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16S rRNA as an applied tool in the molecular characterization of genera and species of bacteria

ARNr 16S como herramienta aplicada en la caracterización molecular de géneros y especies de bacterias

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ABSTRACT

Bacterial identification is carried out by means of conventional methods based on biochemistry characteristics, since its realization and costs are easier to access. However, molecular identification allows us to know the true identity of the genus and the species. For this reason, the molecular identification of 24 strains of bacteria conserved in the Strain Bank of the Francisco de Paula Santander University, Campos Eliseos Experimental Center, was carried out, identified under macroscopic and microscopic phenotypic criteria was performed. Initially, the strains conserved in saline solution were reactivated and characterized macro and microscopically, then DNA extraction was carried out and PCR was performed to amplify the 16S rRNA; The samples were sent to be sequenced and the identity of each bacterium was known through BLAST. 11 strains were identified as *Bacillus cereus*; 1 strain as *Bacillus thurigiensis*; while 3 strains, such as *Bacillus pumilus*; 1 strain as *Bacillus amyloliquefaciens*; 4 strains, formed the group of *Bacillus subtilis*; and it is possible that there are divergent ramifications between *Bacillus* species in phylogenetic trees. Another group that was observed in the phylogenetic tree which correspond 1 strain to *Achromobacter xylosoxidans* and 1 strain to *Alcaligenes faecalis* respectively. Also another group of 2 strains were identified as *Stenotrophomonas maltophilia*. It is important to keep in mind that sometimes the 16S rRNA presents a low discrimination capacity for some genera and species due to recent divergences, it is necessary to complement the identification with the study of other genes.

Palabras clave:

bioinformática, *Bacillus sp. Stenotrophomonas maltophilia*, *Achromobacter xylosoxidans*.

RESUMEN

La identificación bacteriana se realiza por medio de métodos convencionales basados en las características bioquímicas, puesto que su realización y costos son de más fácil acceso. Sin embargo, la identificación molecular permite conocer la verdadera identidad del género y la especie. Por tal motivo se realizó la identificación molecular de 24 Cepas de bacterias conservadas en el Banco de Banco de cepas Universidad Francisco de Paula Santander, Centro Experimental Campos Elíseos, identificadas bajo criterios fenotípicos macroscópicos y microscópicos. Inicialmente se reactivaron las cepas conservadas en solución salina y se caracterizaron macro y microscópicamente, luego se realizó extracción de ADN y se procedió hacer PCR para amplificar el ARNr 16S; las muestras se enviaron a secuenciar y por medio de BLAST se conoció la identidad de cada bacteria. 11 cepas, se identificaron como *Bacillus cereus*; 1 cepa, como *Bacillus thurigiensis*; mientras que 3 cepas como *Bacillus pumilus*; 1 Cepa como *Bacillus amyloliquefaciens*; 4 cepas, conformaron el grupo de *Bacillus subtilis*; y es posible que existan ramificaciones divergentes entre especies de *Bacillus* en arboles filogenéticos. Otra agrupación que se observó en el arbol filogenéticosson las cepas que corresponden una a: *Achromobacter xylosoxidans* y otra cepa a Alcaligenes faecalis respectivamente. También otro grupo de dos cepas fueron identificadas como *Stenotrophomonas maltophilia*. Es importante tener en cuenta que en ocasiones el ARNr 16S presenta una baja capacidad de discriminación para algunos géneros y especies debido a recientes divergencias, es necesario complementar la identificación con el estudio de otros genes.

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Introduction

There are several methodologies used in research to analyze genetic diversity and identify an organism, but the use of each of these methods depends on the objective to be developed and achieved. Therefore, one of the useful techniques for bacterial identification has been the handling of biochemical tests, which are based on the presence or absence of an enzyme, a group of enzymes, or a complete metabolic pathway in one or more microorganisms [1], But these evaluation methods present different limitations, since they do not specify exactly which organism is being studied, nor are they sensitive to genetic mutations (they lead to different characteristics), similarities between different species, or the disparities between strains of the same species [2]. It is important to establish a molecular identification method that will allow us to accurately determine the genus and species of the microorganism, such as the bacteria conserved in the UFPS Strain Bank.

It is very common today to find studies carried out using molecular identification, analyzing specific gene sequences to verify the genus and even the bacterial species, since some do not change their function over time or have very few variations [1]. The 16S ribosomal DNA gene consists of a sequence of approximately 1500 bp, a size that generates sufficient polymorphism to establish differences [1]; A wide variety of genes have been used as molecular targets in taxonomic or phylogeny studies in different genera and different bacterial species, with 16S rRNA analysis being the initial marker and in many situations the sufficient marker to carry out a more precise identification [3]. The sequencing of this gene is a fast and efficient method in the identification of bacteria [4].
In bacterial taxonomy, 16S rRNA gene sequence analysis is the most widely used tool. This housekeeping marker is present in other microorganisms and all bacteria, as a family of multigene or operons [5]. It is also useful to establish phylogenetic relationships within the prokaryotic world, impacting the vision of evolution and, as a consequence, in bacterial classification and identification. For this reason, the fundamental treatises of bacteriology, the Bergey's Manual of Systematic Bacteriology and The Prokaryotes base their structuring of the prokaryotic world on the phylogenetic relationships established with this macromolecule [3]; acts as an efficient marker of evolution; also, it is of an adequate size to perform the analysis. The 16S rRNA, in addition to serving for the detection of bacteria, provides advantageous and rapid information on their identification and phylogeny through comparison with public databases that contain a large number of bacterial sequences. Thus, identification by 16S rRNA is based on its sequence [5].

In this work, the molecular identification of 24 bacterial strains conserved in the strain bank of the Universidad Francisco de Paula Santander, in the Campos Elíseos Experimental Center, was carried out, thus analyzing the sequence of the 16S rRNA gene and in this way, the true identity of each of them.

Materials and Methods

Samples and processing

The strains used were 24, as listed in Table I, preserved in saline solution and initially reactivated in nutrient broth (MERCK, Darmstadt, Germany) at 110 rpm of shaking and an incubation time of 37 °C for 14 hours. Then, it was taken with a loop, and sowing was done by exhaustion in a solid nutrient medium, with incubation conditions of 37 °C for 14 hours. Then chimes were made every 5 days for maintenance.

Gram stain and spore stain

A 1.5 ml microtube was taken containing the culture of each bacterium preserved by the saline method at 4 °C. It was sown in a liquid nutrient medium for 14 hours; then it was seeded by depletion in nutrient agar. Smear was made by taking a colony that was spread in a spiral fashion in a drop of 0.85% saline solution on a slide and then carefully flamed the slide fixed until dry. Subsequently, an excess violet crystal was added to the plate for 1 minute, it was washed with distilled water, and Lugol was added for 1 minute and it was washed with distilled water. Similarly, ketone alcohol was added for 30 seconds to wash with distilled water and finally, safranin was added for 1 minute and the plate was completely cleaned with distilled water to let it dry and observe in the microscope, for Gram staining [6].

Regarding the spore staining, the smear was prepared and fixed by flaming it carefully, then malachite green was added to the plate in a water bath for 5 minutes, then it was washed with distilled water, safranin was added for 1 minute and finally, it was washed with distilled

water allowing to dry under ambient conditions. It was then observed under the microscope, to verify the genus with which they were previously identified [6].

DNA extraction and amplification of the 16s ribosomal gene

For DNA extraction, the culture was left in a liquid medium from the night before. A new culture was obtained in a liquid nutrient medium (37 °C / 14h / 120rpm), in the early stationary phase (taking into account the optical density); 1 ml was placed in a microtube and DNA extraction was performed according to the instructions of the Wizzard Genomic DNA kit (Promega, CO).

The visualization of the DNA, to observe the integrity of the DNA, was performed by agarose gel electrophoresis at a concentration of 0.8% prepared in TBE 0.8X Buffer which was also used as a running buffer and Gel Red as an intercalating agent. The initial run was performed at 120 Volts for 3 minutes followed by 100 volts for 40 minutes and finally, the gel was visualized in the ChemiDoc ™ MP Imaging System photo-documentary from Bio-Rad.

Hot start DNA polymerase mix (Merck, DA) was used for amplification. The primers 27F (AGAGTTTGATCMTGGCTCAG) and 1492R (TACCTTGTTACGACTT) [7], universal primers that amplify the sequence of the 16S rRNA gene, were used. The thermal cycler was programmed as follows for PCR: 1 cycle at 94 °C for 5 min; 30 cycles (94 °C for 30 s, 57 °C for 45 s, and 72 °C for 90 s); and a final cycle at 72 °C for 7 min. The obtained amplicons were sent to Korea for sequencing.

Regarding the quantification of DNA and PCR products, once the genetic material had been obtained, the yield was determined by spectrophotometry, using the NanoDrop 2000 (Thermo Scientific ™). For concentration and purity, the ratio of absorbance at 260/280 nm was considered to estimate the purity of the DNA. The samples were considered concerning values ≥1.8 since these values are accepted as pure DNA and values lower than this indicate the presence of proteins. A second purity assessment was also taken into account using the 260/230 nm ratio, and the accepted values were in the range of 2.0-2.2 since a lower ratio indicates the presence of contaminants such as carbohydrates or phenol. [8].

For the visualization of the amplification products, they were separated by horizontal electrophoresis on 1.5% agarose gel, with an initial run of 120V for 1 minute to continue the run at 90V for 49 minutes, the gels were prepared in 1X TBE buffer (250mM Tris -HCl, 30mM boric acid and 42mM EDTA), also used as a running buffer. Bioline's 1Kb molecular weight marker. The PCR mix (Merck Millipore, 2012). Then 2 µl of the PCR product was added with 3 µl of 1X loading buffer (Buffer 6X, Buffer TE, Glycerol, Gel Red), for electrophoresis and it was visualized in the ChemiDoc ™ MP Imaging System (Bio-Rad) photo documenter..

Sequence analysis and construction of the phylogenetic tree

sequencing, by the SANGER method using the ABI 3730xl sequencer, then the sequences were edited using the DNA BASER version 4.3 program (heracle biosoft) in which consensus sequences were created and The results were analyzed using BLAST (Basic Local Alignment Search Tool) from the National Center for Biotechnology Information (www.ncbi.clm.gov/). The sequences were aligned using MUSCLE 3.8.31, the construction of the molecular phylogeny was performed using PhyML 3.1 / 3.0 aLRT, the SH-like test and visualization in the phylogenetic tree using the TreeDyn 198.3 program (http://www.phylogeny.fr/)

Conservation of strains

Preservation of the strains the preservation of the identified bacteria was carried out with three different protocols established in the Strains Bank of the UFPS, which were: Method in saline solution at 4 °C, Method in glycerol at -80 °C and in a Petri dish with agar nutritious in refrigeration.

Results and Discussion

Reactivation of the strains

According to the macroscopic and microscopic observation (evidence registered in the resume of the microorganisms of the Strain Bank), they were determined: 17 *Bacillus* sp. for: BLB001, BLB003, BLB004, BLB009, BLB010, BLB011, BLB012, BLB013, BLB014, BLB017, BLB018, BLB019, BLB022, BLB023, BLB024, BLB033, BLB037; 1 strain like *Arizona* sp.

(BLB007); 1 strain like *Azotobacter* sp. (BLB016); 1 strain like *Stenotrophomonas* sp. (BLB020); 2 strains of *Pseudomonas* sp. (BLB029, BLB030); 1 strain as: *Burkholderia* sp. (BLB031) and 1 strain of Flavobacterium sp. (BLB032). Identifying the genus, but not the species.

DNA extraction, quantification and amplification

Based on the above, the concentration and quality of the DNA obtained was within the range, showing a representative and acceptable amount of DNA at the purity level. The strain from which the most DNA was obtained was BLB013 with a value of 374.2 ng / µl, and from which the least DNA was obtained was 76.8 ng / µl.

In the next step, the integrity of the DNA was evaluated by 0.8% garose gel electrophoresis using a horizontal electrophoresis chamber under the conditions already described, and finally it was visualized in the photo documenter. Once the electrophoresis was run, it was possible to visualize clear defined bands in most of the samples, reflecting the presence of DNA without RNA residues, as can be seen in figure 1, thus: Lane 1: BLB001, Lane 2: BLB003, Lane 3: BLB004, Lane 4: BLB007, Lane 5: BLB009, Lane 6: BLB0010, Lane 7: BLB011, Lane 8: BLB0012, Lane 9: BLB013, Lane 10: BLB014, Lane 11: BLB016, Lane 12: BLB017, Lane 13: BLB018, Lane 14: BLB019, Lane 15: BLB20, Lane 16: BLB022, Lane 17: BLB023, Lane 18: BLB024, Lane 19: BLB029, Lane 20: BLB030,Lane 21: BLB031, Lane 22: BLB032, Lane 23: BLB033,Lane 24: BLB037, Lane 25: negative control, and in Lane 26: positive control.

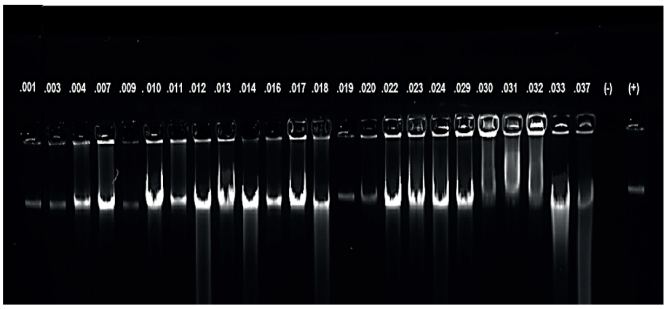

Figure 1 Visualization of the DNA of the 24 extracted strains, in 0.8% agarose gel with their controls.

The integrity of the PCR products obtained from each strain was also evaluated, the highest concentration was obtained for the BLB013 strain amplicon with 399.9 ng / µl and the lowest concentration obtained was for the BLB023 sample amplicon with 125.1 ng / µl and the purity according to the ratio A260 / 280 and A260 / 230 corresponded to values of approximately 1.5 - 2.4 for the strains of study; Likewise, in figure 2, the defined and specific bands are observed according to the amplified region of interest, as follows: Lane 1, 10 and 21: M. Molecular marker. Lane 2: BLB001, Lane 3: BLB003, Lane 4: BLB004, Lane 5: BLB007, Lane 6: BLB009, Lane 7: BLB0010, Lane 8: BLB011, Lane 9: BLB0012, Lane 10: M. Lane 11: BLB013, Lane 12: BLB014, Lane 13: BLB016, Lane 14: BLB017, Lane 15: BLB018, Lane 16: BLB019, Lane 17: BLB20, Lane 18: BLB022, Lane 19: BLB023, Lane 20: BLB024, Lane 22: BLB029, Lane 23: BLB030, Lane 24: BLB031, Lane 25: BLB032, Lane 26: BLB033, Lane 27: BLB037, Lane 28: negative control, and in Lane 29: positive control.

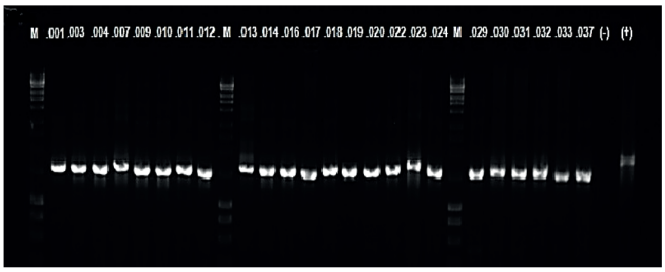

Figure 2. Amplified products of the 16S gene with primers 27F and 1492R, in 1.5% agarose gel; for the 24 bacterial strains with their respective controls.

Molecular identification by 16S rRNA gene

Fragments of approximately 1450 bp were amplified in all 24 bacteria. As mentioned by different studies [9], the most common group of primers used to amplify the entire length of the 16S is 27F / 1492r and was used in this work.

Sequencing was carried out using the forward and reverse primer, obtaining two sequences for each strain, for this reason, they were edited and a consensus sequence was created for each bacterium. The importance of this step is that the formation of false polymorphisms was avoided and it was also possible to achieve a greater length of the sequence when working with fragments of more than 700 bp [10]. After comparing the consensus sequences in the NCBI database, the related species were identified. In table I: the code of the strain, the genus and species with which it was identified molecularly, the accession number, and percentage of identity for each strain, which comprised a range between 85% to 100% of similarity

with access, which is also listed in table I. managing to identify the species, for 20 strains, of the genus *Bacillus*; 2 strains, of the genus *Stenotrophomonas*; 1 strain, of the genus *Achromobacter*; 1 strain, of the genus *Alcaligenes*. Corroborating that indeed not all strains matched their initial identification.

Table I. Molecular identification of bacteria by 16S rRNA sequencing. According to code, genus, and related species; relationship according to the database, access number, and percentage of similarity.

Code	Related genus/species according to rRNA 16s	List of databases	
		Access number	Percentage of similarity
BLB001	*Bacillus subtilis*	KU240496.1	99%
BLB003	*Bacillus cereus*	CP024655.1	100%
BLB004	*Bacillus subtilis*	KC955127.1	99%
BLB007	*Bacillus subtilis*	KX530950.1	99%
BLB009	*Bacillus cereus*	MG561368.1	100%
BLB010	*Bacillus thurigiensis*	KM280648.1	95%
BLB011	*Bacillus cereus*	CP016316.1	100%
BLB012	*Bacillus cereus*	MG516207.1	85%
BLB013	*Stenotrophomonas maltophilia*	MG905289.1	92%
BLB014	*Bacillus cereus*	MG516207.1	89%
BLB016	*Bacillus cereus*	CP008712.1	99%
BLB017	*Stenotrophomonas maltophilia*	CP011305.1	99%
BLB018	*Bacillus cereus*	KP027636.1	100%
BLB019	*Achromobacter xylosoxidans*	GQ409968.1	99%
BLB020	*Bacillus amyloliquefaciens*	KY662296.1	99%
BLB022	*Bacillus cereus*	MH130053.1	99%
BLB023	*Bacillus cereus*	MH041203.1	100%
BLB024	*Bacillus cereus*	KF641858.1	99%
BLB029	*Alcaligenes faecalis*	KX023228.1	99%
BLB030	*Bacillus pumilus*	KX783552.1	100%
BLB031	*Bacillus pumilus*	MF692772.1	99%
BLB032	*Bacillus pumilus*	CP027034.1	99%
BLB033	*Bacillus cereus*	CP024655.1	99%
BLB037	*Bacillus subtilis*	MH045986.1	100%

Furthermore, as reported by different authors [11], this gene is widely used as a molecular target in phylogenetic analysis and has a great advantage as it is available in public databases [12]. For this reason, molecular analyzes are considered to provide the most accurate information on phylogeny and evolution. According to Bou et al., 2011, the ranges of 95 to 99% of similarity are adequate to determine the genus, while similarity of ≥98.5 is accepted to define the species, being> 99% ideal. As can be seen in Table I, the values of similarity or identity obtained for the microorganisms analyzed.

Phylogenetic tree

To know the relationship between the identified microorganisms, a phylogenetic tree was elaborated using TreePhy (figure 3), and the MUSCLE software for the multiple alignments of the sequences. It was observed that the strains were grouped with high similarity according to the comparison of their sequences with the NCBI database. The

vast majority of the bacteria identified belong to the genus *Bacillus* with a large number of phenotypic and genetically diverse species. All *Bacillus* species are rod-shaped and produce highly resistant endospores ranging from facultative to strictly aerobic anaerobic [13]. In the results, it can be seen that the majority of bacteria were conformed into two large groups (I and II). The largest group being II, subdivided in turn into subgroup IIA, led by *Bacillus subtilis* (BLB030, BLB032, BLB031, BLB020, BLB004, BLB007, BLB001, BLB037), and the IIB subgroup made up of *Bacillus cereus* (BLB024, BLB011, BLB018, BLB003, BLB033, BLB010, BLB016, BLB022, BLB009, BLB023) and these strains are characterized by being difficult to discern using standard biochemical and microscopic schemes; The strains were identified in a macroscopic and microscopic way, however when carrying out the molecular characterization it was found that they did not correspond to said identification, according to Smith et al. in 1952, they recognized that the characteristics that each species has can be lost, resulting in strains that they are almost indistinguishable in terms of their phenotype but their genotypic characteristics remain stable. In the case of the group *Bacillus cereus* It is composed of 5 species that are *B. cereus* strictly, *B. thuringiensis*, *B. anthracis, B. mycoides,* and *B. weihenstephanensis* [14]; BLB010 was identified as *Bacillus Thurigiensis*, which is part of the group of *Bacillus cereus* [15] and BLB024, BLB011, BLB018, BLB003, BLB033, BLB010, BLB016, BLB022, BLB009, and the subgroup IIB1, BLB023; identified as *Bacillus cereus*, , corresponding to the bibliography described. However, they were also identified as *Bacillus cereus*, conforming the subgroup IIB2, the strains BLB012 and BLB014 and it is possible as presented here, there are divergent branches between species of *Bacillus* in phylogenetic trees [16], and that present greater genotypic characteristics that make them take a relatively greater distance than the others, but to know which characteristics it comprises, it is necessary to carry out other experiments that involve the presence of specific genes between certain genera and species that help to clarify the differences that may still present under the identity of *Bacillus cereus*, in addition, it must be taken into account that the species of *Bacillus* form multiple conglomerates without great distances that generate disproportion between the genders [17].

On the other hand, the IIA1 subgroup, which made up the majority of bacteria, and were identified as, *Bacillus pumilus*: 3 strains, (BLB030), (BLB032), y (BLB031); and in subgroup IIA 2, *Bacillus amyloliquefaciens* (BLB020), as *Bacillus subtilis*: (BLB004), (BLB007), (BLB001) and (BLB037) which are consistent with the large group of *Bacillus subtilis* (Connor, et al., 2010). This group is made up of closely related species with high genetic and / or biochemical similarities that correspond to *Bacillus amyloliquefaciens*, *B. atrophaeus*, *B. axarquiensis*, *B. licheniformis, B. malacitensis, B. mojavensis, B. pumilus, B. sonorensis, B. tequilensis, B. vallismortis* y *B. velezensis*. Said species, according to the bibliography, were initially named as "spectrum of *Bacillus subtilis*"[18], then they were grouped in "complex of species of Bacillus subtilis" [19] and finally they are referred to as "group Bacillus subtilis" [20]. Similarly, the degree of similarity between Bacillus subtilis and its closely related species is ≥99% for the 16S rRNA sequence level. [twenty-one]. Phenotypic and biochemical differentiation between closely related species is difficult to achieve (Rooney, Price, Ehrhardt, Swezey, & Bannan, 2009) but thanks to molecular tools such as those applied in the present work, they allow to differentiate them between species [22].

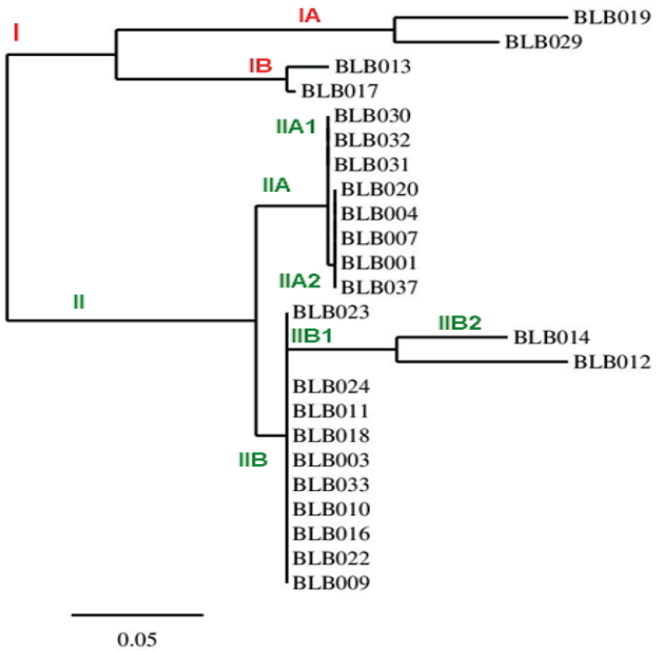

Figure 3. phylogenetic tree obtained from the construction of the molecular phylogeny PhyML 3.1 / 3.0 aLRT with the SH-like test and the visualization in the phylogenetic tree using the TreeDyn 198.3 software, for the 24 sequences analyzed, with the outgroup (*E. coli).*

The grouping observed in the phylogenetic tree as IA, is made up of only two strains: BLB019 and BLB029,

which correspond to *Achromobacter xylosoxidans* and *Alcaligenes faecalis* respectively. Although, *Achromobacter* is one of the 19 genera that belong to the *Alcaligenaceae* family and the *Betaproteobacteria* class [23][24][25], its *Achromobacter* taxonomy has been closely intertwined with the genus *Alcaligenes* [26]; several species of *Alcaligenes* have been reclassified as *Achromobacter*. Thus, *Achromobacter* comprises 15 species: *A. xylosoxidans*, *A. ruhlandii* [26]; *A. piechaudiin* [26]. *A.* [27]; *A. insolitus* [27]; *A. spanius* [25] [27]; *A. marplatensis* [28]; *A. animicus; A. mucicolens*; *A. pulmonis*; and *A. spiritinus* [29]. In addition to the fact that recently, four new species have been described: *A. insuavis* sp. *A. aegrifaciens sp.* A. *anxifer sp.*, And *A. dolens* [30].

In the same sense, it was observed that the IIB2 subgroup constituted by the strains BLB013 and BLB017, identified as *Stenotrophomonas maltophilia*; A microorganism that has had different names, it was isolated for the first time in 1943 and was named Bacterium booker. Later it was renamed *Pseudomonas maltophilia* [31] and later as *Xanthomonas maltophilia* [32]. Finally, due to advances in molecular characterization, it was named *Stenotrophomonas maltophilia* with its own genus name [33]. This genus belongs to the c-proteobacteria and includes ten species: *Sten. Maltophilia, Stenotrophomonas nitritireducens* [34], *Stenotrophomonas acidaminiphila, Stenotrophomonas rhizophila* [35], *Stenotrophomonas koreensis* [36], *Stenotrophomonas humi* and *Stenotrophomonas terrae* [37], *Stenotteinrophomonas* 38].

It is important to bear in mind that sometimes the 16S rRNA has a low discrimination capacity for some genera and species due to recent divergences, it is necessary to complement the identification with the study of other genes. This is the case with different species of the genera *Bacillus (B. cereus* y *B. thuringiensis; B. globisporus* y *B. psychrophilus)*, in *Brucella, Achromobacter, Strenotrophomonas* and *Actinomyces*, In the complex *Acinetobacter baumannii-A. calcoaceticus*, mycobacteria and in the family *Enterobacteriaceae* (especially in *Enterobacter* and *Pantoea*) (Bou, Fernández, Garcia, Sáenz, & Valdezcate, Métodos de identificación bacteriana en el laboratorio de microbiología, 2011), for this reason, the position occupied by strains BLB012 and BLB014 in the phylogenetic tree. Many times the amplification of the 16S region does not provide conclusive results and the 23S region can also be amplified, widely used in the identification of *Stenotrophomonas maltophilia* [40], and currently used as an auxiliary method for taxonomic and phylogenetic purposes.

Conclusions

The bacterial identification provided by the 16S rRNA analysis, made it possible to resolve the identifications as follows: 5 strains identified as *Bacillus subtilis*; 11 strains as, *Bacillus cereus*; 1 strain, *Bacillus thurigensis*; 2 strains, as S*tenotrophomonas maltophilia*; 1 strain as *Achromobacter xilosoxidan*; 3 strains as, *Bacillus pumilus*, and 1 strain as *Alcaligenes faecalis*.

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