bacillus* and related genera were the dominant group in both sites, probably due to their sporulating ability and to the presence in the cell wall of carboxyl groups that mediate the uptake and accumulation of the metal on the cell surface. Also the spores bind a variety of metal by the carboxyl and phosphate groups present on the spore surfaces (Tebo, 1995). The binding of heavy metals by vegetative cells and spores is, however, species- and even strain-specific (Selenska-Pobell et al., 1999).

The long-term exposition to different Cu concentrations could have selected two different bacterial communities with the same biodiversity degree but with different dominance level.

Although molecular determinant sequences for metal resistance are known for many bacteria, we did not find such sequences in the newly metal-resistant isolates, probably because of sequence divergences . It is quite possible that many of these isolates do contain copper-resistant genes or other multiple heavy metal-resistant determinants with sequences sufficiently different from the primer sequences, and no differences were detectable by the approach used.

Despite the amplification of *merRT ΔP* regulatory genes, no *Ralstonia* isolates grew in the presence of 0.01 mM Hg (II), suggesting the absence in these bacteria of a functional Hg resistance system (Mergeay et al., 2003). When aligning the *merR* region only, the gene of *Ralstonia* sp. 12–100, resistant to Cu, Zn, and Ni, showed homologies with *cueR* (32% identity) and the gene of *R. taiwanensis* 181 resistant to Cu, Zn, and Cd showed homologies with *cueR* (28% identity) and *cadR* (34% identity). CopR, CueR, and CadR are members of the Mer family of metal and other stress factor-responsive regulators in different bacteria (Cooksey, 1994; Brown et al., 2003; Mergeay et al., 2003). In *E. coli*, for example, CueR, a Mer-like transcriptional activator, was found to be induced by copper (Stoyanov et al., 2001).

In conclusion, despite the absence of a true control, we detected tendencies concerning the impact of long-term copper exposure on the diversity and Cu-resistant phenotypes of culturable bacterial communities. Similar tendencies were also detected analysing DGGE profiles from directly extracted DNA. Our results support the recently proposed view that readily culturable bacteria are probably the most active prokaryotes in soil and so provide a useful, rapid assessment of biological responses to heavy metal pollution (Bakken, 1997; Kozdroj and van Elsas, 2000; Ellis et al., 2003).

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