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MICROBIOLOGY

Genetic fingerprint and diversity evaluation of halophilic *Bacillus* species by RAPD-PCR

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Abstract: Random amplified polymorphic DNA-PCR (RAPD-PCR) is a technique successfully used to generate characteristic fingerprints of different bacteria. *Bacillus* is a genus that includes heterogeneous species, thus a combination of different techniques is essential for their identification. Here we used RAPD-PCR methodology to distinguish among genetically similar strains and to evaluate the genetic diversity of *Bacillus* species from the Salar del Hombre Muerto, in the Northwest of Argentina. The RAPD-PCR used allowed obtaining different amplification profiles for each *Bacillus* species and strains. By comparing the fingerprint profiles, we could observe that some of the salt flat isolates showed similar profiles than identified strains. As expected, the bacilli group isolated revealed a wide heterogeneity. RAPD-PCR was found to be a quick and reliable technique to evaluate the diversity of *Bacillus* strain and was successfully applied to characterize the genetic diversity present in the Salar del Hombre Muerto.

Key words: Random Amplified Polymorphism DNA-PCR, Halophiles, molecular characterization, biodiversity, polymorphic *Bacillus*, genetic characterization.

INTRODUCTION

The Salar del Hombre Muerto is in the province of Catamarca, northwest of Argentina. It is a typical high-altitude salt flat in which rising groundwater is a saturated brine that forms salt deposits when the water is evaporated (Martínez et al. 2019).

Despite the accumulated salts, which limit nutrient absorption and reduce water availability, some microorganisms known as halophiles, can inhabit and grow under this condition (Ma et al. 2010). As these microorganisms have in general extraordinary activities, they have become the search engine of many work teams (Ma et al. 2010, Sahay et al. 2012, Gupta et al. 2016).

Methods like 16S rRNA gene sequencing and 16S-23S intergenic region sequencing are widely used for the identification of microorganisms in a variety of environments. Other genes like *gyrA*, *recA* and *dnaJ* have been employed when high homology for 16S rDNA was present in some microorganisms (De Clerck et al. 2004, Rodríguez et al. 2007, Shah et al. 2007). Despite all the efforts invested, some microorganisms are indistinguishable even using those sequencing methodologies. This is particularly true for bacteria from *Bacillus*, a large and heterogeneous genus including many species, where combinations of different techniques are essential for their identification (Kim & Park 2009). The random amplified polymorphic DNA-PCR technique (RAPD-PCR) has been proposed initially to detect polymorphisms in several organisms like prokaryotic, fungi and plants (Williams et al. 1990). It was successfully used to generate characteristic fingerprints for different bacterial strains and its power resides

that can discriminate at strain level compared with other PCR-based techniques (Kumar et al. 2010). The aims of this study were to use RAPD-PCR methodology to distinguish between genetically similar strains and to evaluate the genetic diversity of *Bacillus* isolates present in the Salar del Hombre Muerto. The technique proved to be an economical and easy-to-use tool to distinguish between different isolates. This could be very useful as a first screening in laboratories where large numbers of isolations are made saving sequencing costs.

MATERIALS AND METHODS

Bacillus strains and DNA isolation

Fourteen *Bacillus* and one *Micrococcus* strains (Table I) were isolated from soil and water samples from the Salar del Hombre Muerto and previously identified by sequencing 16S ribosomal DNA (Martínez et al. 2019). In addition, five unknown strains isolated from the same salt flat were included in the study. Also, pure cultures of two *B. subtilis* strains, two of *B. licheniformis,* and one of *B. thuringiensis* (Sabaté et al. 2009, Torres et al. 2015) (Table I), were used to evaluate the efficiency of the S30 RAPD primer (5'-GTGATCGCAG-3') to generate differential band patterns. For RAPD-PCR, DNA extraction was performed as described by Pospiech & Neumann (1995). DNA integrity and purity were checked by 1.2 % agarose gel electrophoresis and quantified using an UV spectrophotometer (Biotraza 752 Spectrophotometer UV-Visible).

RAPD-PCR analysis

Genomic DNA extracted from pure culture of *Bacillus* strains was subjected to RAPD-PCR technique using the S30 primer, previously described to rapidly identify *Bacillus* species in fermented food (Kwon et al. 2009 and Lee et al. 2011). RAPD-PCR assays were carried out in 25 µl reaction volume containing a final concentration of 1X KAPA Taq buffer (Biosystem), 0.2 µM of dNTP, 0.4 µM of S30 primer, 3 U KAPA Taq DNA polymerase, and 100 ng of template DNA. PCR was performed using GeneAmp 9600 PCR system (Perkin Elmer, Applied Biosystem, USA) and PCR conditions were: 94 °C for 5 min, 40 cycles of 94 °C for 15 s, 35.5 °C for 15 s, and 72 °C for 2 min, and final extension at 72 °C for 4 min (Kwon et al. 2009). The reproducibility was evaluated amplifying each isolate three times and running each DNA fragment for duplicate.

The presence and absence of RAPD-PCR products were recorded and assembled in a data matrix. The Dice similarity coefficient was used to estimate the genetic similarity (Dice 1945). A dendrogram was generated from a similarity matrix using the Unweighted Pair Group Method with the Arithmetic Mean (UPGMA) algorithm using NTSYS-pc version 2.11 software (Rohlf 2002). The cophenetic correlation coefficient (CCC) was calculated as suggested by Sneath & Sokal (1973).

RESULTS AND DISCUSSION

Band profile for reference strains

The RAPD-PCR of *Bacillus* strains using the S30 primer, allowed obtaining different amplification profiles for each specie and strain, with amplification products displayed between 0.5 and 2.5 Kb (Figure 1). The RAPD-PCR products observed for *Bacillus subtilis* strain C4 were 0.5, 1, and another band near 2 Kb (Figure 1). Nevertheless, for *Bacillus subtilis* Mori2 we could observe 1 and 1.5 kb products. Both strains were previously described as presenting different activities; favoring high honey-bee production and reducing the prevalence of bee diseases (Sabaté et al. 2009, 2013). Probably the different fingerprints observed for these two bacilli could be due to a different genetic base, which was

Table I. Bacterial strains used for the genetic fingerprinting and diversity evaluation. Isolates used as reference strains are in bold.

Species	Strain	Accession number	Reference
Bacillus subtilis* subsp. *subtilis	Mori2	16S EU195329 Gyr HQ828989	Sabaté et al. 2012
Bacillus subtilis	C4	16S EU195328 Gyr HQ828992	Sabaté et al. 2009
Bacillus licheniformis	B63	16S Not determined (ND) Gyr KP893757	*
Bacillus licheniformis	B6	16S KP776730 Gyr ND	*
Bacillus thuringiensis	kurstaki HD-1	CP004870	**
Bacillus sp.	HA120a	MF990750	Martínez et al. 2019
Bacillus sp	HA120b	MF990751	Martínez et al. 2019
Bacillus sp.	HA120c	MF990752	Martínez et al. 2019
Bacillus sp.	FAMB1	MF990749	Martínez et al. 2019
Bacillus sp.	SX139	MF990767	Martínez et al. 2019
Bacillus sp.	SA32	MF990757	Martínez et al. 2019
Bacillus sp.	SA35	MF990758	Martínez et al. 2019
Bacillus sp.	SA39	MF990759	Martínez et al. 2019
Bacillus sp.	SA313	MF990762	Martínez et al. 2019
Bacillus sp.	SA314	MF990774	Martínez et al. 2019
Bacillus sp.	V2	MF990768	Martínez et al. 2019
Bacillus sp.	V3a	MF990769	Martínez et al. 2019
Bacillus sp.	V3B	MF990770	Martínez et al. 2019
Bacillus sp.	V10	MF990772	Martínez et al. 2019
Micrococcus sp.	SX120	MF990765	Martínez et al. 2019
Unknown	FAMB4	Uncharacterized	Martínez et al. 2019
Unknown	SX39	Uncharacterized	Martínez et al. 2019
Unknown	SX310	Uncharacterized	Martínez et al. 2019
Unknown	HX119	Uncharacterized	Martínez et al. 2019
Unknown	HX127	Uncharacterized	Martínez et al. 2019

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reflected in the RAPD-PCR profile. However, a more thorough study would be necessary to test this hypothesis.

For *B. licheniformis* we could observe in both cases (B63 and B6) the same band profile, 0.5 and 1.5 Kb bands, although the intensity of the bands was different. These two bands were previously reported for *B. licheniformis* ATCC 14580 (Kwon et al. 2009). For *B. thuringiensis* serovar kurstaki HD-1, only one product near 0.6 Kb was revealed. The S30 primer was able to generate a repetitive and unique polymorphism for the isolates tested been reliable and strain specific.

Band profile for environmental strains

When DNA extracted from the salt flat isolates was amplified, RAPD-PCR analysis revealed differences in banding pattern for most of the selected isolates (Figure 2). This is not an unexpected outcome; it was reported previously that bacilli´s activities usually correspond to their specific genotype (Freitas et al. 2008). In our case, all isolates were selected based their distinct physiological and morphological characteristics (Martínez et al. 2019).

The fingerprinting analysis showed some of the *Bacillus* isolates like SA35 to *B. subtilis* and HA120b and V2 to *B. thuringiensis* with a band of 0.6 Kb (Figure 2). Also, we could infer that some isolates were similar strains based in their fingerprinting, like SA313 and V10 or HA120a and HA120c, as they presented the same band patterns. Isolate SA314 was identified as *Brevibacterium* by sequencing its 16S RNA; however, by the RAPD-PCR fingerprint it was similar to *B. subtilis*. In a previous report it was suggested that *Brevibacterium halotolerans* DSM8802 should be re-classified into the *Bacillus subtilis* group (Ben-Gad & Gerchman 2017).

In a previous work, we isolated and characterized a diverse group of microorganisms (Martínez et al. 2019). Based in the 16S ribosomal

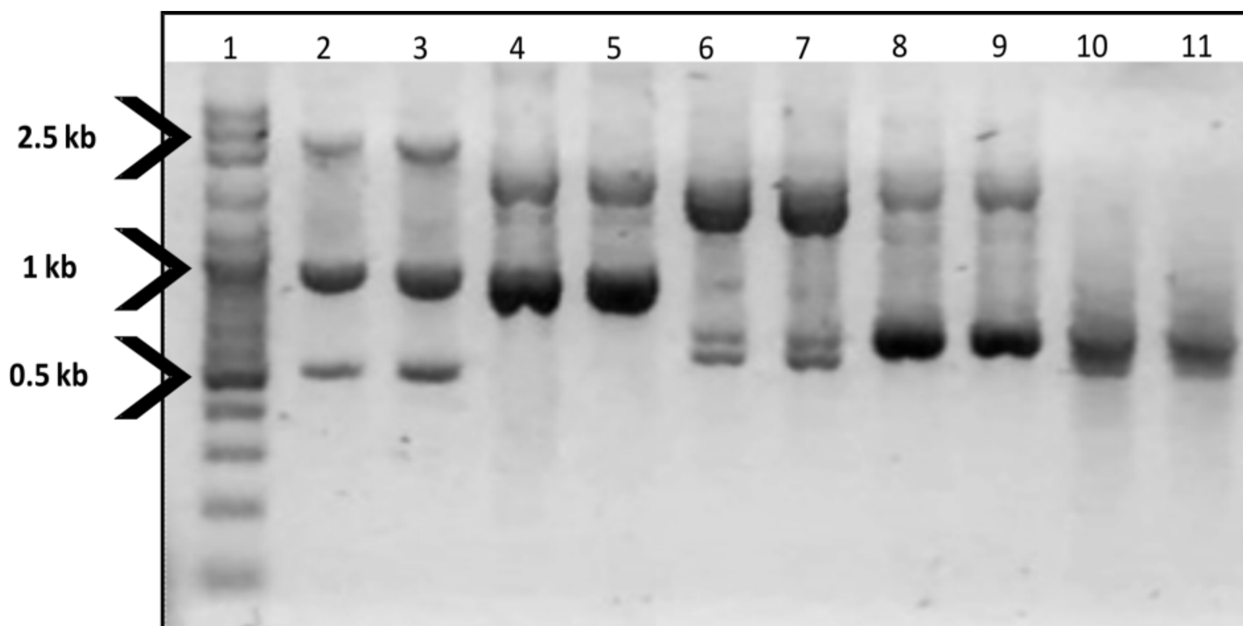

Figure 1. **RAPD-PCR profile of *Bacillus* reference strains. Electrophoresis on 2% agarose gel of DNA extracted from pure cultures of different *Bacillus* sp. DNA amplification was carried out using S30 primer. Lane 1: 100 marker (Genbiotech, Argentina); lanes 2-3: *B. subtilis* C4; lanes 4-5: *B. subtilis* subsp. *subtilis* Mori2; lanes 6-7: *B. licheniformis* B63; lanes 8-9: *B. licheniformis* B6, and lanes 10-11: *B. thuringiensis* serovar kurstaki HD-1.**

DNA identification, the isolates fell into one of the following categories: *B. pumilus*, *B. atrophaeus*, *B. licheniformis* or *Brevibacterium halotolerans*. Nevertheless, by using the RAPD-PCR technique we could discriminate all the isolates throughout their strain specific profile.

The results presented here clearly show that RAPD-PCR technique is a reliable methodology to distinguish genetically similar strains from *Bacillus* group.

Genetic diversity of the strains from the salt flat

To evaluate the genetic diversity between 20 isolates, the RAPD-PCR banding patterns were used to construct a single dendrogram. *Bacillus* sp. pure cultures used as reference were also included (Table I). The dendrogram obtained (Figure 3) displays a clear separation of bacilli group from one of the isolates previously identified as *Micrococcus* sp. SX120 by 16S rRNA sequencing (Martínez et al. 2019) with 0% of similarity.

RAPD-PCR analysis revealed a high degree of genetic diversity of the bacilli group isolated with practically all strains differentiated, showing 100% of polymorphism. Previous studies showed that RAPD-PCR was successfully applied to characterize *Bacillus* strains. Nilsson et al. (1998) described RAPD-PCR as an effective and quick method to differentiate many strains of *B. cereus* because of its highly discriminatory power. Later, Gupta et al. (2016) went a little further describing RAPD-PCR as the best method for molecular typing of *Bacillus* species. More recently, Avsar et al. (2017) also observed 100% of polymorphism throughout the use of M13-10 and OPL-3 primers.

In the dendrogram, the *Bacillus* group was separated into two major clusters (Cluster I and Cluster II) at similarity levels 5% (Figure 3). In Avsar et al. (2017) the two first clusters separated at 5% of similarity when they used RAPD-PCR, conversely to what they observed when Enterobacterial Repetitive Intergenic Consensus PCR (ERIC-PCR) was used, where the similarity ranged from 38 to 90%. In agreement with these results, RAPD-PCR analysis revealed high degree of genetic diversity for all identified bacteria with primer S30.

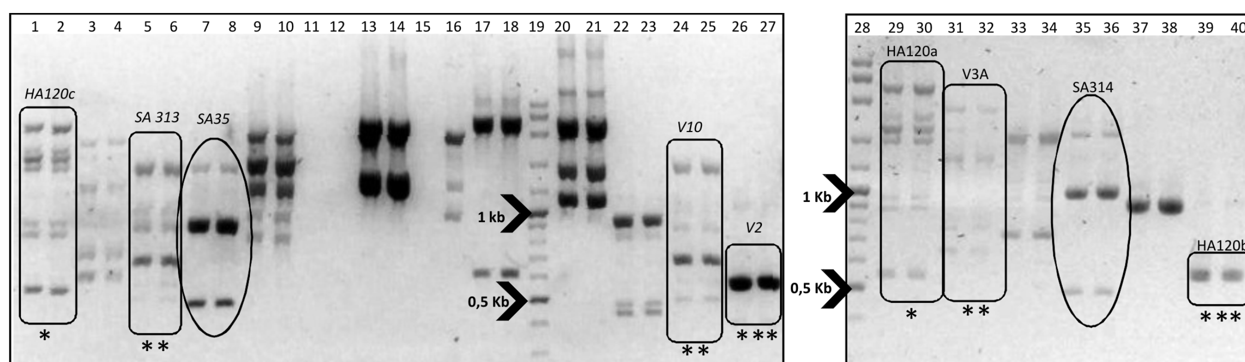

Figure 2. Representative RAPD-PCR profiles showing polymorphisms among halophilic *Bacillus* isolates. Electrophoresis on 2% agarose gel of halophilic bacilli. DNA amplification was done in duplicate using S30 primer. Lines 1-2: HA120c; 3-4: FAMB1; 5-6: SA313; 7-8: SA35; 10-11: SX139; 12-13: HX127; 14-15: V10; 16-17: V2; 19-20: HA120a; 21-22: FAMB1; 23-24: SA32; 25-26: SA314; 27-28: FAMB4; 29-30: HA120b; 32-33: V10; 34-35: SX120; 9, 18 and 31: 100 marker (Genbiotech, Argentina). Isolates presenting the same band profile were indicated in a Box with: *: HA120c and HA120a, **: SA313 and V10, *: V2 and HA120b. SA35 isolate showed the same profile than SA314 and was identical to *B. subtilis* and marked with an ellipse. No template controls, which did not show amplification, were excluded from the figure.**

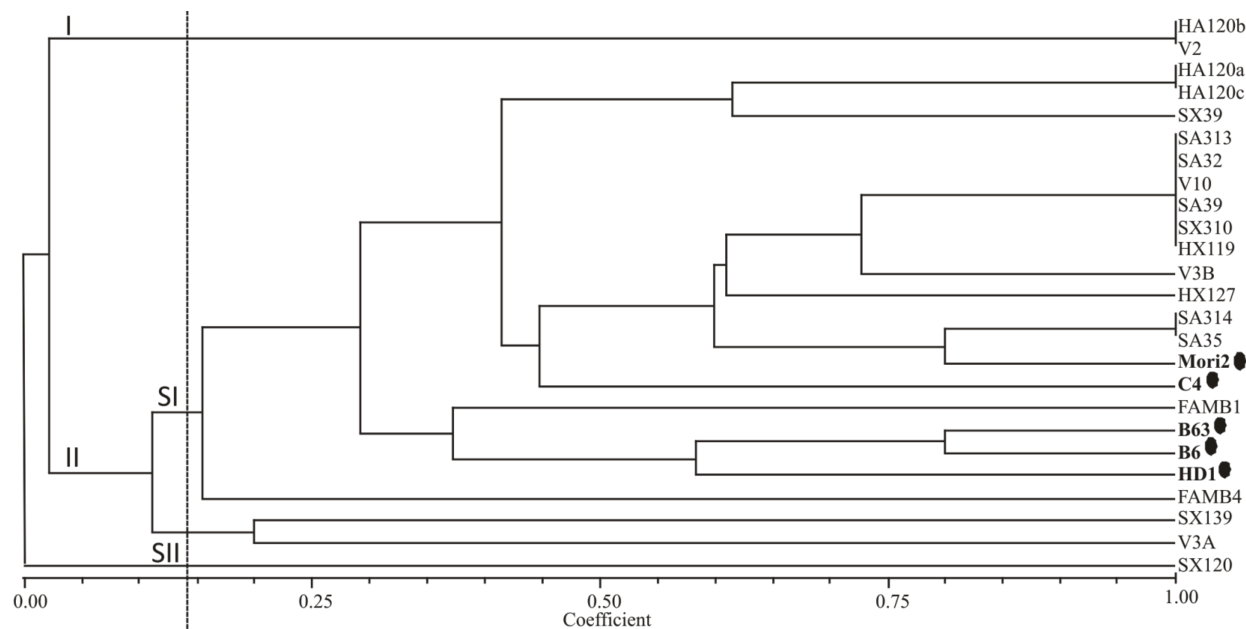

Figure 3. Dendrogram based on RAPD-PCR analysis, generated from the band profile using algorithm of Unweighted Pair Group Method (UPGMA) and clustering using Dice coefficient of similarity generated based on S30 primer. The similarity coefficient is shown at the bottom. Reference strains used in the analysis are marked with a black circle.

In Cluster I only HA120b and V2 grouped together. Cluster II grouped most of *Bacillus* isolates, including those used as reference. It was further divided in two minor sub-clusters: SI and SII (Figure 3, SI and SII), where SI grouped all the bacilli (even the unknown samples) at similarity level between 15% and 100%, except for V3a and SX139 isolates that were grouped together in SII. The fit of the UPGMA to the similarity matrix was significant (CCC = 0.89).

The scan of numerous loci in the genome could be done throughout RAPD technique, making this method particularly interesting to analyze closely related species.

CONCLUSIONS

In this study, RAPD-PCR has been used for genetic and molecular studies. RAPD-PCR was found to be a quick and reliable technique to evaluate the diversity of *Bacillus* strains and was successfully applied to characterize the genetic diversity present in the Salar del Hombre Muerto. The primer chosen, S30, was adequate as a starting point to generate different band patterns and individual strains could be distinguished.

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