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PARTIAL PHENOTYPIC AND 16S rDNA CHARACTERIZATION OF BACTERIA PROMOTING PLANT DEVELOPMENT

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Abstract: Soil microorganisms are closely related to plants, in particular bacteria can help symbiotically or not. The study of promoter bacteria in plant growth is limited, so this work aims to contribute to the knowledge of some phenotypic and genotypic characteristics of bacterial strains for plant development. Two cryopreserved bacterial strains from the FES-Iztacala UNAM Bacterial Culture Collection were requested for analysis. Strain activation and phenotypic analysis were performed; Colonial morphology, Gram stain, biochemical characterization: Oxidation-Fermentation, Catalase, Oxidase and Indole, amplification and sequencing of the 16S rDNA region for BLAST analysis in GenBank. Additionally, genomic DNA of the AAA1, SRA1 and RRJ1 strains previously characterized by morphology and biochemistry and identified as Pseudomonas sp. The RH8530 strain was identified as Rhizobium tropici by traditional tests and 80% similarity with Sinorhizobium meliloti. Molecular analysis determined inconsistency in their characterization in two genomic DNA of the reference strains.
Keywords: BPCV, 16s rDNA, collection of bacterial cultures.

INTRODUCTION

Plants are a vital part of biodiversity, it is an essential resource of the planet of great economic, cultural, nutritional and medicinal importance, worldwide, due to this they have had an anthropocentric development, (Secretary of the Convention on Biological Diversity, 2009). Plants display a wide variety of interactions with soil organisms (bacteria, fungi, protists, and animals), covering the full range of ecological possibilities (competitive, exploitative, neutral, commensal, mutualistic). Throughout modern plant science, most interaction studies have focused on mitigating pathogenic effects such as herbivory and infection (Strange and Scott, 2005; Zhang et al., 2013), or mitigating conditions of abiotic stress (Yaish et al., 2016; Meena et al., 2017). There has also been a longstanding interest in characterizing the positive ecological interactions that promote plant growth.

Plant growth-promoting bacteria (PGPB), or known as "plant growth-promoting bacteria (PGPB)", are a group of different bacterial species that can increase plant growth and productivity (Torriente, 2010). They favor plant growth directly: fixation of atmospheric nitrogen, production and synthesis of siderophores, solubilization of minerals, synthesis of phytohormones (auxins, cytokinins and gibberellins), synthesis of the ACC deaminase enzyme and indirect: biocontrol of phytopathogens, production of antibiotics, iron reduction, induced systemic resistance, and cell wall lytic enzymes (Olanrewaju et al., 2017)

The number of publications associated with microorganisms that promote plant growth has had an exponential increase (Finkel et al., 2017), in particular, on the bacterial genera Rhizobium, Sinorhizobium, Azorhizobium, Allorhizobium, Mesorhizobium, Bradyrhizobium, Frankia, Azoarcus, Achromobacter, Burkholderia and Herbaspirillum (Babalola, 2010; Pérez-Montaño et al., 2014; Turan et al., 2016), all symbiotic nitrogen fixers and the main non-symbiotic bacteria studied are Azotobacter, Azospirillum, Bacillus and Klebsiella sp.

Other bacterial genera studied for their importance in solubilizing inorganic phosphate compounds (phosphate minerals), such as di- and tricalcium phosphate, hydroxyapatite, and rock phosphate, include Pseudomonas, Bacillus, Rhizobium, Burkholderia, Achromobacter, Agrobacterium, Microccocus, Aerobacter, Flavobacterium and Erwinia (Rodríguez et al., 2006). Strains of the genera Pseudomonas,

Bacillus and Rhizobium are considered among the most potent phosphate solubilizers (Banerjee et al., 2010). However, the research work focused on the BPCVs has been based mainly on the recognition of the characteristics of interaction with the plants, making it necessary to study the biochemical, genetic and physiological characteristics of the BPCVs, which would result in an integral management including the possibility of genetic manipulation of "optimal" strains, this according to what was reported by Olanrewajo et al., (2017).

In relation to the above, the establishment of bacterial culture collections is the key to exploring the biological potential of BPCVs. Therefore, the present work intends to contribute to the knowledge about the phenotypic characteristics and by molecular biology from 16S rDNA sequences of two strains deposited for safekeeping and three genomic DNAs used as references, isolated and previously characterized morphologically and biochemically by the group. from the Collection of Bacterial Cultures of the Faculty of Higher Studies Iztacala (CCBIZTA) and presumptively identified as Pseudomonas sp, with possible promoting effects for plant development (BPCV).

METHODOLOGY

The bacterial strains RT, Az and the reference genomic DNA isolated from previous studies corresponding to bacterial strains SRA1, AAA1 and RRJ1, internal key of the Collection of Bacterial Cultures (CCBIZTA) of the Faculty of Higher Studies Iztacala, UNAM, were used.

ACTIVATION OF BACTERIA

Bacteria stored at -20 °C were activated in complete YT medium (0.05g of tryptone, 0.3g of yeast extract, 0.1g of Calcium Chloride and 1% agar for 100 mL of medium) and Ashby 2 medium (Sucrose 0.5g, Mannitol 0.5g, Dibasic Potassium Phosphate 0.1g, Magnesium Sulfate 0.02g, Iron II Sulfate heptahydrate 5mg, Sodium Chloride 0.02g, Calcium Chloride 0.02g and 1.5g of Agar-Agar for 100 mL of medium for R. tropici and A. vinelandii, respectively.After 48 h of incubation at 30°C, the phenotypic characteristics were reviewed, according to Ruiz (2016) and the characterization was performed by the partial sequence of the 16s rDNA (Molina et al., 2014)

First-entry phenotypic characteristics were used:

- Gram: It is considered as a differential technique classifying bacteria according to the composition of their wall.
- Oxidation-Fermentation: it allows differentiating the type of metabolism.
- Catalase: Indicates the presence of catalases, it catalyzes the decomposition of hydrogen peroxide into oxygen and water.
- Oxidase: Determines if the bacterium produces cytochrome.
- Indole: It allows to measure the degradation.

PARTIAL CHARACTERIZATION OF 16S RDNA

DNA COLLECTION

The DNA of the bacterial strains was extracted, by means of the AP method, one batch of each bacterium was mixed in 750 µl of Buffer AP (Urea 7M, NaCl 0.35M, Tris Base 0.05M, EDTA 0.02M and Sarcosine 1%), Vortexed for 30 min; the mixture was centrifuged for 1 min at 12,000 rpm, phenol and chloroform v/v were added to the supernatant for subsequent centrifugation for 3 min at 12,000 rpm (this step was performed twice). The supernatant was taken and added v/v of isopropanol and 0.1 vol of 10Mm ammonium acetate, after centrifuging at 12000 for 10 min, washing the pellet with 500 µl of 70% ethanol and finally drying at room temperature. The

DNA thus obtained was resuspended with 50 µL of nuclease-free water.

POLYMERASE CHAIN REACTION (PCR)

For the PCR reaction, 100 ng of DNA and the primers FD'AGAGTTTGATCCTGGCTCAG 3' and RD 5' AAGGAGGTGATCCAGCC 3' were used. AMPLICON Taq 2X Master Mix was used following the manufacturer's instructions and where each reaction was at a final concentration: 1X buffer, 1.5 mM MgCl, 0.4 mM dNTP's, 0.2 units/uL Amplicon Taq DNA polymerase and each primer at 0.2 m. The final volume of the reaction was 25 L volumetric with molecular grade water. The PCR was carried out in a BIORAD model T100 thermocycler and using the program of one minute at 95°C, followed by 40 cycles consisting of 30 seconds at 94°C, 20 seconds at 51°C, one minute and 10 seconds. at 72°C and finally 8 minutes at 72°C. Genomic DNA and PCR products were visualized on 1% agarose gels with 1X Buffer TAE (40mM Tris-acetate, 1mM EDTA, pH 8) and stained with Midori Green Direct from NIPPON genetics, separated at 80V for 30 min. and observed under ultraviolet light (Poutou et al., 2005).

SEQUENCING

Finally, the PCR products with a weight of 1,500 bp were sequenced by the Sanger method, using the BigDye Terminator version 3.1 Sequencing Kit chemistry, DT3100pop7(BD) v3 polymer support mobility with 50cm capillaries and a sequencing electrophoresis time: 2 h., 30 min, automated sequencing equipment ABI 3130xl Genetic Analyzer (Applied Biosystem HITACHI), Molecular Biochemistry Laboratory FES-Iztacala-UNAM. The sequence obtained was compared by performing BLAST with genome sequences in the database.

RESULTS AND DISCUSSION

Morphological and biochemical characteristics of *R. tropici*.

Bacterial culture collections are constructed from isolates from human, animal, and environmental samples. In the case of environmental bacteria, work has been carried out on various areas of study, one of which is the use of microorganisms to promote plant or plant growth (BPDV). The vast majority of research focuses on the plant-bacteria association in the field or greenhouses (Mwenda et al., 2018; 9. Kontopoulou et al., 2017; Igieton and Babalola, 2018). Knowledge about particular characteristics of bacteria per se is obtained in the laboratory.

In relation to the above, in this work two bacteria were used: *Azotobacter vinelandii* y *Rhizobium tropici*, in addition to three genomic extraction products, of bacterial origin, one of them belongs to: *Pseudomonas fluorescens* considered as a BPDV. The bacteria as the genomic products belong to the Collection of Bacterial Cultures of the FES-Iztacala (CCBIZTA). The bacteria in the collection are stored frozen, so for any test that is requested to be carried out, activation is necessary first. The strain: *A. vinelandii* (Az) it could not be recovered from freezing even when several culture media including one specific for the genus were tested. For the strain corresponding to R. tropici, activation was successful, so genotypic extraction and phenotypic characterization were carried out.

The culture of the Rt strain has the classic morphology of the bacterial colony; small, whitish and mucous colonies (Figure 1). This colonial morphology is similar to the strain: *R. Tropic*, used in industry. The development of colonies on the surface of an agar allows the microbiologist to identify the bacteria because each species and bacterial strain has a characteristic shape and appearance.

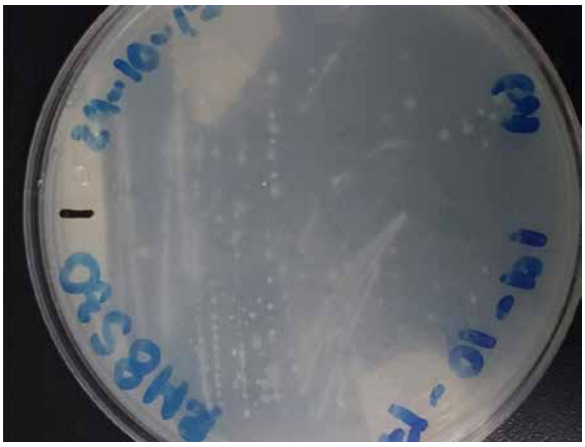

Figure 1: Cultivation of *Rhizobium tropici* in between YB.

The characteristics of the colonial morphology are summarized in Table 1, in which the microscopic morphology and biochemical characteristics can be visualized. *R. tropici* is a Gram negative bacillus that ferments glucose and lactose, therefore it lacks cytochrome oxidase.

Colony Characteristics	
Morphology	Result
Shape	Circular
Edge	Smooth
Elevation	Concave
Surface	Smooth
Size	Small
Color	Whitish
Consistency	Mucoid
Light transmission	Opaque
Reflection of light	Dull
Aspect	Dry
Microscopic morphology	Bacillus
Biochemical tests	Results
Gram stain	Negative
Oxide/Fermentative	Fermentative
Lactose fermentation	Positive
Oxidase	Negative
Catalase	Positive
Indol	Negative

Table 1: Biochemical characteristics, colonial and microscopic morphology of *Rhizobium tropic.*

The presumptive evaluation of a bacterium is based on the colonial morphology, for which the characteristics are taken into account: Elevation: Flat, low convex, convex, rough, crater-shaped and convex and concave, Edges: smooth, toothed, wavy, lobed, cremated, ciliated and branched (more common smooth), Surface: Smooth and Rough, Shape: Circular, wavy and ovulate, Size: punctate, small, medium and large, Light transmission: opaque or translucent, Light reflection: Matte and Shiny, Appearance: Wet and Dry, Consistency: Butyrose (shiny, wet and translucent), Mucoid (like mucus), Vitreous (opaque, dry) and dry (if hard), Pigment: Color (red, green, etc.) and smell. The RT strain, as can be seen in Figure 1 and the description in Table 1, the morphological and biochemical characteristics obtained coincided for the identification of bacteria of the same genus. It must be mentioned that few are the works in which the characteristics of the colonies are reported in detail, one of the most complete in this regard and consistent with the morphology of the RT strain, is the one published by Cuadro, Rubio and Santos (2009).

The recognition of the colonial characteristics made it possible to select the appropriate biochemical tests to determine useful characters in their identification, which allows to ensure their taxonomic location. The biochemical and microscopic characteristics of the RT strain colonies allowed to identify the genus and demonstrate that the culture is pure. To reinforce the identification, the similarity analysis corresponding to the partial sequence of the 16S rDNA was performed.

PCR OF THE 16s rDNA REGION

To avoid interference and therefore errors in a PCR reaction, it is important to consider, among other aspects, the DNA template strand. The purity and concentration is important in

the amplification process (Bolívar et al., 2014). In this sense, the genomic DNA obtained presented a purity in relation to: A_{260}/A_{280} 0.265/0.144= 1.84 and a concentration of 265 ng/uL. The genomic DNA corresponding to the RT, SRA1, AAA1 and RRJ1 samples can be visualized on the gel (Figure 2).

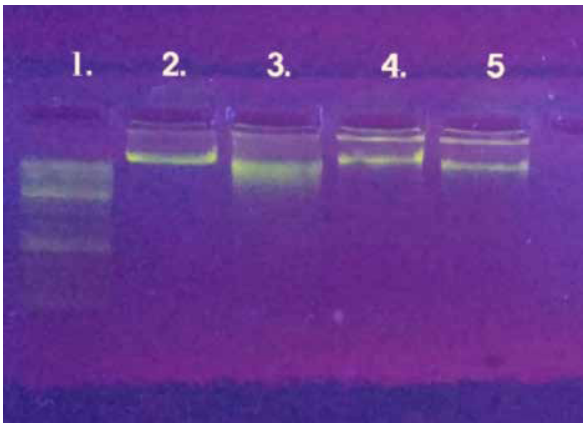

Figure 2. 1% Agarose electrophoresis, Molecular weight marker: 1. MPM, and genomic DNA lane strains: 2. RT, 3. SRA1, 4.AAA1 and 5. RRJ1.

The amplicons correspond to the expected fragment size of 1600 bp using the RD and FD primers (Figures 3). The SRA1 sample was performed in duplicate, increasing the amount of DNA twice, because in the image.

The obtained sequences were analyzed for similarity using BLAST in the Gen Bank database. Sequences with 99% identity were obtained for: *Pseudomonas azotoformans* in the reference sample (SRA1) and *Pseudomonas fluorecens* for the reference sample (AAA1). The two remaining sequences showed a similarity of 80% identity with Paracoccus in the reference sample (RRJ1), and *Sinorhizobium meliloti* for the strain (RH8530). However, a value below 90% indicates that it is a different organism (Table 2).

The strain with the SRA1 code according to the genetic analysis corresponds to: *Pseudomona azotoformans*. In 2016 Fang et al., determined that *P. azotoformans* is a bacterium that infects cereal grains, especially rice. Therefore, due to this characteristic, it is not an organism with possible use in the application for plant growth.

According to Ruiz in 2016, the bacterial strain under the code RRJ1 corresponds to *Bordetella parapertusis*, but in the sequencing carried out for this work, only 80% similarity with the genus Paracoccus was obtained, as can be seen in the table, the number of nucleotides is very small, so it would be convenient to repeat the PCR and sequence.

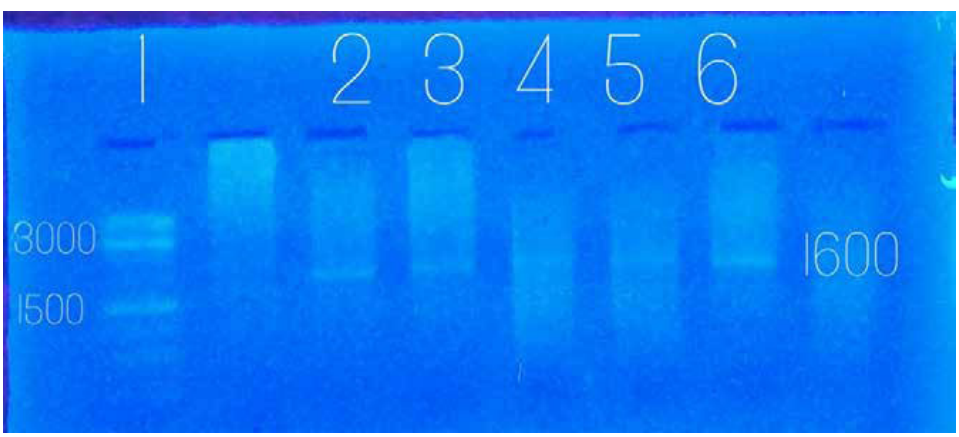

Figure 3. PCR products corresponding to 1600 bp strains: RT (2), SRA1 (3), AAA1 (4), RRJ1 (5) and SRA1' (6), with the RD and FD primers corresponding to region 16. srDNA.

Sample	First	pb	BLAST NCBI	IDENTITY (%)	CAPS	ID
SRA1	FD	746	*Pseudomonas azotomorfas*	99	7/783	HF572854.1
AAA1	FD	865	*Pseudomonas fluorescens*	99	2/876	MF838680.1
RRJ1	FD	101	*Paracoccus*	80	6/126	CP020447.1
RH8530	FD	609	*Sinorhizobim meliloti*	80	24/636	EI256451.1

Table 2: Pure sequence alignments.

The AAA1 strain according to the genetic analysis corresponds to: *Pseudomonas fluorescens* with 99% identity. In the work prepared by Pérez et. al., in 2015, describe *P. fluorescens* with the ability to infect plant tissues due to the fact that it has the rpoB gene, which functions as a natural pathogen controller in plants, inhibiting or increasing resistance to infections, which allows it to be classified as a BPCV. In another investigation RamanaThan et. to the. In 2002 they concluded that it has beneficial effects on crops, contributing to Nitrogen fixation, synthesis of phytohormones and promotion of root growth; thus protection against the growth of fungi on the plant such as Magnaporthe grisea. Other studies in 2014 conducted by Dey et. al and Dell'Amico et. al., in 2008, cited by Hayat et al 2010, found that P. fluorescens produces siderophores and indole-3 acetic acid, promotes nodulation and production in peanuts, and protects canola plants against the inhibitory effects of calcium.

The RT strain belonging to the collection of bacterial cultures of the FES Iztacala, has the classic morphology of the genus Rhizobium. When performing the genetic analysis and BLAST analysis, only an 80% identity coincidence was obtained with *Sinorhizobium meliloti*, this result indicates that it is a different organism.

However, *S. Meliloti* is also of great importance as it is a BPCV of the Fabaceae family, particularly alfalfa (Draghi et al., 2017; Santos et al; 2001).

It is important to highlight that the PCR products obtained were 1600 bp, however when performing the sequencing it was not possible to obtain the complete sequences, the probable causes are due to possible contamination with proteins or phenolic residues in the DNA extraction, DNA degradation, low concentration of the product due to degradation effect. It is important to highlight that in the sequencing of the gene that codes for the 16S rRNA, used for the taxonomic location of bacteria, the criteria used to determine the identification of a bacterium depend on the percentage of similarity of the sequences deposited in the GENBank when making the BLAST.

Those bacteria that have a similarity ≥99% are determined as taxonomic species, the percentage is between 95-98% is assured up to the genus level and < 95%, the taxonomic location was at the family level. There must be 99% identity to locate 90 to 98% species and genus. In the case of: *Pseudomonas fluorescens* AA1, identification is confirmed. The partially characterized bacterial strains will express genes with importance for plant development, so knowing their genomic sequence is expected to coincide with strains already known from the NCBI.

CONCLUSIONS

With the exception of the RRJ1 strain, it is confirmed that the strains used in this study and stored in the Collection of Bacterial Cultures of FES Iztacala UNAM (CCBIZTA) are bacteria belonging to the group of Plant Growth Promoting Bacteria (BPCV).

Molecular analysis is a usable tool for the study of biology that allows taxonomic classifications to be made with greater certainty and reliability in conjunction with classical microbiology techniques. Likewise, the fact of having the bacterial cultures preserved in a collection allows us to continue working on the laboratory and field characterization of the bacteria with the consequent advantage of better use.

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