Environ Sci Pollut Res (2016) 23:19825–19837
DOI 10.1007/s11356-016-7184-1

RESEARCH ARTICLE

Screening beneficial rhizobacteria from *Spartina maritima* for phytoremediation of metal polluted salt marshes: comparison of gram-positive and gram-negative strains

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Received: 14 March 2016 / Accepted: 4 July 2016 / Published online: 14 July 2016

Abstract The aim of our work was the isolation and characterization of bacteria from the rhizosphere of *Spartina maritima* in the metal contaminated Odiel estuary (Huelva, SW Spain). From 25 strains, 84 % were identified as gram-positive, particularly *Staphylococcus* and *Bacillus*. Gram-negative bacteria were represented by *Pantoea* and *Salmonella*. Salt and heavy metal tolerance, metal bioabsorption, plant growth promoting (PGP) properties, and biofilm formation were investigated in the bacterial collection. Despite the higher abundance of gram-positive bacteria, gram-negative isolates displayed higher tolerance toward metal(-loid)s (As, Cu, Zn, and Pb) and greater metal biosorption, as deduced from ICP-OES and SEM-EDX analyses. Besides, they exhibited better PGP properties, which were retained in the presence of metals and the ability to form biofilms. Gram-negative strains *Pantoea agglomerans* RSO6 and RSO7, together with gram-positive *Bacillus aryabhattai* RSO25, were selected for a bacterial *consortium* aimed to inoculate *S. maritima* plants in metal polluted estuaries for phytoremediation purposes.

Responsible editor: Robert Duran

Electronic supplementary material The online version of this article (doi:10.1007/s11356-016-7184-1) contains supplementary material, which is available to authorized users.

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Keywords Heavy metals · Phytoremediation · Estuaries · Odiel River · *Spartina maritima* · Plant growth promoting rhizobacteria (PGPR) · Metal biosorption · Biofilms

Introduction

Heavy metals accumulate continuously in estuaries and coastal sediments (Soliman et al. 2015; Xia et al. 2011) affecting water quality, while their bioaccumulation in aquatic organisms causes long-term impact on human health and ecosystems (Abdel-Baki et al. 2011; Fernandes et al. 2007; Soliman et al. 2015).

In the case of the Odiel estuary (Huelva, SW Spain), both natural and anthropogenic causes determine the high metal accumulation in its sediments (Ruiz 2001). On the one hand, the Iberian Pyrite Belt (IPB), considered as the largest and most important metallogenic sulfide province in the world, forms a belt about 250 km long and up to 60 km wide, trending westward form Seville in SW Spain to South Portugal (Fernández-Caliani et al. 1997; Nieto et al. 2007). Mining activity, today abandoned, dates back to prehistoric times but still generates intense acid mine drainage (Galán et al. 2003; Sáinz and Ruiz 2006) into the Tinto and Odiel rivers. The acid pH (2–4) provides the environment for exceptional microbial communities (Amils et al. 2013). On the other hand, the city of Huelva is surrounded by one of the largest chemical and basic industries complexes in Spain, which is situated between the Tinto and Odiel estuaries. The proximity of urban centers to these pollution sources, together with the presence of agricultural areas and faunal collection (fish, shellfish and crustaceans) in adjacent zones, poses a potential risk to the population, as well as to the ecosystem of protected natural areas nearby (Iriarte et al. 2007). Previous studies showed that in the Odiel estuary, the concentrations of heavy metals (As, up to 700 ppm; Cu, up to 3000 ppm; Pb, up to 3570 ppm; and Zn, up to 4800 ppm) (Ruiz 2001; Sáinz and Ruiz 2006) are far above the threshold for natural parks, according to regional regulations (Junta de Andalucía 1999).

Odiel marshes provide an environment dominated by high salt concentrations adequate to the establishment of salt-tolerant plants known as halophytes (Gómez-Mercado et al. 2012; Redondo-Gómez et al. 2011; Shabala 2013). One of these plant species is *Spartina maritima*. Besides being resistant to heavy metals, this plant tolerates other stress conditions, such as drastic temperatures changes, drought, floods, tidal submergence, and high radiation (Cambrollé et al. 2008; Reboreda et al. 2008; Redondo-Gómez 2013). Moreover, being a grass, it tends to limit the translocation of inorganic pollutants to shoots as much as dicot species, minimizing exposure of wildlife to toxic elements (Reboreda et al. 2008; Redondo-Gómez 2013).

Phytoremediation is being consolidated as a green technology used in the reclamation of soils and sediments, in which the plants and their associated microbes degrade and/or sequester organic and inorganic pollutants for environmental cleanup. It is based on strategies including phytoextraction, rhizoremediation, phytovolatilization, and phytostabilization (Laghlimi et al. 2015; Tangahu et al. 2011; Vamerali et al. 2010). Inorganics cannot be degraded, but they can be phytoremediated via stabilization (immobilization of metals in the rhizosphere of plants) (Mendez and Maier 2008); or sequestration in harvestable plant tissues (hyperaccumulation of metals in aerial parts of plants) (Krämer 2010; Verbruggen et al. 2009). These techniques are improved by the interaction of plants with soil bacteria (Germaine et al. 2013; Pajuelo et al. 2014; Tak et al. 2013), which play a crucial beneficial role in plant responses to heavy metal stress (Burd et al. 2000; Gamalero et al. 2009). Plant growth promoting rhizobacteria (PGPR) are a group of bacteria that colonize the rhizosphere of plants and promote plant growth through mechanisms that include solubilization of mineral phosphates and other nutrients, biological nitrogen fixation, antagonism against phytopathogens by production of siderophores, antibiotics, enzymes and/or fungicidal compounds, stress alleviation through production of phytohormones and of 1-aminocyclopropane-caboxylate (ACC) deaminase, etc. (Ahemad and Khan 2011; Bhattacharyya and Jha 2012; De-Bashan et al. 2012; Glick 2012; Shaharoona et al. 2006). The molecular mechanisms by which rhizobacteria help plants to tolerate stress are not fully understood, but there is increasing information on the induction of the plant systemic tolerance to several types of stress through modulation of gene expression (Sarma et al. 2012; Verhagen et al. 2004; Vurukonda et al. 2016; Yang et al. 2009). Many of these activities are dependent on the bacterial population, known as "quorum sensing." In this particular, formation of biofilms onto the root surface is a key process in order to achieve a minimal bacterial population and also in the exchange of bacteria–bacteria and bacteria–plant signals for cross-talk (Badri et al. 2009; Danhorn and Fuqua 2007).

Rhizosphere bacteria also play a decisive influence on metal accumulation in plants. In this particular, the isolation and characterization of PGPR from *S. maritima* were previously approached (Mesa et al. 2015a, 2015b; Reboreda and Caçador 2008). However, plant inoculation assays have shown an increase in plant metal accumulation upon inoculation with PGPR (Mesa et al. 2015b), whereas inoculation with endophytes led to no changes in plant metal uptake (Mesa et al. 2015c). Conversely, in some other cases, bacteria were able to positively promote plant growth by reducing at the same time, either plant metal uptake or its translocation to the shoot (Dary et al. 2010; El Aafi et al. 2012), and hence, preventing the entry of heavy metals into the food chain (Mendez and Maier 2008).

The objective of the present study was prospecting rhizosphere bacteria associated with *S. maritima* from the Odiel contaminated estuary. These isolates were selected for high heavy metal resistance, metal biosorption, plant growth promoting properties, and the capacity to form biofilms in order to propose a suitable inoculant for phytoremediation in "tailored" restoration programs.

Material and methods

Rhizosphere samples

Samples of *S. maritima* rhizosphere were collected from the Odiel estuary at a depth of 10 cm in February 2014. The location of the sampling site is depicted in Supplementary Information Fig. S1. As for comparison, a sample of the Piedras estuary (situated near the Odiel estuary but not affected by metal pollution) was taken as a control of non-polluted sediment (Supplementary Information Fig. S1). All sediment samples were stored into individual plastic bags at 4 °C with enough aeration until the next morning when appropriate protocols for the study began (Redondo-Gómez et al. 2007).

Characterization of soil samples

Following the methodology used by Cambrollé et al. (2008), physicochemical parameters of sediment, such as redox potential, pH, and conductivity, were measured in sediment samples. pH and redox potential were determined in situ, using a portable meter Crison pH/mV p-506 (Spain). After diluting the sediments with distilled water (1:1) in the laboratory, the conductivity was determined with a conductivity meter (Crison-522, Spain). Homogeneous portions of 5-g soil of the Odiel and Piedras estuaries ($n = 5$ samples) were dried in an oven for 72 h at 70 °C. The concentrations of heavy metals in the samples were determined by inductively coupled plasma–optical emission spectrometry (ICP-OES).

Isolation of rhizosphere bacteria

The roots of *S. densiflora* plants were shaken vigorously in order to separate the bulk soil (not directly adhered to the roots). To collect the rhizosphere soil, pieces of roots were incubated in 25 mL of sterile saline solution for 15 min in a sterile Falcon tube under continuous shaking and allowed to stand for 3 min (Barillot et al. 2013). One hundred microliters of supernatant was plated onto tryptic soy agar (TSA) medium (Thompson et al. 1993) in triplicate. Plates were incubated at 28 °C for 24 h.

Furthermore, in order to isolate possible halophilic bacteria, 100 μL of supernatant was plated on triplicate on Petri dishes containing medium TSA-SW, consisting in TSA medium supplemented with 17 % (*v*/*v*) of the stock solution SW30 (containing 30 % (*w*/*v*) of the sea water salt composition) per liter, NaCl 234.0 g, $MgCl_2 \cdot 6H_2O$ 39.0 g, $MgSO_4 \cdot 7H_2O$ 61.0 g, $NaHCO_3$ 0.2 g, NaBr 0.7 g, KCl 6.0 g, $CaCl_2$ 1.0 g, and H_2O e.q. to 1 L (Mesa et al. 2015a). Plates were incubated at 28 °C for 48 h. Colonies with distinctly different morphologies were isolated. Gram staining of every single colony was made. The collection of rhizosphere bacteria was preserved in 15 % sterile glycerol at −80 °C and recovered whenever necessary on TSA medium or in TSA-SW, depending on their original provenance.

Identification of rhizosphere bacteria

DNA extraction

Bacteria were grown in liquid tryptic soy broth (TSB) medium for 24 h at 28 °C under continuous shaking at 200 rpm. Extraction of DNA was performed using the commercial Kit "G-spin™ Total" (iNtRON Biotechnology, Inc.) following the manufacturer's instructions.

Amplification of 16S ribosomal DNA

The primers used for the PCR amplification of the almost full-length 16S ribosomal DNA (rDNA) sequences were 16F27 5′-AGAGTTTGATCCTGGCTCAG-3′ and 16R1488 5′-AAGGAGGTGATCCAGCCGCA-3′ (Weisburg et al. 1991) synthesized by Isogen Life Science™. PCR reactions were carried out using 17.3 μL of ultrapure water, 2.5 μL of PCR 10× buffer (without Mg), 1 μL $MgCl_2$ (50 mM), 1 μL dNTPs (10 mM), 0.2 μL ECO-Taq (Ecogen), and 100 ng of genomic DNA. The PCR reaction was consisted in an initial denaturation step at 95 °C for 5 min, followed by 35 cycles (95 °C for 30 s, 58 °C for 30 s, 72 °C for 1 min), and a final extension of 10 min at 72 °C. The thermocycler used was SENSQUEST Labcycler®.

Sequencing 16S rDNA PCR products and sequence analysis

PCR samples were electrophoresed on 1 % agarose gels in TAE buffer at 100 V for 20–30 min. For visualization of DNA, the reagent RedSafe™ Nucleic Acid Staining Solution (20,000×) was added to the gels. The electrophoretic bands corresponding to 16S rDNA PCR products were excised from the gel, and DNA were extracted using the kit Speedtools PCR Clean-Up from Biotools B&M Labs, following the recommendations of the provider. DNA was sent for sequencing to Stabvida (Portugal), and sequence analysis was made after comparison with database EzTaxon (http://www.Eztaxon.org) (Chun et al. 2007).

Evaluation of tolerance toward heavy metals and NaCl in rhizosphere bacteria

Tolerance toward heavy metals was determined on plates containing TSA medium supplemented with increasing concentrations (2–10 mM) of heavy metals of $CuSO_4$, $NaAsO_2$, $Pb(NO_3)_2$, or $ZnCl_2$ from stock solutions. In the case of $Pb(NO_3)_2$, addition of the same concentration of ETDA was necessary in order to prevent Pb precipitation. Controls in medium TSA containing only EDTA 2 mM and without Pb were performed simultaneously in order to rule out the inhibitory effect of EDTA on bacterial growth (Supplementary Information Fig. S2). The tolerance was expressed as the maximum tolerable concentration (MTC) for each element tested, considered as the maximum concentration of a metal or metalloid not affecting bacterial growth. Similarly, NaCl tolerance was evaluated on TSA medium supplemented with increasing concentrations of NaCl (0–4 M).

Plant growth promoting abilities of resistant rhizobacteria

Detection of bacteria producing siderophores

Siderophores production by bacteria was investigated by using solid-plate agar medium with chromium-azurol-S (CAS) indicator (Machuca and Milagres 2003). One hundred microliters of fresh cultures (24 h incubation at 28 °C in TSB) of all strains was added to wells excavated in CAS medium. Siderophores production was detected by the formation of orange halos around the wells after 72 h incubation at 28 °C. The halo diameter (in mm) was used as a semiquantitative estimation of the amount of siderophores produced.

Detection of phosphate solubilizing bacteria

The detection of phosphate solubilizing bacteria was performed using the National Botanical Research Institute's phosphate growth medium (NBRiP) (Pikovskaya 1948). One hundred microliters of fresh cultures (24 h incubation at 28 °C in TSB) of all strains was added to wells excavated in NBRiP. The appearance of a transparent halo around the well after 48–72 h incubation at 28 °C was indicative of phosphate solubilization. The halo diameter (in mm) was used as a semi-quantitative estimation of the phosphate solubilization ability.

Detection of auxin (indole acetic acid)-producing bacteria

A colorimetric method was utilized in order to determine the secretion of indole acetic acid (IAA). Test tubes containing 4 mL of TSB supplemented with L-tryptophan (0.5 mg/mL) were inoculated with selected strains from fresh TSA plates. The tubes were incubated for 48 h at 28 °C under continuous shaking at 200 rpm. After incubation, 1 mL of the bacterial culture was centrifuged for 2 min at 14,000 rpm, and the supernatant was transferred to a new test tube. Four milliliters of Salkowski reagent (Patten and Glick 2002) was added, and tubes were incubated for 20 min at room temperature until the development of a pink color in IAA-producing strains. Optical density at OD530 was determined using a spectrophotometer (Lambda 25; PerkinElmer, USA) and compared with a standard curve of pure IAA.

Detection of nitrogen-fixing bacteria

The ability of bacteria to fix nitrogen was evaluated in plates containing minimal medium (Mesa et al. 2015a) without any nitrogen source. After 72 h of incubation at 28 °C, growth in this medium indicated that the bacteria were able to fix nitrogen.

Bioabsorption of heavy metals onto bacterial biomass: analysis by ICP-OES and SEM-EDX

Bioaccumulation of Pb, Cu, Zn, As, and Cd was determined in the following bacterial strains: RSO6, RSO7, and RSO25, according to Rodríguez-Llorente et al. (2010). Flasks with 150 mL of TSB medium containing 2 mM of $Pb(NO_3)_2$ + 2 mM EDTA (added to prevent precipitation of metal), 2 mM $CuSO_4$, or 2 mM $ZnCl_2$ plus a flask without metal (growth control) were inoculated with 1 mL of an overnight culture of each strain. The flasks were incubated for 5 days at 28 °C under continuous shaking at 100 rpm. After incubation, cultures were centrifuged at 8000×*g* for 20 min at room temperature. One sample of bacterial cells of each metal treatment was washed twice for 5 min with distilled water, and another sample was washed with 0.2 M EDTA pH 8.0. Between washing steps, cells were centrifuged at 8000×*g* for 20 min at room temperature. The water-washed sample corresponds to the total bioaccumulation of heavy metal in the bacterial pellet (bioadsorption + bioabsorption), while the sample washed with EDTA corresponds only to heavy metal accumulation within cells (bioabsorption), since EDTA washes out the metal adsorbed onto the bacterial surface. Finally, the pellets were dried in the oven for 72 h at 60 °C, and metal accumulation in the digestive solutions of cells with different treatments was determined by inductively coupled plasma–optical emission spectrometry (ICP-OES).

Capacity for biofilms formation

The capacity for biofilms formation was evaluated through the adhesion to the surface of wells of 96-well microtiter plates, as described by Del Castillo et al. (2012). Cultures of the strains RSO6, RSO7, and RSO25 were grown in TSB medium at 28 °C for 48 h. The $OD_{600\ nm}$ of the cultures was adjusted and normalized to 1.0 by addition of sterile medium. Twenty microliters of the normalized cultures was added to the wells of microtiter plates. For the control, 20 μL of medium was added. The plates were sealed with parafilm and incubated at 28 °C during 4 days under static conditions. After this time, the medium was removed by inverting the plate, and the wells were washed five times with 200 μL distilled water. For staining the biofilms, 200 μL 0.01 % crystal violet was added to the wells and incubated for 20 min. Plates were rinsed three times with 200 μL sterile water. The dye was solubilized in 100 μL of 96 % ethanol; glacial acetic acid (65 %:35 % *v*/*v*) and the absorbance at 570 nm were quantified.

Statistical analysis

The concentrations of metals in soils are means ± standard deviations of five independent samples. The biosorption of metals onto the surface of bacteria and the formation of biofilms onto the surface of microtiter plates were determined in three independent experiments. Results given are the means ± standard deviations. Differences among treatments were assessed by one-way analysis of variance followed by LSD test. Significant differences at $p < 0.05$ are indicated by an asterisk.

Results

Characterization of soil samples

Physicochemical properties of soil (texture, pH, conductivity, and redox potential) were measured in the Odiel and Piedras sediments (Table 1). pH and redox potential are similar in both soils, but remarkable differences were observed in conductivity (the estuary of Odiel had a higher salt content) and also in soil texture (while the Odiel sediment had a higher percentage

Table 1 Physicochemical properties and total content of relevant metals in soils from the Odiel and Piedras salt marshes at the sampling sites

Physico-chemical properties						
Soil	Texture[a] (%)	Redox potential (mV)	Conductivity (mS cm^{-1})	pH	Organic matter (%)	Nitrogen (%)
Odiel	60/16/24	262 ± 10	15.62 ± 0.5	6.88 ± 0.1	13.5 ± 0.2	0.27 ± 0.1
Piedras	20/14/66	150 ± 15	1.2 ± 0.4	7.6 ± 0.3	11.4 ± 0.5	0.23 ± 0.1
Heavy metal concentration in soil (mg/kg)						
Soil	Fe	As	Pb	Zn	Cu	Cd
Odiel	71,026 ± 3436	339.8 ± 12.0	406.7 ± 29.4	2522.7 ± 65.3	1318.4 ± 26.8	4.02 ± 0.13
Piedras	12,669 ± 229	6.5 ± 0.1	16 ± 0.3	78 ± 0.2	19 ± 0.17	0.22 ± 0.08
Level for intervention[b]	–	>100	>1000	>1000	>500	>15

Values are mean ± SE of five replicates

[a] Texture (silt/clay/sand percentage)

[b] Threshold for metal concentration in soils of natural parks according to regional regulation (Junta de Andalucía 1999)

of silt, sand was more abundant in the Piedras sediment). Concerning heavy metals, the concentration of all the elements studied was much higher in the Odiel estuary than in the Piedras estuary (between 25 and 70 folds, depending on the element). Several metal(loid)s, including As, Cu, and Zn, were above the threshold established by regional authorities for natural parks (Table 1), which means that intervention is compulsory. The concentration of Pb was 25-fold higher than that of the Piedras estuary but still below the threshold. Cadmium pollution was not relevant in this site. It is worth to notice that the Odiel salt marshes are a reserve of the Biosphere (Morillo et al. 2008).

Identification of rhizosphere bacteria

A total of 25 colonies with different morphology were isolated and identified by 16S rDNA sequencing (Supplementary Information Table S1). Gram staining revealed that 88 % of the cultivable bacteria isolated from the rhizosphere of *S. maritima* were gram-positive and belonged to the genera *Staphylococcus* (two species; *S. saprophyticus*, and *S. warneri*) and *Bacillus* (three species; *B. thuringiensis, B. cereus*, and *B. aryabhattai*). One of the gram-positive isolates could not be identified due to no significant homology of the 16S rDNA sequence. Only 12 % of the isolates were gram-negative, belonging to the *genera Pantoea* (two isolates) and *Salmonella* (one isolate).

Evaluation of resistance toward heavy metals and NaCl in rhizosphere bacteria

Using MTC as the pattern of evaluation, resistance toward heavy metals Cu, Zn, As, and Pb was determined (Supplementary Information Table S2). Most of the

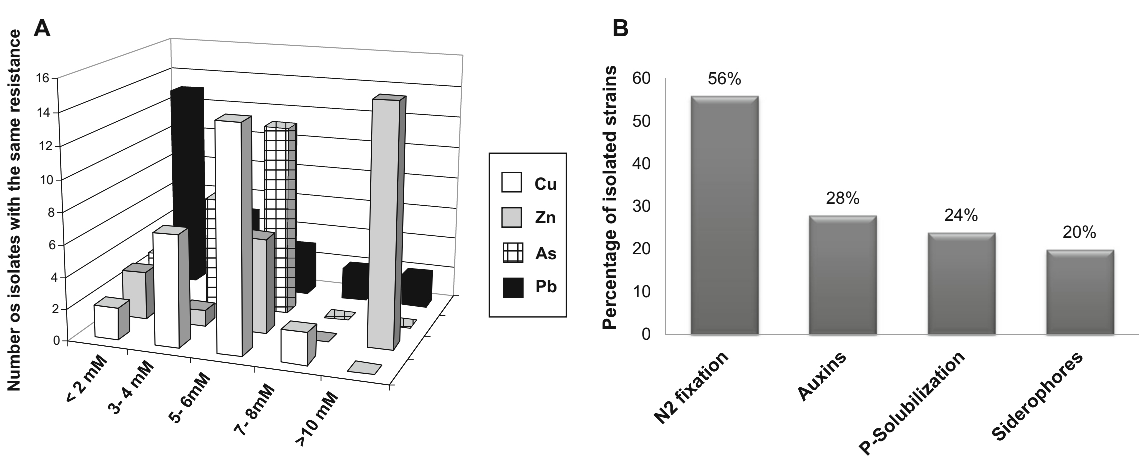


Fig. 1 Analysis of metal resistance and plant growth promoting properties in bacteria from the rhizosphere of *S. maritima*. **a** Number of isolates grouped according to Cu, Zn, As, and Pb resistance range. **b** Percentage of isolated strains with different PGP properties in the absence of heavy metals

isolates from the rhizosphere of *S. maritima* were resistant to intermediate Cu concentrations (4–6 mM), (Fig. 1). The strains RSO6, RSO7, and RSO21 were the most resistant toward Cu, reaching an MTC of up to 8 mM and no one was able to grow at the maximal concentration (10 mM). The resistance of the bacterial collection toward As showed that most of the strains (50 %) displayed an intermediate level of resistance (toward 4–6 mM); 30 % tolerated between 2 and 4 mM and no strains were able to grow over 6 mM As. Conversely, the resistance toward Zn showed a completely different profile; most of the strains (60 %) were able to grow at the maximum concentration of 10 mM Zn, with RSO5, RSO16, and RSO17 being the most resistant strains; 25 % of the bacteria showed an intermediate Zn resistance (4–6 mM), and only a small percentage (12 %) was not able to grow at the minimum concentration tested (2 mM). The analysis of the bacterial collection showed low resistance toward Pb (14 out of 25 strains only tolerated ≤2 mM), whereas only 12 % were resistant toward the highest concentrations 8–10 mM, with RSO6., RSO7, and RSO25 being the best strains (Fig. 1).

Finally, the resistance of isolates toward NaCl was determined. It was observed that the collection of bacteria growing on medium TSA-SW also did on TSA medium without additional salt. These results suggested that the bacteria were halotolerant, not halophiles, since the presence of salt was not a requirement for growth. Forty-six percent of the bacteria showed tolerance toward over 4 mM NaCl; 12 % of isolates tolerated 4 mM, and 42 % displayed resistance toward 3 mM NaCl (Supplementary Information Table S1).

Plant growth promoting abilities of resistant rhizobacteria

Since the final aim of this work was proposing an inoculant for plant growth in polluted estuaries, it was interesting to test whether the isolated bacteria displayed plant growth promoting (PGP) properties, including nitrogen fixation, phosphate solubilization, siderophores production, and auxins production. The complete information of PGP properties for all the strains is given in Supplementary Information Table S3. The most extended ability was nitrogen fixation (over 50 % of the bacteria were potential N_2 fixers), about one fourth of the isolates (28 %) produced siderophores and 24 % were able to solubilize phosphate, whereas one fifth of the bacterial collection secreted auxins.

Further characterization of gram-negative strains RSO6 and RSO7 and gram-positive strain RSO25

On the basis of both metal resistance and multiple PGP properties, three bacterial strains (RSO6, RSO7, and RSO25) were selected for further characterization. First, the taxonomic identification of these strains was done by sequencing the nearly complete 16S rDNA. The amplicon sequences corresponding to RSO6 and RSO7 had 1399 and 1456 bp, respectively. They have been deposited in GenBank under the accession numbers KU052705 (RSO6) and KU052706 (RSO7). The results indicate that the strains RSO6 and RSO7 belong to the species *Pantoea agglomerans*, with a sequence identity of more than 99 % in both cases. The sequence of the 16S rDNA from the strain RSO25 had a length of 1485 bp, and it has been deposited in database under the accession number KU052707 (RSO25). The comparison of the sequence with database indicated that it showed 100 % identity with *Bacillus aryabhattai*. The taxonomic positions of the strains are shown in Supplementary Information (Fig. S3).

The strains *P. agglomerans* RSO6 and RSO7 showed resistance toward Cu (8 mM), Zn (6 mM) and Pb (more than 10 mM), as well as to the metalloid As (2–4 mM) (Table 2). The difference in arsenic resistance (2 mM vs. 4 mM) in both *Pantoea* isolates was suggestive of different isolates of the same species. The strain *B. aryabhattai* RSO25 displayed less resistance toward heavy metals and arsenic as compared to the *Pantoea* strains. Regarding the PGP properties, all the three strains were able to fix nitrogen and to solubilize phosphate, while only the *Pantoea* strains secreted siderophores. Concerning auxins secretion, the *Bacillus* strain RSO25 produced a limited amount of IAA, whereas the *Pantoea* strains produced high levels of auxins (Table 2). Again, minor

Table 2 Values of maximal tolerable concentration (MTC) and plant growth promoting PGP properties of RSO6, RSO7, and RSO25 strains

Strain	Metal resistance MTC (mM)					PGP properties			
	Cd	Cu	Zn	As	Pb	N_2 fixation	Auxins mg/L	P-solubilization halo size (mm)	Siderophores halo size (mm)
RSO6	0.3	8	6	2	>10	+	22.02 ± 2.18	35 ± 2	15 ± 2
RSO7	0.3	8	6	4	>10	++	14.07 ± 1.67	30 ± 2	15 ± 2
RSO25	0.1	6	6	2	8	++	4.81 ± 0.53	30 ± 2	0

Data are means of three independent determinations. Standard deviations are indicated

Table 3 Percentage of increase of PGP properties in the presence of 1 mM of each of the metals Cu, Zn, As, and Pb

PGP property	Strain	Cu[a]	Zn	As	Pb
N_2 fixation	RSO6	−	−	+	*n.d.*
	RSO7	−	+	+	*n.d*
	RS25	−	−	++	*n.d*
Auxins production	RSO6	90 %	29 %	73 %	**119 %**
	RSO7	**203 %**	57 %	**168 %**	**167 %**
	RS25	**260 %**	23 %	**166 %**	**250 %**
P-solubilization	RSO6	67 %	43 %	67 %	50 %
	RSO7	60 %	40 %	53 %	0 %
	RS25	50 %	0 %	0 %	0 %
Siderophores	RSO6	**200 %**	87 %	**133 %**	100 %
	RSO7	**180 %**	80 %	100 %	0 %
	RS25	0 %	0 %	0 %	0 %

For each bacterial strain, 100 % corresponds to the level of the PGP property in the absence of any metal. Cu (0.5 mM) was used for siderophore production due to interference with the CAS medium. Numbers in bold indicate properties that increase in the presence of stress conditions

[a] Nitrogen fixation in the presence of Pb could not be determined due to the utilization of $Pb(NO_3)_2$ as the Pb source

differences were observed in some PGP properties between RSO6 and RSO7, suggesting that they probably correspond to different isolates of the same species. Besides, there were morphological differences among them, with RSO7 being much more mucous than RSO6 (not shown).

Plant growth promoting properties in the presence of heavy metals

Because of high concentrations of metals in the collection site, it was necessary to know whether the selected bacteria were able to maintain their PGP properties in the presence of metals (Cu, Zn, As, and Pb—the most relevant elements in the polluted area). Regarding nitrogen fixation, all the strains were able to fix nitrogen in the presence of 1 mM As and no one did in the presence of 1 mM Cu (Table 3 and Fig. 2). Only *P. agglomerans* RSO7 did fix nitrogen in the presence of 1 mM Zn. Nitrogen fixation in the presence of Pb could not be evaluated, since $PbNO_3$ was used as the source of Pb. Other salts of Pb could be neither used due to low solubility.

Concerning siderophores production, the highest concentration tested for Cu was 0.5 mM because higher concentrations interfered with chromium-azurol-S (data not shown). *B. aryabhattai* RSO25 was the most sensitive strain toward the presence of heavy metals, since it displayed no halo in the presence of any metal, indicative that this ability was abolished by the presence of toxic elements (Table 3). By contrast, *P. agglomerans* RSO6 was the only strain maintaining siderophores production in the presence of all four metals and even it increased the halo diameter in the presence of Cu and As (Fig. 2). On its side, *P. agglomerans* RSO7 had an unequal behavior: it lost its ability to secrete siderophores in the presence of Pb but showed a bigger halo diameter in Cu (Table 3).

With regard to phosphate solubilization, again, *B. aryabhattai* RSO25 was the strain with the highest

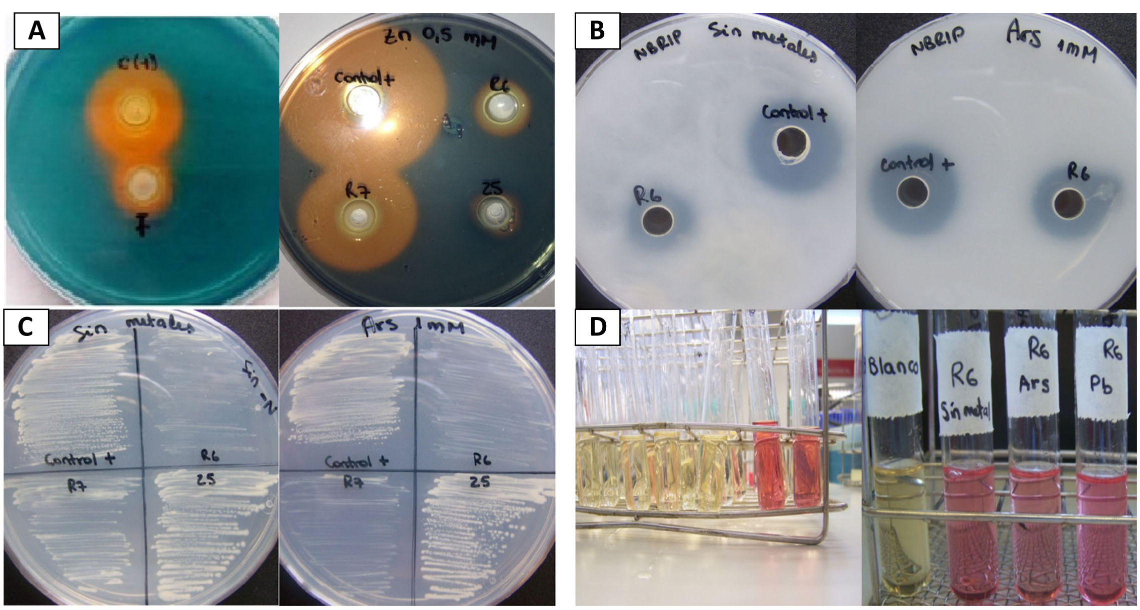


Fig. 2 Best performing strains based on their PGP properties. Siderophores in the presence and the absence of Zn (**a**). P-solubilization in the presence and the absence of As (**b**). N-fixation in the presence and the absence of As (**c**). IAA production in different selected strains in the presence of As and Pb (**d**)

sensitivity toward metals (Table 3 and Fig. 2). It lost the capacity to solubilize phosphate in the presence of As, Pb, and Zn and only maintained 50 % of phosphate solubilization ability in Cu. The *Pantoea* strains showed reduced phosphate solubilization (between 40 and 60 % depending on the metal; Table 3), with the exception of RSO7, which lost its phosphate solubilization capacity in contact with Pb.

Concerning auxins production, the results showed that Zn had a great deleterious effect on this property, since all strains drastically reduced auxins production in the presence of this element (30 %) (Table 3 and Fig. 2). Conversely, RSO25 and RSO7 were able to produce more auxins in the presence of Cu, Pb, and As than without added metal, and RSO6 showed increased auxins production in the presence of Pb. Considering all these data, it could be concluded that the *Pantoea* strains kept the best PGP properties both in the absence and in the presence of heavy metals.

Bioabsorption of heavy metals onto bacterial biomass: analysis by ICP-OES and SEM-EDX

The accumulation of heavy metals Cu, Zn, and Pb in dried biomass of RSO6, RSO7, and RSO25 strains was measured. Two treatments were performed: washing with water (in order to detect total metal accumulated inside the cells plus metal adsorbed onto the cells surface) and washing with EDTA (in order to remove metals adsorbed onto the cell surface and determine only accumulated metals) (Table 4). Clear differences were observed between the behavior of gram-negative RSO6 and RSO7, and the gram-positive RSO25. The *Pantoea* strains accumulated most of Cu and Zn adsorbed onto the bacterial surface (between 79 and 98 %). By contrast, Pb was equally distributed inside and outside the cell of gram-negative strains. The strain RSO25 accumulated, in general, much less metals than the gram-negative bacteria. Moreover, while most of Cu and Pb were adsorbed onto the bacterial surface (75–90 %), most of Zn (80 %) was accumulated inside the cell.

Since the accumulation of Pb reached the highest values in the bacterial pellets of *P. agglomerans* RSO7, a more detailed analysis was done by using scanning electron microscopy coupled to an X-ray detection probe (SEM-EDX) in order to compare the cellular morphology of the strain in the presence and absence of metal and also in order to observe the distribution of this element in the bacterial pellet at the microscopy level (Fig. 3). Our results showed that there was no apparent effect on the morphology of the cells by the presence of Pb (Fig. 3a, b). Moreover, the accumulation of Pb in the pellet was not uniform; by contrast, there were big white zones (corresponding to high electron density), in which the metal was accumulated and others with little metal content (Fig. 3c). A semiquantitative determination of Pb was done by using the EDX probe. The results indicated that, in white zones, the percentage of Pb could reach up to 37 % of dry weight, equivalent to almost 10 % of the total number of atoms (Fig. 3d).

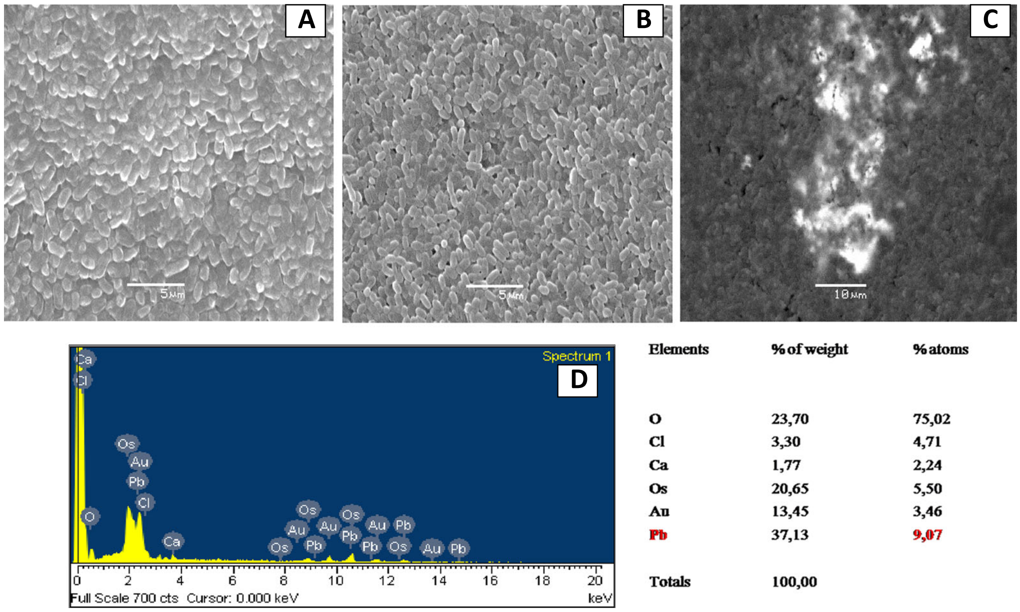
Capacity for biofilms formation

The capacity for biofilms formation was evaluated on microtiter plates (Fig. 4). The strain *B. aryabhattai* RSO25 was not able to develop a biofilm on the surface of the wells, whereas the gram-negative strains of *Pantoea* did. Significant differences were found between both *Pantoea* strains, with RSO7 being the best strain concerning the ability for biofilms formation in vitro.

Table 4 Accumulation of metals in bacterial pellets

Strain	Metal	Biosorption (μg/g)		
		Wash with water	Wash with EDTA	% of metal adsorbed onto the cell surface
RSO6	Cu	3478.43 ± 219.27	179.38 ± 38.05	94.8
	Zn	11,256.76 ± 625.29	149.58 ± 33.02	98.7
	Pb	6252.14 ± 424.56	2636.77 ± 186.48	57.8
RSO7	Cu	1754.40 ± 152.82	364.37 ± 76.29	79.2
	Zn	9533.55 ± 751.72	148.26 ± 29.44	98.4
	Pb	26,154.59 ± 1707.71	13,636.63 ± 814.07	47.9
RSO25	Cu	2921.85 ± 149.57	745.33 ± 55.59	74.5
	Zn	663.49 ± 72.24	544.68 ± 69.01	19.7
	Pb	5094.47 ± 405.68	460.16 ± 53.88	90.9

Biosorption of Cu, Zn, As, and Pb in cells of RSO6, RSO7, and RSO25 strains incubated in the presence of 2 mM Cu, 2 mM Zn, or 2 mM Pb and washed either with water or with 0.2 M EDTA. Data are means ± SD of three independent determinations



Elements	% of weight	% atoms
O	23,70	75,02
Cl	3,30	4,71
Ca	1,77	2,24
Os	20,65	5,50
Au	13,45	3,46
Pb	37,13	9,07
Totals	100,00	

Fig. 3 Analysis of Pb biosorption in RSO7 by SEM-EDX. **a**, **b** Scanning electron microscopy (SEM), showing the morphological aspect of RSO7 cells in the absence (**a**) or the presence (**b**) of Pb. **c** SEM-EDX of RSO7 corresponding to **b** showing areas with high metal content. **d** Spectrum data of the white area as shown in **c**

Discussion

The Odiel salt marshes are affected by an environmental problem of pollution due to the occurrence of several sources of metals, as a consequence of both natural geochemical processes and anthropic origin (Morillo et al. 2008). Our data also confirm the contamination of the Odiel estuary by metals; the concentrations of several of them were found relevant to the threshold established for natural parks by regional regulations. In particular, three elements (As, Cu, and Zn) are the main contaminants of this estuary.

The *genus Spartina* is composed of C4, perennial grasses in the family *Poaceae*. Most species are salt tolerant and colonize coastal or inland salt marshes. Special emphasis is placed on some hyperaccumulator species, such as *S. alterniflora* and *S. maritima*, envisioning their potential use in phytoremediation due to their high metal accumulation in aboveground tissues (1000 mg g^{-1}), while yielding a large plant biomass (Caçador et al. 2009; Redondo-Gómez 2013). By contrast, *S. densiflora* immobilizes a higher concentration of metal in soil, preventing the translocation from rhizosediment to roots (Cambrollé et al. 2008).

The use of microbes in phytoremediation approaches might exert a positive influence, by lowering the toxicity of metals to the plant or aiding plant growth, by increasing the bioavailability of metals to achieve better uptake, and by minimizing the risk for ground and surface waters (Phieler et al. 2013). Soil microbes may substitute the effects otherwise provided by the application of chemical amendments to enhance the solubility and availability of metals in the soil (Salt et al. 1995). In the case of *Spartina*, there are reports that show the importance of rhizosphere bacteria for phytoremediation assays of both organic pollutants and metals (Hong et al. 2015; Reboreda and Caçador 2008; Su et al. 2016). Several works suggest that halophyte–microorganisms interactions could be potentially useful to accelerate phytoremediation of salt marshes by enhancing plant growth and/or increasing plant metal accumulation (Andrades-Moreno et al. 2014; Mesa et al. 2015a, b, c). The disadvantage of this technique is the enhanced transfer of metals to the aboveground plant tissues, so halophytes could be involved not only in metal

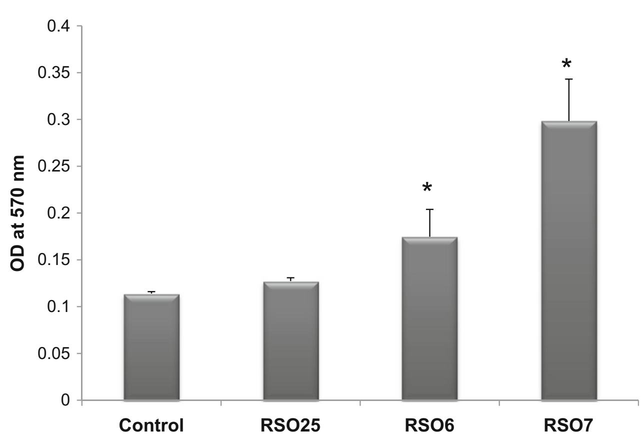


Fig. 4 Evaluation of biofilm formation on a microtiter plate assay. Bacterial strains were grown in the presence of TSB in the wells of a 96-well microtiter plate for 4 days at 28 °C. After this time, the formation of biofilms on the surface of the plate was evaluated by a colorimetric determination. Values are means ± SE (n = 3)

cycling in coastal systems (Caçador et al. 2009; Reboreda and Caçador 2008) but also in being a source of metals in non-polluted estuaries (Couto et al. 2013).

In this work, the cultivable bacteria present in the rhizosphere of *S. maritima* have been isolated and characterized. Our results indicate that gram-positive bacteria are the predominant microbial community in the rhizosphere of *S. maritima*, specifically the *genera Bacillus* and *Staphylococcus*, which are well known as salt tolerant (Parfentjev and Catelli 1964; Sharma et al. 2015). The environment associated to tidal cycles favor the growth of gram-positive bacteria, which are more resistant toward desiccation (Potts 1994; Billi and Potts 2002). Concerning gram-negative bacteria, only the genera *Pantoea* and *Pseudomonas* were found. All the *genera* isolated are commonly found in the rhizosphere and as endophytes of plants (Lugtenberg et al. 2001; Santoyo et al. 2012; Sergeeva et al. 2007; Del Castillo et al. 2015; Mesa et al. 2015a; b).

Our data show a high level of resistance toward Cu, Zn, As, and Pb in the bacterial collection. The overall behavior was different depending on the metals; while the overall resistance toward Zn was very high (over 50 % of the strains displayed resistance toward 10 mM Zn), the average resistance toward Pb was low (<2 mM), and the overall resistance toward As and Cu was intermediate (4–6 mM). Three strains were selected due to multiple resistances, i.e., the two *Pantoea* strains RSO6 and RSO7 and the gram-positive strain *B. aryabhattai* RSO25. The strains also displayed a high capacity for metal bioaccumulation. Differences were observed again between gram-positive and gram-negative strains. In the latest ones, 70–90 % of Zn and Cu were adsorbed onto the cell surface, whereas Pb was equally distributed inside and outside the cell. Moreover, up to 26,000 μg Pb per mg dry biomass was accumulated in the strain RSO7, with equal distribution inside and outside the cell, as determined by SEM-EDX. In the gram-positive RSO25, the metal biosorption capacity was much reduced and percentage of Zn adsorbed onto the cell surface lowered down to 20 %, being most part of this metal accumulated inside the cell. Conversely, Pb was mainly accumulated outside the cell. It is known that differences in the cell walls of organisms (gram-positive and gram-negative bacteria, algae, fungi, cyanobacteria, actinomycetes, etc.) lead to different biosorption properties (Abbas et al. 2014).

When selecting a bacterial inoculant for plants, it is important to check out that the bacteria have plant growth promoting (PGP) properties, and which is more important, that they are retained under stress conditions. The presence of PGP properties in the selected strains was demonstrated. Some of these properties are related to the acquisition of nutrients. Nitrogen and phosphate are the two most important macronutrients in agricultural ecosystems. Bacteria of the *genera Pantoea* and *Bacillus* are reported to fix atmospheric N_2 and make it accessible to plants (Bhattacharyya and Jha 2012). This property seemed not to be affected by As, but conversely, it was very sensitive toward Zn, since no one of the strains, but RSO7, was able to fix nitrogen in the presence of this element.

After nitrogen, phosphorus is an essential plant nutrient. Low levels of this element cause deficiencies that severely restrict crop yield. Phosphate is often found in soil in insoluble forms; therefore, the use of microbial inoculants represents a friendly alternative to chemical fertilizers (Burd et al. 2000). In the case of the *genus Pantoea*, there are many species with the ability to solubilize phosphates by mechanisms that include either the ability to produce and excrete gluconic acid or by reducing the pH of the medium upon secretion of large amount of organic acids (Castagno et al. 2011; Park et al. 2011). In our work, in spite of the three strains were able to solubilize phosphate in the absence of metals, only the strain RSO6 retained this ability in presence of all the metals. RSO7 maintained this capacity—except in Pb—and the strain RSO25 only in Cu.

In general, iron is a scarce element and it is difficult to assimilate by plants and microorganisms. In spite that Fe seems not to be a limiting element in our soil sediments, according to total concentration (Morillo et al. 2008; Sáinz and Ruiz 2006), the bioavailability of this metal could be limited. Many PGPR are able to produce iron chelators with a high Fe^{3+} affinity known as siderophores. This ability helps the plants to acquire enough iron in the presence of staggering amounts of other toxic metals, which has implications not only in plant nutrition but also in the control of phytopathogens (Burd et al. 2000). Another possible functions of siderophores is to act as elicitors of induced systemic resistance (ISR) through molecules as cell wall lipopolysaccharides (LPS), bacterial flagellins, antibiotics, or volatile compounds, such as 2,3-butanediol (Bakker et al. 2007). In our case, only the *Pantoea* strains were able to produce siderophores both in the absence and the presence of metals.

The production of phytohormones, especially auxins, is an important and well-studied mechanism that contributes to plant growth by stimulation of root growth, which also provides resistance to biotic or abiotic stress and helps seed germination (Lugtenberg and Kamilova 2009). Most auxins in the rhizosphere of a plant come from the conversion of tryptophan secreted by plant roots in IAA by rhizobacteria (Spaepen and Vanderleyden 2011). Studies by Del Castillo et al. (2015) showed 26 isolates of the *genus Pantoea* with high capacity to produce auxins. In our results, all the strains showed production of IAA without metals, particularly the *Pantoea* strains. In general terms, PGP properties in the selected strains were similar to those of bacteria isolated from the rhizosphere of *S. densiflora* and *S. maritima* grown on contaminated estuaries (Andrades-Moreno et al. 2014; Mesa et al. 2015a). The increase of ten times of auxin production in the presence of heavy metals is of outstanding interest, since large root systems are a condition for efficient phytoremediation (Gamalero et al. 2009; Sergeeva et al. 2007).

Finally, the ability to form biofilms has been also tested. Biofilm formation is a strategy used by microorganisms in order to survive toxic metals by the combined action of chemical, physical, and physiological mechanisms linked to cellular diversification within the biofilm (Harrison et al. 2007; Das et al. 2012). Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) for different metals have been reported to increase up to 100-fold for biofilms cells with regard to planktonic cells. In this particular, it is hypothesized that biofilms have the capacity to protect the cellular targets of metal-ion toxicity, shielding cells from oxidative stress involved in planktonic cell killing (Harrison et al. 2007). In our case, the strain RSO25 could not form biofilms in our conditions, whereas a gradation was observed for strains RSO6 and RSO7, being the last one the best concerning this particular ability. Noticeably, this strain was also the one producing the greatest amount of polysaccharide. This characteristic, together with the good biosorption properties of *Pantoea* strains, could suggest a possible application in metal immobilization/phytostabilization in the rhizosphere of *S. maritima*, taking into account that an effect of the bacterial rhizosphere populations in metal cycling and speciation has been reported (Duarte et al. 2009; Reboreda and Caçador 2008).

Considering the results obtained, we can conclude that, in spite of the greatest number of isolates belong to the group of gram-positive bacteria, the two isolates of *P. agglomerans* RSO6 and RSO7 exhibited the best results for resistance to and bioaccumulation of heavy metals, for PGPR properties and for the capacity to form biofilms. These abilities make them very interesting as inoculants for phytoremediation processes. At present, greenhouse experiments are being developed in order to test their effect on *Spartina* plants grown in polluted sediments of the Odiel estuary.

Acknowledgments This work has been financed by Junta de Andalucía (Proyecto de Excelencia P11-RNM-7274). K. Paredes-Páliz has been supported by a pre-doctoral grant (SENESCYT, Ecuador). The students Rafael Gil-Baena, Yoely Lantigua, and Reyes Serrano are acknowledged for their collaboration in the isolation and preliminary characterization of the strains. Thanks are given to the Microscopy Service of the CITIUS (University of Sevilla, Spain) and particularly to Cristina Vaquero for her help in the preparation and analysis of samples by SEM-EDX.

Conflict of interest The authors declare that they have no conflict of interest.

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