Article **ECORFAN-Bolivia Journal**
December 2018 Vol.5 No.9 10-16

Molecular characterization of *Bacillus safensis* from cane rhizosphere

Caracterizacion molecular de *Bacillus safensis* a partir de rizosfera de caña

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Received: July 25, 2018; Accepted: November 20, 2018

Abstract

Sugar cane (Saccharum officinarum) is a very important crop for Mexico mainly in the municipalities of Tamaulipas Mante and González. 50% of the nutrients supplied are used by the plants, the rest being lost by leaching, causing serious effects to the environment. The use of bacteria specifically endophytes-diazotrophs, could contribute to economic and environmental sustainability, since they contribute to the restoration of soil conditions to obtain good results in the field. In the present work, the objective was to identify an isolate from the rhizosphere of the sugarcane crop, amplifying the 16S gene of the rRNA and its sequencing. The isolate sequence obtained was compared and made Blast in the NCBI database and was identified as *Bacillus safensis*, showing a 97% coverage and 93% similarity with several strains previously reported. It is important to molecularly characterize the beneficial microorganisms that could later be used as plant growth promoters (BPCV), because they contribute favorably to crops, especially if they are isolated and used in the same crop, such as sugarcane. South region of Tamaulipas.

Cane, Gene, Molecular

Resumen

La caña de azúcar (*Saccharum officinarum*) es un cultivo muy importante para Mexico principalmente en los municipios de Mante y González en Tamaulipas. El 50 % de los nutrientes suministrados son utilizados por las plantas, perdiéndose el resto por lixiviación, causando graves efectos al ambiente. El empleo de bacterias específicamente endófitas-diazotrofas, podría contribuir a la sustentabilidad económica y ambiental, ya que contribuyen al restablecimiento de las condiciones del suelo para la obtención de buenos resultados en el campo. En el presente trabajo tuvo como objetivo identificar un aislado a partir de rizosfera del cultivo de caña de azúcar, amplificando el gen del 16S del rRNA y su secuenciación. La secuencia del aislado obtenido se comparó y se hizo Blast en la base de datos del NCBI y fue identificada como *Bacillus safensis*, mostrando un 97 % de cobertura y 93 % de similitud con varias cepas reportadas previamente. Es importante caracterizar molecularmente a los microorganismos benéficos que podrían ser empleados posteriormente como promotores del crecimiento vegetal (BPCV), debido a que contribuyen favorablemente a los cultivos sobre todo si son aislados y utilizados en el mismo cultivo, como el de caña de azúcar de la región sur de Tamaulipas.

Caña, Gen, Molecular

Citation: SÁNCHEZ-VARELA, Alejandro, RODRÍGUEZ-LUNA, Isabel Cristina and GARCIA-OLIVARES, Jesus Gerardo. Molecular characterization of *Bacillus safensis* from cane rhizosphere. ECORFAN Journal-Bolivia. 2018. 5-9: 10-16.

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1. Introduction

Sugarcane (Saccharum officinarum) contains high concentrations of sucrose, currently it is a source of sugar worldwide (Martin, 2005) (*Rojas et al., 2015*). Also in Mexico it is a very important crop especially for the southern region of Mante and González, in Tamaulipas, where a hot and semi-dry extreme climate prevails, with an average annual temperature ranging from 22◦ C to 26◦ C, with rainfall that occur in summer, which reach 800 to 1000 millimeters and with an altitude of 80 meters above sea level, which favors this crop (Jimenez Cordova *et al*., 2004).

Due to the biology of the plant, the yield is decreasing, considering that cane cultivation presents high production costs related to applied nitrogen fertilization and that only 50% of the nutrients supplied are used by the plants, losing the rest by leaching, which causes an imbalance of soils and low availability of available nutrients for plants, which is why it is important to consider strategies to identify nutritional problems, not only to improve crop yields, but also to prevent the development of diseases, as well as reduce damage caused by insects or physiological damage (Vitousek *et al*., 1997) (Montealegre *et al*., 2018) (Rodríguez *et al*., 2018).

Therefore, the use of agrobiotechnological techniques that support the management of crops, including cultural practices, that provide the basis for the implementation of sustainable agriculture, for which the bacteria that promote plant growth are studied, which include bacteria that inhabit the rhizosphere, such as rhizobia and endophytic bacteria, which provide benefits to crops (Acemad and Kribet, 2014) (Peoples and Craswell, 1992) (Raymond *et al*., 2004) (Hernández *et al*., 2018) (Méndez-Úbeda *et al*., 2018). It is important the microbiological characterization of the isolates, such as knowing the specific nutritional requirements, their metabolic abilities, as well as their capacities to survive in different environments, However, it is necessary to complement this characterization with molecular methods, since in this last composition of the bacterium, the genes that encode enzymes or metabolites that provide resistance to pathogens are contained, with the ability to degrade pollutants in the environment or as nifH genes with the ability to fix nitrogen or other genes that encode other enzymes that solubilize other elements (Rojas *et al*., 2015).

(Stacey *et al*., 1992) (Chulia *et al*., 2018). On the other hand, in terms of molecular identification there are sequences conserved in bacteria such as the 16S rRNA, which is widely used to perform phylogenetic analysis, as well as for the identification of bacterial groups (Soares-Ramos *et al*., 2003) (Kim *et al*., 2014) (Valenzuela *et al*., 2015). In the present study, a rhizosphere bacterial isolate of sugarcane was characterized, by means of nifH genes, which codes for a nitrogenase enzyme involved in the fixation of nitrogen and this as a promoter of plant growth.

It was also identified by PCR amplifying the 16S rRNA gene and sequencing. This proposal for identification and molecular characterization is used as a tool, in order to complement the microbiological tests and as support in the agronomic area contributing to improve sugarcane crops in the southern region of Tamaulipas.

2. Methodology

Collection of samples

20 plants were collected at random from a sugar cane crop with everything and soil from the region near the city of Mante, Tamaulipas. These were transported to the Experimental Biotechnology laboratory of the Genomic Biotechnology Center of the National Polytechnic Institute, where 2 unique plants containing nodules were selected, since it is known that very likely they may contain beneficial bacteria associated with the rhizosphere. (Grinder *et al*., 2002).

Obtaining the isolated

0.5 cm pieces of cane roots were cut with a scalpel, the roots were washed with 3% sodium hypochlorite solution and then three times with sterile distilled water, each sample of each plant was identified as M1 and M2. To obtain plant growth promoting bacteria, specifically endophytes-diazotrophs. It was inoculated in semi solid medium WAT4C, to isolate nitrogen fixing bacteria at 25 ° C for 24 hours. From which the isolates were obtained being of the following way of M1 samples were obtained (7, 8 and 10) and of M2 they were sample (3, 4, 5, 6, and 11).

ISSN-On line: 2410-4191

Obtaining genomic DNA

Once there was growth, an isolated colony was resected in Luria Bertani broth at 25 ° C for 18 hours, then DNA extraction was performed, using the Wizard Genomic Cat. A1120 Kit. Which was made according to the instructions of the commercial case.
The quality and concentration of each of the samples of the isolates were determined by reading in a nanodrop 2000 and by electrophoresis in a 1% agarose gel. Electrophoresis was carried out at 80 Volts for 1 hour. Once the sliding time was over, the gel was visualized with UV light in a photodocument coupled with a Kodak Molecular Imaging Software program., (Version 5.0., Park West, New Haven).

PCR of nifH genes and the 16S rRNA gene

The nifH (nitrogenase enzyme) gene from the endophyte-diazotrophic isolates was amplified by PCR, and the 16S rRNA gene was carried out under the same conditions. The PCR was performed under standardized conditions in a Perkin Elmer Biosystems Gene Amp thermal cycler. PCR System 9700 (Singapore Norwalk). For visualization of the amplified products, an electrophoresis was performed to visualize the PCR products of the nifH gene of the isolates and the 16S gene of the rRNA. This is under the same conditions similar to that for genomic DNA.

Sequencing of the rRNA 16S gene

It is worth mentioning that only the identification of an isolate of M2 sample (3) was carried out and the PCR product of the 16S rRNA gene was then cleaned with an enzyme from ExoSAP-IT (USB Corporation 2000) and then the reaction of the sequencing was carried out according to the instructions of the BigDye Terminator V3.1 Cycle Sequencing Kit, Cat. 4311320 and runs on the automated sequencer AB 3130 (Applied Biosystems) of the Service Laboratory of the Genomic Biotechnology Center, of the Polytechnic Institute National Park in Reynosa Tamaulipas, Mexico. Once obtained the sequences in format. ab1, these were analyzed with the software package Chromas version 2.4.4 copyright 1998-2016 Technelysium Pty Ltd. They were then analyzed and compared in the BLAST Nucleotide Standard of the National Center for Biotechnology Information (NCBI).

For the elaboration of the dendrogram, the program CLC sequence viewer 8.0.0 was used. Text written inTimes New Roman No.12, single space.

3. Results

We obtained eight isolates from the rhizosphere of sugarcane of the two plants that were identified as M1 and M2, and its distribution was as follows, M1 three isolates were obtained (7, 8 and 10) and M2 were five isolated (3, 4, 5, 6 and 11) obtained as pure colonies, from the semi-solid medium WAT4C. The genomic DNA of each of the isolates was obtained, an electrophoresis was run and in figure 1, the bands representing their nucleic acids are shown

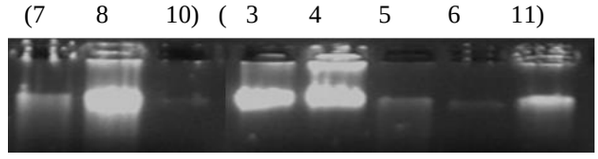

Figure 1 Image showing the genomic DNA bands of each one of the isolates obtained, for M1 (7, 8 and 10) and for M2 (3, 4, 5, 6 and 11)

The DNA concentration was obtained for most of the samples was found in a range of 50 to 100 ng of concentration. The amplification of the nifH gene was carried out, which codes for one of the structural subunits of the nitrogenase enzyme, key in the process of biological nitrogen fixation [4]. Figure 2 shows the PCR amplification products of the nifH gene, from the genomic DNA, of each of the isolates.

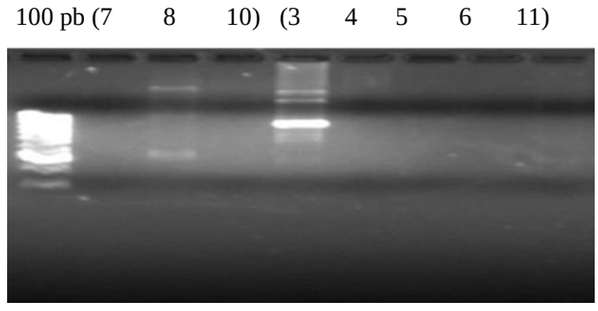

Figure 2 The order of left to right of the lanes is shown, Marker 100 bp of promega, lanes for M1 all negative and of M2 only the isolated one (3), it was positive by PCR for the nifH gene

Amplification of the expected nifH gene fragment of about 800 bp was obtained, at least for 1 sample. For M1 all were negative and for M2 the sample (3) was positive.

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Therefore, it was decided as a strategy to complete its characterization and identification, since only this isolate presented this characteristic.

Then the 16S rRNA gene was amplified by means of the Polymerase Chain Reaction (PCR), from the genomic DNA of each one of the isolates, the image was shown with the amplified products of the 16S rRNA in the Figure 3.

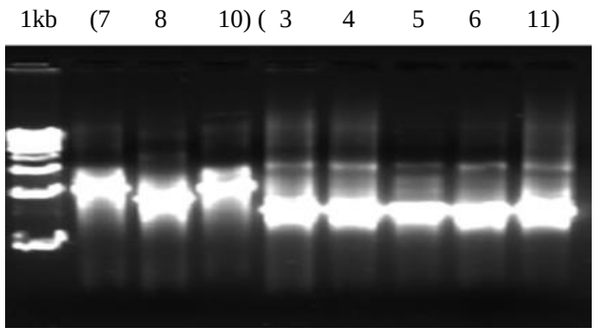

Figure 3 The order of left to right of the lanes is shown, Marker 1 kb of promising, lanes for M1 samples were obtained (7, 8 and 10) and of M2 were the samples (3, 4, 5, 6 y 11), positive by PCR for the 16S rRNA gene

All samples showed a fragment of 1500 bp, both M1 and M2. Therefore, the latter was selected, sequenced and analyzed from the 16S gene of the rRNA, showed a coverage percentage in its sequences of 97% and a percentage of identity of 93%, with those previously reported in the base of NCBI data. An alignment was performed with the Bacillus safensis strain JQ818355.1 previously reported in the genetic data bank of the National Center for Biotechnology Research (NCBI). In the following image you can see the Blast made in the NCBI database showed its coverage and identity for the 16S rRNA gene, Figure 4.

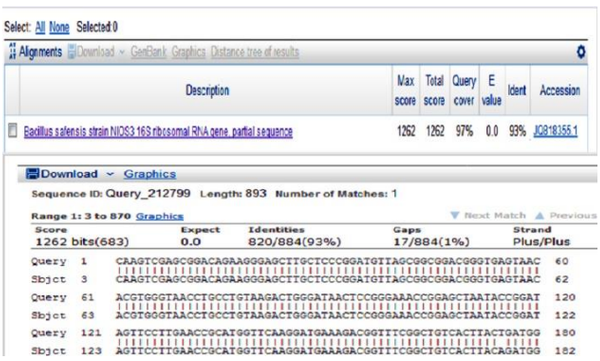

Figure 4 Image of the sequence obtained with those reported in the NCBI Blast, two showed around 93% identity for the 16S rRNA gene, showing few differences between their sequences, when performing a multi-alignment

After having made an alignment of the own sequence, with one reported by other authors in the NCBI, few variable regions were observed between both analyzed sequences, having this coincidence between their sequences. To confirm the genus and species in our work we proceeded to perform molecular analysis of the isolates obtaining different similarities within the genus Bacillus. With the application of molecular techniques and 16S amplification of the rRNA, the similarity of the bacteria was confirmed, presenting 99% in 6 of the samples and 2 of 100% of homology, based on the NCBI data.

Subsequently these sequences were submitted with the CLC program sequence viewer 8.0.0. Obtaining the genetic distances between the strains analyzed (Figure 5).

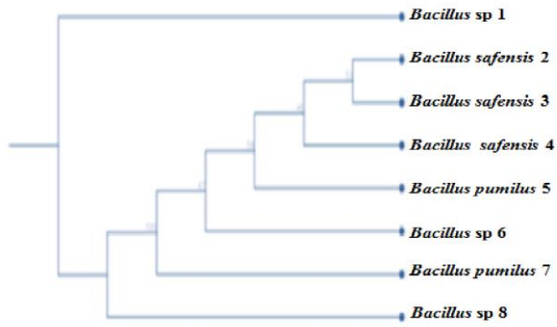

Figure 5 Dendogram obtained from the 16S rRNA gene sequences

4. Acknowledgement

To Instituto Politécnico Nacional, the Secretaria de Investigación y Posgrado of the Instituto Politécnico Nacional and to the Comisión de Operación y Fomento de Actividades Académicas.

5. Discussion

Biologically, nitrogen fixation is catalyzed by nitrogenogens, is a complex of enzymes that encode the nifD and nifK genes and dinitrogensase reductase subunit encoded by the nifH gene, the latter reduces N2 to NH3, leaving this element available for the plants to assimilate and grow more vigorous (Cleveland *et al*., 1999) (Gaby and Buckley, 2014) (Kim *et al*., 2014) (Valenzuela-González *et al*., 2015). For the nifH gene, at least 1 sample was positive.

ISSN-On line: 2410-4191

For M1 all were negative and for M2 sample 3. The fact of finding nifH genes in the isolates indicates that they may have the capacity to fix nitrogen. Therefore, the application of this bacterium could be feasible that could be able to metabolize nitrogen and provide it more assimilable for plants. It is worth mentioning that this isolate was applied to *B. safensis*, in another experimental work demonstrating to have a very good effect on the growth and development of a sugarcane crop in the south of Tamaulipas.

On the other hand, the use of molecular methods as strategies for the characterization of bacteria of biotechnological importance, could contribute enormously to the development of a sustainable agriculture. There are bacteria in various environments with the ability to fix nitrogen, because they have the nifH genes, very likely they can be applied in different crops, as breeders in production (Farnelid *et al*., 2011) (Montealegre *et al*., 2018) (Rodríguez *et al*., 2018). As well as some bacteria of the genus Bacillus sp produce proteases and other enzymes that inhibit and antagonize phytopathogenic fungi such as Fusarium sp, which produce rot in stems and roots of crops (Hernández *et al*., 2018) (Méndez-Úbeda *et al*., 2018).

There are currently databases of genes for nitrogen-fixing bacteria with which ecological and evolutionary studies are carried out (Gaby and Buckley, 2014), which will serve to understand the abilities and abilities of various bacteria and for the improvement of field crops (Hebert *et al*., 2003) (Martin, 2005) (Sidnei *et al*., 2003). On the other hand, the sample that was selected from the 16S rRNA for sequencing for identification purposes, showed a 97% coverage percentage in its sequences and a 93% identity percentage, which indicated that the molecular marker is reliable for the identification of the bacterium, most likely as B. safensis. Very similar results have been reported with the use of rRNA 16S, in the identification of bacteria by several authors (Soares *et al*., 2003) (Yarza *et al*., 2014). In addition, this molecular tool is completely standardized for the identification of existing bacteria in different environments (Grinder *et al*., 2002). As well as the relatively long size of the sequences of the 16S rRNA of 1500 bp of nucleotides minimizes the fluctuations and in the conservation of the sequences, which favors the precise alignment during the comparison of sequences (Velázquez *et al*., 2008).

In a more recent study revealed values of 98.65% similarity of the 16S rRNA gene can be used as a threshold for the differentiation of two species (Jimenez *et al*., 2004) (Vitousek *et al*., 1997) (Chulia *et al*., 2018). On the other hand, it has been established that a similarity between two 16S rRNA equal or less than 94.5%, 86.5%, 82.0% or 75.0% establishes the distinction of gender, family, order, class and edge, respectively (Velázquez *et al*., 2008). As noted, there are distinctions to the use of the 16S rRNA marker, depending on the specific use, so to be able to carry out more in-depth studies in the search for specific genes of the species identified as *B. safensis*, it is necessary to work with the particular sequences associated to characteristics of interest that could lay the foundations of later studies.

6. Conclusion

In this study we found the presence of nifH genes, from a sugarcane plant called M2, from where the isolate 3 came from.

Which was identified, by sequencing the 16S gene of rRNA as B. safensis. In addition, the genetic distance, based on their sequences, was obtained from our strain of B. safensis, compared with other strains of Bacillus sp, previously reported in the NCBI database. It is worth mentioning that this is the first time that this species of *B. safensis* has been reported, as an endophytic strain that improves the cultivation of sugarcane in the Northeast of Tamaulipas region. In addition, this proposal for identification and molecular characterization was used as a tool, in order to complement the microbiological tests and as support in the agronomic area contributing to improve sugarcane crops in the southern region of Tamaulipas.

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ISSN-On line: 2410-4191