MOLECULAR CHARACTERIZATION OF *Bacillus subtilis* GROUP STRAINS ISOLATED FROM AN IMMUNOBIOLOGICAL PHARMACEUTICAL FACILITY

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ABSTRACT

In the pharmaceutical industry, the isolation of species that have biotechnological potential have been reported, as species from the *Bacillus subtilis* group. Its potential applications include biotechnology, agriculture, medical fields, among others. The aim of this study was to characterize strains previously identified as *Bacillus subtilis* group by molecular techniques. Fifty strains isolated from an immunobiologicals pharmaceutical facility previously identified as *Bacillus subtilis* group by phenotypical techniques were selected and characterized by partial 16S rRNA sequencing and two species-specific PCR methods targeting *aroE* and *pycA* genes. *In silico* analysis was performed to verify the specificity of *aroE* and *pycA* primers. Partial 16S rRNA sequencing identified all strains at genus level, with 16 possible species. Twenty-nine (58.0%) strains were positive for *aroE* and *pycA* PCR. *In silico* analysis showed the specificity of *aroE* primers, but *pycA* PCR predicted the fragment for two *Bacillus spizizenii* strains. The findings suggest that partial 16S rRNA gene sequencing alone is insufficient for species differentiation within the *Bacillus subtilis* group. However, PCR targeting the *aroE* gene proves satisfactory for *B. subtilis* identification. Further, these strains will be deposited in a Culture Collection and can be available for studies to contribute to the Brazilian Health Industrial Complex.

Keywords: *Bacillus subtilis*. 16S rRNA. Identification. Pharmaceutical industry.

1 INTRODUCTION

Microbiological control is one of the key points in the pharmaceutical industry to guarantee quality and safety of the products [1]. Quality control methods in laboratories in pharmaceutical industries must be capable of detecting and identifying contaminants from various sources, including production environments. The identification of isolates at the species level and possibly at the subspecies level can be necessary [1,2].

On a global scale, data on microorganism strains from the pharmaceutical industry are still scarce. Previous studies have shown that *Bacillus* and related genera are among the most common contaminants of these environments [3,4,5]. However, difficulties in identifying the species of these groups with phenotypical methods, like VITEK®2, and proteotypic, as Matrix-Assisted Laser Desorption Ionization–Time of Flight/Mass Spectrometry (MALDI-TOF MS), have already been described [3,4]. In this cases, genotyping methods including 16S rRNA and/or specie-specific genes sequencing can be applied to identify these isolates.

Advances in molecular techniques have led to periodic reviews, making it possible to realize that some species share close genetic and ecological relationships, making it difficult to identify them at the species level. An example of this is *Bacillus subtilis*, which shares genetic and phenotypic similarities with its close relatives. *Bacillus amyloliquefaciens* is part of the *B. subtilis* group, i.e. they are closely related, which has led to a growing need to identify and typify these microorganisms beyond the genus level, contributing to the discovery of their biotechnological properties [6].

B. subtilis is a Gram-positive, rod-shaped bacterium. It forms endospores and is well adapted to adverse environmental conditions through robust response mechanisms to extreme temperatures, limited nutrition, desiccation, and exposure to toxins. They are widely present in various environments around the world, such as soil, plants, and the digestive tracts of animals, showing great tolerance to environmental stress [6]. With great scientific importance, it is one of the best-explored bacteria and considered one of the most popular microorganisms in microbiology and industry. Its potential applications include biotechnology, agriculture, medical fields, among others. In green microbiology, *B. subtilis* contributes to the study of ecological processes and the advancement of sustainability practices. Some factors contribute to these microorganisms having great biotechnological potential, such as their genetic accessibility, metabolic versatility, stress tolerance, role in sustainable bioproduction, environmental bioremediation, plant-microbe interactions, and ability to produce enzymes and biofuels [7].

For the commercial use of *B. subtilis* it is necessary to ensure the specific isolation of this species from other related species to obtain it in pure form. *B. subtilis* is generally considered a safe bacterium due to its history of use in the food industry, through fermented foods, probiotics, and industrially important enzymes such as xylanase, cellulose and pectinase. From these conventional applications of *B. subtilis,* many innovative applications have emerged in various areas of technology such as the generation of stable and recyclable enzymes, synthetic biology, drug delivery and material sciences. Some examples of applications of *B. subtilis* spores are, enzyme expression and delivery platforms, expression and delivery of mucosal vaccines, versatile and stable platforms to produce nanobodies (single domain antibodies), cancer drug delivery vehicles, metal ion capture and self-healing of concrete, among others. All of these show the use of spores to address global difficulties such as food shortages, environmental protection, and healthcare [8].

Due to the potential biotechnological application of bacterial species that can be found in pharmaceutical environments, the aim of this study was to characterize *Bacillus subtilis* group strains by molecular techniques.

2 MATERIAL & METHODS

One hundred and twenty-nine strains isolated from different types of samples from the production chain of immunobiologicals from 2016 to 2022, previously identified by VITEK®2 (bioMérieux, France) as *Bacillus subtilis* group were evaluated. These strains were further analyzed by MALDI Biotyper® (Bruker, USA) [9]. The strains identified as belonging to the *Bacillus subtilis* group (n=50) were selected for molecular characterization by partial 16S rRNA sequencing and two species-specific PCR methods.

The strains were cryopreserved in skim milk (Difco, USA) with glycerol (Merck, USA) at 30%, and stored in an ultrafreezer at ≤ -70 ºC. For analysis, the strains were grown on sheep's blood agar (ASC) or Tryptone Soy Agar (TSA) and incubated at 30-35°C for 24-48 h.

The partial 16S rRNA gene sequencing of the strains was performed using the MicroSEQ™ 500 16S rDNA Bacterial Identification System (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. The identification results were obtained from the website https://www.ezbiocloud.net/ (database update: 2023.08.23) [10]. All sequences were deposited at https://www.ncbi.nlm.nih.gov/. Only species that had an identification percentage ≥ 98.7 % were classified as valid [10]. A phylogenetic analysis was carried out by aligning the sequences using the BioEdit [11]. MEGA 11, software version 11.0.13 [12], was used to construct a Neighbor-joining phylogenetic tree, employing the Kimura-2 parameter model with branching support based on 1,000 bootstrap replicates.

Species-specific primers were used to differentiate *B. subtilis* from other *Bacillus* species, targeting the two genes, shikimate dehydrogenase (*aroE*) and pyruvate carboxylase (*pycA*) [13]. The genomic DNA of the 50 strains was extracted using the DNeasy tissue kit (Qiagen, Germany). The DNA concentration was measured using NanoDrop 2000c Spectrophotometer (Thermo Scientific, USA) and the purity was checked by agarose gel electrophoresis. In order to verify the primers specificity, an *In silico* analysis [14] was performed with the *aroE* and *pycA* primers with the 8 *Bacillus* spp. strains sequences available at the software database (last access 06/04/2024).

3 RESULTS & DISCUSSION

The 50 *Bacillus subtilis* group strains submitted to partial 16S rRNA gene sequencing provided fragments ranging from 494 to 540 base pairs (bp). Analysis in the EzBioCloud database identified all strains at genus level, with 16 possible species, showing that the partial 16S rRNA gene sequencing cannot differentiate the closely related strains of the *Bacillus subtilis* group. The Neighbor-joining tree is shown in Figure 1.

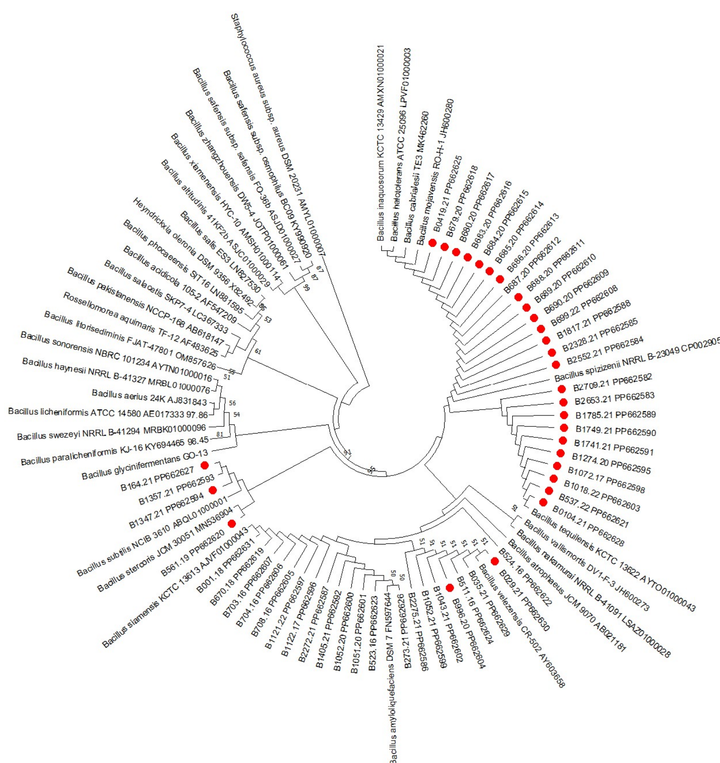

Figure 1. Neighbor-joining tree based on partial sequences of the 16S rRNA gene (477 bp) showing the phylogenetic position of the 50 strains with the closest species of the genera *Bacillus*. *Staphylococcus aureus* DSM 20231 was used as an outgroup. Numbers at nodes indicate the percentage of 1,000 boot-strap replicates. The scale bar represents 0,01 substitutions per nucleotide position. The GenBank accession number is provided in parentheses. Strains that presented positive results for PCR targeting *aroE* and *pycA* genes are marked in red.

Due to the proximity shared between some species, it was necessary to sequence other more specific genes from the studied group due to the great difficulty in identifying them at the species level. According to Lee et al. [13], the *pycA* and *aroE* genes were selected for the discrimination of *B. subtilis* due to their high number of polymorphic sites and because they present less homology between them than the other housekeeping genes described in Mullti-locus sequence type (MLST) scheme, proving to be an alternative to conventional methods for the detection and identification of *B. subtilis* [13].

As a result of the PCR fragments specifying the *B. subtilis* species of the *pycA* and *aroE* genes, from the 50 strains, 29 (58.0%) amplified the fragment of both genes. These strains were clustered in the clade with *B. inaquosorum*, *B. halotolerans*, *B. cabrialesii*, *B. mojavensis*, *B. spizizenii*, *B. tequilensis*, *B. vallismortis*, *B. nakamurai*, *B. atrophaeus*, *B. velezensis*, *B. amyloliquefacies*, *B. siamensis*, *B. stercoris* and *B. subtilis* species (Figure 1).

After *In silico* analysis, *aroE* primers amplified the 278 bp fragment for 11 strains identified in the database as: 54 - *Bacillus* sp. JS, 55 - *Bacillus subtilis*, 56 - *Bacillus subtilis* BEST7613 DNA, 57 - *Bacillus subtilis* BSn5, 58 - *Bacillus subtilis* PY79, 59 - *Bacillus subtilis* QB928, 60 - *Bacillus subtilis* XF-1, 61 - *Bacillus subtilis* subsp. *spizizenii* TU-B-10, 62 - *Bacillus subtilis* subsp. *spizizenii* str. W23, 63 – *Bacillus subtilis* (previously classified as *Bacillus subtilis* subsp. *subtilis*) 6051-HGW, 64 - *Bacillus subtilis* (previously classified as *Bacillus subtilis* subsp. *subtilis*) RO-NN-1, 65 - *Bacillus subtilis* (previously classified as *Bacillus subtilis* subsp. *subtilis*) str. BAB-1, and 66 - *Bacillus subtilis* (previously classified as *Bacillus subtilis* subsp. *subtilis* str. BSP1). *pycA* primers amplified the 233 bp fragment for 13 strains identified in the database, all the 11 cited above, plus the two strains: 61 - *Bacillus spizizenii* (previously classified as *Bacillus subtilis* subsp. *spizizenii*) TU-B-10 and 62 - *Bacillus spizizenii* (previously classified as *Bacillus subtilis* subsp. *spizizenii*) str. W23. These results indicate that PCR targeting *pycA* gene is not specific for *B. subtilis* species, since it can be amplified in *Bacillus spizizenii* species*. Bacillus spizizenii* was previously classified as a subspecies of *B. subtilis*, but it was further reclassified as a new species of the genus [15].

4 CONCLUSION

The partial 16S rRNA gene sequencing was not sufficient to differentiate the species belong to *B. subtilis* group. The PCR targeting *aroE* gene was considered satisfactory for *B. subtilis* species identification, since it amplified only strains of this species after *In silico* analysis. Further, these strains will be deposited at the Coleção de Bactérias do Ambiente e Saúde (CBAS) hosted at Fiocruz (www.cbas.fiocruz.br). So, this strains with biotechnological applications can be available to be required by the scientific community to further studies in order to contribute to the Brazilian Health Industrial Complex.

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