Microbial Ecology

Genetic Diversity of Bacterial Communities of Serpentine Soil and of Rhizosphere of the Nickel-Hyperaccumulator Plant *Alyssum bertolonii*

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Abstract

Serpentine soils are characterized by high levels of heavy metals (Ni, Co, Cr), and low levels of important plant nutrients (P, Ca, N). Because of these inhospitable edaphic conditions, serpentine soils are typically home to a very specialized flora including endemic species as the nickel hyperaccumulator *Alyssum bertolonii*. Although much is known about the serpentine flora, few researches have investigated the bacterial communities of serpentine areas. In the present study bacterial communities were sampled at various distances from *A. bertolonii* roots in three different serpentine areas and their genetic diversity was assessed by terminal restriction fragment length polymorphism (T-RFLP) analysis. The obtained results indicated the occurrence of a high genetic diversity and heterogeneity of the bacterial communities present in the different serpentine areas. Moreover, TRFs (terminal restriction fragments) common to all the investigated *A. bertolonii* rhizosphere samples were found. A new cloning strategy was applied to 27 TRFs that were sequenced and taxonomically interpreted as mainly belonging to Gram-positive and α-Proteobacteria representatives. In particular, cloned TRFs which discriminated between rhizosphere and soil samples were mainly interpreted as belonging to Proteobacteria representatives.

Introduction

Serpentine (ultramafic) outcrops are distributed all over the world and, for their natural geological origin, are characterized by high levels of cobalt, chromium, and especially nickel [5]. The vegetation adapted to survive in these soils [22] can include the so-called nickel-hyperaccumulating plants [3] that concentrate metal in stems and leaves to levels higher than the substrate concentration and far in excess from any physiological requirement (more than 1000 μg g^{-1} shoot dry matter). *Alyssum bertolonii* Desv. (Brassicaceae) is a nickel hyperaccumulator, endemic to the serpentine outcrops of Central Italy [51], belonging to a genus which recently has stirred new and increasing attention due to its practical application for phytoremediation [8, 41].

The study of metal-hyperaccumulating plant effects on soil microorganisms is an important topic. Microorganisms can have a great impact on the performances of revegetation of polluted soils [37]. Studies on serpentine soils may provide new insight into bacterial diversity under unfavorable conditions, new isolates, and probably new genetic information on heavy-metal resistance, which could be exploited.

Bacteria present in serpentine soils and their interaction with hyperaccumulating plants have focused the attention of several investigators in past years [9, 15, 16, 26, 31, 36, 39, 44, 52, 53]. These authors found that serpentine bacterial communities tolerated spiking of metals, such as nickel, more than those from unpolluted soils and that the presence of hyperaccumulating plants as *Sebertia acuminata, Thlaspi caerulescens*, and *Alyssum bertolonii* led to an increase in metal-resistant bacteria proportion in the soil samples collected near the plants. Moreover, metal-resistant bacteria present in the plant rhizosphere may play an important role in regulating the availability of metal for the plant [46, 53].

Nickel-resistant rhizosphere bacteria have recently been shown to increase nickel uptake into the shoots of the nickel hyperaccumulator *Alyssum murale* [1].

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DNA-based techniques have become a powerful tool for studying diversity and composition of soil bacterial communities in cultivation-independent ways [49]. One of the most important methods for soil bacteria surveys is the analysis of a 16S rDNA clone library [14, 17]. However, because of the complexity of soil communities and the effort required for this type of analysis, 16S rDNA clone libraries have been restricted to the analysis of a single or a few samples in an environment.

Terminal Restriction Fragment Length Polymorphism (T-RFLP) is a recently introduced PCR-based tool for studying the genetic diversity of bacterial communities [20, 25, 28]. T-RFLP analysis is based on the detection of a single restriction fragment in each sequence amplified directly from the environmental sample of DNA and is capable of surveying dominant members comprising at least 1% of the total community [10]. In recent years T-RFLP has been widely used for the analysis of bacterial communities in different conditions (for examples, see [11, 13, 27, 33–35, 40]) and to assess spatial heterogeneity of bacterial communities in soils [23], sediments, and water environments [4, 7, 21, 43].

The aim of this work was to investigate the genetic diversity of bacterial communities present in serpentine soils and in the rhizosphere of the nickel-hyperaccumulating plant *A. bertolonii* by using T-RFLP analysis.

Materials and Methods

Sampling Procedure and Soil Parameters. Soil samples (at ~10 cm of depth) of ~200 g were taken during winter 2001/2002 with a clean steel spatula sterilized with ethanol. Samples were shaken and kept in sterile plastic boxes at 4°C for a few hours before DNA extraction. Samples were collected from three serpentine outcrops, located in Tuscany (Italy): Galceti, Impruneta, and Pieve Santo Stefano. The three localities had different plant coverage of the serpentine outcrop; in particular, the Galceti outcrop was a rocky hill covered by few pines (*Pinus maritima*), whereas the Impruneta outcrop was covered by a wood of cypress (*Cupressus sempervirens*), and Pieve Santo Stefano outcrop was in a cliff covered by a few grasses. Soil chemical characteristics were determined on soil samples taken at 10 cm from the stem of *Alyssum bertolonii* following the methods described by Sparks [47]. For the metal analysis, samples were dried at 80°C for 1 day, weighed, and digested by wet ashing with HNO_3:$HClO_4$ (5:2 v/v). The soil metal extractable fraction was determined following the method of Escarré et al. [12]. Metal concentration in the samples was determined by atomic absorption spectroscopy (PE Biosystems 370, PerkinElmer, Norwalk, CT, USA). All the measurements were performed in triplicate. In each location three soil samples were taken at various distances from the hyperaccumulating plant *Alyssum bertolonii* Desv. Soil samples were labeled as A, B, and C portions to indicate absence of the plant (soil free from plants in an area of 50 cm of diameter), presence of the plant at a distance of 10 cm, and plant shoot at 5 cm, respectively. No plant species other than *A. bertolonii* were present in an area of 1 m of diameter from the sampling site. For the collection of the rhizosphere soil particles, the main root of the plant with a diameter of ~0.2 cm was cut from 5 cm to 10 cm under the collar in sterile conditions and the larger soil particles were shaken away. These rhizosphere samples were labeled as D portion.

DNA Extraction and PCR Conditions. DNA was extracted with the Fast Prep DNA Kit for Soil (BIO101, Qbiogene, Carlsbad, CA, USA) following manufacturer's instructions. For the rhizosphere samples, the roots were mixed in a sterile tube with 40 mL of 10 mM $MgSO_4$ and shaken for 2 h on a slowly rotating plate; then soil particles were collected by centrifugation and used for DNA extraction. Extractions were performed in duplicate.

16S rDNA was amplified in a 50-μL volume with 20 ng of template DNA and 2 U of *Taq* DNA polymerase (Dynazyme II, Finnzyme, Espoo, Finland) using 27f primer labeled with TET (4,7,2′,7′-tetrachloro-6-carboxyfluorescein) and FAM (6-carboxyfluorescein) and 1495r primer [24], as described in Mengoni et al. [31]. The use of two fluorescent dyes allowed two different restriction digestions to run together in the same capillary, reducing cost and time of the analysis. PCR reactions were repeated three times on each DNA sample (technical replicate).

16S rDNA T-RFLP. The amplified products were purified with a Qiaquick PCR purification kit (Qiagen Inc. Chatsworth, CA, USA), and 600 ng of amplified 16S rDNA was digested with 20 U of *Msp*I, *Hha*I, *Rsa*I, or *Hinf*I (New England Biolabls, Beverly, MA, USA) for 3 h at 37°C. A 200-ng aliquot of the digested products was resolved by capillary electrophoresis on an ABI310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) using TAMRA 500 (Applied Biosystems) as size standard for GenScan (Applied Biosystems) analysis. Fragment sizes from 35 to 500 bp were considered for profile analysis.

Analysis of T-RFLP Profiles. By comparison of T-RFLP profiles from the duplicate DNA samples and the three technical replicates a derivative profile was created following the same criteria used by Dunbar et al. [10]. Only fragments with fluorescence intensity ≥50 arbitrary units of fluorescence were considered, and the total DNA quantity represented by each profile was checked by summing all peak intensities. Alignment of the profiles was performed directly on the output table of the soft-

Table 1. Chemical characteristics of serpentine soil from the three localities

	Impruneta		*Galceti*		*Pieve S. Stefano*	
	Total	*Extractable*	*Total*	*Extractable*	*Total*	*Extractable*
Nickel (μg g^{-1} d.w.)	1774 ± 200	97.3 ± 7.2	1214 ± 229	129 ± 16	2412 ± 99	11.5 ± 1.8
Cobalt (μg g^{-1} d.w.)	152 ± 12	n.d.	104 ± 48	6.5 ± 3.2	112 ± 5	n.d.
Chromium (μg g^{-1} d.w.)	2321 ± 475	n.d.	1080 ± 177	n.d.	1180 ± 142	n.d.
Magnesium (mg g^{-1} d.w.)	195 ± 11	1.78 ± 0.29	174 ± 10	1.72 ± 0.31	159 ± 82	3.56 ± 0.28
Calcium (mg g^{-1} d.w.)	2.1 ± 0.3	0.422 ± 0.005	3.3 ± 1.3	0.406 ± 0.028	3.8 ± 0.8	0.228 ± 0.081
pH	7.52 ± 0.09		7.26 ± 0.04		7.15 ± 0.06	
CEC (meq 100 g^{-1} d.w.)	24.5 ± 4.9		38.1 ± 0.2		28.1 ± 5.1	
Organic matter (%)	4.00 ± 0.74		8.26 ± 1.26		1.66 ± 0.04	

Values are means of triplicate sample measurements. Errors are standard deviations. CEC: cation exchange capacity. pH was measured at a dilution of 1:2.5 soil: H_2O. d.w: Dry weight; n.d.: not detectable.

ware GenScan and ±0.5 bp was allowed to discriminate peaks of consecutive sizes. Derivative T-RFLP profiles of the different enzymes were then combined together and a binary vector, in which presence or absence of peaks were scored as strings of ones or zeros, was prepared. The vectors of binary profiles of each soil portion and location were then compared to compute the community similarity values using the Dice index as implemented in the software NTSYSpc 2.0 [38], The matrix of Dice similarity values was then used for construction of a UPGMA dendrogram and for Principal Component Analysis using the modules present in NTSYSpc 2.0 [38].

Cloning and Sequencing of TRFs. TRFs of interest were cloned by means of adapter as described in Mengoni et al. [32]. The sequences of double strand adapter and selection primer for the *Hha*I restriction site were: 5′-GAGCATCTGACGCATGGTTAA-3′, 5′-CCATGCGTCAGATGCTCCG-3′, selection primer: 5′-CCATGCGTCAGATGCTCCGC-3′; and for the *Hinf*I restriction site, 5′-ANTCTCGTAGACTGCGTACC-3′, 5′-GGGGGGTACGCAGTCTACGAG-3′, selection primer: 5′-GGGGGGTACGCAGTCTACGAG-3′. Clones were sequenced from M13 forward primer external to the insertion site of cloning plasmid [32] using the DYEnamic ET Terminator Cycle Sequencing Kit (Amersham Biosciences Europe GmbH, Freiburg, Germany), and sequences were run on an ABI310 genetic analyzer (Applied Biosystems). Sequences were compared with those present in the GenBank database by using the BLAST tool [2] to obtain similarity matches.

Results

Metal Concentration and Soil Characteristics. The three serpentine locations showed high concentrations of heavy metals as Ni, Co and Cr and a higher level of Mg in relation to Ca (Table 1). The soils had very similar and slightly basic pH values. The CEC values were similar, too, whereas the organic matter concentration varied from 1.66% (Pieve Santo Stefano) to 8.26% (Galceti).

Ribotype Richness of the Soil Samples. The ribotype richness of serpentine soil samples was estimated determining the number of terminal restriction fragments (TRFs). The four restriction endonucleases applied to 16S rDNAs amplicons yielded a total of 138 different peaks or TRFs. *Msp*I produced the highest number of peaks (48), while *Hha*I gave the lowest (27); the other two enzymes, *Rsa*I and *Hinf*I, yielded 35 and 28 peaks, respectively. Figure 1 shows the overall trends of the total number of TRFs for the three localities. Impruneta and Galceti showed slightly more peaks in soil portions B, C and D than in A.

Similarities of T-RFLP Community Profiles. The similarity of the 12 samples based on UPGMA analysis of T-RFLP profiles is shown in Fig. 2. Samples from the same locality did not form homogeneous clusters. Comparison of the samples, with respect to the soil portion they were derived from, revealed that soil portions A and B of the different localities were the most

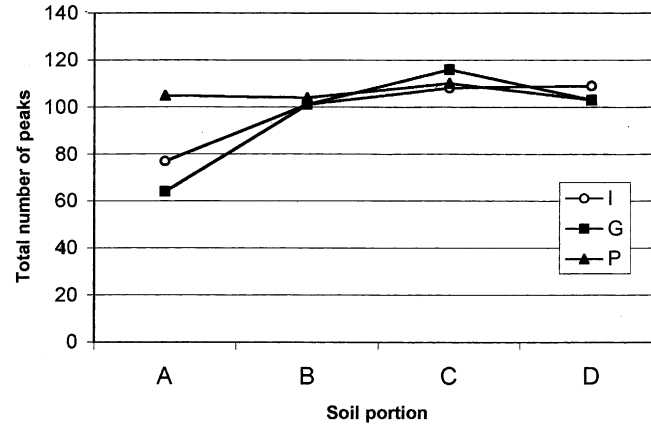

Figure 1. Total number of TRFs derived from amplified 16S rDNA genes within the soil portions in the different localities. Plotted values represent the sums of the number of TRFs from the restriction digestions with four endonucleases (*Msp*I, *Rsa*I, *Hha*I, *Hnf* I) obtained in soil portions A, B, C, and D for the localities of Impruneta (I), Galceti (G), and Pieve Santo Stefano (P).

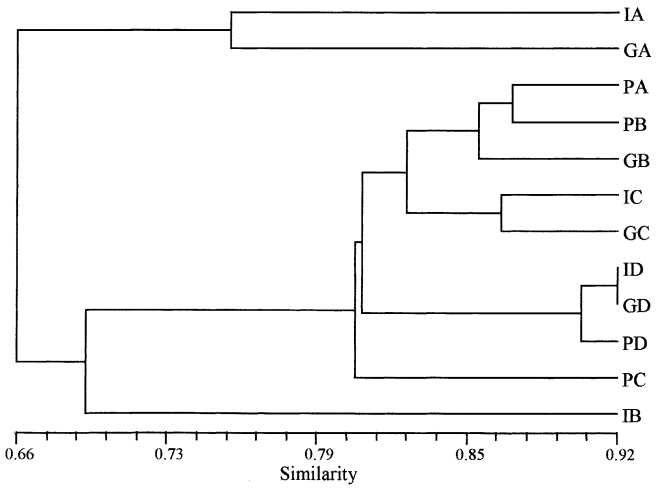

Figure 2. UPGMA dendrogram computed from Dice similarity matrix among T-RFLP profiles of amplified 16S rDNA genes from the different soil portions and locations. Scale bar indicates Dice similarity values.

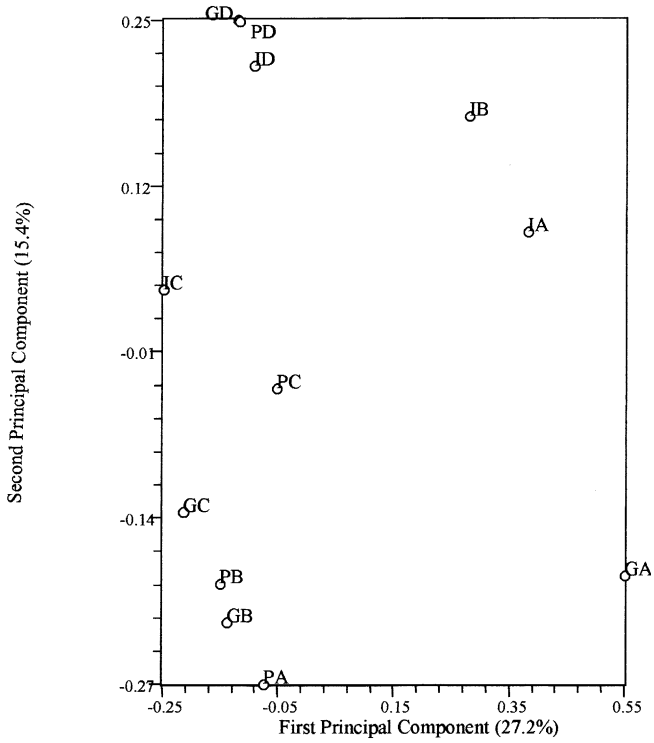

Figure 3. Principal component analysis of the 12 T-RFLP profiles. In parentheses, percentage of variance due to the displayed component.

variable (average similarity: 0.71 and 0.74, respectively). Soil portions C were more similar (average similarity: 0.82) and rhizosphere samples (portions D) were the most similar (average similarity: 0.91), forming a homogeneous cluster. Principal component analysis (Fig. 3) illustrated, on the first two components (explaining the 42.6% of total variance), a similar pattern with the samples from the same locality being interspersed, while the same soil portions (especially D and C) formed homogeneous groups. In particular the first component mainly separated the free soil samples (A) from samples nearer to the plant (B, C, and D). The second component separated the rhizosphere samples (D) from B and C samples.

Taxonomic Interpretation of TRFs and Dominant Eubacterial Groups. Among the 138 TRFs obtained, 27 were selected on the basis of their distribution. In particular, eight TRFs present in all samples, seven TRFs exclusive of rhizosphere samples, and 12 distributed in more than one soil portion were chosen for the analysis. These TRFs had a fluorescence intensity >200 fluorescence units in our experimental conditions. Nineteen TRFs derived from *Msp*I digestion, six from *Hha*I digestion, and two from *Hinf*I digestion were sequenced using a previously described procedure [29], and the sequences compared to sequences present in GenBank to obtain a taxonomic interpretation of T-RFLP profiles. Sequences are presented in the Appendix. The results of the analysis are shown in Table 2. Most of the sequenced TRFs showed similarities with Gram-positive bacteria (10) and α-Proteobacteria (6). The distribution of the sequenced TRFs indicated that most of the TRFs common to all portions could be interpreted as belonging to non-Proteobacteria representatives, while most of TRFs exclusive of all portions D (rhizosphere) were interpreted as belonging to Proteobacteria representatives, mainly from the Alpha subdivision.

Discussion

The studies on bacterial communities present in heavy-metal-contaminated ecosystems have mainly focused on the effect of anthropogenic metal pollution in shaping the genetic structure of the community [42]. In recent years the attention has been focused on metal-resistant bacterial communities present in the serpentine soil naturally rich in heavy metal and on bacteria associated with the roots of serpentine-endemic metal hyperaccumulating plants [9, 15, 26, 31, 44, 52, 53]. In this work, by using the T-RFLP technique, we provided a first description of the total bacterial communities isolated from serpentine soils along a distance gradient from the nickel-hyperaccumulating plant *Alyssum bertolonii*. In particular, the same rationale and the same soil samples which we previously used for the analysis of nickel-resistant bacteria [31] were investigated to possibly correlate the results obtained from the previous analysis with those from the total community profiles.

Chemical characteristics of the sampled soils were similar to those of other serpentine soils [5].

Table 2. Taxonomic interpretation of TRFs

Size (nt)	*Soil portion*[b]	*Best matched sequence (accession number, bit score, E-value, % of similarity)*[c]
*Msp*I[a]		
Gram-positive bacteria[d]		
49	B, C	Bacillales (AF227850, 48, 7e-4, 91%)
64	All	Actinomycetes (X68451, 119, 2e-25, 100%)
66	All	Micrococcace (AP353696, 119, 3e-25, 98%)
79	B, C	Actinomycetes (X68458, 117, 2e-24, 100%)
136	B, C, D	Actinobacteria (AF388343, 188, 3e-45, 93%)
145	A, B, C	Bacillales (AY214769, 236, 7e-60, 95%)
170	D	*Bacillus* sp. (AB021194, 281, 2e-73, 98%)
159	D	*Paenibacillus* sp. (AF407677, 220, 5e-55, 93%)
217	G, C	*Bacillus* group (X70312, 244, 4e-62, 95%)
Proteobacteria[d]		
71	All	β-Proteobacteria (AF447801, 40, le-8, 95%)
95	D, C	α-Proteobacteria (AB054157, 96, 2e-19, 98%)
149	D, $A^{(1)}$, $B^{(1)}$	α-Proteobacteria (AJ292621, 285, 7e-75, 98%)
152	D	α-Proteobacteria (X65571, 168, le-39, 87%)
153	D	α-Proteobacteria (X97076, 184, 3e-44, 92%)
175	D	*Metylosinus* sp. (AJ318115, 200, 4e-49, 97%)
212	D, $C^{(2)}$, $B^{(2)}$	β-Proteobacteria (AF447801, 64, 9e-8, 95%)
Other groups[d]		
100	B	Bacteroidetes (AY214775, 72, 6e-41, 97%)
118	All	Verrucomicrobia (AF010081, 157, le-35, 95%)
202	D	*Acidobacteria* (AJ006027, 321, le-85, 96%)
*Hha*I[a]		
Proteobacteria[d]		
62	C	Rhizobiaceae (AE0096656, 121, le-25, 100%)
66	B, C, D	β-Proteobacteria (AF422666, 115, 7e-24, 98%)
89	B	*Azospirillum* group (M59061, 129, 4e-28, 100%)
Other groups[d]		
90	All	Chlorobi (AY118151, 105, le-20, 91%)
95	All	Acidobacteria (AY281355, 143, 5e-32, 100%)
99	All	Verrucomicrobia (AY214734, 87, 3e-15, 92%)
*Hinf*I[a]		
41	All	β-Proteobacteria (AF447801, 46, 3e-3, 90%)
49	D	γ-Proteobacteria (AF114581, 48, 9e-4, 91%)

(1): Present in Impruneta sample only; (2): present in Galceti sample only; (3): present in Galceti and Pieve Santo Stefano samples only.
[a]The enzyme used for the generation of the TRF.
[b]The occurrence of the TRF in the different soil portions is reported. Unless indicated the TRFs were present in all locations.
[c]The bacterial group shown is that comprising the best matched sequences after BLAST search. In brackets the accession numbers, bit score, *E*-value, and percentage of similarity of the best matched sequences are reported. The bit score is an alignment score of the query sequence against the subject sequence present in the database (the higher the score the better the homology), while the *E*-value or expectation value is the number of different alignments with scores equivalent to or better than that shown that are expected to occur in a database search by chance. The lower the *E*-value, the more significant the homology.
[d]The taxonomic division of the best matched sequences is reported.

A high genetic diversity was detected, especially in the samplings at 10 cm (B) and 5 cm (C) distant from the plant. Actually, average numbers of TRFs for the four different enzymes were consistent, or slightly less, with numbers reported for forest soil [10] and marine sediments [4, 50], but higher than numbers reported for copper-contaminated lake sediments [21]. The number of TRFs in Galceti and Impruneta samples showed a slight increase from A to D, particularly from A to B, suggesting an effect of the plant in increasing the microbial diversity. A plant-driven effect on bacterial community was also suggested by the comparison of T-RFLP profiles. Both UPGMA and principal component analyses indicated plant, more than locality, as the main factor in shaping the community profile. In particular, both first and second principal components divided the overall variance mainly in relation with the proximity to the plant. Furthermore, it was possible to recognize a gradient of similarities among samples belonging to different localities, from the most different soil portions A, to the more similar B and C portions, to the rhizosphere in which differences between samples of the three localities appeared very low. The effect of plant roots on bacterial communities is a well-established topic [19]. Plant rhizosphere has been recognized by several authors [18, 30, 45] as harboring a complex and differentiated bacterial community. Recently the rhizosphere bacteria of the Zn hyperaccumulator *Thlaspi caerulescens* have stir-

red much attention about their possible role in increasing metal accumulation by the plant. In particular, two papers [9, 26] reported that *T. caerulescens* rhizosphere was rich in metal-resistant bacteria, also exhibiting multiple heavy-metal resistances. Recently a cultivation-independent approach was used for the analysis of *T. caerulescens* rhizosphere (gremion) showing a high proportion of Actinobacteria in the metabolically active bacterial community. Finally, Whiting et al. [53] demonstrated that the addition of rhizosphere bacteria to axenic plant cultures increased plant Zn uptake. For the nickel hyperaccumulator *A. bertolonii* an effect of the rhizosphere was previously shown for the culturable fraction of microbial community [31], showing that as well as for *T. caerulescens*, a high number of metal-resistant isolates harbored multiple metal resistances (Ni, Co, Cr). Recently, bacterial strains isolated from the rhizosphere of the congeneric nickel hyperaccumulator *Alyssum murale* have been shown to increase plant nickel uptake up to 32% [1]. These strains seemed to facilitate the release of nickel from the nonlabile phase in the soil (by organic acids or siderophore production and phosphate solubilization), thus enhancing the availability of nickel to *A. murale*.

T-RFLP analysis has mainly been applied as a tool for the comparison of microbial communities [20, 25, 28], limiting the taxonomic interpretation of the profile either to clone libraries screening (for a review see [20]) or to *in silico* prediction [29]. In this work we applied a recently developed technique [32] which provides a partial taxonomic interpretation of TRFs by their sequencing. Taxonomic interpretation of some of the common and exclusive TRFs by this technique showed an uneven distribution of bacterial groups in the samples. Rhizosphere-exclusive TRFs were mainly interpreted as due to Proteobacteria (mainly α-Proteobacteria), whereas TRFs present in all samples were mainly due to non-Proteobacteria representatives. In a previous analysis on nickel-resistant bacteria present in the soil and in the rhizosphere of *A. bertolonii* [31], we found that several *Pseudomonas* strains were present in the plant rhizosphere, whereas on free soil several exclusive *Streptomyces* strains were found. It could be possible that most rhizosphere-specific α-Proteobacteria representatives found in T-RFLP profiles did not harbor Ni-resistant determinants or were not culturable under the aerobic and heterotrophic conditions applied. A similar result was found for the rhizosphere of other members of the family Brassicaceae (*Brassica napus* and *Thlaspi caerulescens*) in which rhizosphere representatives from α-Proteobacteria and Cytophaga–Flavobacterium–Bacteroides subdivisions were found in 16S rDNA clone libraries, while representatives from β- and γ-Proteobacteria subdivisions were found among cultured isolates [15, 18]. Several studies have provided evidence that heavy-metal-resistant Proteobacteria may protect plants or other bacteria from the toxic effects of heavy metals or even enhance metal uptake by hyperaccumulator plants [6, 48, 53]. Recently, an α-Proteobacteria representative (*Sphingomonas macrogoltabidus*) was isolated from the rhizosphere of the nickel hyperaccumulator *Alyssum murale* and has been shown to increase plant nickel uptake [1].

Summarizing, our results indicated that (i) the bacterial community present in serpentine soils is highly differentiated with members from Gram-positive species, Proteobacteria, Acidobacteria, Verrucomicrobia, and Chlorobi groups; (ii) the presence of the Ni-hyperaccumulating plant *A. bertolonii* seems to shape the community composition along a distance gradient at least at a 5-cm distance; and (iii) most of the genetic diversity distinguishing plant roots from free soil appears to be related to Proteobacteria strains.

Acknowledgments

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Appendix

Restriction enzyme	*Length (nt)*	*Sequence*
*Msp*I	49	GAGAGTTTGATCCTGGCTCAGTATATCGTAACAAGGTAGCCGTAGACCT
*Msp*I	64	GTTTGATCCTGGCTCAGGACGAACGCTGGCGGCGTGCTTAACACATGCAAGTGGAGCG ACAAAC
*Msp*I	66	GAGTTTGATCCTGGCTCCAGGATGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGA ACGATGAT
*Msp*I	71	GAGAGTTTGATCCTGGCTCAGTATATCGTAACAAGGTAGCCGTAGACCTGAGAGTTTGA NNCTGGCTCAGT
*Msp*I	79	GAGAGTTTGATCCTGGCTCAGGGCAAAGCCGCGAACGGGTGAGTAACACGTGGGTAAC CTGCCCCGATGATTGGGACAA
*Msp*I	95	GAGAGTTTGATCCTGGCTCAGAGTCGAACGAGACctTCGGGTTAGTGGCGCACGGGTGA GTAACGCGTGGGAACctGCCTGTGGTACGGAATAAC
*Msp*I	100	GAGAGTTTGATCCTGGCTCAGGAtGAACGCTAGCGGCAGGCTTAATACTGCAAGTCGAG GGGCAGCATGTcTTGTAGCAATACAGGAtGATGGCGACCGC

Continued

Appendix continued

Restriction enzyme	*Length (nt)*	*Sequence*
*Msp*I	118	GAGAGTTTGATCTGGCTCAGAACGAACGCTGGCGGCGTGGAtAAGACTGCAAGTCGAA CGATCACTATTGGGTAGCAATATTCGGTAGTGGTAGTGGCGCAAGGGTGCGTAACAC GTG
*Msp*I	136	GAGAGTTTGAtCcTGGctcAGGACGAACGCTNGGCGGCGTGCCTAACACATGCAAGTGGA GCGACGAACCAGGGCTTGCCTAGGGGCCAAGCCGCGAACGGGTGAGTAACACGTGG GTAACCTACCCCGATGACCG
*Msp*I	145	GAGAGTTTGATCCTGGCTCAGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCG AGCGTATCGATAGGAGCTTGCTCCTGTTGGTTAGCGGCGGACGGGTGAGTAACACG TGGGCAACCTGCCTGTAAGACTGGGATAACA
*Msp*I	149	GAGAGTTTGATCCTGGCTCAGAACGAACGCTGGCGGCATGCCTAACACATGCAAGTCG AACGAGACCTTCGGGTCTAGTGGCGCACGGGTGCGTAACGCGTGGGAATCTGCCCT TGGGTTCGGGATAACAGTTAGAAATGACTGCTAAT
*Msp*I	152	GAGAGTTGGATCCTGGCTCAGTGCGAACGCTGGCGNNAGGCCTAANNCATGCAAGTC GAGCGCCGNAGCAATACCGGAGTGGCAGACGGGTGAGNANCACGTGGGANCGNGN CTTTTGNTTCNGAACAACCCAGGGAAACTTGGGCTAATAC
*Msp*I	153	GAGAGTTTGATCCTGGCTCAGAACGAACGCTGGCGGCAGGCTTTAACTACATGCTAAG TCGAACGCACCGCAAGGTGAGTGGCATACGGGTGAGTAACGCGTGGGAACGTGCCC TTCAGTTCGGGATAACCCAGGAAACTTGGGCTAATACCG
*Msp*I	159	GAGAGTTTGATCCCGGCTCAGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCG AGCGGATTTATCCCTCGGGATAAGTTAGCGGCGGACGGGTGAGTAACACGTAGGTA ACCTGCCTATAAGACTGGGATAACACCGGGAAACGAATGCTAAGA
*Msp*I	170	GAGAGTTTGATCCTGGCTCAGGACGAACGCTGGCGGCGTGCCTAATACATGCCAAGTC GAGCGAATCTTGAGGTGCTTGCACCATCTTGGTTAGCGGCGGACGGGTGAGTAACA CGTGGGCAACCTGCCTGTAAGACTGGGATAACTTCGGGAAACCGAAGCTAATACCG
*Msp*I	175	GAGAGTTTGATCCTGGCTCAGTCGTAACAAGGTAGCCGTAGACCTGAGAGTTTGATCC TGGCTCAGACGAACGCTGGCGGCAGGCCTAATACATGCAAGTCGAACGCTGTAGCA GATACAGAGTGGCAGACGGGTGAGTAACGCGTGGGAATCTACCCATCACTACGGAA CAACT
*Msp*I	202	GAGAGTTTGATCCTGGCTCAGAATCAACGCTGGCGGCGTGCCTAACACATGCAAGTCG AACGCGAGAGAGGCTTCGGCCTCGATTGAGTGGCAAACGGGTGAGTAACACGTGGG TGACCTACCTTCGAGTGGGGGATAACGTCCCGAAAGGGACTGCATAATACCGCATAA CATCCTGCCTTTGAACAGGCGGAGATCAAAG
*Msp*I	214	GAGTTTGATCCTGGCTCAGTATATCGTAACAAGGTAGCCGTAGAGGCGAGAGTTTGAT CCTGGCTCAGTCGTAACAAGGTAGCCGTAGACCTGAGAGTTTGATCCTGGCTCAGT TCGTAACAAGGTAGCCGTAGAACCGTAGGGCTGAGAGTTTGATCCTGGCTCAGTCG TAACAAGGTAGCCGTAGACCTGAGAGTTTGATCCTGGCTCAG
*Msp*I	217	GAGAGTTTGATCCTGGCTCAGTcAGGACGAACGCTGGCGGCGGCCTAATACATGCAAG TCGAGCGAATCGATGGGAGCTTGCTCCTGAGATTAGCGGCGGACGGGTGAGTAACA CGTGGGCAGCCTGCCTATAAGACTGGGATAACTTCGGGAAACCGCTCGTAGACTGC GTACCAATCTGGAATTCGTCGACAAGCTTCTACGAGCCTAGGCTAAG
*Hha*I	62	GAGAGTTTGATCCTGGCTCAGAACGAACGCTGGCGGCAGGCTTAACACATGCAAGTCG AGCG
*Hha*I	66	GAGAGTTTGATCCTGGCTCAGAGTGAACGCTGGCGGAATGCTTTACACATGCAAGTCG AGCGGTAG
*Hha*I	89	GAGAGTTTGATCCTGGCTCAGAACGAACGCTGGCGGCATGCCTAACACATGCAAGTCG AACGAGGTTTCCGCTTCGGTGGTTGCCTAGT
*Hha*I	90	GAGAGTTTGATCCTAGGCTCAGGACGAACGTTGGCGGCGTGCTTAATACATGCAAGTC AACTGAGGCAGCGGTAGTAATACCGCTGTGGA
*Hha*I	95	GAGAGTTTGATCCTGGCTCAGAATCAACGCTGGCGGCGTGCCTCAGACATGCAAGTCG AACGCGAAAGTCCCTTTGGGGGATAAGTATGAGTGGC
*Hinf*I	41	GAGAGTTTGATCCTGGCTCAGTCGTAACAAGGTAGCCGTAG
*Hinf*I	49	GAGTTTGATCCTGGCTCAGTAAATCGTAACAAGGTAGTCTCGTAGACTC

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