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Molecular Study Of Brevibacillus Laterosporus

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Abstract

Public health is seriously threatened by the development and spread of antibiotic resistance among harmful bacteria, especially given the dearth of newly discovered antibiotics. New antimicrobials that can be utilised as bacteriostatic substitutes for traditional antibiotics are thus needed. Brevibacillus strains are well-known antibacterial and antifungal agents. Antibacterial substances produced by Brevibacillus bacteria have been researched recently. They are a crucial piece of equipment for biological control. Brevibacillus species have a wide range of antimicrobial activity, including activity against fungus and bacteria.Proteins and antibiotics are just two examples of the many compounds whose apparent pathogenicity and modes of action have been linked.Previously, it was thought of as a biological control agent against diseases, and some Brevibacillus species' antifungal and antibacterial capabilities sparked interest in medicine due to its connection to the creation of antibiotics with therapeutic effects. This study aims to identify and isolate Brevibacillus species from soil.DNA was taken from the bacteria that were isolated from the soil and identified phenotypically and genetically. PCR and electrophoresis were then carried out on the extracted DNA.

Keywords: Molecular , Brevibacillus , laterosporus

الدراسة الجزيئية لمرض Brevibacillus Laterosporus

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خلاصة

تتعرض الصحة العامة لتهديد خطير بسبب تطور وانتشار مقاومة المضادات الحيوية بين البكتيريا الضارة ، خاصة بالنظر إلى ندرة المضادات الحيوية المكتشفة حديثًا. وبالتالي ، هناك حاجة إلى مضادات جرثومية جديدة يمكن استخدامها كبدائل للجراثيم للمضادات الحيوية التقليدية. سلالات Brevibacillus هي عوامل معروفة مضادة للجراثيم ومضادة للفطريات. تمت دراسة المواد المضادة للبكتيريا التي تنتجها بكتيريا Brevibacillus مؤخرًا. إنها قطعة مهمة من المعدات للمكافحة البيولوجية. تمتلك أنواع Brevibacillus نطاقًا واسعًا من النشاط المضاد للميكروبات ، بما في ذلك النشاط ضد الفطريات والبكتيريا ، والبروتينات والمضادات الحيوية ليست سوى مثالين على العديد من المركبات التي تم ربط

مسببات الأمراض الظاهرة وأنماط عملها. ضد الأمراض ، وأثارت القدرات المضادة للفطريات والبكتيريا لبعض أنواع Brevibacillus الاهتمام بالطب بسبب ارتباطه بإنتاج المضادات الحيوية ذات التأثيرات العلاجية. تهدف هذه الدراسة إلى تحديد وعزل أنواع Brevibacillus من التربة ، حيث تم أخذ الحمض النووي الريبي من البكتيريا التي تم عزلها من التربة وتم التعرف عليها من الناحية المظهرية والوراثية. ثم تم إجراء تفاعل البوليميراز المتسلسل والرحلان الكهربي على الحمض النووي المستخرج.

الكلمات المفتاحية: جزيئي ، Brevibacillus، laterosporus

Introduction

Inan et al., 2019; Johnson and Dunlap, 2019; Brevibacillus is a genus of rod-shaped, oval, Gram-positive or Gram-variable bacteria that thrive in a range of conditions. According to Nazina (2001) and Logan & De Vos (2009), the numerous strains of the genus Brevibacillus are notable for their ability to perform facultative anaerobic and aerobic fermentation.

The majority of species build uniform, flat, yellowish-gray colonies on common media. It is particularly difficult to distinguish between species of Brevibacillus because of the large variation within the genus and the low responsiveness to conventional biochemical identification approaches (Goto et al., 2004). Even Logan et al. (2002) had trouble distinguishing between all the species using a molecular technique like rDNA restriction analysis.

The hypervariable (HV) region from the Brevibacillus members is interesting because it is well preserved within a species but has diverged enough between species to allow identification and clustering of Brevibacillus species by sequence comparisons of the HV area (Allan et al., 2005). According to Nazina et al. (2001) and Ammanagi et al. (2021), the phylogenetic genus Brevibacillus is quite diverse.

Isolate Brevibacillus spp. and choose the isolation environment

In the months of August and September 2022, forty-eight samples were collected, sixteen samples at a time, from the north, south, east, and west of the Al-Diwaniyah governorate at eight sites of the sanitary landfill areas for waste, each site at two depths of five and ten centimetres. The last sample collection took place on October 18/2022.

The study bacteria were isolated in accordance with Brown (2001) exhaustive isolation from soil, and the method is summed up as follows:. They were kept in plastic containers, labelled, and kept in the incubator at 37° C, which is the appropriate temperature for the growth of bacteria, until they were used in the study.

One gramme of soil was taken and mixed with 9 ml of distilled water in a plastic test tube using a shaker device (Shaiker) for 1 - 15 minutes in order for the soil to be completely dissolved, where the dilution here was (101), before 1 ml of the previous mixture was taken and added to 9 ml of distilled water and mixed. The soil was then transferred to the laboratory to be cultivated.

Then the dilution turned into (10^2) then, as the dilution at this point became (10^3), 1 ml was taken from the second mixture and added to 9 ml of distilled water. It was then thoroughly mixed for 15 minutes using the shaker to create 3. Finally, 1 ml was taken from the third mixture and added to 9 ml of distilled water. The dilution in this case was (10^4).

These four dilutions were carried out until the sixth dilution (10^6) was reached with the intention of lowering the number of microorganisms present in the soil. The samples were then activated with BHI broth before being planted on plates of Muller-Hinton agar medium, where the majority of the isolates, bacillus subtilis and bacillus cereus, showed signs of growth. In terms of phenotypic diagnosis, samples No. 2 and No. 4, which were extracted from a depth of 5 cm, were most similar to Brevibacillus.

Bacterial isolates' identification

Morphology

The diagnosis was made based on the morphological characteristics of the colonies, including size, colour, edges, and rising of the colonies. Pure and sparse colonies were chosen based on observing the phenotypic characteristics of each growth present on the culture medium of the primary culture. The shape of the bacterial cell, how they are arranged next to one another, and how Gramme staining affects them are all examples of the phenotypic traits of the cells.

On Brevibacillus spp., numerous biochemical assays have been carried out. These are the outcomes:

1-Gram stain : positive

2-Motility : positive

3-Catalase test : positive

4-Methyl red test : positive

5-Blood hemolysis : positive

6-Oxidase test : negative

It has been grown Brevibacillus spp. These results surfaced on various cultural media.

Muller Hinton agar : Growth

Blood agar : Growth

MRS agar : Growth

MacConky agar : No Growth

2.3-Molecular study

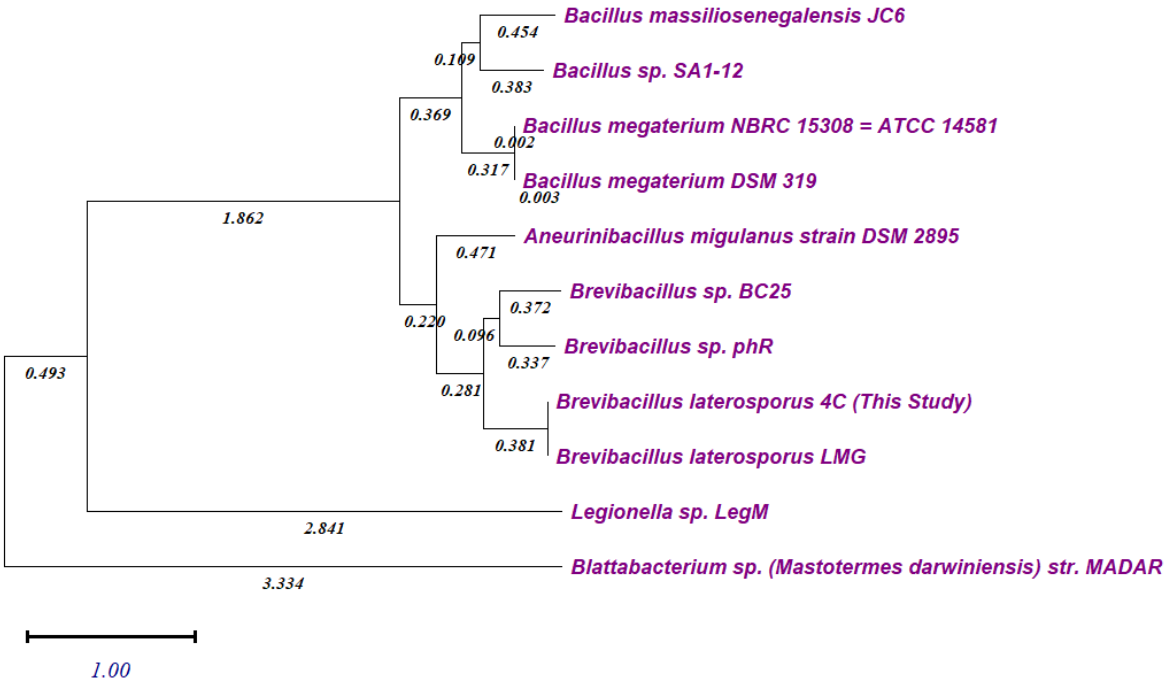

Fig (1): Phylogeny analysis based on 500 gene homology to identify the phylogenetic placement and the closest representative genomes to *Brevibacillus laterosporus* 4C genome. red numbers represent branch length.

2.3.1-Bacterial DNA extraction protocol:

To identify *Brevibacillus laterosporus* in metagenomic DNA of soil samples, A sample with 1g of soil was extracted by two methods.

Method 1: Directly from soil samples according to Presto™ Soil DNA Extraction Kit Quick Protocol (Genaid, USA, SLD050).

Method 2: DNA extraction from cultured soil samples.

In this method, 1g of soil sample was suspended in Brain heart infusion broth and incubated overnight 37C, and then cultured on Brain heart infusion agar that support growth all soil microorganisms. Bacterial metagenomic DNA was extracted from culture using a special extraction kit using the Presto Mini g DNA bacteria set from Geneaid company USA (No. GBB 101) and according to the manufacturer's instructions as follows:

1-We transferred one mL of BHI cultured sample directly into a 1.5 ml micro centrifuge tube. Centrifuge at 14-16000xg for 1 min, then discard the supernatant.

2- We added 200 µl of GT buffer and then re-suspended the cell pellet by vortexing. Add 20 µl proteinase K, incubate at 60 for at least 10 min in a water bath, and during incubation invert the tube every 3 min.

3- We added 200µl GB buffer to the sample and mix by vortex for 10 seconds. Then the tubes were incubated at 70ºC for 10 minutes in a water bath and inverted every 3 minutes through incubation periods.

4-Absolute ethanol (200µl) was added to the sample and immediately mixed by shaking vigorously then precipitates broke it up by pipetting. A GD column (DNA filter column) was placed in a 2 ml collection tube and transferred all of the mixture (including any precipitate) to the GD column. Then centrifuged at 14-16,000 x g for 2 minutes. And the 2 ml collection tube containing the flow-through was discarded and placed the GD column in a new 2 ml collection tube.

5- We added W1 buffer (400µl) to the GD column, then centrifuged it for 30 seconds at 14-16,000x g. The flow-through was removed and the GD column was reinserted into the 2 ml collection tube.

6- We added Wash Buffer 600µl to the GD column. Then centrifuged at 14-16,000x g for 30 seconds. The flow-through was discarded and placed the GD column back in the 2 ml collection tube. And the tubes were centrifuged again for 3 minutes at 14-16,000x g to dry the column matrix.

7-We transferred The dried GD column was transferred to a clean 1.5 ml micro-centrifuge tube and70µl of pre-heated elution buffer were added to the centre of the column matrix. The tubes were let stand for at least 3 minutes to ensure the elution buffer was absorbed by the matrix. Then centrifuged at 14-16,000x g for 30 seconds to elute the purified DNA.

PCR amplification

This assay used to identify *Brevibacillus laterosporus* by using genus specific primers (Shida et al., 1996) and species-specific primers (Ruiu et al. 2017). all primers in table (1-7). PCR amplification of DNA was carried out in final

reaction mixture volume of 25 µl (GoTaq® Green Master Mix, Promega. USA) and within 30 cycles.

Table (1-7): Primer sequences of genus (*Brevibacillus)* **and species (***Brevibacillus laterosporus*).

Primer	**Sequence**	**PCR product**
16SrRNA -F	AGACCGGGATAACATAGGGAAACTTA	1.2kb
16SrRNA-R	GGCATGCTGATCCGCGATTACTAGC	
BL-F	CTGCTACTAGTTGATCTAAG	709bp
BL-R	CTGATTGGTAGCTTAGGTA	

Table (2-7): Uniplex PCR mixtures and conditions for identification of genus-specific Identification.

PCR mixtures		**PCR conditions**		
Contents	**Volume**	**Type of cycle**	**Condition**	**No. of cycles**
Master Mix	12.5 µl	Initialization	94 °C for 5 min	1
Forward Primer	2.5 µl	Denaturation	94 °C for 1 min	30
Reverse Primer	2.5 µl	Annealing	59 °C for 1 min	
Template DNA	3 µl	Extension	72 °C for 1 min	
Nuclase-Free Water	4.5 µl	Final Extension	72 °C for 10 min	1

Table (2-7): Uniplex PCR mixtures and conditions for identification of species-specific identification.

PCR mixtures		**PCR conditions**		
Contents	**Volume**	**Type of cycle**	**Condition**	**No. of cycles**
Master Mix	12.5 µl	Initialization	94 °C for 5 min	1
Forward Primer	2.5 µl	Denaturation	94 °C for 45s	30
Reverse Primer	2.5 µl	Annealing	57 °C for 45s	
Template DNA	3 µl	Extension	72 °C for 45s	
Nuclase-Free Water	4.5 µl	Final Extension	72 °C for 10 min	1

2.3.3.3- Agarose gel electrophoresis:

The Agarose was prepared according to the following steps:

1- We prepared a 2% agarose gel with 1X TBE and dissolved it in a water bath at 100 °C for 15 min, after which, it was left to cool at 50 °C.

2- Then 250µL of Green star stain was added into an agarose gel solution.

3-After setting the comb in place, we poured the agarose gel solution onto the tray and then left it to freeze for 15 minutes at room temperature until the comb was gently removed from the tray.

4- We then placed the gel tray in an electrophoresis chamber and 1X TBE buffer solution was added.

5- We added 5µl of products PCR in each well of the comb and added 5µl of the (100bp Ladder) in one well of the comb. Then electric current was performed at 100 volts for 45 min.

6- PCR products were visualized using the Gel Documentation System.

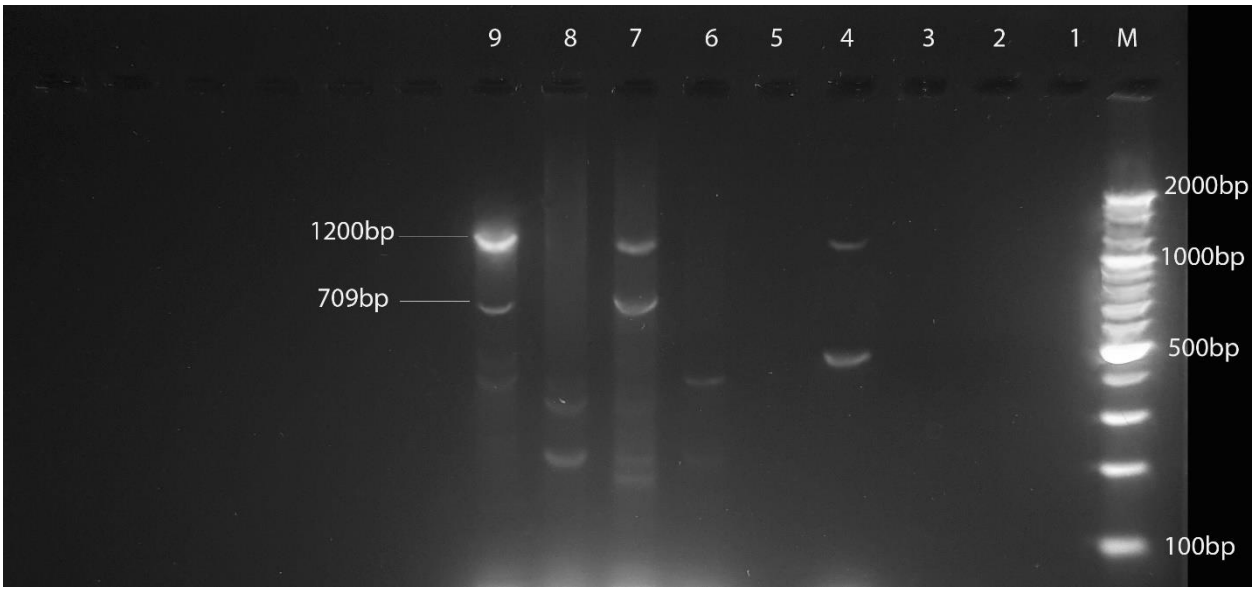

Fig. (3-1): Agarose gel electrophoresis of multiplex-PCR products obtained by using two specific primers: the first pair specific for *Brevibacillus* genus targeting 16SrRNA with 1.2 kp product, the second pair primer is specific for *Brevibacillus laterosporus* that targeting SC-CSPB complex protein with 709bp product. lanes 1-4 represent the direct Extracted-DNA of 5cm-soil samples, while lanes 6-9 represent culture-extracted DNA of 5cm-soil samples. Lane M represent 100bp DNA ladder. Lane 5 is an empty.

Table ( ): PCR identification of *Brevibacillus laterosporus* extracted directly or from culturing of 5-cm Soil samples

5-cm Soil samples	Direct extracted DNA	Culture extracted DNA
1	-	-
2	-	+
3	-	-
4	+*	+
%	25	50

* this sample showed positive for *Brevibacillus genus* only.

Fig. (3-1): Agarose gel electrophoresis of multiplex-PCR products obtained by using two specific primers: the first pair specific for *Brevibacillus* genus targeting 16SrRNA with 1.2 kp product, the second pair primer is specific for *Brevibacillus laterosporus* that targeting SC-CSPB complex protein with 709bp product. lanes 1-4 represent the direct Extracted-DNA of 10cm-soil samples, while lanes 6-9 represent culture-extracted DNA of 10cm-soil samples. Lane M represent 100bp DNA ladder. Lane 5 is an empty.

Table ( ): PCR identification of *Brevibacillus laterosporus* extracted directly or from culturing of 10-cm Soil samples

5-cm Soil samples	Direct extracted DNA	Culture extracted DNA
1	-	-
2	-	-
3	-	-
4	-	-
%	0	0

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