Comptes Rendus Biologies

Microbiology: bacteriology, mycology, parasitology, virology/Microbiologie : bactériologie, mycologie, parasitologie, virologie

Characterization of efficient plant-growth-promoting bacteria isolated from *Sulla coronaria* resistant to cadmium and to other heavy metals

Manel Chiboub, Omar Saadani, Imen Challougui Fatnassi, Souhir Abdelkrim, Ghassen Abid, Moez Jebara, Salwa Harzalli Jebara *

Laboratoire des légumineuses, centre de biotechnologie Borj Cedria, university Tunis El Manar, BP 901, 2050 Hammam Lif, Tunisia

A B S T R A C T

The inoculation of plants with plant-growth-promoting rhizobacteria has become a priority in the phytoremediation of heavy-metal-contaminated soils. A total of 82 bacteria were isolated from *Sulla coronaria* root nodules cultivated on four soil samples differently contaminated by heavy metals. The phenotypic characterization of these isolates demonstrated an increased tolerance to cadmium reaching 4.1 mM, and to other metals, including Zn, Cu and Ni. Polymerase Chain Reaction/Restriction Fragment Length Polymorphism (PCR/RFLP) analysis showed a large diversity represented by genera related to *Agrobacterium* sp., *R. leguminosarum*, *Sinorhizobium* sp., *Pseudomonas* sp., and *Rhizobium* sp. Their symbiotic effectiveness was evaluated by nodulation tests. Taking into consideration efficiency and cadmium tolerance, four isolates were chosen; their 16SrRNA gene sequence showed that they belonged to *Pseudomonas* sp. and the *Rhizobium sullae*. The selected consortium of soil bacteria had the ability to produce plant-growth-promoting substances such as indole acetic acid and siderophore. The intracellular Cd accumulation was enhanced by increasing the time of incubation of the four soil bacteria cultivated in a medium supplemented with 0.1 mM Cd. The existence of a cadmium-resistant gene was confirmed by PCR. These results suggested that *Sulla coronaria* in symbiosis with the consortium of plant-growth-promoting rhizobacteria (PGPR) could be useful in the phytoremediation of cadmium-contaminated soils.

© 2016 Académie des sciences. Published by Elsevier Masson SAS. All rights reserved.

1. Introduction

Metal-contaminated soils pose a severe threat to environment, agriculture, and consequently via the food chain to human and animal health [1].

Cadmium is one very prevalent heavy metal and is one of the most toxic pollutants of the surface soil layer. It is introduced into soils by synthetic fertilizers and pesticides, widespread due to mining, smelting and power station industry activities [2]. This metal is non-essential, but poisonous for plants, animals, and humans [3]. In plants, cadmium inhibits shoot and root growth and affects nutrient uptake, homeostasis and physiological processes such as photosynthesis [4].

This is why cadmium pollution attracts the most attention of environmentalists around the world. Like other heavy metals, cadmium affects the qualitative and quantitative structure of microbial communities, resulting in decreased metabolic activity and diversity [5]. Remediation of soils contaminated by heavy metals can be done

using chemical, physical, and biological techniques [6]. Chemical and physical methods are expensive and cause secondary pollutants [7]. Phytoremediation is the biological technique which refers to the use of some plants for bioremediation. Considerable amounts of toxic metals can be accumulated through bioremediation, and it can play a significant role in cleaning up metal pollutants [8]. Recently, legumes associated with plant-growth-promoting rhizobacteria have been extended to bioremediation of both organic and metal pollutants [9–11]. PGPRs have the exceptional ability to promote the growth of plants by different mechanisms such as nitrogen fixation, solubilisation of minerals, transformation of nutrient elements, production of phytohormones, and sequestration of iron by siderophores [12]. In addition, PGPRs had different heavy metal tolerance mechanisms; they involve biosorption, precipitation or bioaccumulation in external and intracellular spaces. They contribute in the accumulation of heavy metals in forage plants especially in roots and minimize their translocation for shoots [11].

The aim of this work was to identify a cadmium-tolerant soil bacteria isolated from nodules of *Sulla coronaria* cultivated in heavy metal contaminated soil samples and to form a symbiotic system useful for the phytostabilization of Cd-contaminated soils, being known that this plant is one of the most important for fodder; it is cultivated in several countries of the western Mediterranean basin, especially in Spain, Italy and North Africa [13,14]. It is a species well-adapted to a large range of environmental conditions and actually shows a marked resistance to extreme conditions of drought, salinity, and alkaline pHs as high as 9.3 [15].

2. Materials and methods

2.1. Determination of toxic metals in soil samples

Four composite soils were collected from the north of Tunisia: S1 Jerissa (35°51′N, 8°38′E), which is an agricultural site not contaminated by heavy metals and used as a control. S2 Ghezela (37°05′02″N, 9°32′08″E), S3 Manzel Bourguiba (37°9′N, 9°47′E) located in Bizerte governate and near a large iron mine and S4 Jebal Ressas (36°36′N, 10° 20′E) is an agricultural area located near an old lead–zinc (Pb–Zn) mine. Three samples were taken at a depth of 15 cm from the soil surface and separated by 10 m. The collected soil samples were air dried at 40 °C for 16 h and sieved into coarse and fine fractions. Total soil heavy metals were extracted by dissolving 0.5 g of soil sample in 6 mL of hydrochloric acid and 2 mL of 2% nitric acid at 95 °C for 75 min, filtered and then diluted to 50 mL with distilled water. Extractable heavy metals were extracted with 1 M NH_4OAc [16]. The above heavy metal concentrations in the extracts were determined by inducted coupled plasma optical spectrophotometer (ICP-OES) after aqua regia treatment in a microwave system.

The soil pH was measured in distilled water with a soil/solution ratio of 1:2.5 (W:V). The organic matter content and the total phosphorus in the soil were determined according to the methods described in [16].

2.2. Isolation of strains and culture conditions

Seeds of *Sulla coronaria* Bikra 21 were obtained from UTAP (« Union tunisienne de l'agriculture et de la pêche ») station of Manouba, located to the northwest of Tunis. Its surface was sterilized with 70% ethanol for 1 min then rinsed five times with distilled water. Seeds were germinated on 9 ‰ agar plates at 28 °C in darkness and grown in pots of 1 kg of soil from the contaminated sites (3 plants/pot) and 10 pots from each soil were placed in a controlled plant-growth chamber (16 h light, 8 h dark/relative humidity 60%) and watered with distilled water. The experiment has been performed for 2 months.

The collected nodules of *Sulla Coronaria* were washed with sterile water and then surface sterilized using 70% ethanol and 0.2% $HgCl_2$, then thoroughly washed with sterile distilled water. After surface sterilization, the nodules were crushed and then streaked in YEMA medium (Yeast Extract Mannitol Agar) [17]. Single colonies were selected and checked for purity by repeated streaking. All isolates were incubated at 28 °C and maintained in a 20% (v/v) glycerol solution at –80 °C.

2.3. Screening test for cadmium and heavy-metal-resistant strains

To isolate cadmium-resistant strains, the isolates were screened on YEMA plates, supplemented with different concentrations of Cd in the form of cadmium chloride (0.02, 0.06, 0.1, 0.4, 0.8, 1, 1.2, 1.6, 2, 2.4, 3, 4.1, 4.5 mM). Ni (in the form of Nickel chloride) was used at concentrations of 0.008, 0.016, 0.024, 0.06, 0.08, 0.12, 0.2 and 0.28 mM, Pb (in the form of lead chloride) was used at concentrations of 0.27, 0.675, 0.94, 1.35, 2.025, 2.7, 3.375, and 4.05 mM, Zn (in the form of zinc sulfate) was used at concentrations of 0.6, 0.8, 1, 1.2, 1.28, 1.36, 1.4 and 1.6 mM, and Cu (in the form of copper sulfate) was used at concentrations of 0.01, 0.02, 0.05, 0.075, 0.1, 0.15, 0.25, and 0.35 mM. Thus, the maximal concentration used for resistance testing was similar to those of the corresponding elements found in contaminated soils.

Multi-point inoculators were used to add a 20-μL aliquot of cell suspension from each sample isolate to the YEMA plates, and then the culture was incubated at 28 °C for 3–5 days. The minimum inhibitory concentration (MIC) was defined as the lowest concentration at which no viable colony-forming units (CFU) were observed after 3 days of incubation at 28 °C.

2.4. PCR amplification and RFLP of the 16S rRNA gene

Regarding DNA extraction, the method described by [18] was used. Primers fD1 5′-GGAGAGTTAGATCTTGGCTC-3′ and rD1 5′-AAGGAGGTGATCCAGCCGCA-3′ were used for the amplification of almost full length 16S rRNA genes, around 1530 base pairs per region using the Bio-RAD Thermal Cycler model (C1000).

PCR reactions were done in a final volume of 25 μL containing the following components: 2.5 μL of 10X reaction buffer (10 mM Tris-HCl, 50 mM $MgCl_2$), 1 μM of DNA primer, 1.5 mM of $MgCl_2$, 200 μM of dNTP, 1.5 U of

Taq polymerase (Appligene 5U μL^{-1}), 1.5 μl (10 ng) of bacteria DNA template.

DNA amplification was carried out as follows: 1 cycle at 94 °C for 3 min; 35 cycles at 94 °C for 50 s, 54 °C for 45 s, and 72 °C for 90 s; 1 cycle at 72 °C for 8 min.

DNA fragments resulting from PCR reactions were electrophoresed in 1% (w/v) agarose gels for 20 min and visualized by ethidium bromide staining.

Sixteen rDNA PCR/RFLP analyses were carried out by digesting the respective PCR products with the endonucleases *Msp*I, *Nde*II. DNA fragments resulting from PCR reactions, treated with endonucleases, were electrophoresed in 3% (w/v) agarose gels for 3 h and visualized by ethidium bromide staining.

Species assignation was done according to 16S rDNA typing and comparison with the published database of mapped restriction sites in the 16S rRNA genes of rhizobia [18].

2.5. Nodulation assay

Inoculation and seed treatment were performed by using the method of Vincent [17]. The plants were cultured in a growth chamber at a constant temperature of 23 °C and a light/dark photoperiod of 16/8, hand watered with a nitrogen-free nutrient solution [17]. Six repetitions in six different pots were used for every strain, and sterile sand was used as the non-inoculated control (negative control).

2.6. Phylogenic analysis of the 16S rRNA gene

The 16S rRNA gene of the four selected strains was amplified with the same primers and procedure as in the restriction analysis, and was sequenced directly by using a PCR-based method. Nucleotide sequence similarity searches were conducted by Genbank BLAST (N).

2.7. Production of Indole Acetic Acid (IAA) and siderophores

Each bacterial strain selected was incubated in 10 mL of an SMS medium (sucrose, 10 g.L^{-1}; $[NH_4]_2SO_4$, 1 g.L^{-1}; K_2HPO_4, 2 g.L^{-1}; $MgSO_4 \cdot 7H_2O$, 0.5 g.L^{-1}; yeast extract, 0.5 g.L^{-1}; $CaCO_3$, 0.5 g.L^{-1}; NaCl, 0.1 g.L^{-1} and pH 7.2), supplemented with 0.5 mg.mL^{-1} of tryptophane for 5 days at 28 °C with shaking at 150 rpm. Cells and supernatants were separated by centrifugation at 10,000 g for 5 min and 1 mL of supernatant was mixed with 100 μL of orthophosphoric acid (10 mM) and 2 mL of Salkowsky reagent (2% 0.5 M $FeCl_3$ in 35% perchloric acid) and incubated at 28 ± 2 °C in darkness for 30 min. The absorbance of the pink color developed was read at 530 nm. The IAA concentration in the supernatant was determined using a standard IAA solution [19]. The experiments were repeated three times on different time intervals. Siderophores production was detected using Chrome Azurol S (CAS) plates [20].

2.8. Phosphate solubilisation

Plates containing Trypticase Soya Agar medium supplemented with $Ca_5(PO_4)_3OH$ were inoculated with 20 μL of a LB pure bacterial culture. Plates were incubated at 28 °C and observed daily during 7 days until the formation of transparent "halos" around each colony [10].

2.9. Bioaccumulation of Cd in bacteria

The cells (0.1 g of dry weight, DW) were inoculated into 100 mL of a TY medium containing 0.1 mM Cd^{2+} and incubated at 28 °C with agitation at 150 rpm. The cells were harvested at 24 h and 48 h after inoculation by centrifugation at 6000 g for 25 min. After washing three times with sterilized ddH$_2$O, the cell pellets were agitated with 10 mM of sterilized EDTA at 28 °C at 150 rpm for 10 min in order to remove the cadmium ions adsorbed on the cell surface. After further centrifugation at 14,000 g for 25 min, the cells were suspended in 5 mL of 0.1 M HNO$_3$, and the solution was centrifuged at 14,000 g for 30 min. The supernatant was used for the determination of cell wall-bound Cd^{2+} with Flame Atomic Absorption Spectrometry. After measurement of the dry weights, the cell pellets were digested with H_2SO_4–$HClO_4$ (V:V = 3:1) at 110 °C for 3 h and diluted with ddH$_2$O, for the determination of intracellular accumulated Cd^{2+} [21].

2.10. Amplification of Cd-resistant gene partial sequence

The strains were incubated in TY medium, and the total DNA was extracted using the method reported by [22]. Four primers: P1: Cad A1 forward (GAAAGAAGATAAAGTGCCGTTTT), Cad A1 reverse (ATGCACAAGGACAACCAACA), P2: Cad A3 forward (TGAAAAATCTGCTCGCCAAG), Cad A3 reverse (ATGCACAAGGACAACCAACA), P3: Cad A2 forward (CTCGCCAAGCTTCACAAGAG), Cad A2 reverse (ATGCACAAGGACAACCAACA), P4: CZC1 forward (AACCAGATCTCGCGCGAGAAC), CZC1 reverse (CGGCAACACCAGTAGGGTCAG) for the amplification of Cd-resistant genes were utilized. The conditions for PCR amplification were: pre-denaturalization at 95 °C for 3 min, then 30 cycles of denaturing at 94 °C for 1 min, annealing for 1 min for extension at 50 °C (P2), 52 °C (P1, P3) and 55 °C (P4) for 2 min, and a final step for extension at 72 °C for 10 min. The PCR product was detected as described by [23].

2.11. Statistical analysis

All experiments were done with at least three replications and repeated independently. The average and standard deviations were calculated (±SD) by Microsoft Excel 2007 and Statistica 6.0

3. Results

3.1. Determination of toxic elements in soil samples

In the S1 soil, all concentrations of heavy metals were under the French norm for agricultural soil [24]. The S2 soil samples were severely contaminated by Pb (10,100 mg.kg$_{DW}^{-1}$) and Cd (16.5 mg.kg$_{DW}^{-1}$), and their levels were higher than those defined by the French norm (Table 1). The S3 soil sample was mainly characterized by its Pb contamination (426 mg.kg$_{DW}^{-1}$). In the S4 soil sample, Pb

Table 1
Characteristics and heavy metal concentration analyzed in the soils used in the experiment; S1: Jerissa, S2: Ghzela, S3: Manzel Bourguiba, S4: Jbel Ressas. Results are in mg.kg^{-1}. FN: French norms for agricultural soils.

Heavy metals concentration (mg.kg$_{DW}$$^{-1}$)	S1	S2	S3	S4	FN
Cd total	0.27	16.5	0.27	0.841	0.7–2
Cd extractible	< 0.27	< 0.27	< 0.27	< 0.27	
Cu total	10.6	21.6	20.0	40.3	35–100
Cu extractible	< 0.97	< 0.97	< 0.97	< 0.97	
Pb total	27.2	10100	426	116	60–100
Pb extractible	< 1	143	50	< 1	
Zn total	31.2	235	84.5	211	150–300
Zn extractible	–	121	–	48	
Soil pH	8.1	9.04	7.5	8.23	5.5–7.5
% Carbon	0.55	0.65	1.1	2.32	No limit
Phosphorus (P$_2$O$_5$, ppm)	21	16.5	31	541.7	70–200
%Organic matter	1.5 ± 0.3	1.11 ± 0.6	1.15 ± 0.2	4.02 ± 0.6	No limit
‰ n total	1.87	0.33	1.68	2.37	
Potassium (K$_2$O, ppm)	822	84	877.2	878.4	

contamination was moderate (116 mg.kg$_{DW}$$^{-1}$), but higher than those defined by the French norm. This site was richer in organic matter and phosphorus; it is currently used as an agricultural zone for viticulture.

3.2. Tolerance of the isolates to heavy metals

A total of 82 strains were isolated from the nodules of the *Sulla coronaria* grown in the four soil samples. The phenotypic characterization of the isolates for tolerance to heavy metals was done by measuring the MIC of the strains (Table 2). Our study showed that 73 isolates tolerated 4 mM of Pb and 48 resisted to 1.6 mM of Zn. The majority of the isolates were sensitive to Cd and Cu: only 21 of them could grow in a medium supplemented with 2.6–4.1 mM of Cd and 34 isolates tolerated 0.25–0.35 mM of Cu. Nickel was especially lethal and only 16 were resistant to 0.12–0.2 mM of Ni. Some strains were particularly resistant to several elements at the same time (Table 2).

3.3. Molecular diversity of the strains

The 16S rDNA region of the 82 isolates, selected from *Sulla coronaria*, was amplified by PCR. Electrophoresis revealed from one to five profiles for each enzyme used. The number of bands varied from 70 bp to 690 bp for *Nde*II (Fig. 1). Despite the high diversity, restriction enzymes produced polymorphic banding patterns, the most discriminative of which was that obtained with *Msp*I.
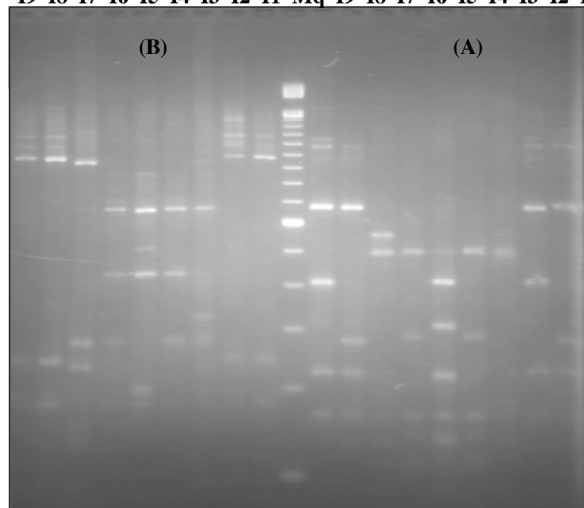

Fig. 1. PCR/RFLP profiles of 16S rRNA genes of 9 isolates (I1...I9) digested with (A) *Msp*I and (B) with *Nde*II and separated by electrophoresis; Mq: molecular marker (100 pb).

The collection was represented by different bacterial genera. Thirty-four percent of the isolates were related to *Rhizobium* sp. The genus *Agrobacterium* was represented by 8%, whereas only 2% of isolates belonged to the genus *Sinorhizobium*. Finally, PCR-RFLP of the 16S rDNA gene

Table 2
Tolerance to heavy metals (Pb, Zn, Cu, Ni and Cd) of isolates isolated from nodules of *Sulla Coronaria*.

		Heavy metals				
		Pb	Zn	Cu	Ni	Cd
Sensible	Concentration (mM)	(0.2–1)	(0.6–1)	(0.01–0.07)	(0.008–0.032)	(0.01–1)
	Strains number	4	6	27	19	47
Tolerant	Concentration (mM)	(1.3–2.7)	(1.2–1.3)	(0.1–0.2)	(0.04–0.08)	(1.1–2.5)
	Strains number	5	28	21	47	14
Very tolerant	Concentration (mM)	(2.8–4)	(1.3–1.6)	(0.25–0.35)	(0.12–0.2)	(2.6–4.1)
	Strains number	73	48	34	16	21

Table 3

Growth and nodulation test of *Sulla coronaria* inoculated by the 19 isolates (SDW: shoot dry weight; RDW: root dry weight; Nod·plant^{-1}: nodule per plant). The values are the average of three repetitions in three independent pots) and the molecular characterization of 19 isolates by PCR/RFLP of the 16rDNA gene.

	Locality	Nod·plant^{-1}	% SDW	% RDW	PCR/RFLP characterization
Control	–	0	100	100	–
I1	S4	0	77.01	94.94	*Agrobacterium*
I2	S1	0	73.39	78.4	*Agrobacterium*
I3	S2	4	168.7	72.18	*No identified*
I4	S2	3	104.03	106.5	*R. leguminosarum*
I5	S2	0	112.4	97.07	*Rhizobium* sp.
I6	S3	4	139.2	103.95	*R. leguminosarum*
I7	S2	0	79	57.68	*No identified*
I8	S2	15	153.8	150	*Rhizobium* sp.
I9	S2	3	173.33	151.39	*No identified*
I10	S2	3	162.1	89.09	*No identified*
I11	S3	0	104.33	59	*S. melliloti*
I12	S1	7	131.21	76.35	*No identified*
I13	S1	0	91.98	54.39	*R. phaseoli*
I14	S2	0	81.65	71.52	*Agrobacterium*
I15	S1	0	124.22	85.79	*No identified*
I16	S2	5	110.33	92	*Rhizobium* sp.
I17	S1	0	71.19	52.48	*Rhizobium* sp.
I18	S2	0	126.48	86	*R.leguminosarum*
I19	S1	2	53.44	48.17	*Agrobacterium*

could not discriminate 56% of the isolates, which were not identified.

3.4. Plant nodulation test and symbiotic effectiveness

Among the 82 isolates, 19 were selected and tested for their ability to nodulate *Sulla coronaria*. These strains represent the different profiles of digestion obtained by PCR/RFLP (Table 3). The nodulation test showed that only 9 strains nodulated the host plant.

Inoculation of *Sulla coronaria* by I8 and I9 enhanced SDW by 153% and 173%, respectively, and RDW increased up to 150 and 151%, respectively. In addition, inoculation with I3 and I10 improved SDW by 169% and 162%, respectively, whereas inoculation with the strain I19 conferred the lowest growth to *Sulla coronaria* (Table 3).

3.5. Selection of the inoculum

Taking into consideration the isolates tolerance to Cd and nodulation capacity, four strains were chosen. The I3 strain had the lowest MIC (0.25 mM Cd), but it improved plant growth. The I8 and I9 strains tolerated 1 mM Cd, enhanced the SDW of plants, and it was noted that I8 gave

the largest number of nodules per plant. I3 and I9 had little white nodules, and I8 strains had red nodules. Among the selected strains, I10 was considered the most tolerant to Cd, due to its resistance up to 4.1 mM.

The nucleotide sequence of the amplified DNA was determined and compared with available 16S rRNA sequences to construct a phylogenetic tree. The subsequent BLAST analysis in GenBank affirmed that the strain I9 have 96% homology with *pseudomonas fluorescens* strain MFN 1032, I10 showed 88% similarity with uncultured *pseudomonas* sp., clone A6B-D10, I3 have 96% homology with *Rhizobium sullae* strain IS123 and I8 has 97% similarity with *Rhizobium sullae* IS 123.

3.6. Production of indole acetic acid (IAA), siderophores and solubilization of P

Our results showed that I8 (*Rhizobium sullae*) showed a higher production of IAA (64.18 μg.μL^{-1}) compared to the rest of the studied bacteria, whereas the production of siderophores was positive in I3, I8 and I10 (Table 4).

The isolates I3 and I8 were not able to solubilize the P contrarily to I9 and I10 (Table 4).

3.7. Estimation of Cd bioaccumulation in the screened bacteria

The accumulation of cadmium varied between cells sites and incubation times; in addition it increased with Cd tolerance. The highest cadmium accumulation appeared in the whole cells of I10 isolates at 48 h, reaching 14737.54 μg.g$_{DW}$$^{-1}$ (Table 5). The Concentration of cadmium in cell walls were the lowest in I3 at 24 h (0.57 μg.g$_{DW}$$^{-1}$), therefore it enhanced significantly at 48 h (23 μg.g$_{DW}$$^{-1}$). The highest level was in I10 at 24 h (70 μg.g$_{DW}$$^{-1}$), but it declined by 65% at 48 h, whereas it was enhanced by 67% in I9. The intracellular Cd accumulation were enhanced by increasing the time of incubation in the four strains (Table 5); the maximum content was obtained at 48 h in I10 (14513 μg.g$_{DW}$$^{-1}$).

3.8. Detection of resistance genes in the screened bacteria

In the four selected soil bacteria, four primers were selected and identified by primer Blast from NCBI. The amplification of the Cd-resistant gene showed different bands with different size (Table 6). The alignment of Cd-resistant gene PCR showed a high homology with Cd-resistant genes from other bacteria. In I3 (*Rhizobium sullae*), a band of 1200 bp was found with primer1, which had 90% homology with *Pseudomonas fluorescens*.

Table 4

Indole acid acetic (IAA), siderophores production and P solubilization of isolates. The values are the average ± SD of three repetitions.

Strain	CMI (mM Cd)	IAA (μg/μL)	Siderophores	Solubilization of P
I3 (*Rhizobium sullae*)	0.25	21.58 ± 1.35	+	–
I8 (*Rhizobium sullae*)	1	64.18 ± 0.35	+	–
I9 (*P. fluorescens*)	1	2.30 ± 0.11	–	+
I10 (*Pseudomonas* sp.)	4.1	0.68 ± 0.15	+	+

Table 5
Cadmium accumulations in two compartments of isolates: cell and cell wall in (μg.g.DW^{-1}) at two times, 24 and 48 h. The values are the average $\pm$ SD of three repetitions.

Strain	Intracellular Cd		Cell wall Cd	
	24 h	48 h	24 h	48 h
I3 (*Rhizobium sullae*)	7035 ± 34	7844 ± 25	0.57 ± 0.1	23.44 ± 3
I8 (*Rizobium sullae*)	387 ± 18	3703 ± 35	37.64 ± 4	33.47 ± 5
I9 (*P. fluorescens*)	2.56 ± 1	2118 ± 47	19.9 ± 3	33.34 ± 6
I10 (*Pseudomonas* sp.)	264 ± 23	14713 ± 65	70 ± 12	24.54 ± 3

In I8 (*Rhizobium sullae*), we evidenced a band of 1600 pb with primer 1, the comparison of the Cd gene sequence indicated 85% homology with *Sinorhizobium meliloti* plasmid pHRC017.

In I9, one band was revealed with each tested primers (P2:500 pb; P3: 1200 pb and P4: 500 pb, Fig. 2) and the sequence analysis showed homology with *Pseudomonas*

Table 6
PCR amplification of Cd-resistant genes using different primers.

| Strain	Primer1	Primer2	Primer3	Primer4
I3	1200 pb	–	–	–
I8	1600 pb	–	–	–
I9	–	500 pb	1200 pb	500 pb
I10	1300–600 pb	1300 pb	–	–

Fig. 2. PCR Amplification of a Cd-resistant gene in the two selected bacteria (I9 and I10) with different primers. A: I9with Primer P4; B: I9 with primer P3; C: I100 with primer P2; D: molecular marker (100 pb).

fluorescens by 90%. In I10, a band of 1300/600 pb was found with primer1 and a band of 1300 pb with primer 2 (Fig. 2); these sequence showed 99% similarity with *Pseudomonas putida* (Table 6).

4. Discussion

4.1. Tolerance of the isolates to heavy metals

In the present study, we have obtained a collection of soil bacteria isolated from nodules of *Sulla coronaria* cultivated in soils contaminated by heavy metals, which even influence the growth, abundance, tolerance, qualitative and quantitative structure of microbial communities [25]; it is well known that the bacteria isolated from polluted soil are resistant to higher concentrations of heavy metals than those isolated from unpolluted area [26]. The Pb-tolerance study of soil bacteria indicated that the majority of isolates tolerated 2.8–4 mM Pb; these values were comparable to Pb resistant PGPRs isolated from *Lens culinaris* cultivated in Pb contaminated soils [27], whereas *Bradyrhizobium* and *Sinorhizobium* can grow in media containing up to 2 mM Pb [11]. The Ni and Zn tolerance of isolates was lower than that of *Rhizobium leguminosarum* isolated from a sewage-sludge-treated soil and having a tolerance of 0.24 mM Ni and 6–8 mM Zn [28]; elsewhere, the tested soil bacteria were sensitive to Cu comparatively to others such as *Agrobacterium tumefaciens* [23] and *Sinorhizobium meliloti* [21], which can tolerate up to 2 mM Cu. The level of resistance to Cd in these bacteria is comparable to those of other bacteria from different genera such as *Azorhizobium caulinodans*, *Staphylococcus aureus*, *Bacillus subtillis*, and *E. coli* [29].

Even though metals exert their toxic effects on the diversity of microorganisms [1], the most Cd-resistant soil bacteria were isolated from nodules of *Sulla coronaria* cultivated in Cd-contaminated soil S2, heavy metal tolerant bacteria could survive in these sites and were selected for their potential application in the phytoremediation of contaminated soils [25].

4.2. Molecular diversity and symbiotic effectiveness of heavy-metal-resistant strains

The molecular study of strains isolated from nodules of *Sulla coronaria* showed a large diversity represented essentially by *Rhizobium* sp., *R. leguminosarum*, *Agrobacterium* sp., *Pseudomona* sp., and *Rhizobium sullae*. The genus *Sinorhizobium* sp. was the least represented. In many studies, heavy-metal-contaminated soils can serve as a
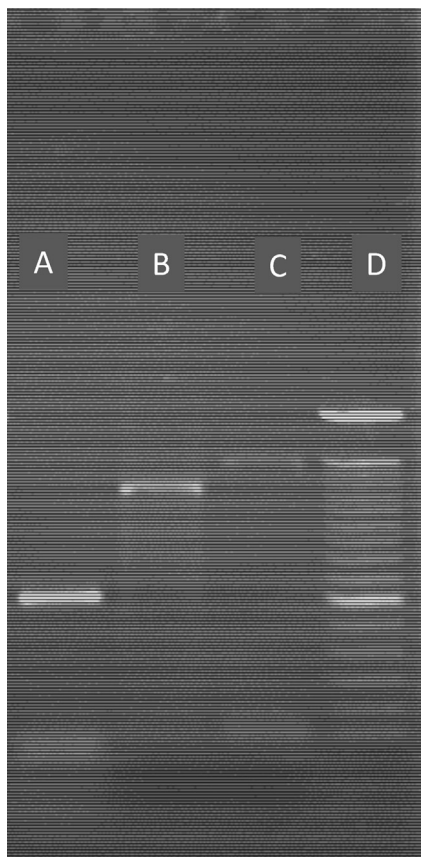
source for the isolation of heavy-metal-resistant bacteria [21,30] and *Rhizobium* can be used as an organism indicating contamination by several heavy metals as mentioned in [31]. The isolation and characterization of *Rhizobium* strains from nodules of legumes grown in heavy-metal-contaminated soils were examined in many works, such as *Sinorhizobium meliloti* nodulating alfalfa [10] and *Medicago lupilina* [21], *R. leguminosarum* biovar *trifolii* isolated from clover nodules [1], and *Agrobacterium tunefaciens* found in the nodules of *Lespedeza cuneata* [23,32]. Nodulation tests suggested the existence of functional nodules in inoculated *Sulla coronaria* with rhizobial strains; it could be associated with symbiotic and nitrogen fixation abilities. In fact, nodulation of *Sulla coronaria* by *Rhizobium sullae* was demonstrated by [33] and [34] affirm that Gamma proteobacteria can nodulate the genus *Hedysarum*. The presence of many symbiotically effective *Rhizobium* in highly polluted soils seems to be very important [10]. In this work, four bacterial strains belonging to *pseudomonas* and *Rhizobium sullae* were selected and characterized in order to use them as symbiotic inoculums for *Sulla coronaria*, ameliorating the potential of phytostabilization of the plant in contaminated soils. The application of bacterial a consortium containing *Pseudomonas* as an endophytic bacteria and *Rhizobium* to improve the productivity of legumes was tested by [35], and its efficiency in the phytoremediation process had been previously demonstrated in many other works [11,27,36].

4.3. Accumulation of cadmium in cells of selected bacteria

The accumulation of cadmium in the whole cell was highest in the most Cd-resistant bacteria I10 (*Pseudomonas* sp.), suggesting the possible relationship with a mechanism of tolerance to heavy metals [23]. The intracellular Cd accumulation was greater than in the cell wall and it was enhanced with the time of incubation in the four strains; these phenomena demonstrated that Cd accumulation was related to the cell growth or to the increase in the biomass [21]. Moreover, other mechanisms of heavy metal tolerance were discovered such as biosorption and bioaccumulation both in internal and external spaces; these processes influence the bioavailability of heavy metal to plant [26].

4.4. Production of plant-growth-promoting substances by selected bacteria

In this work, the screened soil bacteria had the ability to produce IAA essentially in *Pseudomonas* sp. and *Rhizobium sullae* more than those reported in other heavy-metal-resistant bacteria [1,25]. The main effect of bacterial IAA is the enhancement of mineral and nutrient uptake, inducing bacterial proliferation on the roots [12].

In addition, these soil bacteria produced siderophores that indirectly affect the growth of plants; generally siderophores form a stable complex with iron and other heavy metals, resulting in an increase in metal concentration [26]. This phytohormone played an important role in plant–bacteria interactions and plant growth in metal contaminated soils [37]. *Pseudomonas* bacteria, especially *P. fluorescens* and *P. Putida*, are the most important kind of PGPR, which produce auxin and promote the yield [38]. The consortium of selected soil bacteria can be designed as a Cd-resistant PGPR that have the exceptional ability to ameliorate the growth of host plant in contaminated soils by various mechanisms such as nitrogen fixation, solubilisation of minerals, production of phytohormones and siderophores [37], as confirmed for *Vicia faba* by dual inoculation with rhizobium and PGPRs under copper stress [39].

4.5. Detection of Cd-resistance genes in the screened bacteria

PCR amplification and electrophoresis of some Cd-resistant genes selected showed a positive amplification of the Cd resistance gene with one or more primers in the four selected soil bacteria, suggesting that some primers were inappropriate. The sequence comparison showed a similarity with Cd-resistant genes of other bacteria, indicating that these genes were acquired in the selected soil bacteria by lateral gene transfer from other soil bacteria as has been proposed for some symbiotic genes [40,41]. These genes encoded certain enzymes of the mechanism of tolerance to Cd in bacteria.

5. Conclusion

This study suggested that the selected PGPRs, including *Pseudomonas* and *Rhizobium sullae*, were efficient and heavy metal tolerant; they accumulate Cd essentially in the intracellular compartment, produced plant-growth-promoting substances and revealed the presence of some genes responsible for the resistance to Cd. These characteristics suggest that this consortium could be a useful inoculum for *Sulla coronaria* to phytostabilize Cd-contaminated soils, and further studies are needed to clarify the mechanism. The prospective symbiotic system should be tested in contaminated areas.

References

[1] V. Stan, E. Gament, C.P. Cornea, C. Voaides, et al., Effects of heavy metal from polluted soils on the *Rhizobium* diversity, Not. Bot. Hort. Agrobot. Cluj. 39 (1) (2011) 88–95.

[2] V. Ganesan, Rhizoremediation of cadmium soil using a cadmium-resistant plant growth-promoting rhizopseudomonas, Curr. Microbiol. 56 (2008) 403–407.

[3] U.C. Gupta, S.C. Gupta, Trace element toxicity relationships to crop production and livestock and human health: implications for management, Commun. Soil. Sci. Plant 29 (1998) 1491–1522.

[4] M. Bibi, M. Hussein, Effect of copper and lead on photosynthesis and plant pigments in black gram (*Vignamungo* L.), Bull. Environ. Contam. Toxicol. 74 (2005) 1126–1133.

[5] K. Giller, E. Witter, S.P. Mc Grath, Toxicity of heavy metals to microorganisms and microbial processes in agricultural soils, Soil Biol. Biochem. 30 (1998) 1389–1414.

[6] S.Y. Koo, C. Kyung-Suk, Isolation and characterization of a plant growth-promoting rhizobacteria, Serratia sp. SY5, J. Microbiol. Biotechnol. 19 (11) (2009) 1431–1438.

[7] T. Kumino, K. Seaki, K. Nagaoka, H. Oyaizu, et al., Characterization of copper-resistant bacteria community in rhizosphere of highly copper-contaminated soil, Eur. J. Soil Biol. 37 (2001) 95–102.

[8] M. Beladi, A. Kashani, D. Habibi, F. Paknejad, et al., Uptake and effects of lead and copper on three plant species in contaminated soils: Role of phytochelatin, Afr. J. Agric. Res. 6 (2011) 3483–3492.

[9] S.P. Ghosh, H. Sing, A review on phytoremediation of heavy metals and utilization of its byproduct, Appl. Ecol. Environ. 3 (2005) 1–18.

[10] J.A. Carrasco, P. Armario, E. Pjuelo, A. Burgos, et al., Isolation and characterisation of symbiotically effective Rhizobium resistant to arsenic and heavy metal safter the toxic spill at the Aznalcollar pyrite mine, Soil Biol. Biochem. 37 (2005) 1131–1140.

[11] M. Dary, M.A. Chamber-Perez, A.J. Palomares, E. Pajuelo, In situ phytostabilisation of heavy metal polluted soils using *Lupinus luteus* inoculated with metal resistant plant-growth promoting rhizobacteria, J. Hazard. Mater. 177 (2010) 323–330.

[12] B.R. Glick, Using soil bacteria to facilitate phytoremediation, Biotechnol. Adv. 28 (2010) 367–374.

[13] H. Baatout, Comparison of phenotypic variation in self-fertilizing and outcrossing subspecies of *Hedysarum spinosissimum*, a Mediterranean herb, Plant Genet. Res. Newslett. 105 (1996) 23–28.

[14] N. Trifi Farah, H. Baatout, M. Boussaïd, D. Combes, et al., Évaluation des ressources génétiques des espèces du genre *Hedysarum* dans le Bassin méditerranéen, Plant Genet. Res. Newslett. 130 (2002) 1–6.

[15] A. Ben Guedouar, V. Corich, A. Giacomini, A. Squartini, et al., Characterization of symbiotic bacteria from the Mediterranean legume crop *Hedysarum coronarium* (Sulla) by multilocus enzyme electrophoresis, Agr. Med. 127 (1997) 173–177.

[16] SSICA (Soil Sci. Ch. Acad.), Physical and chemical analyses of soils, Changhai Academic Press, China, 1980.

[17] J.M. Vincent, 1A manual for the practical study of root-nodule bacteria. IBP Handbook, Blakwell Scientific Publications, Oxford, 1970.

[18] G. Laguerre, M.R. Allard, F. Revoy, N. Amarger, Rapid Identification of Rhizobia by Restriction Fragment Length Polymorphism Analysis of PCR-Amplified 16S rRNA Genes, Appl. Environ. Microbiol. 60 (1994) 56–63.

[19] J.M. Bric, R.M. Bostock, S.E. Silverstone, Rapid in situ assay for indole acetic acid production by bacteria immobilized on nitrocellulose membrane, Appl. Environ. Microbiol. 57 (1991) 537–538.

[20] B. Schwyn, J.B. Neilands, Universal chemical assay for the detection and determination of siderophore, Anal. Biochem. 160 (1987) 47–56.

[21] L.M. Fan, Z.Q. Ma, J.Q. Liang, H.F. Li, et al., Characterization of a copper-resistant symbiotic bacterium isolated from *Medicagolupina* growing in mine tailings, Bioresour. Technol. 102 (2011) 703–709.

[22] S.N. Terefework, L. Suomalainen, K. Paulin, Lindstrom phylogeny of Rhizobium galegae with respect to other rhizobia and agrobacteria, Int. J. Syst. Bacteriol. 48 (1998) 349–356.

[23] G. Wei, L.M. Fan, W. Zhu, Y. Fu, et al., Isolation and characterization of the heavy-metal-resistant bacteria CCNWRS334-2 isolated from root nodule of *Lespedeza cuneata* in gold mine tailings in China, J. Hazard Mater. 162 (2009) 50–56.

[24] D. Baize, T. Sterckeman, The necessity of knowledge of the natural pedogeochemical background content in the evaluation of the contamination of soils by trace elements, Sci. Total Environ. 264 (2001) 127–139.

[25] L.Y. He, Y.F. Zhang, H.Y. Ma, L.N. Su, Z.J. Chen, Q.Y. Wang, Characterization of copper-resistant bacteria and assessment of bacterial commu-

nities in rhizosphere soils of copper-tolerant plants, Appl. Soil Ecol. 44 (2010) 49–55.

[26] M.Y.M. Rajkumar, H. Freitas, Improvement of plant growth and nickel uptake by nickel resistant-plant-growth promoting bacteria, J. Hazard Mater. 166 (2009) 1154–1161.

[27] H.S. Jebara, S. Abdelkrim, C.I. Fatnassi, M. Chiboub, O. Saadani, et al., Identification of effective Pb resistant bacteria isolated from *Lens culinaris* growing in lead-contaminated soils, J. Basic Microbiol. 55 (3) (2015) 346–353.

[28] D. Purchase, R.J. Miles, Survival and nodulating ability of indigenous and inoculated *Rhizobium leguminosarum trifolii* in sterilized and un-sterilized soil treated with sewage sludge, Curr. Microbiol. 42 (2001) 59–64.

[29] Z. Zhengwei, W. Fang, L. Helen, Z. Yang, Responses of *Azorhizobium-caulinodans* to cadmium stress, FEMS Microbiol. Ecol. 54 (2005) 455–461.

[30] I.C. Fatnassi, M. Chiboub, M. Jebara, S.H. Jebara, Bacteria associated with different legumes species grown in heavy metal contaminated soils, Int. J Agric. Policy Res. 2 (12) (2014) 460–467.

[31] J.L. Bostford, A simple method for determining the toxicity of chemicas using a bacterial indicator organism, Environ. Toxicol. 14 (1999) 285–289.

[32] J. Yu, L. Fan, S. Yang, M. Tang, et al., Characterization of copper-resistant Agrobacterium isolated from legume nodule in mining tailing, Bull. Environ. Contam. Toxicol. 82 (2009) 354–357.

[33] A. Squartini, P. Struffi, H. Doering, S. Selenska-Pobe, et al., *Rhizobium sullae* sp. nov. (formerly *Rhizobium "hedysari"*): the root-nodule microsymbiont of *Hedysarum coronarium* L, Int. J. Syst. Evol. Microbiol. 52 (2002) 1267–1276.

[34] S. Saidi, S. Chebil, M. Grari, R. Mhamdi, Characterization of root-nodule bacteria isolated from *Vicia faba* and selection of plant growth promoting isolates, World J. Microbiol. Biotechnol. 29 (6) (2013) 1099–1106.

[35] Y. Benhizia, H. Benhizia, A. Benguedouar, R. Muresu, A. Giacomini, A. Squartini, Gamma proteobacteria can nodulate legumes of the genus *Hedysarum*, Syst. Appl. Microbiol. 27 (2004) 462–468.

[36] I.C. Fatnassi, M. Chiboub, O. Saadani, M. Jebara, S.H. Jebara, Phytostabilisation of moderate copper contaminated soils using co-inoculation of *Vicia faba* with plant growth promoting bacteria, J. Basic Microbiol. 55 (3) (2015) 303–311.

[37] B.R. Glick, C.L. Patten, G. Holguin, D.M. Penrose, Biochemical and Genetic Mechanisms Used by Plant Growth-Promoting Bacteria, Imperial College Press, London, 1999.

[38] B.S. Saharan, V. Nehra, Plant Growth Promoting Rhizobacteria: A critical Review, Life Sci. Med. Res. 2011 (2011) LSMR–LSMR21.

[39] I.C. Fatnassi, M. Chiboub, O. Saadani, M. Jebara, S.H. Jebara, Impact of dual inoculation with Rhizobium and PGPR on growth and antioxidant status of *Vicia faba* L. under copper stress, C. R. Biol. 338 (4) (2015) 241–254.

[40] F.G. Barcellos, P. Menna, J.S. da Silva Batista, M. Hungria, Evidence of horizontal transfer of symbiotic genes from a *Bradyrhizobium japonicum* inoculant strain to indigenous *Sinorhizobium* (*Ensifer*) *fredii* and *Bradyrhizobium elkanii* in a Brazilian savannah soil, Appl Environ Microbiol. 73 (2007) 2635–2643.

[41] C.T. Zhao, E.T. Wang, W.F. Chen, W.X. Chen, Diverse genomic species and evidences of symbiotic gene lateral transfer detected among the Rhizobia associated with *Astragalus* species grown in the temperate regions of China, FEMS Microbiol. Lett. 286 (2008) 263–273.