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Isolation And Identification Of Paenibacillus Bacteria By Biochemical Tests And Molecular Techniques

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Abstract

The current study aims to isolate and diagnose the bacteria Paenibacillus from different types of local yogurt in the city of Mosul. (80) Yogurt samples were collected, (20) which constitute of each of (sheep, cows, buffaloes and goats), 10 isolates of Paenibacillus were obtained from them. (12.5%) of the total population of the samples under study, the bacterial isolates were distributed in to (5) isolates from sheep's yogurt, two isolates from cow's yogurt, and (3) isolates from buffalo yogurt with percentages of (50%, 20%, 30%), respectively, However, no isolate was obtained from goat's yogurt. Bacterial isolates were diagnosed based on morphological and biochemical tests, diagnosis was confirmed by polymerase chain reaction (PCR) using the 16S rRNA gene of the bacteria under study. In order to decide on the strain type and the isolates, the bacterial isolates that were undergone PCR test, were sent to Macrogen biotechnology company affiliated to the Japanese Hitachi company using the Genetic analysis 3130 device, the sequence of nitrogenous bases of the 16S rRNA gene of Paenibacillus was identified, and after comparing it with its globally classified analogues on the NCBI website, (3) different types of Paenibacillus were diagnosed, two of which were diagnosed and registered in the genebank as Paenibacillus lautus and Paenibacillus ginsengagri, and the third type was diagnosed and registered as a new local isolate in the genebank within the current study under the name Paenibacillus sp.RAN.

Introduction:

According to the opinion of most of the regulatory authorities yogurt in most parts of the world, is known as a fermented milk product, as it provides digested lactose sugar in appropriate quantities, and contains multiple live bacterial species within its environment, which work to increase the shelf life of the product as a result of causing a decrease in pH, including Bifedobacterium, Paenibacillus, Streptococcus, Lactobacillus and others (Hendrati et al., 2017; Treven et al., 2015).

Paenibacillus is one of the probiotic bacteria, and it is one of the genera originally belonging to the genus Bacillus, however, it was separated from it and considered a new genus by Ash et al. (1993).

The term Paenibacillus is derived from the Greek word (Paene), which means (almost) in Arabic, and thus the literal translation is (almost bacillus). The members of this genus are

characterized by being bacillus Gram-positive aerobic or endospores forming facultative anaerobic (Sanghamitra et al., 2013).

Bacterial species of the mentioned genus were isolated from various environments such as soil, water, insect larvae, food and clinical samples. Note that the genus Paenibacillus includes (211) isolate, diagnosed and recognized species, of which (22) species were isolated from human clinical samples (Saez et al., al. 2017; Sharm et al., 2015).

Members of this genus represent one of the natural biological control factors due to their ability to produce different types of biologically active bacterial antimicrobials against a wide group of pathogenic bacteria, Gram positive and Gram negative, through their effect on biofilms, which are thin films consisting of living materials secreted by the bacterial cells and can be formed on non-living surfaces such as metals and dead organisms bodies, in addition to the possibility of being secreted on living surfaces such as plants, animals and humans (Macia and Oliver, 2014; Lohans et al., 2012). Based on that, the environment remains an important reservoir of microorganisms capable of the producing natural antibiotics, including Paenibacillus bacteria (Guo et al., 2012).

Materials and Methods:

samples were collected using sterile glass vials ,one-tenth dilution were prepared from samples with normal saline, then (0.1) cm^3 of the last dilution was transferred to MRS agar medium and incubated at (37)c° for (48) hours in aerobic conditions. the different shapes of the growing colonies were noted and recorded (Hikmat, 2011; Koneman et al., 2006). The bacterial isolates were diagnosed based on the following examinations and tests:

- **Cultural characteristics microscopic examination and Biochemical tests.**

These tests were conducted according to (Procop et al.,2016 ; Koneman et al.,2006; MacFaddin,1985).The tests included detection of Catalase, Oxidase, Urease, Indole test, Voges proskauer, Citrate , Sugars fermentation.

- **Molecular diagnostics:-**
- **Extraction of DNA from Paenibacillus sp .**

Relying on the analysis kit supplied by Geneaid Company, DNA was extracted from bacterial isolates of the genus Paenibacillus.

- **Polymerase Chain Reaction (PCR)**

Table (1): demonstrates the components of the PCR reaction mixture and their quantities.

component	Size (Microliter)
DNA template	**4.0**
Forward primer	**1.0**
Revers primer	**1.0**
Distilled water	**4.0**
Master mix	**10.0**

Table (2) demonstrates cycles and intervals of each (PCR) reaction**.**

NO	Stage	Temperature	Time	Cycle number
1	Initial denaturation	95	6 min.	1
2	Denaturation	95	45 sec.	35
3	Annealing	56	1 min.	
4	Extension	72	1 min.	
5	Final extension	72	5 min.	1

To detect the presence of 16S rRNA gene in DNA samples of Paenibacillus used in the study, 16S rRNA gene primers were used, the sequences of which are shown in table (3) according to the designed program supplied by Biolaps company to the contents of the Master mix.

Table (3): Primers used in the study

Primer	Sequence
Forward	GACCTCGGTTTAGTTCACAGA
Revers	CACACGCTGACGCTGACCA

DNA bundles were extracted from the agarose gel, resulting from the polymerase chain reaction, using the analysis kit supplied by Geneaid company, to purify them and send them to the Macrogene biotechnology company, Hetachi, Japan, in order to read and analyze the sequences of nitrogenous base the 16S rRNA gene of Paenibacillus sp. for the purpose of identifying their types and determining their strains, based on the Genetic analysis 3130 device.

Results and Discussion:

Samples Collection and Bacterial Isolation

Eighty Samples of different types of yogurt were collected of local market the city of Mosul during the period from (20/1/2020 - 20/3/2020). The samples yogurt (Sheep, Cow, buffalo and goat); (20) samples were collected for each of the mentioned types. After the isolation and morphological and biochemical and molecular diagnosis. (10) isolates of the genus Paenibacillus were obtained, with a percentage of (12.5%) . The bacterial isolates were distributed as (5) isolates from sheep yogurt, (2) two isolates of cow yogurt, (3) Isolates from buffalo yogurt, and no isolates of Paenibacillus were obtained from goat yogurt. This may be due to some of the constitute available in goat yogurt compared to other types, as it contains a high percentage of medium and short-chain fatty acids, as indicated by (Al -Fekaiki et al.,2017) and the table (4) shows this.

Table (4): Number of samples and percentages of each isolate of Paenibacillus bacteria in various samples of Yogurt

Type of yogurt sample	Before Diagnosis		After Diagnosis	
	Number of samples	Percentage %	Number of bacterial isolates	Percentage %
Sheep	20	25	5	50

Cow	20	25	2	20
Buffalo	20	25	3	30
Goat	20	25	---	---
Total	80	100	10	100

(-----) indicates no Isolates of bacteria from goat yogurt.

Diagnosis of Bacterial Isolates

- **Cultural characteristics and Microscopic Examination**

Bacterial isolates were identified after they were cultured on MRS agar medium, and those isolates had grown in the form of convex circular-shaped colonies which had creamy color. Cram stained smears from isolated colonies showed that the bacteria appeared as Gram-positive bacilli, and this is due to the nature of the composition of their cell wall; it contains a small layer of fat compared to the thick layer of peptidoglycan, and the bacilli were of medium length with rounded ends, singly or in pairs (Shida et al., 1997) image (1) demonstrates . the microscopic appearance of the isolate.

A B

Image (1)

A - Colonies of Paenibacillus bacteria on MRS solid medium.

B - Paenibacillus bacteria cells after staining with Gram stain and examining them with a compound-light microscope using a 100× oil lens.

- **Biochemical Tests**

Pure cultures of Paenibacillus sp. species on agar slants, biochemical tests for laboratory diagnosis were carried out. The results of these tests agreed with what was mentioned by (Sharma et al., (2015); Ouyang et al., (2008); Djordjevic et al., (2000)), and Table (5) demonstrates that.

Table (5): Results of Biochemical tests of the three species of Paenibacillus bacteria

Type of Bacteria / Type of Test	**Paenibacillus lautus**	**Paenibacillus ginsengagri**	**Paenibacillus Sp.RAN**
Catalase	+	+	+
Oxidase	+	+	+

Urease	-	-	+
Indole	-	-	-
Voges proskauer	+	+	+
Citrate	-	-	-
Esculin	+	+	-
Sorbitol	+	+	+
Maltose	+	+	-
Mannose	-	+	+
Glucose	+	+	+

(+) Positive test result (-) Negative test result

* **Molecular Diagnostics**

After bacterial isolates were diagnosed using morphological and biochemical tests, DNA was isolated from the ten obtained isolates. The isolated DNA agarose gel ware electrophoresed on to ensure its presence, purity, and the similarity of its molecular weight in all the isolates under study; it was found that they all distances moved the same in agarose gel and this indicates that they were of the same size, Image (2) shows the results of electrophoresis of the genetic material on agarose gel with a potential difference of (70) volts and for (60) minutes.

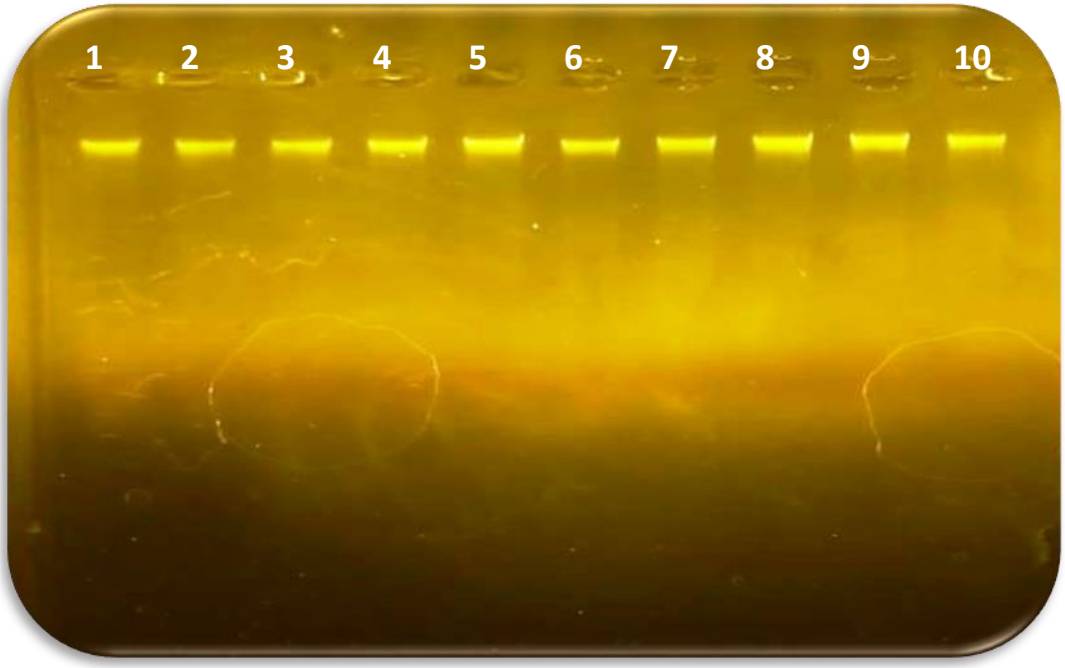

Image (2)

Results of electrophoresis of DNA isolated from Paenibacillus bacteria on agarose gel. Numbers from (1-10) represent the samples used in electrophoresis

*** Polymerase Chain Reaction (PCR)**

The diagnosis of bacteria based on physical characteristics and biochemical tests is often unreliable, and the reason for this may be due to the similar morphological and nutritional requirements of different species of the same genera. Therefore, researchers have resorted to adopting molecular diagnostic methods, including diagnosis using the sequence of nitrogenous bases of the 16S rRNA gene as a complement to the phenotypic diagnosis and not a substitute for it (Baradaran et al., 2012).

Also, the process of distinguishing the bacterial species of the genus Paenibacillus by traditional methods and biochemical tests is differential in some cases, so the researchers relied on the use of the reference standard for molecular diagnosis by using special primers for the 16S rRNA gene (Nieto et al., 2017).

The genetic sequence of the 16S rRNA region relied upon to diagnosing the bacteria Paenibacillus after the genetic material was amplified by PCR technique and the desired gene was targeted with a known sequence of primers in order to associate with them and to ensure that the DNA samples electrophoresed on the agarose gel belong to the genus Paenibacillus (image3), all DNA bands appeared at the molecular size (1300 pb) for all samples and this indicates the association of the 16S rRNA gene primer with the complementary sequence for one of the two DNA strands, which represents the gene in question, and this indicates that all samples under study have the target gene, which indicates the specificity and diversity of the gene sequence. This indicates that the phenotypic diagnosis is congruent with the genetic diagnosis by (100%) for the bacterial isolates.

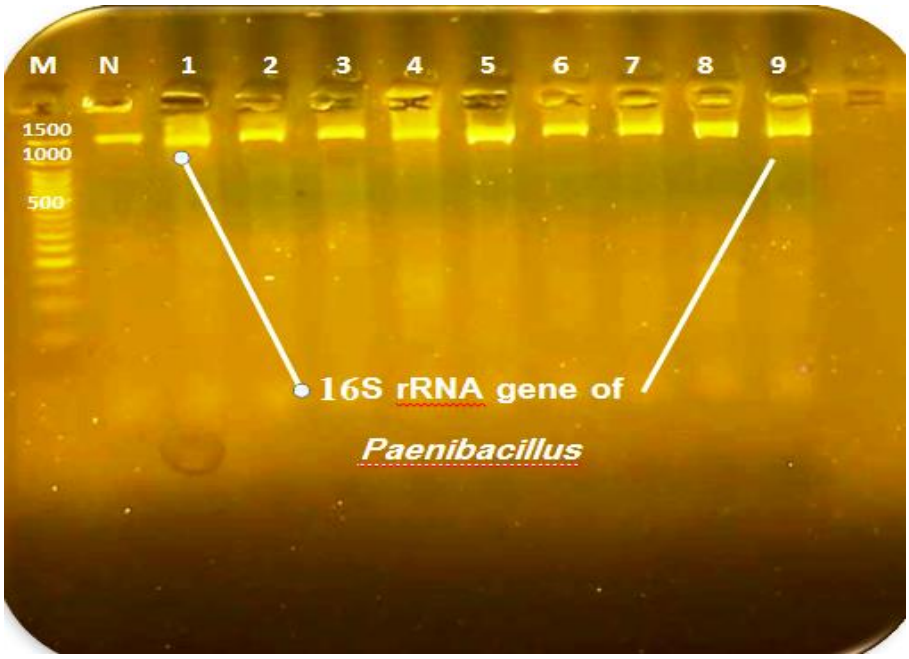

Image (3)

Results of PCR for Paenibacillus bacteria, specifically for 16S rRNA region

- The letter (M) represents the molecular volume index path consisting of (100) base pairs.
- The letter (N) represents the pathway of the negative control coefficient (PCR reaction material and primer without template)..
- The numbers (1-9) represent the pathways of 16S rRNA gene amplification product for the bacterial isolates under study.

The target genes that were amplified using specialized primers were analyzed by detecting the sequence of nitrogenous bases for each gene in order to obtain the required sequence and to compare it with the genes classified on the website of the National Center of Biotechnology Information. The results showed that all the selected isolates belong to the genus Paenibacillus.

*** DNA Sequencing for the Gene 16S rRNA**

After the DNA samples ware obtained from the polymerase chain reaction were sent to the center of the Japanese company Hitachi / Macrogen Biotechnology Company, sequence analysis of the 16S rRNA gene was carried out using the Genetic Analysis 3130 device. The results of the sequence analysis, after they were compared with the globally classified genes on the NCBI website, showed that three different species belongs to the genus Paenibacillus were diagnosed and they fulfilled the requirements of the said genus by using the Sequence chain analysis technique for the target gene, and two isolates of them were previously classified and known, which were Paenibacillus lautus and Paenibacillus ginsengagri, and the two figures (1) and (2) show this.

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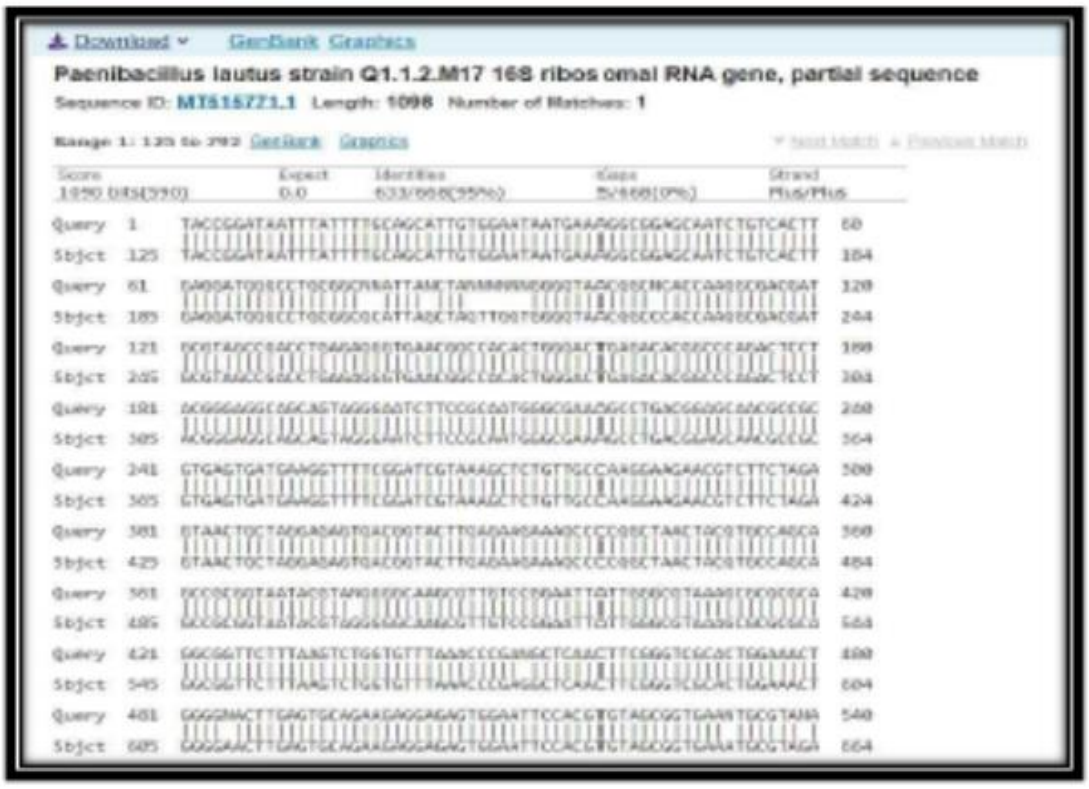

Figure (1) :Sequence chain of 16S rRNA gene of Paenibacillus lautus bacteria

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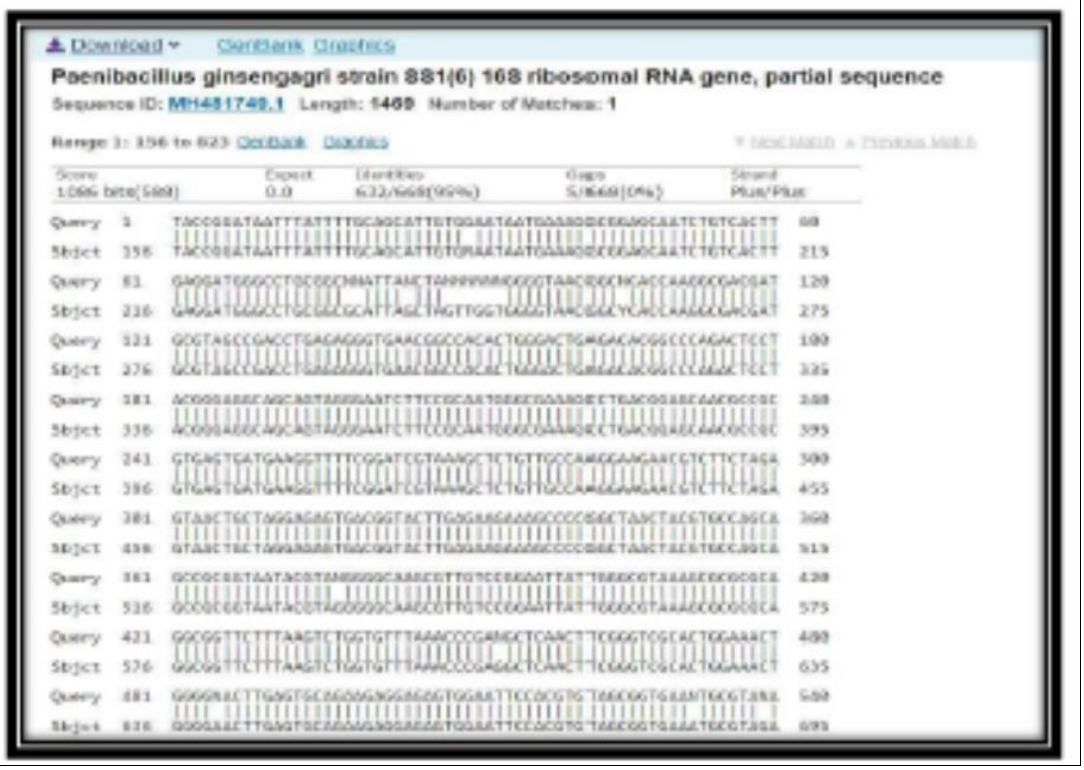


Figure (2)

Sequence chain of 16S rRNA gene of Paenibacillus ginsengagri bacteria

When analyzing the 16S rRNA gene segment for the purpose of detecting the sequence of its nitrogenous bases and comparing them with the sequences of globally classified genes, the diagnosis of Paenibacillus isolates from patients suffering from urinary tract infection was confirmed (Sharma et al., 2015).

As for the third isolate, it was not diagnosed locally neither was it previously registered as a strain belonging to the mentioned genus; it was registered and named in this study in the gene bank under a new strain belonging to the genus Paenibacillus, and named (Paenibacillus sp.RAN), and the letters RAN denote the initials of the researcher's name (R) and the names of the two professors who had supervised the studies (A) and (N), and the figures (3) and (4) show this.

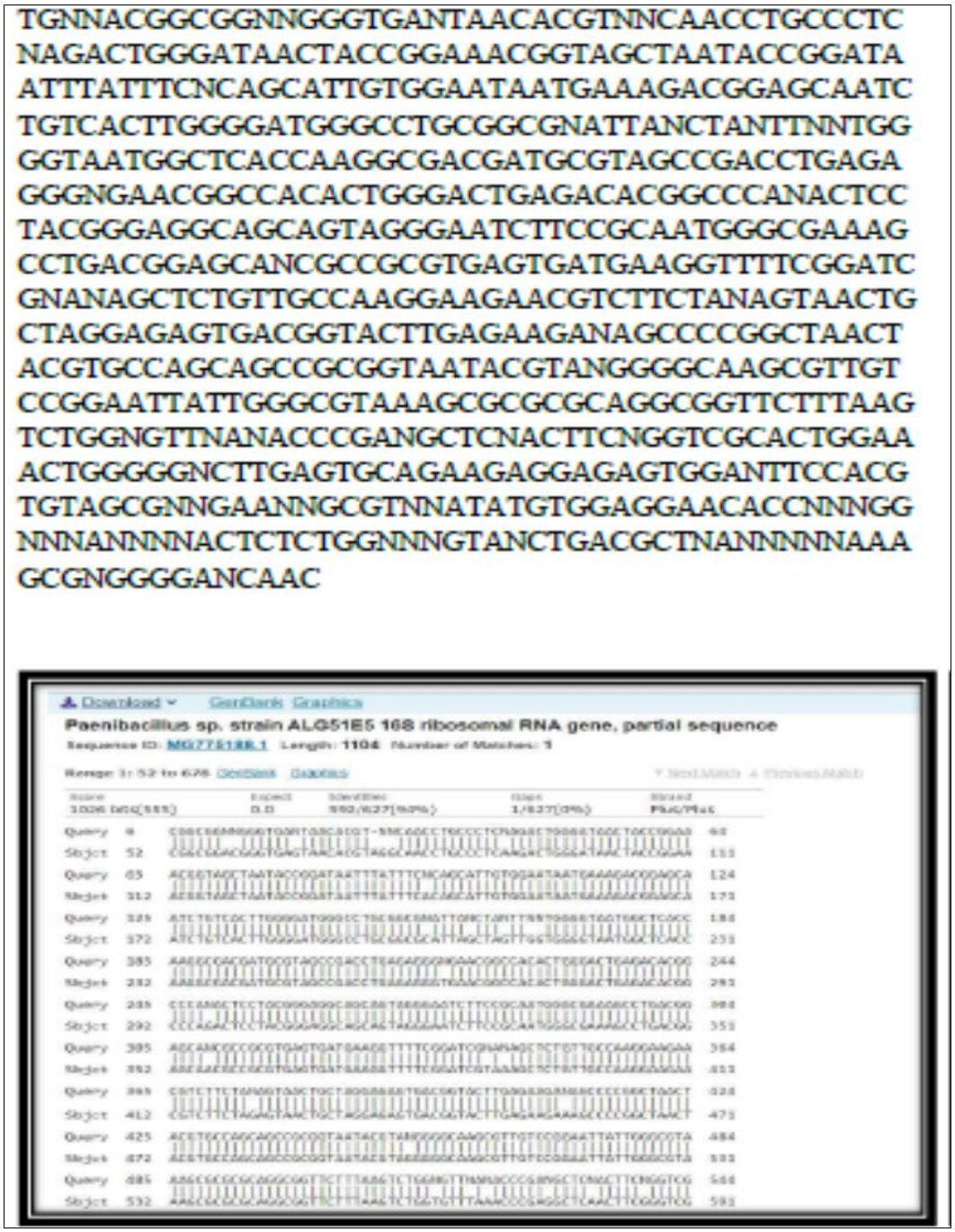
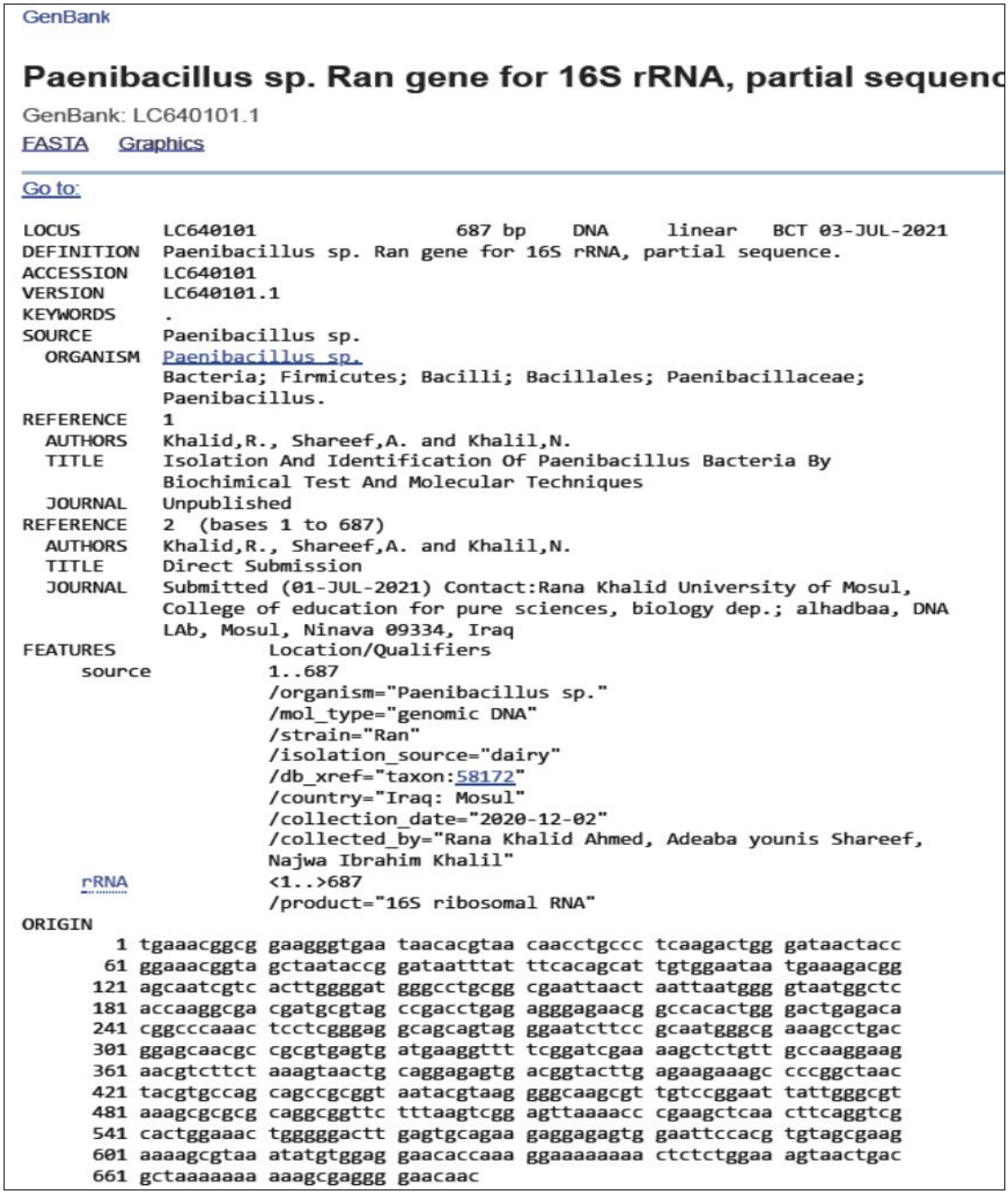


Figure (3) Sequence chain of 16S rRNA gene of Paenibacillus sp.RAN bacteria



GenBank

Paenibacillus sp. Ran gene for 16S rRNA, partial sequenc

GenBank: LC640101.1

FASTA Graphics

Go to:

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            Paenibacillus.
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  AUTHORS   Khalid,R., Shareef,A. and Khalil,N.
  TITLE     Isolation And Identification Of Paenibacillus Bacteria By
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  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 687)
  AUTHORS   Khalid,R., Shareef,A. and Khalil,N.
  TITLE     Direct Submission
  JOURNAL   Submitted (01-JUL-2021) Contact:Rana Khalid University of Mosul,
            College of education for pure sciences, biology dep.; alhadbaa, DNA
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```

Figure (4)

Registration of the new strain of Paenibacillus sp.RAN in the bank of genes

The study of Rodriguez et al, (2019) revealed a new strain of the genus Paenibacillus, where the genetic analysis of the 16S rRNA gene sequence showed that this strain is related to the species of the genus Paenibacillus, as it was very close to the bacteria Paenibacillus chitinolyticus and after comparing it in the phylogenetic tree with other species of Paenibacillus bacteria, it was confirmed that they have the same evolutionary pattern.

16S rRNA gene sequencing was used to study the phylogenetic tree of the bacteria under study, and the results showed the correlation of Paenibacillus with the neighboring bacteria which indicates the location of the isolates diagnosed in the current study and their geneticall identity with the genus Bacillus and its species, and this result agreed with the study of Weselowski et al., (2016) and the study of Nieto et al., (2017).

The study of AL-Sammak (2008) also confirmed that the family Bacillaceae is the largest within the order Bacillales, as many of its species were placed in new genera and families depending on the sequence chain of the 16S rRNA gene. The study also stated that the most important among its genera is Paenibacillaceae, which is one of the new and genetically close genera to the genus Bacillus, and the figure (5) shows this.

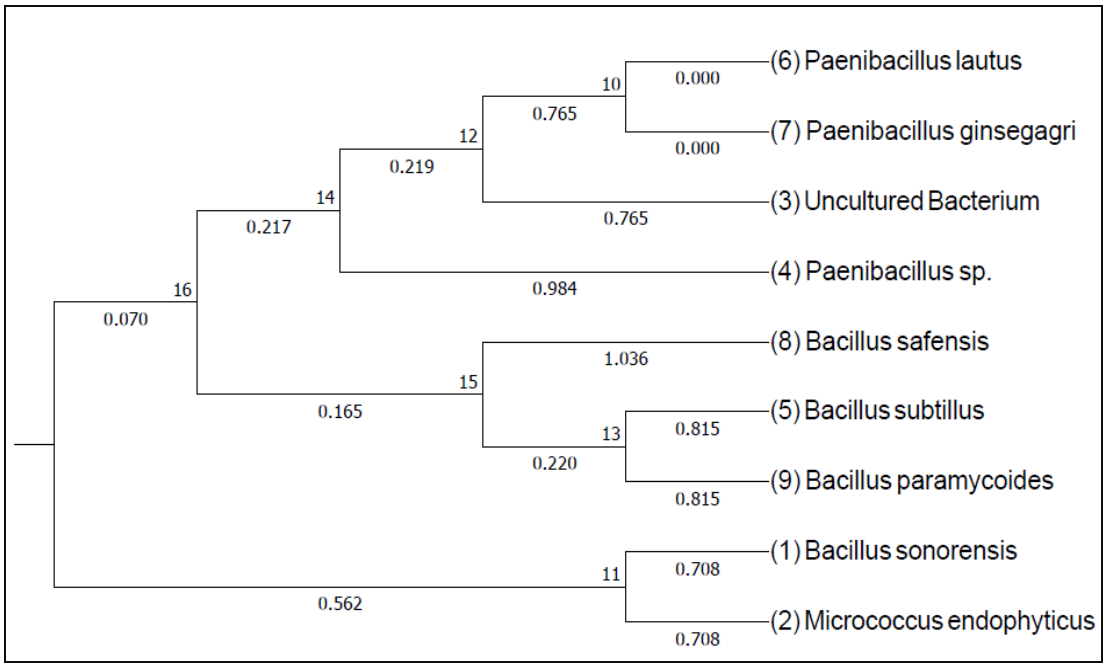

Figure (5)

Phylogenetic tree of Paenibacillus bacteria

The study of Cotta et al, (2011) confirmed the genetic affinity between Paenibacillus and Bacillus bacteria, which appeared in the genetic tree of evolution. Because the conserved genome belonging to the 16S rRNA gene segment of Paenibacillus species is different from that of Bacillus, the species belonging to the genus Paenibacillus were included in a genus independent of the genus Bacillus.

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