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BENEFICIAL ASSOCIATION OF *Pseudomonas aeruginosa* IN ROOTS OF *Typha latifolia* PRESENT IN THE MINING TAILINGS WETLAND

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Abstract: *Pseudomonas aeruginosa* is a bacterium capable of living in diverse environments, producing biosurfactants, fixing nitrogen, tolerating heavy metals, and is capable of transferring resistance genes to Pb, Cd and As by means of plasmids. These characteristics make it an excellent proposal for bioremediation studies in sites contaminated with metals. The objective of this study was to search for bacterial strains associated with the rhizosphere of *T. latifolia* with biotechnological potential. For this purpose, strains were isolated from the rhizosphere of the aquatic plant and only those capable of producing biosurfactants by foam production in PPGAS broth, droplet collapse, IE_{24h} were selected. MIC to Pb, Cd and As was determined, nitrogen fixation was detected on NFb agar, plasmid extraction was performed by Kieser's method, and PCR detection of *arsA, cadA* and *pbrA* genes. Only four strains PAR1, PAB12, J2 and J3 able to produce biosurfactants and fix nitrogen were isolated. They did not present plasmids and only amplified Pb and Cd resistance genes (*cadA* and *pbrA*). These strains are proposed for further study and use in wetland bioremediation processes in sites contaminated with heavy metals.
Keywords: *P. aeruginosa*, biosurfactants, resistance genes, heavy metals, wetland.

INTRODUCTION

Biosurfactants (BS) are biological molecules that decrease surface and interfacial tension, increase solubility, have the ability to mix two immiscible solutions, among others (Satpute *et al*, 2010), are produced by a wide variety of microorganisms such as bacteria, fungi and yeasts, are environmentally compatible and biodegradable, have low toxicity, resist a wide range of salinity, temperature and pH (Vijayakumar and Saravanan, 2015). They have been studied mainly for microbial oil recovery, in bioremediation of contaminated soils and waters (Wu *et al.,* 2008; Amani

et al., 2013). Few microorganisms are capable of producing them, so the interest in studying them has been increasing. Among the genera described with this capacity are *Bacillus sp, Psedumonas sp, Enterobacter sp, Serratia sp, Acinetobacter sp* among others (Jiménez-Islas *et al*., 2010 , Suresh-Chander et al., 2012) . In Mexico the exploitation of mineral deposits has been carried out since the 16th century, but it has also generated a large amount of mining waste, accumulated called jales, which contaminate both soils and water bodies, with soluble salts of potentially toxic elements (PTE) such as arsenic (As), selenium (Se), lead (Pb), cadmium (Cd) and sulfur oxides, among others (Moreno Tovar, et al., 2012). Bacteria capable of producing BS with tolerance to heavy metals have been reported in several mining studies, likewise the association between plants and bacteria with biotechnological potential is well documented (Van de Voorde et al., 2012). Within this group, endophytic bacteria that confer protection and promote plant growth in the plants that host them and can be found within their tissues stand out. They have the ability to fix nitrogen, solubilize phosphates, produce phytohormones or other metabolites, which makes them of interest for their considerable potential as biofertilizers and as biocontrol agents (Beneduzi et al., 2012). The study of sites contaminated with heavy metals, plants and microorganisms yield data that can be used to plan phytoremediation strategies assisted by native bacteria. That is why in this study we focused on the search for biosurfactant-producing bacteria resistant to heavy metals that are associated with the rhizosphere of *Thypha latifolia* present in the El Fraile wetland in Guerrero, Mexico, with the aim of using them in *in vitro* or *in vivo* studies in bacterial-assisted phytoremediation processes of sites contaminated with heavy metals.

MATERIALS AND METHODS

Roots of *Typha latifolia* were collected the El Fraile wetland in 2014, washed with 2% sodium hypochlorite (NaOCl); 1 g of each root was used and macerated in 50 mL of SSI (sterile isotonic saline solution), in order to obtain endophytic strains. The number of CFU/g in each root was determined. The isolated bacteria were evaluated for their ability to produce BS by hemolysis on 10% ram's blood agar, foam production and stability in PPGAS broth (Toribio Jimenez et al, 2014), drop collapse and emulsification index (IE_{24}), oil dispersion (Wild et al., 1997). Those that produced BS in PPGAS broth, extraction of the same was performed with ethyl acetate in 2:1 ratio, and finally the partially purified BS (BPP) were resuspended in buffer PBS pH 7.2 (Kosaric, 2001). Likewise, the temperature stability test (40, 70, 100 and 120 °C) was done for 1 h in water bath, pH of 2.0, 5.0, 9.0 and 12.0, was adjusted with NaOH or HCl as appropriate (Nithya and Pandian, 2010). The effect of salinity was done using 100µL of BPP and 400µL of NaCl solution at concentrations (0-10% w/v). The viability of the biosurfactant in each assay was tested using the oil dispersion technique. At the end, BS was detected by thin layer chromatography (TLC) on preparative plates with 0.25 mm Silicagel 60 F_{254} (Saikia et al., 2012). Strains were identified by conventional biochemistry (Kligler Iron Agar, LIA Agar, MIO medium, Christensen's Urea Agar, Simmons Citrate and Nitrate Broth) including Gram staining and catalase and oxidase detection. On the other hand, the ability to fix molecular nitrogen in NFb Agar for 96 h at 30 °C was determined (Hernández Y, 1998). Tolerance to heavy metals was determined through the Minimum Inhibitory Concentration (MIC). Solutions of Cd $(NO_3)_2$, Pb $(NO_3)_2$ and $As^{5+,}$ were prepared and used at the following concentrations, 640, 320, 160, 120, 80, 40, 40, 20, 10, 5, 2.5 and 1.25 mM, respectively, adding each concentration in Muller-Hinton culture medium, poured into

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Petri dishes; from a fresh bacterial culture, suspensions of each of the bacterial strains were made in 0.9% saline solution, with an optical density at 625 nm, at an absorbance between 0.080-0.130 ($1x10^8$ CFU/ml). With the help of the STEER Replicator, each of the bacterial suspensions was sown by chopping in Petri dishes with metals of all concentrations, once the bacteria were placed on the plates, they were allowed to dry and incubated at 37 °C for 24 hours (Paniagua *et al.,* 2003).

Subsequently, from strains capable of producing BS, fixing N_2 and tolerant to heavy metals, plasmid DNA was extracted by the modified Kieser technique (Kieser *et al.,* 1984), and chromosomal DNA was obtained by heat shock. Polymerase chain reaction (PCR) amplification of the *arsA, cadA* and *pbrA* genes was performed**. The** oligonucleotides used for arsenic (*arsA*) are arsF (5'CCAGATTGGGAAGCAACCT 3') and asrR (5'ACCGTCTAGTGCTGGCTGGTTGGTTGC 3'), and the reaction conditions were previously described by Eckweiler *et al,* (2014), for cadmium, *cadA* gene the following oligonucleotides were used were cadAF (5'GTCAGGTCACGGACGGACATGAT 3') and cadAR (5'GGTGGCTTCCGTTCCACTT 3') with 743 bp , described by Stover *et al* (2000) and for lead the amplified gene was *pbrA* with 940 bp, the conditions and oligonucleotides were described in this study,; *pbrAF* (5' GCGAAYACMGCCATCCACA 3') and *pbrAR* (5' CCTWGACAAGACCGGCAC 3'), the amplification conditions were as follows 5 min at 94°C, followed by 30 cycles of 50 sec at 94°C, 40 sec at 54°C, and 2 min at 72°C, and a final extension of 7 min at 72°C. The amplified products were subjected to 1% agarose gel electrophoresis in a power supply at 120 V for 30 min, after which time they were stained with ethidium bromide and visualized under UV light; *P. aeruginosa* PAO1 was used as a control in all experiments.

RESULTS

Figure 1 shows the wetland where *Typha latifolia* roots were obtained in the mining region of El Fraile, Guerrero, Mexico, 125 strains with different phenotypes were isolated, only four (PAR1, PAB12, J2 and J3) were BS producers (Figure 1b, c and d). As for microbiological identification, the isolated strains were typed as *P. aeruginosa*, and they were able to produce mono- and di-rhamnolipid biosurfactant, and all of them were able to fix nitrogen. The CFU/g obtained in each sample ranged from $2x10^5$ to $3x10^{10}$ respectively. The MIC to Pb, Cd and As obtained for PAR1 and PAR12, for Pb 6mM, Cd 4mM and As was 16mM, in the case of strains J2 and J3, they presented Pb 16mM, Cd 4mM and As was 2mM respectively.

Figure 1. Sample selection and biosurfactant production by the isolated bacteria, a) obtaining roots of *T. latifolia* in the wetland in El Fraile, Guerrero, b) foaming activity, and c) collapsed droplet, d) biosurfactant production by the isolated bacteria, and e) biosurfactant production by the isolated bacteria.

The presence of plasmids was not detected, only the *pbrA* and *cadA* genes were amplified in the four strains, as shown in Figure 2, the *arsA* gene was not amplified in any strain.

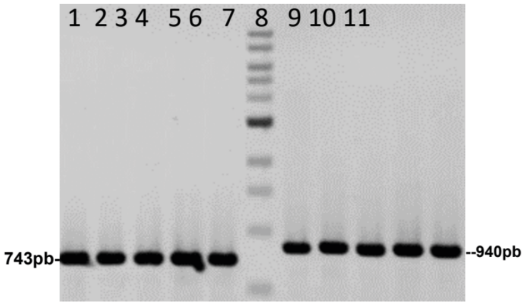

Figure 2. 1% agarose gel electrophoresis of PCR products for amplification of *cadA* and *pbrA* genes, lanes 1-5) PAR1, PAB12, J2, J3, positive control of *cadA* gene amplification (743bp), lane 6) MPM 10Kb. lanes 7-11) PAR1, PAB12, J2, J3, positive control of *pbrA* gene amplification (940bp).

DISCUSSION

Heavy metals, when present in high concentrations, directly affect the health of the population as described by Moreno Godinez et al (2009) and its surroundings, and to a great extent the development and balance of the flora and fauna in these places. In the studies carried out in the sampling in the mining tailings of El Fraile, no strains of *P. aeruginosa* were isolated; they were only isolated in the rhizosphere of *T. latifolia* present in the wetland at the same site. The results provide insight into the resistance mechanism employed by *P. aeruginosa* and its relationship with the roots of aquatic plants in the bioremediation system proposed at the mine. All strains were able to resist Pb and As concentrations up to 16 mM, with Cd the maximum concentration reached 4 mM, Sevgi *et al*., (2010) evaluated strains of *Pseudomonas sp.* isolated from industrial drainage samples reporting a resistance of 0.5-1mM Cd. This difference in resistance could be based on the concentration of metals present in the soil and the habitats from which they were isolated. Armienta *et al*., (2003) quantified the concentration of metals present in the soil of the El Fraile tailings, resulting in 455-22,900 mg/kg of Pb, 201-2,052 mg/kg of As and 427-22,200mg/kg of Zn, while Sevgi *et al*., (2010) reported values of 0.1-0.8 mg/kg of Cd, 16.3-22.8 mg/kg of Co, 25.7-41.5 of Cu and 38.1-65.9 mg/kg of Zn for the industrial drainage samples. We can say that *P. aeruginosa* endophytes of *T. latifolia* are much more resistant to these metals, and the fact that they are exposed to high concentrations in their environment allows them to adapt adequately to contaminated sites.

The absence of plasmids demonstrates that the transfer of resistance is not taking place horizontally. Therefore, the absence of plasmids in the strains evaluated suggests that these strains *cadA, pbrA* genes are in the chromosome. In this case in which the strains did not present plasmids, Marrero *et al*. (2010) report that it is possible to locate the genes in the bacterial chromosome, many of the bacteria usually insert the genes in their chromosome since it is more stable and they do not run the risk of losing their plasmid and therefore their resistance to heavy metals and/or metalloids. Nitrogen fixation is one of the capabilities that many bacteria present in the rhizosphere or in internal plant tissue do, this benefit can make a beneficial association between the plant and the bacteria, and can also be used as bioremediation strategies for contaminated aquatic sites. In the strains it was possible to amplify the genes that confer resistance to Cd and Pb, but not those of As, this allows us to know more about the microorganism-metal interaction, as well as to understand the mechanism by which *P. aeruginosa* carries out its adaptability to extreme habitats.

CONCLUSION

The study on the association of *P. aeruginosa* with roots of *T. latifolia* in the El Fraile wetland will serve to design *in vivo* bioremediation strategies in artificial wetlands to reduce heavy metal contamination.

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