Adaptation and Promotion of in Vitro Growth by Pseudomonas Aeruginosa GU270941.1 A in the Presence of Cadmium

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Abstracts

Cadmium (Cd) is one of the most toxic heavy metals. Its high mobility and bioaccumulation power in plant, animal and human tissues differentiate it from the rest of its group and motivate the interest of scientists to know its effects and interaction with plants and the environment. The aim of the present study was to isolate and evaluate the in vitro tolerance of rhizospheric bacteria associated with rice plants in cadmium-contaminated environments and to evaluate them in vitro capacity to tolerate cadmium and the production of compounds related to their ability to remediate and promote plant growth. Rhizospheric bacteria were isolated, tolerance was found up to 500 mg/L CdCl2 and the ability to produce siderophore and solubilize phosphate at this concentration of cadmium was observed in vitro. The morphospecies with the ability to tolerate cadmium up to 500 mg/L found corresponds to Pseudomonas aeruginosa GU270941.1, a species of rhizospheric bacteria that shows an alternative to reduce cadmium uptake by rice plants and promote the growth of these plants for their adaptation and tolerance to this metal.

Keywords: Rhizosphere, rice, bacteria, tolerance, siderophore.

Introduction

Studies by Abah et al. (2016) indicate that industrial activities generate large-scale pollution with heavy metals (copper, zinc, lead, cadmium, chromium, nickel, mercury, cobalt, silver and gold); which as expressed by Ukpong et al, (2013; Jyothi et al., (2017), heavy metals are non-degradable chemical elements, which once incorporated into the environment, are distributed in the air, water and soil, in some cases changing their oxidation state or in others being incorporated into living beings.

Järup and Åkesson, (2009), indicate that the element cadmium is a highly toxic transition metal at very low exposure levels and has acute and chronic health effects on plants, animals, humans and all living things in general. As a consequence of industrial activity and anthropisation. Similarly, several studies (Ahmad et al., 2010; Olivares et al., 2013; Duressa and Leta 2015; Gimba et al., 2015; Mohod 2015; Abdel-Satar et al., 2017), indicate that cadmium is found in different parts of the planet and in our country Cd levels have been detected in water, soil and plants that exceed the permissible limits established for different uses.

Similarly, research by (Huang et al., 2015; Hédiji et al., 2015; Jinadasa et al., 2016) indicates that cadmium is not degradable in nature, so once released into the environment, it will remain in circulation. This property together with its high mobility, bioaccumulative power and toxicity at very low concentrations make it one of the most important heavy metals. Studies on several crops have shown that it reduces growth, photosynthetic activity, transpiration and chlorophyll content. Other studies (Mysliwa-Kurdziel, et al., 2004; Shaw et al., 2004; Nogueirol et al., 2016; Li et al., 2016), indicate that it causes chlorosis, oxidative stress, nutritional imbalances and modifies the activity of enzymes involved in the metabolism of organic acids and the Krebs cycle. In general, the effects on some physiological processes can be so marked that plants are not able to avoid them and manifest themselves in other processes. Cd toxicity can lead to plant death, and this depends, among other factors, on the exposure time, the metal content and the specific adaptations they develop.

Due to the damage caused by Cd toxicity to plants and the risk of Cd accumulation in plants, several studies have proposed different strategies to mitigate its effects. Most of the strategies include modifications in nutritional management. But other practices have also shown favorable results, such as inoculation with beneficial bacteria.

According to Kumar et al., (2015), some strains of Pseudomonas promote plant growth by making plant available phosphorus, potassium, and zinc from the soil, phytohormone synthesis, HCN, lytic enzymes, and siderophores production. Thus, it might be concluded that the bacterial strains of Pseudomonas sp. with their multifunctional properties will attract more attention in the field of biofertilization. Pseudomonas aeruginosa is commonly found in soil, plants, animals, and humans (Lee et al., 2020).

Based on the experience of rhizospheric bacteria as a biological alternative to contribute to the reduction of cadmium contamination in rice soils, the strategies proposed were to isolate rhizospheric bacteria, evaluate their capacity to tolerate different cadmium concentrations and finally the production of growth-promoting substances as a mechanism to reduce the uptake of the metal by plants.

Materials and Methods

Sampling. Initially, bibliographic information was collected on areas contaminated with heavy metals due to anthropogenic activities in the department of Sucre. Once the areas with soils contaminated with heavy metals had been identified, site visits were made and a diagnosis was made of the current state, crops grown, number of hectares and vulnerable areas. Next, the

contaminated soil sampling stage was carried out. This consisted of a random sampling, which was divided into three plots according to the topographical characteristics of the land. In each of these plots, three samples of rhizospheric soil from established rice crops were taken at random, covering the first 20 cm of depth. Approximately 3000 g of sample were taken per site. These were distributed into two sub-samples of 1500 g each (cadmium concentration and the other for microbiological analysis). The soil samples were then stopped, carefully packed in labelled and identified plastic bags, stored and preserved for transport and processing.

Cadmium concentration in soil. The Cd in the soil Cd was extracted with the Tessier multistage continuous extraction method (Tessier et al., 1979). Afterwards, the Cd concentrations of the samples were determined by an atomic absorption spectrophotometer.

Isolation of rhizospheric bacteria. It was carried out using the methodology proposed and modified by Ayubb et al., (2017), which consisted of: prepare A 1:100 dilution of soil was inoculated on selective medium with 1-carboxylic acid-1-aminocyclopropane (ACC), the bacteria that grew on this medium were characterized for their growth-promoting capacity. This medium contains the mineral salts of the Dworkin and Foster medium whose composition per litre is as follows (4g KH_2PO_4, 6 g $NaHPO_4$, 0.2 g $MgSO_4$, 1 mg $FeSO_4$, 10 µg H_3BO_3, 10 µg $MnSO_4$, 50 µg $CuSO_4$, 10µg MoO_3, 70 µg $ZnSO_4$), glucose 0.2%, gluconic acid 0.2%, citric acid 0.2% and bacteriological agar at 2% and ACC 3 mM (Sigma).

Bacterial population density (CFU/g soil) is determined by direct colony counting on the surface of agar plates. During counting, colonies showing variations in shape, texture, color and size shall be identified and selected (Pérez et al., 2014).

In vitro evaluation of cadmium tolerance by rhizospheric bacteria. Bacterial isolates were seeded on nutrient agar supplemented with $CdCl_2$ at concentrations of 100, 150, 200, 250, 300, 350, 400 up to 1000 mg/L; then incubated at 29°C for 7 days (Sorkhoh et al. 2010).

Determination of phosphate solubilizing bacteria. For the determination of phosphate solubilizing bacteria, NBRIP medium (National Botanical Research Institute Phosphate Medium) was used, whose composition per litre was as follows: 10g Glucose, 5g $Ca_3(PO_4)_2$, 5g $MgCl_2{\cdot}6H_2O$, 0. 025g of $MgSO_4{\cdot}7H_2O$, 0.2g of KCl, 0.1g of $(NH_4)SO_4$ and 0.05g of bromothymol blue. Each of the reagents was added and dissolved in the above order, then 20g of bacteriological agar was added and sterilized at 15lb pressure for 15min. Each of the isolated strains was inoculated onto NBRIP medium by pitting and incubated at 28°C for 24hrs. Solubilizing strains were determined according to the formation of a degradation halo (Perez et al., 2014).

Siderophore production. The qualitative production of siderophores was carried out on the chromium azurol-S (CAS) medium proposed by Schwyn and Neilands (1987), which consisted of dissolving 60.5 mg of CAS in 50 ml of distilled water, to this mixture was added 10 ml of iron (III) solution (1 mM FeCl3.6H2O and 10 mM HCl), under stirring. The solution was mixed with 72,9 mg HDTMA dissolved in 40 ml water. The resulting blue liquid was autoclaved at 121°C for 15 minutes. A mixture of 750 ml of water, 15 g of agar, 30.24 g of pipes, and 12 g of a 50% (w/w) solution of NaOH adjusted to pH 6.8 was also autoclaved in another vessel. To the medium 4 g of glucose was added as a carbon source. Isolates were seeded using wooden sticks and

incubated for 7 days at 30°C. The ability of the bacteria to produce siderophores was evidenced by the formation of a transparent halo around the colonies.

Molecular identification of cadmium-tolerant rhizospheric bacteria. The extraction of genomic DNA from cadmium-tolerant rhizospheric bacteria was performed using the protocol proposed by Oliveira et al. (2013). For the amplification of the 16S rRNA gene, three specific primer sets were used: F BLS342 (5‘ CAGCAGTAGGGAATCTTC 3’) and R 1392 (5‘ ACGGGGGCGGTGTGTGTACA 3’), for the class Firmicutes; F 948β (5‘ CGCACAAGCGGTGGTGGATGA 3’) and R 1492 (5‘ TACGG(C/T) TACCTTGTTACGACTT 3’), for the class Betaproteobacteria; FD2 (5‘ AGAGTTTGATCATGGCTCAG 3’) and RP1 (5‘ ACGGTTACCTTGTTACGCTT 3’), for the gammaproteobacteria class. The conditions used in each amplification reaction were based on the protocol described by Oliveira et al. (2013), using a mastercycler nexus eppendorf thermal cycler. The amplification products obtained were purified and sequenced at Macrogen Korea. The sequences obtained were compared with those stored in Genbank. Base alignment was performed in the Clustal w program, analysis and correction in the Mega 5 program, phylogenetic inferences were obtained by the maximum similarity method, based on the kimura-2-parameter model.

Results and Discussion

Figure 1 shows the amount of rhizospheric bacteria isolated from rice soil in the San Jorge sub-region in the department of Sucre. The presence of rhizospheric bacteria ranged from 2.3 X 10^5 to 9.1 X 10^6 CFU/g of roots.

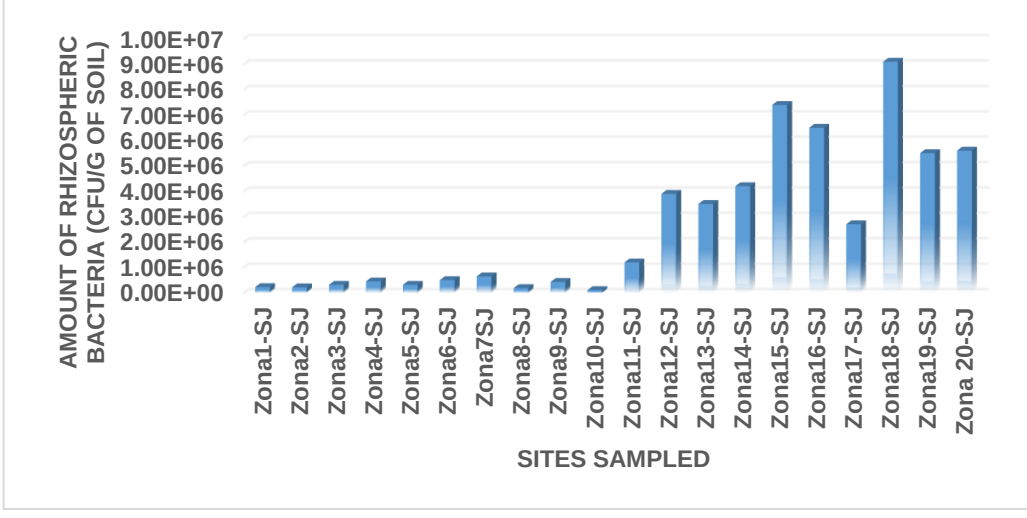

Figure 1. Number of rhizospheric bacteria associated with cadmium-contaminated rice soils in the San Jorge sub-region in the Department of Sucre, Colombia.

Table 1 shows the number of isolates that showed tolerance from 100 to 500 mg/L $CdCl_2$ and the ability to produce siderophore in vitro and solubilize phosphate in vitro. Table 1 shows that isolates C62RLIM and C85RLIM grew up to 400 mg/L, while C85RLIM grew up to 500 mg/L $CdCl_2$ and showed respectively in vitro ability to solubilize phosphate and produce siderophores.

Table 1. Isolates of rhizospheric bacteria with cadmium tolerance and cadmium and plant growth promotion activity.

Isolated	Sample code	$CdCl_2$ (mg/L)					Phosphate solubilization	Siderophore Production
		100	200	300	400	500		
1.	C60RLIM	+	+	+	-	-	+	-
2.	C62RLIM	+	+	+	.	-	+	-
3.	C63RLIM	+	+	+	+	-	+	-
4.	C67RLIM	+	+	+	-	-	+	-
5.	C85RLIM	+	+	+	+	+	+	+
6.	C85RLIM	+	+	+	+	-	+	-

+: Growth and/or in vitro activity; -: No growth and/or activity

Figure 2 shows the growth behaviour of the C85RLIM isolate from 100 to 500 mg/L $CdCl_2$. The growth capacity of the endophytic bacterial isolate to tolerate different cadmium concentrations is also observed.

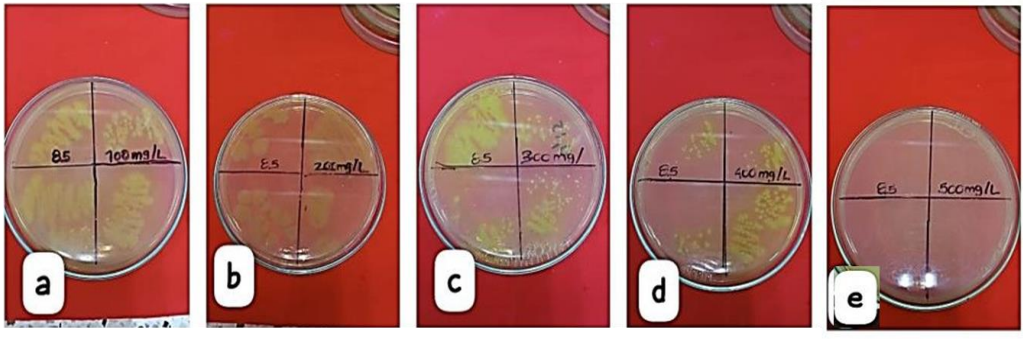

Figure 2. Comportamiento de crecimiento del aislado C85RLIM en medio de cultivo suplementado con diferentes concentraciones de $CdCl_2$.

Figure 3 shows in vitro siderophore production and phosphate solubilisation by the C85RLIM isolate.

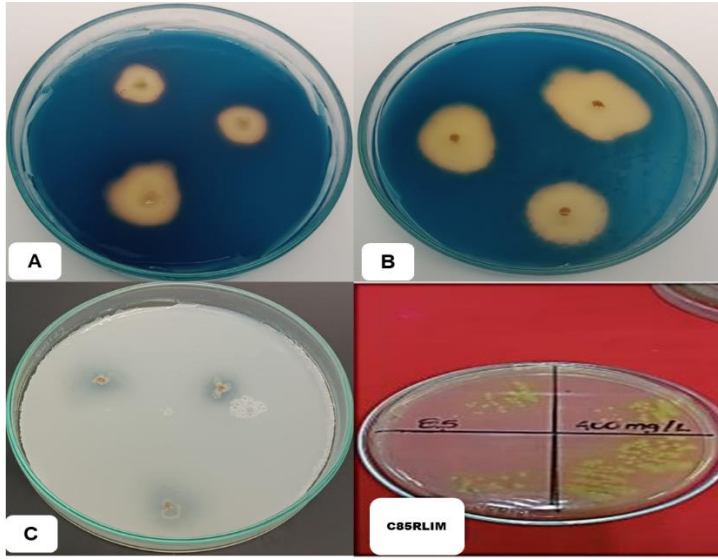

Figure 3. In vitro evaluation of isolate C85RLIM. a) siderophore production at 40 mg/L, b) siderophore production at 500 mg/L $CdCl_2$ concentration and c) phosphate solubilisation.

Figure 4 shows the phylogeny of rhizospheric bacterial strains isolated from soils cultivated with rice in the sub-region of San Jorge in the department of Sucre, Colombia.

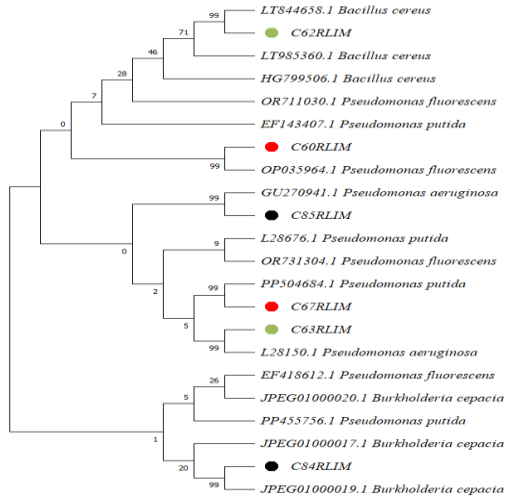

Figure 4. Phylogenetic tree based on a partial 16S rRNA gene sequence of bacteria's rhizospheric.

As shown in Figure 4, at the phylum level of species, the dominant bacteria in the rice rhizosphere soil sample from where Proteobacteria (93.11 %), and Firmicutes (6.99 %), while the dominant bacteria in the rice rhizosphere soil sample from: Pseudomonas aeruginosa, Pseudomonas putida, Pseudomonas fluorescens, Burkholderia cepacia y Bacillus cereus.

The isolate referenced as C85RLIM and identified as Pseudomonas aeruginosa GU270941.1 was the species with the ability to tolerate up to 500 mg/L $CdCl_2$ and to produce siderophore and solubilize phosphate in vitro.

Tolerance refers to the ability of microorganisms to survive and grow optimally under heavy metal stress conditions without influencing their normal reproductive, physiological and biochemical functioning (Abdu et al., 2017). This characteristic of microorganisms involves different and varied mechanisms such as: formation of insoluble metal complexes, volatilization and removal of these from soil and binding of the metal to the bacterial cell wall or proteins. Other mechanisms include the regulation of metal uptake from soil and the transformation of metal to less toxic forms (Riaz et al., 2020).

According to morphological and physiological studies, P. aeruginosa is a Gram-negative bacterium isolated in pure culture from skin wounds for the first time in 1882 by Gessard (Luján, 2014), deriving its name from the word aeruginous (aeruginous), which means 'the color of oxidized copper', due to the characteristic blue-green color of its colonies in culture (Ruiz, 2007). Despite being associated with health problems (Luján et al., 2008), several of its strains have been reported to have a detoxifying capacity for certain organic and inorganic soil pollutants (Das and Mukherjee, 2007; Mayz and Manzi, 2017), and are especially useful for combating heavy metal contamination (Bojórquez et al., 2016).

Biochemical evidence shows positive oxidase and catalase activity, oxidatively degrades glucose and has a temperature optimum of 30°C to 37°C. It is found in different environments, being predominant in aquatic and terrestrial environments and even in animal and plant tissues. Its respiration is aerobic, but some strains can grow anaerobically by denitrification (Özen and Ussery, 2012). It has the ability to use a wide range of organic and inorganic compounds for its metabolism, which allows it to take advantage of many types of substrates, several of which are considered toxic, such as aliphatic and aromatic hydrocarbons, as well as being resistant to heavy metals, antimicrobials and detergents (Ruiz, 2007; Martínez et al., 2010).

According to Awasthi et al. (2015), in the bioremediation processes of environments contaminated with heavy metals, it seems that P. aeruginosa has a high bioremediative capacity, which prevents them, in quantities above certain limits, from being extremely harmful to the soil and the biota that support them.

More than 500 different siderophores have been reported to date (Złoch et al., 2016). Pyoverdine (PVD) is a yellow-green fluorescent siderophore produced by Pseudomonas spp. (Kang et al., 2019; Trapet et al., 2016. PVD consists of three parts: a dihydroxyquinoline chromophore, a specific peptide chain composed of 6 to 14 amino acids, and a side chain that binds to the nitrogen atom at the C-3 position of the chromophore (Budzikiewicz et al., 2007; Han et al., 2018). Compared with other siderophores, PVD has a higher affinity for heavy metals and plays important roles in promoting plant growth under heavy metal stress, so there has been increasing

interest in studying the potential of PVD for the bioremediation of heavy-metal-contaminated soil (Braud et al., 2010; Cornelis et al., 2023). Research has shown that the secretion of PVD by Pseudomonas aeruginosa (P. aeruginosa) KUCd1 under Cd(II) exposure increases the strain's resistance to Cd(II) (Sinha and Mukherjee, 2008).

Currently, many Cd-tolerant rhizosphere bacteria are being screened and used for environmental pollution remediation (Sun et al., 2023; Wang et al., 2020). However, these bacteria are not highly resistant to Cd(Π) (Mishra et al., 2018).

In our study, we isolated a highly effective Cd-tolerant P. aeruginosa bacterium from rice soils in the region of San Jorge, department of Sucre, contaminated with cadmium., which was able to tolerate up to 500 mg/L $CdCl_2$. The results obtained indicate the presence of P. aeruginosa screened has good environmental adaptability, making it a good candidate for environmental remediation.

Research has shown that microbial metabolites forming complexes with metal ions can reduce the concentration of extracellular active heavy metals, thereby reducing the ability of heavy metals to diffuse into cells and their toxicity (Retamal-Morales et al., 2021).

Shixue et al., (2024), indicate that the Cd(Π) resistance mechanism of P. aeruginosa screened is the complexation of Pyoverdine-PVD for Cd(Π) and the adsorption of bacteria for Cd(Π); furthermore, PVD plays an important role in improving the Cd(Π)-resistant ability of bacteria. This study provides a deeper understanding of the highly effective Cd(Π) resistance mechanism of P. aeruginosa and the function of Cd(Π)-induced PVD in bacteria.

Conclusion

In this study, we isolated a highly effective Cd-tolerance P. aeruginosa GU270941.1, which can resist 500 mg/L $CdCl_2$ and grow under various environmental conditions. This P. aeruginosa can produce a type of siderophore up to a concentration of 500 mg/L $CdCl_2$ and additionally solubilizes phosphate.

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Author contribution. Alexander Perez Cordero: experiment execution, data analysis. Donicer Montes V and Yelitza Aguas M, conceptualization, writing - revision and editing. All authors have read and approved the manuscript.

Conflict of interest. All the authors of the manuscript declare that they have no conflictof interest.

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