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Scouting contaminated estuaries: Heavy metal resistant and plant growth promoting rhizobacteria in the native metal rhizoaccumulator *Spartina maritima*

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Spartina maritima is a native endangered heavy metal rhizoaccumulator cordgrass naturally growing in southwest coasts of Spain, where is used as a biotool to rehabilitate degraded salt marshes. Fifteen bacterial strains were isolated from the rhizosphere of *S. maritima* growing in the estuary of the Tinto River, one of the most polluted areas in the world. A high proportion of bacteria were resistant towards several heavy metals. They also exhibited multiple plant growth promoting (PGP) properties, in the absence and the presence of Cu. *Bacillus methylotrophicus* SMT38, *Bacillus aryabhattai* SMT48, *B. aryabhattai* SMT50 and *Bacillus licheniformis* SMT51 were selected as the best performing strains. In a gnobiotic assay, inoculation of *Medicago sativa* seeds with the selected isolates induced higher root elongation. The inoculation of *S. maritima* with these indigenous metal-resistant PGP rhizobacteria could be an efficient method to increase plant adaptation and growth in contaminated estuaries during restoration programs.

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1. Introduction

Heavy metal pollution is a major environmental problem that is rapidly gaining importance due to its impact on human health through the food chain. Industrial and agricultural activities, such as mining and smelting of metalliferous ores, waste water irrigation and chemical fertilizers and pesticides abuse, are increasing heavy metal pollution of the environment (USEPA; WHO). The Rio Tinto system in the province of Huelva, south western Spain, has great historical significance as well as environmental interest. The joint estuary of the Tinto and Odiel rivers is one of the most polluted areas in the world, with high concentrations of heavy metals in the sediments (up to 697 ppm of As; 3000 ppm of Cu, 3570 ppm of Pb and 4800 ppm of Zn) (Nelson and Lamothe, 1993; Ruiz, 2001; Sáinz et al., 2002, 2004), and most likely has been so for thousands of years (Davis et al., 2000). The Tinto and Odiel rivers drain the region of the world's oldest continuously operating mine (Wilson et al., 1981), as it is in the headwaters of Tinto River that mining supporting the Copper Age and Bronze Age took place. The other and recent aspect of the potential pollution of this fluvial-estuarine system is the industrialisation of the Huelva area beginning in 1967 (Davis et al., 2000).

Spartina maritima (Curtis) Fernald, in the family *Poaceae*, is an indigenous cordgrass that naturally grows in the estuary of Tinto and Odiel rivers. This species has a wide distribution along the European and North-African Atlantic coasts. The native *S. maritima* species play an important ecological role in the salt marsh dynamics by protecting the coastline from erosion and modifying the physical structure of intertidal coastal zones. *S. maritima* tolerates a wide range of salinity, tidal submergence, drainage, and anthropogenic pollutants (Mateos-Naranjo et al., 2007, 2010). Furthermore, heavy metals accumulation at different rates in *S. maritima* tissues and rhizosediment allowed concluding that this species could be used for phytostabilisation of estuarine sediments (Cambrollé et al., 2008; Redondo-Gómez, 2013). *S. maritima* is also potentially useful for biomonitoring coastal systems where this plant is abundant (Padinha et al., 2000).

The estuarine sediments constitute complex and interesting ecosystems that can be strongly influenced by plant activity (Almeida et al., 2006). Noninfecting rhizospheric microorganisms (especially bacteria) colonise the roots of plants from the rhizoplane to the apoplasm and cortex. Such microorganisms have a marked contribution to plant growth and physiology, helping plants to tolerate abiotic stress (Yang et al., 2008). In soils with high heavy metal content, such rhizospheric bacterial populations are known to play a crucial beneficial role in plant responses to heavy metal stress (Gamalero et al., 2009). Plant

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growth-promoting rhizobacteria (PGPR) can enhance plant growth by a wide variety of mechanisms, including phosphate solubilisation, siderophores production, biological nitrogen fixation, phytohormone production, production of 1-aminocyclopropane-1-carboxylate deaminase, exhibiting antifungal activity, etc. (Ahemad and Khan, 2011; Bhattacharyya and Jha, 2012). Metal phytoremediation, as well as plant growth, is often facilitated by soil microorganisms living in intimate association with plant roots (Shilev et al., 2001). This contribution of the rhizomicrobial population to phytoremediation is usually referred to as rhizoremediation (Kuiper et al., 2004). A huge amount of reports have described the facilitation of metal phytoextraction through the inoculation of PGPRs. In those reports, bacteria were able to increase plant growth and then, when lower and less toxic levels of metals are present, to increase the amount of metal taken up by the plant (Glick, 2010). When metal phytostabilisation was aimed, bacteria were able to promote plant growth and reduce either plant metal uptake or its translocation to the shoot, thus preventing the impact of metals in plant development and their entrance into the food chain. Thus, several works have reported that phytostabilisation of metal-polluted soils can be positively affected by the inoculation with PGPRs (Glick, 2010; Dary et al., 2010; El Aafi et al., 2012).

Studies of rhizospheric bacteria and their importance for plant development in contaminated soils are an active area of research; however, little is known about microorganisms colonising the rhizosphere of estuarine plants. In particular, *S. maritima* bacterial rhizospherehas never been systematically characterised previously. Thus, the aim of this study was to isolate and characterise rhizospheric bacteria from *S. maritima* growing in a contaminated estuary and study their heavy metal resistance (at different salt concentrations) and traits associated with plant growth promotion (in the absence and presence of metal contamination), as a purpose to use them to ameliorate phytostabilisation of such ecosystems.

2. Methods

2.1. Soil sampling and chemical analysis

Three plant samples of *S. maritima* were harvested together with rhizospheric soil (20 cm depth) in May 2013 from the Tinto River estuary (37°13′N, 6°53′W). Also, sediment cores (10 cm diameter) were taken in areas where vegetation was absent (with 0.5 m spacing from the plant), in order to be analysed. The samples were placed in individual plastic bags, transported to the laboratory and stored at 4 °C until processing. Rhizobacteria were isolated within 24 h. For chemical analysis of the sediment and rhizosediment, samples were dried at 80 °C for 48 h, ground and homogenised by sieving through nylon nets of 2 mm in order to remove large stones and dead material (Redondo-Gómez et al., 2007). Then, samples were acid-digested with concentrated HNO_3 for 15 min at room temperature and placed on a hot plate, until dry. The cool residue was extracted with HF and H_2O_2. Concentration of elements was measured by inductively coupled plasma (ICP-AES) spectroscopy (ARLFisons3410, USA). Finally, the conductivity of the sediments was measured in order to elucidate its salinity. It was determined in the laboratory with a conductivity meter (Crison-522, Spain) after diluting the sediments with distilled water (1:1).

2.2. Isolation of cultivable bacteria from S. maritima rhizosphere

5 g Of fresh roots with rhizospheric soil were washed with 50 ml of sterile distilled water in a sterile Falcon tube, by shaking for 15 min using a vortex mixer. 100 μL of the suspension were plated ten times onto tryptic soy agar (TSA) medium Thompson et al., 1993 and modified TSA medium containing NaCl 0.6 M (approximately seawater salt concentration), as this estuary is located near the mouth of the river and the presence of halophilic bacteria was not dismissed. Following the incubation for 72 h at 28 °C, single colonies were separated according to both morphology type of the colony and microscopic observations of the bacteria (form and motility), and then subsequently re-isolated by plating on TSA and modified TSA NaCl 0.6 M and incubated at 28 °C for 48 h in order to obtain pure cultures. Purified bacterial cultures were preserved in 15% glycerol at −80 °C for further use. TSA NaCl 0.6 M was prepared by replacing the correct amount of distilled water by SW30 solution (per litre, 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, H_2O e.q. to 1 L) when preparing TSA medium before autoclaving at 121 °C during 20 min.

2.3. Genetic diversity by BOX-PCR

Genomic DNA was extracted using the i-genomic BYF and G-spin™ Total DNA extraction kits (Intron Biotechnology Ltd., Korea) according to the manufacturer' instructions. BOX-PCR was performed using the BOX A1R primer (5′-CTA CGG CAA GGC GAC GCT GAC G-3′). The template DNA concentration for PCR reaction was 40 ng and the PCR steps were programmed as follows: enzyme activation at 94 °C for 5 min, 35 cycles of denaturation at 94 °C for 1 min, annealing at 52 °C for 1 min, extension at 72 °C for 1 min and final extension at 72 °C for 10 min. The PCR products were electrophoresed through a 1.5% agarose gel stained with etidium bromide in TAE buffer, at 75 V for 2 h. Gel was visualised under UV radiation and photographed. Images were analysed using Phoretix 1D® Software (TotalLab, UK) by constructing dendograms and BOX-PCR fingerprint similarities were determined by calculating the Pearson's product moment correlation coefficient (Jobson, 1991).

2.4. Identification of cultivable bacteria

Bacteria were identified by PCR amplification and sequencing of the 16S rDNA. Primers and PCR conditions used are described in Rivas et al. (2002). To determine homologies with DNA sequences from other previous described organisms, EzTaxon server was used (Chun et al., 2007). For each bacterial isolate, the corrected 16S rDNA sequences were deposited in GenBank under the accession numbers (from KF962965 to KF962979) presented in Table 2.

2.5. Resistance against NaCl and heavy metals

The resistance of isolated bacteria to different heavy metals and sodium arsenite was determined on plates containing TSA medium and modified TSA 0.2 M NaCl medium (according to soil conductivity), both supplemented with increasing concentrations of heavy metals from stock solutions: 0.5M $NaAsO_2$, 1 M $CdCl_2$, $CuSO_4$ 1 M, 1 M $CoCl_2$, 0.2 M $NiCl_2$, 0.5 M $Pb(NO_3)_2$ (in order to avoid Pb precipitation when mixing with TSA, the same concentration of EDTA needs to be added to the plates) and 1M$ZnSO_4$. Likewise, bacterial growth in the presence of NaCl was evaluated on TSA medium supplemented with increasing concentrations of NaCl, by adding SW30 solution. The resistance was expressed as the maximum tolerable concentration (MTC) for each element, namely the maximum concentration of a metal or metalloid not affecting bacterial growth.

2.6. Screening for plant growth promoting traits

2.6.1. Nitrogen fixation, phosphate solubilisation, production of siderophores and indole-3-acetic acid (IAA) production

These PGP properties were studied as described by Andrades-Moreno et al. (2014). Briefly, nitrogen fixation was determined

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