New isolation method of desiccation-tolerant microorganisms for the bioremediation of arid and semiarid soils

M. Manzanera[1], J. J. Narváez-Reinaldo[1,2], L. SantaCruz-Calvo[1], J. I. Vílchez[1,2], J. González-López[1,2] & C. Calvo[1,2]
[1]*Institute of Water Research, University of Granada, Spain*
[2]*Department of Microbiology, University of Granada, Spain*

Abstract

In arid and semiarid regions, the establishment of microorganisms and plants for the bioremediation of polycyclic aromatic hydrocarbons (PAHs) is further impeded by a number of physicochemical factors including low precipitation, high evaporation, high winds and extreme temperatures. These factors contribute to the extremely low water content in soil and reduce the survival of most of the microbial isolates with a role in the bioremediation of soils. We have developed a new technology based on the ability of anhydrobiotic microorganisms to withstand desiccation and the remarkable stability they display in a dried state. Because of their ability to survive without water and to promote plant growth under water stressing conditions, we have used this tolerance for the isolation of a collection of new desiccation-tolerant microorganisms that could be useful for the treatment of PAH-polluted soils in arid and semiarid regions.

Keywords: anhydrobiosis, polycyclic aromatic hydrocarbons, arid and semiarid soils, bioremediation, PGPR.

1 Introduction

Rhizoremediation combines the use of plants and microorganisms for the removal of pollutants. In this process, the microbes present in the rhizosphere of plants used during the bioremediation process provide an important contribution to the degradation of such pollutants [1, 2]. This technique has been extensively used for the treatment of soils polluted with polycyclic aromatic hydrocarbons

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(PAHs). This type of phytoremediation results from the higher densities and greater activities of microorganisms close to the plant's roots than in the surrounding soil [3, 4]. The combination of plants and microorganisms enhances the efficiency of contaminant removal thanks to the effect of root growth and its subsequent penetration through the different substrates and layers of soil. This allows the removal of entrapped contaminants that might have previously been inaccessible [5].

PAHs are recalcitrant pollutants often found in high residual concentrations in soils of industrial sites. This is a group of compounds of great environmental concern because some are mutagenic and carcinogenic [6]. PAHs are mainly introduced into the environment by emissions from human activities including power plants, industrial boilers, incinerators, ships, aircraft, automobiles and commercial and industrial heating systems [6]. Natural combustion such as forest fires and volcanic eruptions is another significant source of PAHs in soils [7, 8] Therefore, the soils of arid regions such as the Arabic peninsula or semiarid regions such as the Mediterranean basin are especially affected by PAH contamination and a lack of water [9].

Stressful conditions in arid and semiarid soils reduce microbial community diversity, mainly by limiting the horizontal migration of bacteria from the rhizosphere environment to the interspace because of the low moisture content and rapid water drainage in these types of soils [10].

There have been several studies of the most suitable plant species for the rhizoremediation of PAHs [11–13]. However, the presence and survival of beneficial rhizobacterial strains is very limited. The success of these beneficial processes is based on the rhizosphere competence of the microbes [14, 15], which is reflected by the ability of the microbes to survive in the rhizosphere, compete for the exudate nutrients, sustain in sufficient numbers and efficiently colonise the growing root system [14]. In our research group, we have developed a method for coating seeds with bacteria, a technique often used to apply beneficial microbes in a bioinoculant [16–19]. This approach is most successful when the bioinoculant is well adapted to the rhizosphere [20]; therefore, the isolation of well-adapted microorganisms to dry environments is of paramount importance for the successful rhizoremediation of arid and semiarid soils. In this work, we have developed a method for the isolation of desiccation-tolerant rhizobacteria and have shown their benefits as plant growth promoting rhizobacteria (PGPR) and their effect and survival under drought stress conditions.

2 Materials and methods

2.1 Soil samples

Soil samples were taken from the *Nerium oleander* rhizosphere subjected to seasonal drought at Granada (Spain) (37.182 N, 3.624 W) after a period of three months with no registered rainfall or any type of exposition to water. The soil

sample was collected in a plastic bag, air-dried at room temperature, homogenised and sieved (2 mm mesh).

2.2 Microorganism, medium and culture conditions

Bacteria were grown in M9 minimal medium with PAHs as the sole carbon source at 30°C [21]. When PAHs were used, chips of naphthalene, anthracene, phenanthrene or pyrene were placed on the lid to avoid direct contact with cells; therefore, they were provided in vapour form.

2.3 Isolation of bacteria using a standard isolation method

One gram of air-dried soil was mixed with 10 ml sterilised water and thoroughly mixed. After the soil particles settled, serial dilutions were made and a 100 μl aliquot from each dilution were plated on M9 minimal medium plates. After 48 hours of incubation at 30°C, individual colonies were randomly picked and streaked out to obtain pure cultures. After the incubation time, the mixtures were transferred onto sterile glass plates and incubated in sterile conditions for 30 minutes to allow for the complete evaporation of water. The soil was mixed with water and diluted as described above. These tests were performed in triplicate. Alternatively, minimal medium with naphthalene, anthracene, phenanthrene or pyrene as the sole carbon source was used for the isolation of strains with biodegradative properties.

2.4 Sequencing of 16S rRNA genes and phylogenetic analysis

All strains isolated in this study were identified by the analysis of the partial sequence of the gene encoding 16S rRNA. Primers fD1, fD2, rD1 and rD2 [22] were synthesised by Sigma Genosis (UK) and used to amplify almost the full length of the 16S rRNA gene. Total DNA was isolated following the Kado and Liu method [23]. The PCR products were purified and sequenced as described previously by Pozo et al. [24]. The sequences were edited using 4Peaks software (http://mekentosj.com/4peaks/). Closely matched sequences were found in the GenBank database using the BLASTn algorithm [25].

2.5 Air drying: determination of survival rates

To determine survival rates, a colony of each pure culture containing 10^7–10^9 cells was resuspended in 1 ml of M9 minimal medium. Aliquot volumes (100 μl) were placed on sterile Petri dishes and dried under a current of sterile air for 24 hours. Cells were resuspended in 1 ml of sterile water, and serial dilutions of the cell prior and after drying were plated on TSA plates. All manipulations were performed at ambient temperature. The survival rate (%) was calculated as the rate of cells/ml after drying with reference to cells/ml before drying. The assays were performed in triplicate.

2.6 Sporulation test

To determine if the isolates were sporulant strains, a four-day-old colony was resuspended in 1 ml of sterile deionised water and incubated at 72°C for 30 min as described previously [17]. Aliquot volumes (100 µl) were plated before and after incubation. Strains able to grow in both conditions were discarded as sporulant or thermotolerant. Therefore, only temperature-sensitive strains were selected.

2.7 Plant growth promoting tests and plant protection against desiccation

Plants material and growth conditions consisted of a variation of the protocol established by Mayak et al. [26]. In this way, pepper (*Capsicum annuum* L. *cv.* Maor) seedlings were started from seeds that were sown in plastic trays in wet vermiculite. After one week, uniform-sized seedlings (shoot height of approximately 3 cm) were selected and planted in vermiculite, one per 7 cm diameter plastic pot. During the second week, the seedlings were fertilised once with 40 ml of either 1/10 or 1/5 Murashige and Skoog (MS) medium [27] as indicated. Three days after fertilisation, some of the seedlings were treated with 40 ml of bacterial suspension (A_{600nm} = 1.0), whereas others were watered with deionised water. Two weeks later, the seedlings were transplanted and watered. The seedlings were maintained in a growth chamber at a day/night temperature of 25/20°C with 25 µmol photons m^{-2} s^{-1} or 75 mol photons m^{-2} s^{-1} of light supplied for 12 h during the daytime.

For fresh (FW) and dry (DW) weight measurement, pepper plants were measured at 7, 14, 20 and 33 days after watering ceased. The relative water content (RWC) in pepper plants was also determined at the same times. The fully turgid weight (FTW), defined as the weight of the shoot after the plant had been held in 100% humidity conditions in the dark at 4°C for 48 h, of each plant was recorded. The RWC was calculated as follows:

$$RWC = \frac{FW - DW}{FTW - DW}$$

2.8 Statistical analysis

Data were analysed by analysis of variance (ANOVA), and pairwise comparisons were done using a student's *t* test. All hypotheses were tested at the 95% confidence level.

3 Results and discussion

The main objective of this study was the isolation of a collection of desiccation-tolerant microorganisms to study their effect on plants subjected to drought stress and their potential use for the removal of PAHs in rhizoremediation treatments.

3.1 Isolation of desiccation-tolerant microorganisms

For the isolation of desiccation-tolerant microorganisms a novel technique was developed by Narvaez-Reinaldo et al. [28] based on the differential survival of anhydrobionts in the desiccated state [17, 18]. Three different field samples of dry soil were collected from non-irrigated soils after three months without rain in Granada (South of Spain). In this area, there are two dry seasons, one during winter and another much drier season during summer. It was assumed that some of the environmental samples collected would be a good source of natural desiccation-tolerant microorganisms. Collections were made depending on the soil origin, including bulk soil, rhizoplane and rhizosphere. Soil samples were extensively dried as described in Materials and Methods. After the complete evaporation of water and suspension of the soil sample, serial dilutions were plated onto PAHs minimal medium plates. More than 36 strains from each plate were tested for their tolerance to withstand high temperature (as described in Materials and Methods), rendering 420 strains. Quick sporulation tests were performed for the selection of non-sporulating strains.

Because our main objective was to select desiccation-tolerant microorganisms, a series of tests to determine desiccation tolerance were performed to select 13 strains with a remarkable tolerance to drought and a survival of 4% or above to complete desiccation for 48 hours. Figure 1 shows the results of the desiccation tolerance tests.

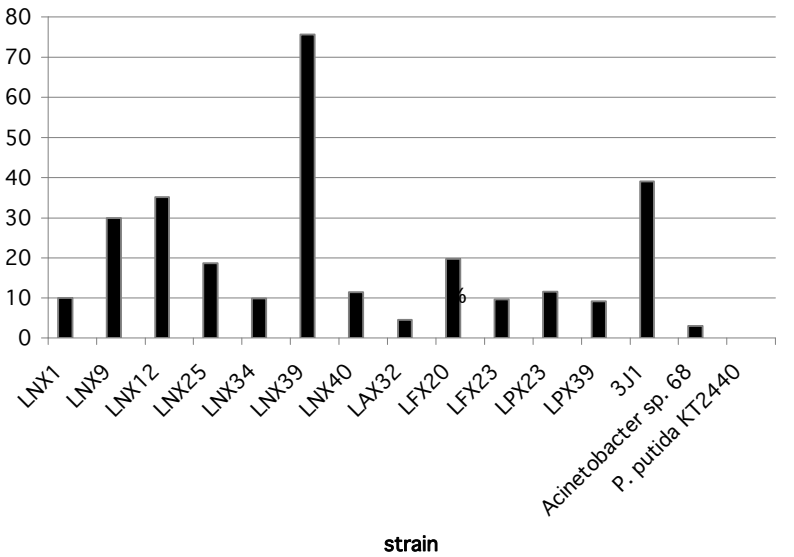

Figure 1: Desiccation tolerance of the different isolates. Survival rate, in percentage, is shown the y-axes. Name of the isolates is shown in the x-axes.

The molecular characterisation of the 14 different isolates based on the 16S rDNA homology of a partial sequence (1,440 bp) with the sequences in GenBank, the nucleotide sequencing of amplified 16S rDNA fragments obtained after colony PCR and the comparative analysis with the DNA databases allowed us to identify the desiccation-tolerant strains as members of the *Actinobacteria* group.

3.2 Plant growth promoting effect

A second desirable characteristic of this collection should be their ability to positively interact with a plant's roots. Some bacteria produce substances that stimulate the growth of plants through the production of phytohormones (i.e. auxins, gibberellins or cytokinins), ion uptake processes (such as iron that has been sequestered by bacterial siderophores or soluble phosphate), nitrogen fixation or the synthesis of plant development modulators such as the enzyme ACC deaminase, which can lower plant ethylene levels [29, 30].

We focused on selecting microorganisms promoting plant growth, in particular those protecting plants from desiccation. We tested the 13 desiccation-tolerant strains for their potential for growth promoting the three isolates that showed the highest level of desiccation tolerance: *Arthrobacter* sp., *Microbacterium* sp. and *Rhodococcus* sp. As a positive control, *Pseudomonas putida* KT2440, a saprophytic bacterium used as a model system for studying the biodegradation and interactions of a nonsymbiotic microorganism with plants,

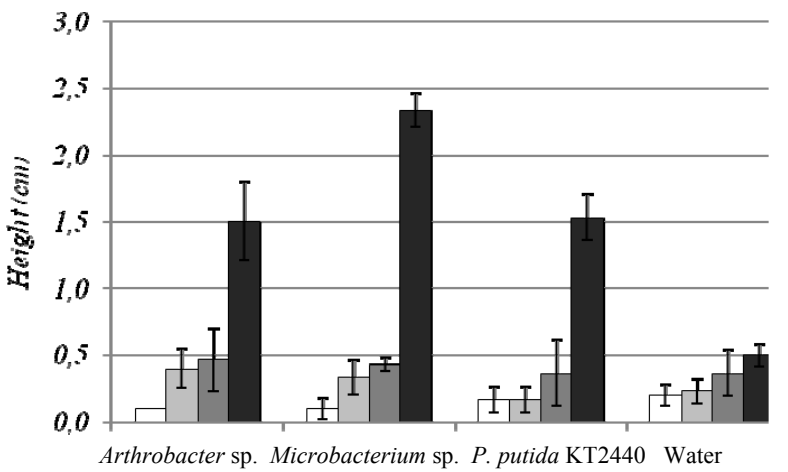

Figure 2: Stem size of pepper plants inoculated with the isolates *Arthrobacter* sp. and *Microbacterium* sp. Water was included as negative control and *P. putida* KT2440 as positive control. Measures were taken at 7 (white bars), 14 (light grey bars), 20 (dark grey bars) and 33 days (black bars).

was included [31, 32]. As a negative control, an equal volume of water was added instead. Figure 2 shows the effect of these three microorganisms on pepper plant growth. The addition of *Microbacterium* sp., *Arthrobacter* sp., and *P. putida* KT2440 produced an increase in plant growth determined by longer roots and stems and higher dry and fresh weights than negative controls. Plant growth was especially remarkable for plants inoculated with *Microbacterium* sp.

3.3 Plant protection against desiccation by bacterial strains

A similar test was performed with plants subjected to drought stress. Plants were inoculated with *Arthrobacter* sp., *Microbacterium* sp. and *Rhodococcus* sp. Additionally, plants inoculated with *P. putida* KT2440 and non-inoculated were also included. This time plant growth (roots and stem length and fresh and dry weight) was associated with time (7, 14, 20 and 33 days) in absence of water. Similarly, the FTW and RWC values were estimated. The results of these tests showed that only plants inoculated with *Arthrobacter* sp. and *Microbacterium* sp. presented a notable desiccation tolerance (Figure 3). These plants showed longer roots and aerial part of the plant, a higher fresh and dry weight and higher RWC, which is a value established by Mayak and co-workers to determine the tolerance of a plant to desiccation [26]. Therefore, these two strains were selected for future tests of rhizoremediation in arid and semiarid regions.

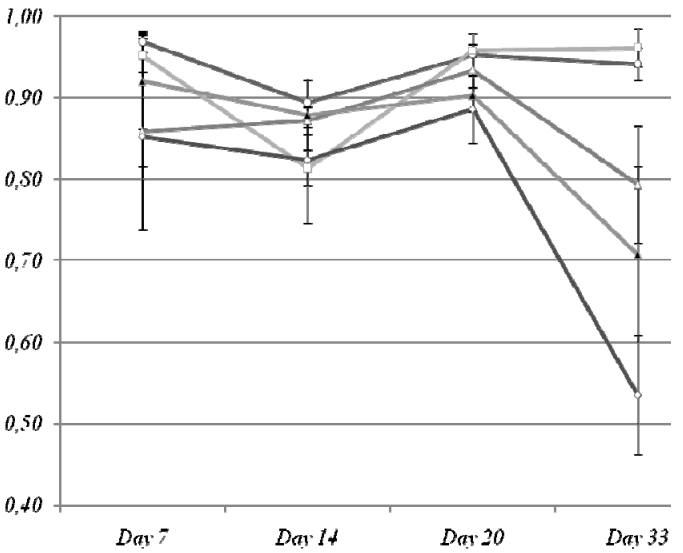

Figure 3: RWC values of pepper plants at 7, 14, 20 and 33 days of the drought stress inoculated with *Arthrobacter* sp. (circles), *Microbacterium* sp. (squares), *Rhodococcus* sp. (open triangles), *P. putida* KT2440 (diamonds), and water (solid triangles).

3.4 PAH removal by desiccation tolerant microorganisms.

Different isolates of *Arthrobacter* and *Microbacterium* have previously being used for the bioremediation of PAH-polluted soils [33, 34]. Therefore, we decided to test the ability of xerotolerant strains to grow on minimal media with naphthalene, anthracene, phenanthrene and pyrene as the only carbon source. The results of this test showed that the selected strains could grow on these PAHs. Therefore, the isolation of new strains was performed following the above mentioned method by plating in minimal medium with PAHs as the only carbon source. Using this method, 26 different strains were isolated in minimal medium with naphthalene as the sole carbon source, 10 strains were isolated using anthracene as the sole carbon source, 14 strains were isolated using phenanthrene as the sole carbon source and 13 strains were isolated using pyrene as the sole carbon source. We are currently assessing the efficiency of PAHs removal in liquid and in microcosms tests of those desiccation tolerance strains, as well as studying their ability to colonise roots. Future tests to establish the efficiency of the PAH removal of the bacterial strains and plant–microorganisms with the high and low presence of water are needed to determine which pair plant–microorganisms are the most appropriate for the efficient treatment of PAH-polluted arid and semiarid soils.

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