Annals of Microbiology, **59 (1)** 17-23 (2009)

Occurrence of multiple metal-resistance in bacterial isolates associated with transgenic white poplars (*Populus alba* L.)

Alma BALESTRAZZI[1*], Martina BONADEI[1], Emanuele QUATTRINI[2], Daniela CARBONERA[1]

[1]Dipartimento di Genetica e Microbiologia, Università di Pavia, via Ferrata 1, 27100 Pavia; [2]C.E.T.A.S., Università degli Studi di Milano, via Emilia, 26838 Tavazzano, Lodi, Italy

Received 1 September 2008 / Accepted 17 December 2008

Abstract - The occurrence of multiple metal-resistance was assessed in two bacterial collections, named Herbicide Resistant Bacteria (HRB) and Nuclease-Producing Bacteria (NPB) respectively, consisting of 15 and 11 isolates obtained from a loamy sand cultivated with transgenic white poplars (*Populus alba* L., cv 'Villafranca') engineered for herbicide resistance. A third collection of 11 bacterial isolates, named Leaf-Associated Bacteria (LAB), obtained from the leaves of transgenic white poplars expressing the *StSy* gene for resveratrol production and from untransformed plants was evaluated. Resistance to Cd, Co, Cu, Pb and Zn was tested. As for the HRB collection, nine different phenotypes were monitored, which included tetra-, tri- and double-resistance. Tri- and double-metal resistance occurred also within the NPB and LAB collections. In both cases five different phenotypes were recovered. An additional investigation was carried out on the HRB-1c isolate, resistant to Cd, Co, Pb and Zn, which was previously demonstrated to produce indoleacetic acid, a plant-growth-promoting trait. Colorimetric assays, performed on the cell-depleted medium of HRB-1c liquid cultures grown in presence of heavy metals, confirmed that this trait was not affected. A 19-kb plasmid, possibly involved in the maintenance of the multiple metal-resistant phenotype, was detected in the HRB-1c cells.

Key words: leaf-associated bacteria; multiple metal-resistance; transgenic poplar.

INTRODUCTION

Nowadays heavy metal pollution in soils represents a severe problem for environmental and human health and, unfortunately, remediation technologies still require consistent improvement (Lone *et al*., 2008; Mendez and Maier, 2008). Although phytoremediation is currently considered a promising strategy (Lone *et al*., 2008), a further optimization might be achieved by exploiting the beneficial effects of plant-associated bacteria (Jing *et al*., 2007). Microorganisms can enhance the remediation ability of plants, reduce the phytotoxicity of polluted soils and participate in heavy metal mobilization/immobilization (Siciliano *et al*., 2001; Jing *et al*., 2007; Kuffner *et al*., 2008).

Multiple metal-resistance, widespread in rhizobacteria (Diaz-Ravina *et al*., 1994; Ryan *et al*., 2008), involves different mechanisms such as enzymatic detoxification of the metal, binding of the metal to cell wall and to other specific cell components, blocking of metal uptake by cells and metal extrusion by means of molecular pumps (Mergeay, 1991; Silver, 1992; Nies, 1999).

To date, fast growing trees with high transpiration rates, such as poplar, represent a suitable system with a great potential in phytoremediation (Peuke and Rennenberg, 2006). Thus, the availability of bacterial isolates able to establish optimal tree/microbe combinations is considered as a valuable tool to enhance the phytoremediation potential of elite clones. From this point of view, the white poplar (*Populus alba* L.) cultivar 'Villafranca' used in this work represents an ideal system, due to the high biomass production and resprouting ability (Confalonieri *et al*., 2000). In recent years, the same cultivar has been modified by gene transfer in order to acquire several agronomically relevant traits (Giorcelli *et al*., 2004; Zelasco *et al*., 2006; Balestrazzi *et al*., 2006), tested with innovative marker-free gene-transfer technologies (Zelasco *et al*., 2007) and recently utilized for phytoremediation purposes (Castiglione *et al*., 2007; Lingua *et al*., 2008).

A study on the environmental impact of transgenic white poplars belonging to the 'Villafranca' cultivar has been also carried out, focusing at the soil level. Such an investigation was possible since a greenhouse trial was established using two different classes of genetically modified (GM) white poplars: two transgenic lines expressing the *bar* gene from *Streptomyces hygroscopicus*, encoding an acetyltransferase able to inactivate phosphinothricin (PPT, the active component of Basta®) (Confalonieri *et al*., 2000) and two transgenic lines expressing the *StSy* gene, from *Vitis vinifera* L., encoding stilbene synthase required for resveratrol biosynthesis (Giorcelli *et al.*, 2004).

* Corresponding Author. Phone: +39 0382 985435;
Fax: +39 0382 528496; E-mail: almbal04@unipv.it

Two collections consisting of bacterial isolates obtained from the loamy sand cultivated with GM white poplars engineered for herbicide tolerance are currently available. The Herbicide Resistant Bacteria (HRB) and Nuclease-Producing Bacteria (NPB) collections have been already characterized in previous works (Balestrazzi *et al*., 2007, 2008).

The HRB collection, isolated on selective medium containing PPT consisted mainly of *Pseudomonas* and *Bacillus* species and some of the tested HRB isolates showed useful properties for biotechnological and agronomical applications, such as swarming motility and indoleacetic acid production (Balestrazzi *et al*., 2008). The NPB collection, isolated on a selective medium able to reveal the presence extracellular DNase activity, included mainly *Bacillus* species and a few members of other genera, such as *Brevibacillus*, *Microbacterium*, *Pseudomonas* and *Stenotrophomonas* (Balestrazzi *et al*., 2007).

Besides this, a third collection, named Leaf-Associated Bacteria (LAB), has been produced starting from the leaf tissues of transgenic white poplars expressing the *StSy* gene for resveratrol synthesis (Balestrazzi *et al*., unpublished results). The LAB collection is made of leaf epiphytic and endophytic bacteria, belonging to the *Bacillus* genus, which have been extensively characterized for their tolerance to oxidative stress and to the antioxidant compound resveratrol.

Endophytic bacteria are considered promising tools in the field of bacteria-assisted phytoremediation and a deeper knowledge of heavy metal-resistant endophytic bacteria represents an essential prerequisite for effective phytoremediation of heavy metal-contaminated soils (Siciliano *et al*., 2001).

The present work reports on the occurrence of multiple metal-resistance in the HRB, NPB and LAB collections. This investigation will help identifying novel combinations of plant-growth promoting traits and multiple metal-resistant phenotypes for bacteria-assisted phytoremediation.

MATERIAL AND METHODS

Bacterial isolates. The HRB collection used in this study was recovered from an agricultural soil cultivated with transgenic white poplars (*Populus alba* L. cv 'Villafranca') engineered with the *bar* gene for herbicide tolerance as described by Balestrazzi *et al*. (2008). Briefly, soil samples (1 g) were resuspended in 10 ml of 0.85% NaCl and maintained under constant shaking (200 rpm) for 30 min. The soil suspensions were serially diluted and plated onto Plate Count agar (PCA, Oxoid) supplemented with cycloheximide (100 mg ml^{-1}, Duchefa Biochemicals) and phosphinothricin (PPT, 400 mg ml^{-1}). Plates were incubated at 28 °C for two days.

As for the NPB collections, the same procedure was followed, except for the fact that the soil suspensions, serially diluted, were plated onto Difco™ DNase Test Agar with Methylgreen (DGM Medium, Becton, Dickinson and Company Sparks, USA) (Balestrazzi *et al*., 2007).

The LAB collection, including epiphytic and endophytic bacteria associated with the leaves of transgenic white poplars expressing the *StSy* gene for resveratrol production, was obtained from the GM lines 5EAC1 and 12EAC1 and from the untransformed line as previously reported. Leaves were collected from two-year old white poplars during the vegetative growth season (summer). Leaves were transferred to sterile tubes containing 5 ml of 0.85% NaCl and incubated at room temperature for 30 min, under constant shaking (200 rpm) in order to remove the epiphytic bacteria living on the leaf surface. For each poplar line, leaves were collected from three different plants. Isolation of epiphytes was carried out on PCA medium as previously reported. The same leaves were subjected to surface sterilization by shaking in 2% NaClO for 10 min, then washed five times with sterile distilled water. Leaves were then grounded with a pestel in small mortars containing 0.85% NaCl. Extracts were transferred to sterile tubes, debris were removed by centrifugation (1500 rpm, 5 min) and the liquid supernatant was treated as previously described in order to isolate the endophytic population.

All the HRB, NPB and LAB isolates examined in this study were maintained at -70 °C in vials containing liquid LB medium supplied with 50% glycerol. Aliquots (100 μl) were transferred from the stock vials onto Petri plates containing fresh Luria Bertani (LB) medium and bacteria were grown for two days at 28 °C.

Heavy metal-resistance. Resistance to heavy metals was evaluated on solid LB medium supplemented with 0.25 mM $CdSO_4$, 0.3 mM $Co(NO_3)_2$, 10 mM $CuCl_2$, 50 mM $PbCl_2$ and 3 mM $ZnSO_4$ (Sigma-Aldrich). The salts were all dissolved in distilled water, sterilized by membrane filtration (Millipore Corporation, pore size 0.45 μm) and added to previously sterilized LB medium. The bacterial isolates were transferred onto the selective plates by means of replica plating. For each isolate, the ability to withstand a single heavy metal was first tested. Subsequently, the multiple metal-resistant phenotypes were further assessed by combining all the heavy metals in the same Petri plate. Bacterial growth was evaluated after incubation for 2 days at 28 °C. For each isolate, three independent experiments with three replicated samples for each treatment were carried out.

Indoleacetic acid (IAA) production. IAA production was measured as follows: 4-day-old cultures of the HRB-1c isolate grown in liquid LB medium in presence/absence of heavy metals were collected by centrifugation; aliquots (1 ml) of cell-depleted medium were mixed with 4 ml of Salkowsky's reagent (150 ml of concentrated H_2SO_4, 250 ml of distilled H_2O, 7.5 ml of 0.5 mol l^{-1} $FeCl_3.6H_2O$) (Gordon and Weber, 1951) and incubated at room temperature for 20 min. OD_{535} was measured and IAA concentrations were calculated by comparison with a standard curve, obtained using purified IAA (0.5, 1.0, 2.5, 5.0, 10.0 and 20.0 μg ml^{-1}; Duchefa Biochemicals). Uninoculated medium with the reagent added was used as control. Three independent experiments, with three replicated samples for each treatment, were carried out.

Extraction of plasmid DNA. Exponentially growing HRB-1c liquid cultures incubated in LB medium supplemented with each single metal (0.25 mM $CdSO_4$, 0.3 mM $Co(NO_3)_2$, 50 mM $PbCl_2$ and 3 mM $ZnSO_4$) were utilized. The HRB-1c cells were also exposed simultaneously to all the toxic metals while, as control, they were grown in absence of pollutants. Heat was used as a plasmid-curing agent (Gonzalez and Carlton, 1984). An exponentially grown HRB-1c culture was used to inoculate pre-warmed LB medium at 43 °C. The samples were then incubated at the same temperature for 24 h. The cultures were spread on agar plates containing the above reported heavy metals. Those colonies lacking metal tolerance were selected for plasmid extraction. To this purpose the plasmid-cured HRB-1c cells were grown as previously described. Cell growth was monitored by measuring the optical density (OD_{600}). Bacterial cells were collected by centrifugation and plasmid DNA was extracted using the QIAGEN Plasmid Maxi Kit, according to the manufacturer's instructions. Conventional agarose gel electrophoresis was carried out with 0.8% (w/v) agarose (Duchefa Biochemicals), using the standard procedure described by Sambrook *et al*. (1989).

TABLE 1 - Heavy-metal resistant phenotypes detected in the Herbicide Resistant Bacteria (HRB) collection

Genus[a]	HRB isolate	Multi heavy-metal resistance[b]				
		Cd	Co	Cu	Pb	Zn
Pseudomonas	HRB-1	+	+	-	-	+
	HRB-2	+	+	-	-	+
	HRB-3	+	+	-	-	+
	HRB-4	-	+	+	-	-
	HRB-5	+	-	+	-	-
	HRB-6	+	+	-	-	-
	HRB-7	+	+	-	+	+
	HRB-8	+	+	-	+	+
	HRB-9	+	+	-	+	+
	HRB-10	+	+	-	+	-
	HRB-11	-	+	-	+	-
	HRB-12	+	-	-	+	-
Bacillus	HRB-1a	+	+	-	+	-
	HRB-1b	-	+	+	-	-
	HRB-1c	+	+	-	+	+

[a] For each HRB isolate, the genus has been determined in a previous work (Balestrazzi *et al*., 2008).

[b] Metals were supplied to LB medium as $CdSO_4$ (0.25 mM), $Co(NO_3)_2$ (0.3 mM), $CuCl_2$ (10 mM), $PbCl_2$ (50 mM) and $ZnSO_4$ (3 mM).

RESULTS

Occurrence of multiple metal-resistant phenotypes in the HRB collection

The HRB isolates (Table 1) were grown in the presence of toxic metals supplied in the form of $CdSO_4$, $Co(NO_3)_2$, $CuCl_2$, $PbCl_2$ and $ZnSO_4$. In a preliminary experiment, different doses for each elemental pollutant were used, based on the current literature (data not shown). The results reported in Table 1 refer to the highest concentration of heavy metal that allowed the growth of HRB isolates after two days. All the isolates revealed multiple-resistant phenotypes which survived the following doses: 0.25 mM $CdSO_4$, 0.3 mM $Co(NO_3)_2$, 10 mM $CuCl_2$, 50 mM $PbCl_2$ and 3.0 mM $ZnSO_4$. Nine different phenotypes were monitored. As shown in Table 1, four HRB isolates out of 15 showed tetra-resistance. The HRB-7, HRB-8, HRB-9 and HRB-1c isolates could survive Cd, Co, Pb and Zn. Another group of HRB isolates was characterized by tri-resistance. This included the HRB-1, HRB-2 and HRB-3 isolates which showed resistance to Cd, Co and Zn while the HRB-10 isolate could withstand Cd, Co and Pb. Finally, the HRB-1a isolate was able to survive Cd, Co and Pb. The double-resistant phenotype was common to five HRB isolates: HRB-4 (Co, Cu), HRB-5 (Cd, Cu), HRB-6 (Cd, Co), HRB-11 (Co, Pb) and HRB-12 (Cd, Pb).

Occurrence of multiple metal-resistant phenotypes in the NPB collection

As previously reported for the HRB collection, the NPB isolates (Table 2) were grown in the presence of different metals supplied in the form of $CdSO_4$, $Co(NO_3)_2$, $CuCl_2$, $PbCl_2$ and $ZnSO_4$. Results from these experiments, described in Table 2, represent the highest heavy metal concentration that allowed growth after two days. All the NPB isolates showed resistance to cobalt since they were able to survive 0.3 mM $Co(NO_3)_2$. The tri-resistant phenotype was found in the case of NPB-2, NPB-3 (Co, Pb, Zn) and NPB-11 (Cd, Co, Pb) isolates. A variegated range of double-resistant NPB isolates was observed: NPB-1, NPB-4, NPB-6, NPB-8 and NPB-10 (Co, Pb), HRB-7 and HRB-9 (Co, Zn), HRB-5 (Co, Cu).

TABLE 2 - Heavy-metal resistant phenotypes of the Nuclease-Producing Bacteria (NPB) collection

Genus[a]	NPB isolate	Multi heavy-metal resistance[b]				
		Cd	Co	Cu	Pb	Zn
Bacillus	NPB-1	-	+	-	+	-
	NPB-2	-	+	-	+	+
	NPB-3	-	+	-	+	+
	NPB-4	-	+	-	+	-
	NPB-5	-	+	+	-	-
	NPB-6	-	+	-	+	-
Brevibacillus	NPB-7	-	+	-	-	+
	NPB-8	-	+	-	+	-
Microbacterium	NPB-9	-	+	-	-	+
Pseudomonas	NPB-10	-	+	-	+	-
Stenotrophomonas	NPB-11	+	+	-	+	-

[a] For each NPB isolate, the genus has been determined in a previous work (Balestrazzi *et al*., 2007).

[b] Metals were supplied to LB medium as $CdSO_4$ (0.25 mM), $Co(NO_3)_2$ (0.3 mM) and $CuCl_2$ (10 mM), $PbCl_2$ (50 mM) and $ZnSO_4$ (3 mM).

TABLE 3 - Heavy-metal resistant phenotypes in the Leaf Associated Bacteria (LAB) collection. Epiphytic and endophytic bacteria were isolated from the leaves of the GM lines 5EAC1 and 12EAC1 and untransformed (CTRL) white poplars. All the LAB isolates are members of the genus *Bacillus*

Poplar line	Leaf-associated bacteria[a]	Multi heavy-metal resistance[b]				
		Cd	Co	Cu	Pb	Zn
CTRL	EP-C-1	-	+	+	+	-
	EP-C-2	-	+	-	+	+
	EN-C-1	-	+	-	+	+
5EAC1	EP-5-1	-	+	-	-	+
	EP-5-2	-	+	+	-	-
	EP-5-3	-	+	-	+	-
	EN-5-1	-	+	+	-	-
	EN-5-2	-	+	-	-	+
12EAC1	EP-12-1	-	+	-	+	-
	EP-12-2	-	+	-	+	-
	EN-12-2	-	+	+	+	-

[a] EP: epiphytic, EN: endophytic.

[b] Metals were supplied to LB medium as $CdSO_4$ (0.25 mM), $Co(NO_3)_2$ (0.3 mM) and $CuCl_2$ (10 mM), $PbCl_2$ (50 mM) and $ZnSO_4$ (3 mM).

Occurrence of multiple heavy metal-resistant phenotypes in the LAB collection

The leaf-associated bacteria (Table 3) were tested for their ability to grow in the presence of different metals which were supplied as $CdSO_4$ (0.25 mM), $Co(NO_3)_2$ (0.3 mM), $CuCl_2$ (10 mM), $PbCl_2$ (50 mM) and $ZnSO_4$ (3.0 mM). Also in this case, results shown in Table 3, refer to the highest concentration of toxic metal that allowed growth after two days. The LAB collection included isolates from untransformed (CTRL) and GM leaf tissues (5EAC1 and 12EAC1). Tri-resistance (Co, Pb, Zn) was recovered in two isolates deriving from untransformed white poplars, namely the EP-C-2 and EN-C-1 and in one of the isolates obtained from the GM line 12EAC1 (EN-12-2; Co, Cu, Pb). The double-resistant phenotypes occurred in all the LAB isolates associated with the 5EAC1 GM line: isolates EP-5-1 and EN-5-2 showed resistance to Co and Zn while isolates EP-5-2 and EN-5-1 were resistant to Co and Cu. Finally, the double-resistance to Co and Pb was a feature of EP-5-3, EP-12-1 and EP-12-2.

Indoleacetic acid production in presence of heavy metals

Since in a previous work the HRB-1c isolate was found to be the most active IAA producer, additional experiments were carried out in order to verify whether this specific trait was maintained in the presence of heavy metals. Results from these analyses are shown in Table 4. The IAA content in the cell-depleted medium of 4-day-old HRB-1c liquid cultures was 21.35 ± 0.79 μg ml^{-1} (untreated control). In presence of heavy metals the rate of IAA production was always reduced. When single metals were supplied, the lowest IAA amount was recorded for Pb and Co (16.51 ± 3.72 and 16.97 ± 2.15 μg ml^{-1}, respectively). Sligthly higher was the production in cultures containing Zn, 18.30 ± 1.43 μg ml^{-1}. In presence of cadmium, IAA production corresponded to 20.14 ± 1.60 μg ml^{-1}. Finally, when all the four heavy metals (Cd, Co, Pb, Zn) were added simultaneously to the medium, the resulting IAA biosynthesis, 14.65 ± 3.27 μg ml^{-1}, was significantly affected.

Plasmid profile of HRB-1c isolate

In the attempt to assess the molecular basis of the tetra-resistant phenotype detected in the HRB-1c isolate, plasmid DNA was extracted from exponentially growing liquid cultures exposed to each single pollutant (Cd, Co, Pb and Zn) and to all of them simultaneously. Conventional agarose gel electrophoresis revealed the occurrence of a large plasmid (19.0 kb in size) (Fig. 1, lane 1). The same plasmid was absent in heat-cured cells (Fig. 1, lane 2). Differently from the wild type cells, the heat-cured cells were not able to withstand Cd, Co, Pb and Zn when supplied separately and in combination (Table 5).

TABLE 4 - Indoleacetic acid production by HRB-1c isolate in presence and absence of heavy metals

Heavy metals[a]	IAA production (mg ml^{-1})
Untreated control	21.35 ± 0.79
Cd	20.14 ± 1.60
Co	16.97 ± 2.15
Pb	16.51 ± 3.72
Zn	18.30 ± 1.43
Cd, Co, Pb, Zn	14.65 ± 3.27

[a] Metals were supplied to LB medium as $CdSO_4$ (0.25 mM), $Co(NO_3)_2$ (0.3 mM), $PbCl_2$ (50 mM) and $ZnSO_4$ (3 mM).

TABLE 5 - Growth ability of wild type and plasmid-cured HRB-1c isolate in presence and absence of heavy metals

Heavy metals[a]	HRB-1c	
	Wild type	Plasmid cured
Untreated control	+	+
Cd	+	-
Co	+	-
Pb	+	-
Zn	+	-
Cd, Co, Pb, Zn	+	-

[a] Metals were supplied to LB medium as $CdSO_4$ (0.25 mM), $Co(NO_3)_2$ (0.3 mM), $PbCl_2$ (50 mM) and $ZnSO_4$ (3 mM).

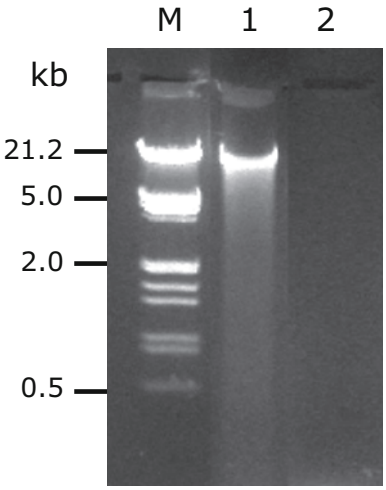

FIG. 1 - Plasmid-profile of HRB-1c isolate. Line 1: wild type cells, line 2: plasmid-cured cells, line M: Lambda DNA/ *Eco*RI + *Hind*III Marker 3 (M-Medical s.r.l.).

DISCUSSION

Aim of the present work was to fulfil the phenotypic characterization of two different collections of bacterial isolates deriving from a loamy sand cultivated with transgenic white poplars expressing the *bar* gene for herbicide tolerance (Confalonieri *et al*., 2000). A third collection, including the epiphytic and endophytic bacteria isolated from the leaves of transgenic white poplars expressing the *StSy* gene for resveratrol production (Giorcelli *et al*., 2004; Balestrazzi *et al*., unpublished results) was also examined.

All the heavy metals investigated in the present work cause severe environmental pollution (Lone *et al*., 2008). When the HRB collection was analysed, resistance to several metal combinations was evidenced. The widest range of resistance included four heavy metals (Cd, Co, Pb and Zn) and it was found also in the HRB-1c isolate, belonging to the *Bacillus* genus. HRB-1c is characterized by traits of agronomical relevance, such as a remarkable swarming ability, the consistent production of indoleacetic acid and the ability to stimulate the *in vitro* growth of white poplar explants (Balestrazzi *et al*., 2008). It has been reported that Cd can suppress auxin production of plant-growth-stimulating bacteria (Kamnev *et al*., 2005). The finding that the HRB-1c isolate could maintain IAA biosynthesis also in the presence of Cd and other heavy metals enhances the biotechnological value of this microorganism as a tool for bacteria-assisted phytoremediation.

Resistance to Co was a common feature of NPB isolates and it associated with resistance to Pb, Cu or Zn, thus producing different double-resistant phenotypes in isolates classified as *Bacillus*, *Brevibacillus*, *Microbacterium* and *Pseudomonas*. Tri-resistance, involving Co, Pb and Zn and also Cd, Co and Pb, was detected in NPB isolates belonging to the genera *Bacillus* and *Stenotrophomonas* (Balestrazzi *et al.*, 2007).

Multiple metal-resistance occurred in both endophytic and epiphytic bacteria. Tri-resistance was observed for all LAB isolates deriving from the leaves of untransformed white poplar and in the case of the EN-12-2 associated with the GM line 12EAC1. As for the GM line 5EAC1, all the tested isolates, epiphytic and endophytic, showed a double-resistant phenotype. Based on the reported data and considering the limited number of LAB isolates available, a discussion on the possible differences between GM and untransformed trees, in terms of leaf-associated microorganisms would be premature. However, it is worth noting that the LAB isolates associated with the GM lines 5EAC1 and 12EAC1 revealed a significant degree of resistance to oxidative stress conditions when exposed to hydrogen peroxide and UV-C radiation, respectively, compared to isolates derived from untransformed leaves (Balestrazzi *et al*., unpublished results).

It has been hypothesized that endophytic microorganisms harbouring traits for the effective removal of pollutants can significantly help metal mobilization through the plant vascular system. This might be particularly relevant in poplar trees, where a prolonged time might be required in order to move contaminants from roots to leaves. Notwithstanding the several studies carried out on soil bacteria-assisted phytoremediation (Zaidi *et al*., 2006; Dell'Amico *et al*., 2008), to date there is limited information on the potential of endophytic bacteria on the phytoremediation of heavy metal-contaminated soils.

It has been suggested that the use of LB media supplemented with heavy metals could not represent the optimal choice to investigate the bacterial response, due to the possible chelation/ precipitation reactions occurring in micronutrient-rich culture media. This approach has been however reported by different authors in the case of cadmium, lead, zinc and copper (Yoshida *et al*., 1998; Shakoori and Muneer, 2002; Zhigang *et al*., 2006).

Since the HRB-1c isolate represents the most intriguing product so far obtained from our investigation, further analyses were carried out to gain information on the molecular determinants of its tetra-resistant phenotype. A single extrachromosomal element was evidenced by conventional gel electrophoresis in the HRB-1c cells. The plasmid (19.0 kb in size) was removed by heat-mediated curing and resistance to Cd, Co, Pb and Zn disappeared. The occurrence of larger plasmids, visible only using more sophisticated electrophoretic approaches, cannot be excluded. In a recent work, Pereira *et al.* (2006) reported that cadmium resistance was linked to high molecular weight plasmids while the *czc* cation efflux system, able to actively extrude cadmium, zinc and cobalt from the cell is encoded by a large plasmid (Nies, 1992). Moreover, plasmids which harbour genes involved in biotransformation of lead have been detected in *Staphylococcus aureus* and *Alcaligenes eutrophus* (Mergeay, 1991).

When considering the total number of isolates examined in this study, resistance to cobalt showed the highest frequency, since it was recovered in 94.59% of isolates. This trait is generally dependent on cobalt-transporting CPx-Type ATPases (Rutherford *et al*., 1995) and on cobalt efflux proteins (Thorgensen and Downs, 2007). Lead resistance occurred in 62.16% of the total bacteria examined. It has been reported that, in some cases, resistance to lead may be connected with production of extracellular polymers or with the intracellular sequestration of this metal by bacterial cells (Roane, 1999).

In contrast, resistance to Cd was found only in 35.13% of the isolates. Exposure to cadmium induces synthesis of proteins that export or chelate the heavy metal while the intracellular glutathione-mediated detoxification of Cd has been also described in the genus *Rhizobium* (Figueira *et al*., 2005). Finally, resistance to zinc and copper occurred with a frequency of 43.24 and 18.91%, respectively.

Detailed molecular analyses of the HRB, NPB and LAB isolates will be required to better understand the cellular and molecular mechanisms responsible for the variegated patterns of multiple metal-resistance so far described. Additional studies will help distinguishing between the resistance mechanisms which rely

on chromosomal genes, plasmids or transposons and the ubiquitary metal tolerance ascribed to general metabolic responses. Mechanisms responsible for metal resistance might include exclusion by permeability barrier, intra- and extracellular sequestration, efflux pumps, enzymatic detoxification, proteome and metabolome changes (Silver, 1992; Nies, 1999; Baker-Austin *et al*., 2006).

Several studies have documented the predominance of Gram-negative bacteria in metal-contaminated soils and this finding might be explained by the fact that the Gram-negative cell wall is a more efficient barrier to toxic metals than the Gram-positive one (Duxbury and Bicknell, 1983). In accordance with this hypothesis, although most of the environmental isolates (62.1%) examined in this study were Gram-positive, the tetra-resistant phenotypes were more frequent among the Gram-negative bacteria belonging to the genus *Pseudomonas*.

The wide range of heavy metal resistant phenotypes routinely isolated from soil bacteria might be related to the common agricultural practices which rely upon the use of metal-containing products, such as fertilizers, fungicides, pesticides and herbicides, routinely adopted to enhance crop production (He *et al*., 2005). It is worth noting that both the herbicide-tolerant and resveratrol-producing white poplars grown in greenhouses underwent intensive fertilization treatments and nutrients solutions enriched in microelements were supplied to support growth (Balestrazzi *et al*., 2008). The heavy metals tested in this work include micronutrients such as Co, Cu and Zn which are essential components of redox processes and cofactors of relevant enzymes (O'Halloran, 1993). It is known that bacteria possess copper resistance operons which display metal-responsive transcriptional regulation with membrane-spanning proteins involved in monitoring changes in copper concentrations in periplasm (O'Halloran, 1993). On the other hand, zinc is found in enzymes involved in transcription and in transcription factors responsive to a variety of intra- and inter-cellular signals (O'Halloran, 1993). Cadmium is the only non essential element evaluated in the present work. Resistance to essential elements is usually chromosome-based while the resistance to non essential trace elements is associated with plasmids and transposons (Silver, 1992). This is in accordance with the reported data linking the resistance to cadmium and the 19-kb plasmid detected in HRB-1c cells. Within this context the correlation between this extracellular element and resistance to Co, Pb and Zn remains difficult to explain. However, in case of plasmid-determined copper resistance mechanisms involving bioaccumulation, sequestration and efflux have been documented (Silver, 1992).

Finally, it has been reported that heavy metal toxicity might be lowered in soils with high clay, since the charged clay particles limit metal availability by sequestration (Collins and Stotzky, 1989). The medium-textured loamy sand utilized for white poplar cultivation in greenhouse was characterized by a reduced clay content (5.62%) (Balestrazzi *et al*., 2007, 2008) and this might have contributed to the high frequency of multiple metal-resistance detected in the bacterial isolates.

The response of HRB, NPB and LAB isolates to a wider range of trace elements, including As, Se and Hg, will possibly reveal additional resistance traits useful for waste bioremediation or biometallurgy. To date, microbial studies are required to identify novel soil culturable microorganisms able to support the phytoremediation performace of specific plant systems (Lone *et al*., 2008). The present work presents a list of novel environmental isolates with multiple metal-resistance which will be useful in future studies on bacteria-assisted phytoremediation with trees.

The investigation focused on the capacity of HRB, NPB and LAB isolates to withstand significant heavy metal concentrations. This ability would suggest for potential applications of such bacteria in the field of *in situ* bioremediation of polluted environments. Specific isolates with multiple metal-resistance might be useful to target different elemental pollutants at contaminated sites. Furthermore, the ability to interact with perennial trees, e.g. those included in the genus *Populus*, might help to obtain novel plant-microbe remediation systems.

Acknowledgments

This research was supported by a grant from Regione Lombardia (Divisione Generale Agricoltura). M.B. received a doctoral fellowship from Regione Lombardia.

REFERENCES

Baker-Austin C., Wright M.S., Stepanauskas R., McArthur J.V. (2006). Co-selection of antibiotic and metal resistance. Trends Microbiol., 14: 176-182.

Balestrazzi A., Allegro G., Confalonieri M. (2006). Genetically modified trees expressing genes for insect pest resistance. In: Fladung M., Ewald D., Eds, Tree Transgenesis: Recent Developments, Springer-Verlag Berlin Heidelberg, pp. 253-273.

Balestrazzi A., Bonadei M., Carbonera D. (2007). Nuclease-producing bacteria in soil cultivated with herbicide resistant transgenic white poplars. Ann. Microbiol., 57: 531- 536.

Balestrazzi A., Bonadei M., Zelasco S., Quattrini E., Calvio C., Galizzi A., Carbonera D. (2008). Recovery of useful traits from isolates inhabiting an agricultural soil cultivated with herbicide-resistant poplars. Can. J. Microbiol., 54: 201-208.

Castiglione S., Franchin C., Fossati T., Lingua G., Torrigiani P., Biondi S. (2007). High zinc concentrations reduce rooting capacity and alter metallothionein gene expression in white poplar *(Populus alba* cv. Villafranca). Chemosphere, 67: 1117-1126.

Collins Y.E., Stotzky G. (1989). Factors affecting the toxicity of heavy metals to microbes. In: Beveridge T.J., Doyle R.J., Eds, Metal Ions and Bacteria, John Wiley and Sons Inc., New York, pp. 31-90.

Confalonieri M., Belenghi B., Balestrazzi A., Negri S., Facciotto G., Schenone G., DelleDonne M. (2000). Transformation of elite white poplar (*P. alba*) cv Villafranca and evaluation of herbicide resistance. Plant Cell Rep., 19: 978-982.

Dell'Amico E., Mazzocchi M., Cavalca L., Allievi L., Andreoni V. (2008). Assessment of bacterial community structure in a long-term copper-polluted ex-vineyard soil. Microbiol. Res.,163: 671-683.

Diaz-Ravina M., Baath E., Frostegard A. (1994). Multiple heavy metal tolerance of soil bacterial communities and its measurement by a thymidine incorporation technique. Appl. Environ. Microbiol., 60: 2238-2247.

Duxbury T., Bicknell B. (1983). Metal-tolerant bacterial populations from natural and metal polluted soils. Soil Biol. Biochem., 9: 105-112.

Figueira E.M.A.P., Lima A.I.G., Pereira S.I.A. (2005). Cadmium tolerance plasticity in *Rhizobium leguminosarum* bv. *viciae*: glutathione as a detoxifying agent. Can. J. Microbiol., 51: 7-14.

Giorcelli A., Sparvoli F., Mattivi F., Balestrazzi A., Tava A., Vrhovsek U., Bollini R., Confalonieri M. (2004). Expression of stilbene synthase (StSy) gene from grapevine in transgenic white poplar results in high accumulation of the antioxidant compounds resveratrol glucosides. Trans. Res., 13: 203-214.

Gonzalez J.M.Jr, Carlton B.C. (1984). A large transmissable plasmid is required for crystal toxin production in *Bacillus thuringiensis* variety *israelensis*. Plasmid, 11: 28-38.

Gordon S.A., Weber R.P. (1951). Colorimetric estimation of indoleacetic acid. Plant Physiol., 26: 192-195.

He Z.L., Yang X.E., Stoffella P.J. (2005). Trace elements in agroecosystems and impacts on the environment. J. Trace Elem. Med. Biol., 19: 125-140.

Jing Y., He Z., Yang X. (2007). Role of soil rhizobacteria in phytoremediation of heavy metal contaminated soils. J. Zhejiang Univ. Sci. B, 8: 192-207.

Kamnev A.A., Tugarova A.V., Antonyuk L.P., Tarantilis P.A., Polissiou M.G., Gardiner P.H. (2005). Effects of heavy metals on plant-associated rhizobacteria: comparison of endophytic and non-endophytic strains of *Azospirillum brasilense*. J. Trace Elem. Med. Biol., 19: 91-95.

Kuffner M., Poschenrieder M., Wieshammer G., Gorfer M., Sessitsch A. (2008). Rhizosphere bacteria affect growth and metal uptake of heavy metal accumulating willows. Plant Soil, 304: 35-44.

Lingua G., Franchin C., Todeschini V., Castiglione S., Biondi S., Burlando B., Parravicini V., Torrigiani P., Berta G. (2008). Arbuscolar mycorrhizal fungi differentially affect the response to high zinc concentrations of two registered poplar clones, Villafranca (*Populus alba* L.) and Jean Pourtet (*Populus nigra* L.). Environ. Pollut., 153: 137-147.

Lone M.I., He Z., Stoffella P.J., Yang X. (2008). Phytoremediation of heavy metal polluted soils and water: progresses and perspectives. J. Zhejiang Univ. Sci. B, 9: 210-220.

Mendez M.O., Maier R.M. (2008). Phytostabilization of mine tailings in arid and semiarid environments - an emerging remediation technology. Environ. Health Perspect., 116: 278-283.

Mergeay M. (1991). Towards an understanding of the genetics of bacterial metal resistance. Trends Biotechnol., 9: 17-24.

Nies D.H. (1992). Resistance to cadmium, cobalt, zinc and nickel in microbes. Plasmid, 27: 17-28.

Nies D.H. (1999). Microbial heavy metal resistance. Appl. Microbiol. Biotechnol., 51: 730-750.

O'Halloran T. (1993). Transition metals in control of gene expression. Science, 261: 717-725.

Pereira S.I., Lima A.I., Figueira E.M. (2006) Screening possible mechanisms mediating cadmium resistance in *Rhizobium leguminosarum* bv. viciae isolated from contaminated portuguese soils. Microb. Ecol., 52: 176-186.

Peuke A.D., Rennenberg H. (2006). Heavy metal resistance and phytoremediation with transgenic trees. In: Fladung M., Ewald D., Eds, Tree Transgenesis: Recent Developments, Springer-Verlag Berlin Heidelberg, pp. 253-273.

Roane T.M. (1999). Lead resistance in two isolates from heavy metal-contaminated soils. Microb. Ecol., 37: 218-224.

Rutherford J.C., Cavet J.S., Robinson N.J. (1995). Cobalt-dependent transcriptional switching by a dual-effector MerR-like protein regulates a cobalt-exporting variant CPx-type ATPase. J. Biol. Chem., 274: 25827-25832.

Ryan R., Ryan D., Dowling D. (2008). Multiple metal resistant transferable phenotypes in bacteria as indicators of soil contamination with heavy metals. J. Soils Sedim., 5: 95-100.

Sambrook J., Fritsch E.F., Maniatis T. (1989). Molecular Cloning: A Laboratory Manual, 2nd edn., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Shakoori A.R., Muneer B. (2002). Copper-resistant bacteria from industrial effluents and their role in remediation of heavy metals in wastewater. Folia Microbiol., 47: 43-50.

Siciliano S.D., Fortin N., Mihoc A., Wisse G., Labelle S., Beaumier D., Ouellette D., Real R., Whyte L.G., Banks M.K., Schwab P., Lee K., Greer C.W. (2001). Selection of specific endophytic bacterial genotypes by plants in response to soil contamination. Appl. Environ. Microbiol., 67: 2496-2475.

Silver S. (1992). Plasmid-determined metal resistance mechanisms: range and overview. Plasmid, 27: 1-3.

Thorgensen M.P., Downs D. (2007). Cobalt targets multiple metabolic processes in *Salmonella enterica*. J. Bacteriol., 189: 7774-7781.

Yoshida N., Murooka Y., Ogawa K. (1998). Heavy metal particle resistance in *Thiobacillus intermedius* 13-1 isolated from corroded concrete. J. Ferment. Bioeng., 85: 630-633.

Zaidi S., Usmani S., Singh B.R., Musarrat J. (2006). Significance of *Bacillus subtilis* strain SJ-101 as a bioinoculant for concurrent plant growth promotion and nickel accumulation in *Brassica juncea*. Chemosphere, 64: 991-997.

Zelasco S., Reggi S., Calligari P., Balestrazzi A., Bongiorni C., Quattrini E., Delia G., Bisoffi S., Fogher C., Confalonieri M. (2006). Expression of the *Vitreoscilla* hemoglobin (VHb)-encoding gene in transgenic white poplar: plant growth and biomass production, biochemical characterization and cell survival under submergence, oxidative and nitrosative stress conditions. Mol. Breed., 17: 201-216.

Zelasco S., Ressegotti V., Confalonieri M., Carbonera D., Calligari P., Bonadei M., Bisoffi S., Yamada K., Balestrazzi A. (2007). Evaluation of MAT-vector system in white poplar (*Populus alba* L.) and production of *ipt* marker-free transgenic plants by 'single-step transformation'. Plant Cell Tiss. Org. Cult., 91: 61-72.

Zhigang A., Cuijie L., Yuangang Z., Yeljie D., Watcher A., Gromes R., Rausch T. (2006). Expression of BjMT2, a metallothionein 2 from *Brassica juncea*, incresaes copper and cadmium tolerance in *Escherichia coli* and *Arabidopsis thaliana*, but inhibits root elongation in *Arabidopsis thaliana* seedlings. J. Exp. Bot., 57: 3575-3582.