JAP&T TPA-Timişoara

Available online at http://journal-of-agroalimentary.ro

Journal of Agroalimentary Processes and Technologies 2021, *27*(4-Supplement), 565-569

Journal of Agroalimentary Processes and Technologies

Diversity of 16S rRNA gene and its GC contents in *Bacillus* species

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Abstract

The 16S ribosomal RNA (rRNA) gene is widely used for estimating the composition of bacterial communities. In this work, the phylogenetic diversity of 40 *Bacillus* sp. strains (3 *B. atrophaeus*, 20 *B. subtilis*, 2 *Paenibacillus polymyxa*, 10 *B. amyloliquefaciens*, 1 *B. simplex* and 4 *B. tequilensis*) were investigated based on 16S rRNA gene and its guanine (G) and cytosine (C) contents. 16S rRNA partial sequencing assigned all the strains to the *Bacillus* genus, with close genetic relatedness to the *B. subtilis* and *B. amyloliquefaciens* groups, and to the *B. tequilensis*. The phylogenetic tree did not result in any clusters/clades specific to origin or morphological criteria. In addition, the percentage of GC contents for 16S rRNA gene sequences ranged between 54.1% - 56.9%, and no clear correlation were observed between the GC content and distribution of species strains within clusters. This work demonstrated many of the same general features seen in the *Bacillus* species 16S rRNAs sequences.

Keywords: *Bacillus*, 16S rRNA, GC content, genetic variation

1. Introduction

Bacillus is a diverse genus of Gram-positive comprises of more than 200 species with a broad genetic diversity, and classification these species is complex due to similarities among closely related species [18]. The use of molecular biological markers have become common method for *Bacillus* species identification [4]. 16S rRNA region has been employed as an alternative method for bacillus taxonomic classification, and several *Bacillus* species were reclassified using this gene sequences and separated into different phylogenetically distinct clusters [3,15].

Moreover, since ribosomes are necessary component of the protein biosynthesis system with highly conserved, the small ribosome subunit has been proved to be a useful molecular tool for investigating evolutionary relationships among organisms [9]. From these characteristics, the sequences of 16S rRNA gene have become powerful tools in the taxonomic classification of microorganisms with the increasing use of PCR technology. Nowadays 16S rDNA is a vital standard for taxonomy of *bacteria* [8,19].

On the other hand, measuring guanine and cytosine (GC) content is known to be a major subject in genetics as genomic base composition contributes to the fitness of organisms such that identifying the base composition is vital for reliable discovery of selection [5]. It has been reported that the most important factor contributing to the thermal constancy of double-stranded nucleic acids was depending on the base stackings of adjacent bases rather than the hydrogen bonds numbers among bases. It has found more appropriate stacking energy for GC pairs than for adenin - citosin (AC) and adenin- uracil (AU) pairs due to the relative sites of exocyclic groups [16]. However, the ratio of GC content for bacterial genomes has been reported to be ranged between 25% and 75% [12,17], therefore, this wide GC variation in bacteria including *Bacillus* species have many important questions for the scientists.

Despite its substantial importance, a limiting factor for taxonomic and diversity characterization of *Bacillus* species, there is a lack of adequate information concerning 16S rRNA structure of *their strains*.

During a polyphasic experiments, more than 525 bacilli were isolated from different regions of Syria, and in this work the genetic diversity of 40 *Bacillus* sp. strains was investigated based on 16S rRNA gene and its GC contents.

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2. Materials and Methods

2.1. Bacterial strains

Soil samples were obtained from diverse regions of Syria (Table 1). They were shaken in 9 ml of sterilized water at 160 rpm for 5 min. Serial dilution was prepared from 10^{-3} to 10^{-7} (Ammouneh et al. 2011), and then 0.5 ml of each one was spread onto Nutrient Agar (NA) medium and incubated all night, the colonies of *Bacillus* sp. were identified due to Wulff et al. (2002) [20], and 40 out 525 strains were selected for this study (Table 1). Six *Bacillus* species, specifically, *atrophaeus, subtilis, P. polymxa, amyloliquefaciens, simplex* and *tequilensis*, were used in this work. The *Bacillus* sp. isolates were grown on NA and kept for 24 h at 30 °C.

2.2. 16S rRNA gene amplification

16S rRNA was amplified using two primers BacF (5'-GTGCCTAATACATGCAAGTC-3') and BcaR (5'-CTTTACGCCCAATAATTCC-3') flanking a highly variable sequence region of 545 bp towards the 5'end of the 16S rDNA region were used [14]. The 50 µl reaction mixture contained 2 µl genomic DNA, l × PCR buffer, 2 mM $MgCl_2$, 0.2 mM dNTP, 1 µM of each primer and 5U/µl Taq DNA polymerase (Fermentas). PCR amplification was done by the following parameters: An initial denaturation step at 95 °C for 5 min followed by a second denaturation step at 95 °C for 1 min, annealing for 1 min at 54 °C, an extension at 72 °C for 90 s, and a final extension step of 72 °C for 10 min. A total of 30 serial cycles was achieved. PCR products were electrophoresed on a 1.5% agarose gel that were stained with ethidium bromide and visualized under UV light. PCR products were purified with QIAgen gel extraction kit (28704) according to the manufacturer´s recommendations.

2.3. Sequencing and Phylogeny Analysis

16S rRNA partial sequences from 40 strains were analyzed using a Genetic Analyzer (ABI 310, Perklin-elmer, Applied Biosystems, USA). The 16S rRNA sequences were matched up with the known sequences using the NCBI database (http://www.ncbi.nlm.nih. gov).

A phylogenetic tree was generated by performing distance matrix analysis using the NT system. The experiments were performed in triplicate.

2.4. GC% determination

GC % content was calculated as a percentage value according to the *Madigan and Martinko (2003)* [13] as*:* (C+G/A+T+C+G) x 100.

3. Results and Discussion

In the present study, PCR amplification yielded single DNA fragments of ~ 545 bp, that found in all *Bacillus sp.* (Fig. 1). *Bacillus* sp. strains were recognized as *B. atrophaeus*, *P. polymyxa*, *B. subtilis*, *B. amyloliquefaciens*, *B. tequilensis* and *B. simplex* with similarities ≤ 98% in their 16S rRNA sequences as compared with their closely related species. The obtained sequences has been deposited in GenBank (accession numbers MT159352 to MT159391; Table 1) [11].

Comparative analysis of the sequences discovered two major phylogenetically distinct clusters divided into several sub-clusters in each one. Group 1 (included 4 *B. amyloliquefaciens*, 6 *B. subtilis*, 2 *Paenibacillus polymyxa* and one of each *B. atrophaeus*, *B. tequilensis* and *B. simplex*. Group 2 consisted of 6 *B. amyloliquefaciens* 14 *B. subtilis*, 2 *B. atrophaeus* and 1 *B. tequilensis.* The phylogenetic tree did not result in any clusters/clades specific to origin or morphological criteria (Fig 2).

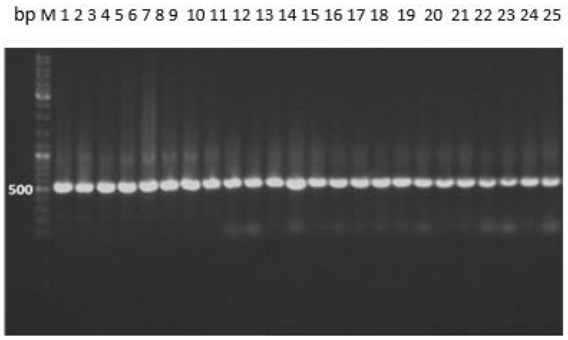

Figure 1. Electrophoresis of 16S rRNA of some *Bacillus sp.* strains used in this work. M represents the 100-bp DNA marker (*Hin*fI; MBI Fermentas, York, UK).

Table 1. *Bacillus* species used in this work

No.	Strain no.	Accession number	Location		Morphology
1	*B. subtilis SY118C*	MT159362	040.54°E	34.29°N	Dry surface with irregular edges
2	*B. subtilis SY130D*	MT159364	040.58°E	34.24°N	Dry surface have tree edges
3	*B. subtilis SY134D*	MT159368	040.54°E	34.28°N	Dry surface with irregular edges
4	*B. subtilis SY190E*	MT159374	039.17°E	39.20°N	Crimped surface with almost-regular edges
5	*B. subtilis SY168C*	MT159373	037.56°E	36.30°N	Smooth surface with smooth edges
6	*B. subtilis SY139D*	MT159370	040.27°E	35.01°N	Dry surface with irregular edges
7	*B. subtilis SY124B*	MT159363	040.39°E	35.53°N	Smooth surface, polished, smooth edges
8	*B. subtilis Sy41B*	MT159356	038.44°E	35.29°N	Dry surface with irregular edges
9	*B. subtilis SY133D*	MT159367	040.53°E	34.43°N	Dry surface with irregular edges
10	*B. subtilis SY132E*	MT159366	040.43°E	34.33°N	Dry surface with irregular edges
11	*B. subtilis SY35A*	MT159355	039.17°E	36.39°N	Dry surface with irregular edges
12	*B. subtilis SY151C*	MT159371	039.46°E	35.37°N	Dry surface with irregular edges
13	*B. subtilis SY44A*	MT159357	039.21°E	35.52°N	Dry surface and wrinkled
14	*B. subtilis SY113C*	MT159360	040.42°E	34.48°N	Dry surface with irregular edges
15	*B. subtilis SY116C*	MT159361	040.42°E	35.37°N	Dry surface with irregular edges
16	*B. subtilis SY132C*	MT159365	040.42°E	34.48°N	Dry surface, sticky with irregular edges
17	*B. subtilis SY160C*	MT159372	038.50°E	35.29°N	Crimped surface with smooth ends
18	*B. subtilis SY135D*	MT159369	040.27°E	35.01°N	White crinkled surface
19	*B. subtilis SY73B*	MT159359	036.46°E	36.39°N	Dry surface with irregular edges
20	*B. subtilis SY60A*	MT159358	038.21°E	35.58°N	Wrinkled surface with smooth edges
21	*B. atrophaeus SY15.B*	MT159352	034.56°E	36.66°N	Brown-black, opaque. Circular, smooth
22	*B. atrophaeus SY63.E*	MT159354	035.59°E	38.26°N	Brown-black, opaque. Circular, smooth
23	*B. atrophaeus SY199.A*	MT159353	038.23°E	34.35°N	Brown-black, opaque. Circular, smooth
24	*P. polymyxa SY53.C*	MT159375	035.01°E	38.65°N	Milky white, then often with amoeboid spreading
25	*P. polymyxa SY55.B*	MT159376	035.01°E	38.65°N	Milky white, then often with amoeboid spreading
26	*B. amyloliquefaciens SY82.C*	MT159377	033.22°E	35.83°N	Creamy white with irregular margins, sticky texture
27	*B. amyloliquefaciens SY96.C*	MT159378	034.82°E	33.33°N	Creamy white with irregular margins, sticky texture
28	*B. amyloliquefaciens SY96.E*	MT159379	034.82°E	33.33°N	Creamy white with irregular margins, sticky texture
29	*B. amyloliquefaciens SY123.A*	MT159380	040.44°E	36.03°N	Creamy white with irregular margins, sticky texture
30	*B. amyloliquefaciens SY128.B*	MT159381	040.33°E	35.28°N	Creamy white with irregular margins, sticky texture
31	*B. amyloliquefaciens SY134.C*	MT159382	040.54°E	34.28°N	Creamy white with irregular margins, sticky texture
32	*B. amyloliquefaciens SY159.D*	MT159383	040.14°E	36.38°N	Creamy white with irregular margins, sticky texture
33	*B. amyloliquefaciens SY177.C*	MT159384	039.00°E	36.46°N	Creamy white with irregular margins, sticky texture
34	*B. amyloliquefaciens SY190.D*	MT159385	039.17°E	36.39°N	Creamy white with irregular margins, sticky texture
35	*B. amyloliquefaciens SY200.D*	MT159386	038.08°E	34.18°N	Creamy white with irregular margins, sticky texture
36	*B. tequilensis SY69.A*	MT159387	032.97°E	35.96°N	Yellowish with irregular margins, sticky texture
37	*B. tequilensis SY145.D*	MT159388	040.02°E	35.25°N	Yellowish with irregular margins, sticky texture
38	*B. tequilensis SY149.C*	MT159389	039.49°E	35.40°N	Yellowish with irregular margins, sticky texture
39	*B. tequilensis SY150.D*	MT159390	039.57°E	35.28°N	Yellowish with irregular margins, sticky texture
40	*B. simplex SY198.B*	MT159391	039.53°E	36.45°N	Small, creamy, smooth, glossym, circular, sticky texture

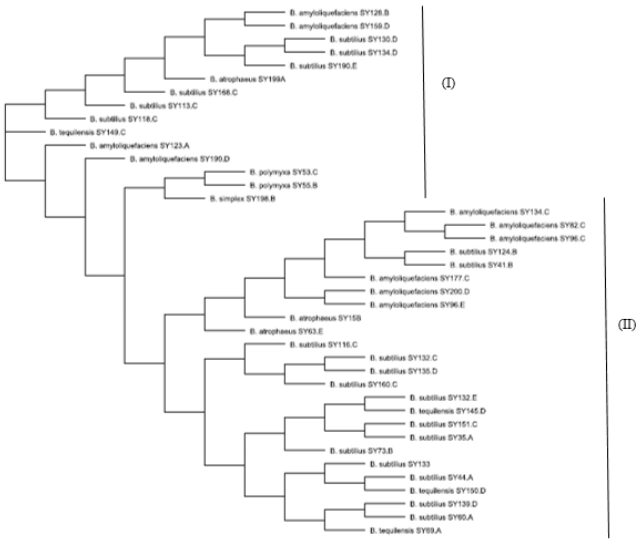

Figure 2. Phylogenetic tree of members of the genus *Bacillus*, based on 16S rRNA gene sequences.

Table 2. GC content of *Bacillus* species according t o *Madigan* and *Martinko* (2003) [13]

No.	Species	GC content %
	B. atrophaeus	
1	SY15B	55.3
2	SY199A	55.5
3	SY63E	55.2
	B. subtilis	
4	SY35A	54.1
5	Sy41B	56
6	SY44A	55
7	SY60A	55.2
8	SY73B	54.9
9	SY113C	54.3
10	SY116C	54.4
11	SY118C	53.6
12	SY124B	55.4
13	SY130D	55.2
14	SY132E	55.1
15	SY133	54.8
17	SY132C	55.6
19	SY134D	55.4
20	SY135D	55.4
21	SY139D	56.5
22	SY151C	54.6
23	SY160C	55.5
24	SY168C	55.4
25	SY190E	55.5
	Paenibacillus polymyxa	
24	SY53C	56
25	SY55B	55.6
	B. amyloliquefaciens	
26	SY82C	56.4
27	SY96C	55.9
28	SY96E	55.9
29	SY123A	55.6
30	SY128B	56.1
31	SY134C	56.2
32	SY159D	56.9
33	SY177C	55.1
34	SY190D	55.3
35	SY200D	55.6
	B. tequilensis	
36	SY69A	54.9
37	SY145D	54.9
38	SY149C	55
39	SY150D	54.8
	B. simplex	
40	SY198B	54.7

In both groups *B. atrophaeus, B. amyloliquefaciens, B. tequilensis* and *B. subtilis* species are phenotypically very similar according to Chun and Bae (2000), and share almost identical 16S rRNA gene sequences (Ash et al., 1991) that can support our results in this investigation and might explain their distribution within phylogenetically tree (Fig 2). A similar cladogram has been published [7] suggesting that the *B. subtilis* species complex which need further characterization. On the other hand, *Bacillus* strains from both within and between regions of Syria showed similar16S rRNA gene sequences and were not concentrated in one region of the country (Fig 2). This can be attributed that strains might have derived from the same source *Bacillus* population and disseminated from one area to another by different factors.

Moreover, the presence of *Bacillus* in all sampled locations might be attributed to its ability to move at high rates under harsh natural conditions [6], as well as to its adaptation under hot climates [1].

On the other hand, the GC content % of 16S rRNA was ranged between 54.1% - 56.9% (Table 2), this can reflect the number of hydrogen bonds between the paired strands which is reasonable to interpret these ratios as *Bacillus* species adaptations to various regions temperatures, but this can't provide an idea about how quickly such adaptation can happen. Similar GC ration was found among *Bacillus* species as reported by Sabir et al. (2013) [15]. On the other hand, Galtier and Lobry (1997) [9] reported that the GC contents of several structural RNAs in prokaryotes positively linked with the favorable growth temperature.

4. Conclusion

This work showed that 16S rRNA sequencing classified the collected strains under *Bacillus* genus, with close genetic relatedness between *B. subtilis* and *B. amyloliquefaciens* groups and *B. tequilensis*. The phylogenetic tree couldn't separate species into individual clusters, and no any clusters/clades were specific to origin or morphological criteria. GC% for 16S rRNA gene sequences ranged between 54.1% - 56.9%, and no clear correlation between the GC content and distribution of species strains within clusters was observed. This study showed many of the same general features seen in the *Bacillus* species 16S rRNAs sequences.

Compliance with Ethics Requirements. Authors declare that they respect the journal's ethics requirements. Authors state that they have no conflict of interest and all procedures connecting human or animal topics (if exist) respect the precise regulation and standards.

Acknowledgements. The authors would like to thank the Director General of AECS and the Head of the Molecular Biology and Biotechnology Department for their support throughout this work. We would also like to extend our thanks to Dr. H. Ammouneh for his assistance in achieving the experiments, and to Mr. H. Khalaf for *his help in statistical analysis.*

References

1. Aanniz, T.; M. Ouadghiri, M.; Melloul, et al., "Thermophilic bacteria in Moroccan hot springs, salt marshes and desert soils," *Braz. J. Microb.* **2015**, *46*, 443–453.
2. Ammouneh, H.; Harba, M.; Idris, E.; Makee, H. Isolation and characterization of native *Bacillus Thuringiensis* isolates from Syrian soil and testing

under insecticidal activities against some insect pests. *Turk. J. Agri. Forest.* **2011**, *35*, 421-431.
3. Ash, C.; Farrow, J.A.E.; Wallbanks, S.; Collins, M.D., Phylogenetic heterogeneity of the genus *Bacillus* revealed by comparative analysis of small-subunit-ribosomal RNA sequences. *Lett. Appl. Microbiol.* **1991**, 13: 202-206.
4. Bailón-Salas, A. M.; Medrano-Roldán, H.; Valle-Cervantes, S.; Ordaz-Díaz, L. A.; Urtiz-Estrada, N.; Rojas-Contreras, J.A. (2017). Review of molecular techniques for the identification of bacterial communities in biological effluent treatment facilities at pulp and paper mills. *BioRes.* **2017,** *12*, 4384-4409.
5. Browne, P.D.; Nielsen, T.K.; Kot, W.; et al. GC bias affects genomic and metagenomic reconstructions, underrepresenting GC-poor organisms. *Gigascience*. **2020**, *9*(2):giaa008.
6. Connor, N.; Sikorski, J.; Rooney, A. P.; et al., Ecology of speciation in the genus *Bacillus*. *Appl. Envir. Microb.* **2010,** 76, 1349–1358.
7. Fan, B.; Blom, J.; Klenk, H.P.; Borriss, R., *Bacillus amyloliquefaciens, Bacillus velezensis*, and *Bacillus siamensis* Form an Operational group *B. amyloliquefaciens*" within the *B. subtilis* species complex. *Front. Microbiol*. **2017,** *8*, 22.
8. Fatma, H.G.; Seufi, A.M., Molecular characterization of cultivable bacteria associated with adult *Schistocerca gregaria*, using 16S rRNA. J. *Taib. Uni. Sci.* **2020,** *14*,605-614.
9. Galtier, N., Lobry, J.R., Relationships between genomic G+C content, RNA secondary structures, and optimal growth temperature in prokaryotes. J. Mol. Evol. **1997,** *44*, 632–636.
10. Goto, K.; Omura, T.; Hara, Y.; Sadaie, Y., Application of the partial 16S rDNA sequence as an index for rapid identification of species in the genus *Bacillus*. *J. Gen. Appl. Microbiol.* **2000,** *46*, 1-8.
11. Harba, M.; Jawhar, M.; Arabi, M.I.E., *In vitro* Antagonistic activity of diverse *Bacillus* species against *Cochliobolus sativus* (common root rot) of barley, *Acta Phytopathol. Entomol. Hung.* **2020,** *55, 139–146.*
12. Lassalle, F., Périan, S., Bataillon, T., Nesme, X., Duret, L., et al. GC-content evolution in bacterial genomes: the biased gene conversion hypothesis expands. *PLoS Genet.* **2015**, *11*, e1004941.
13. Madigan, M.T.; Martinko, J.M., Brock biology of microorganisms (10th ed.). *Pearson-Prentice Hall.* **2003,** *ISBN* 84-205-3679-2.
14. Nair, J.R.; Singh, G.; Sekar, V., Isolation and characterization of a novel *Bacillus* strain from coffee phyllosphere showing antifungal activity. *J. F. Appl. Microbiol.* **2002**, *93*,772–780.
15. Sabir, J.S.M.; Salah, E.M.; Abo-Aba, A.; Refaei M.; Hussein, A.B.; Nabeeh A.B., Isolation, identification and comparative analysis of 16S rRNA of *Bacillus subtilis* grown around *Rhazya stricta* roots. Life Sci. J. **2013**, *10*.
16. Silva, C.A.; Bertran, J.; Oliva, A., Mutual relationship between stacking and hydrogen bonding in DNA. Theoretical study of guanine-cytosine, guanine-5-methylcytosine, and their dimers. *The J. Phys. Chem. B.* **2010**, *114*,10217-27.
17. Sueoka, N., On the genetic basis of variation and heterogeneity of DNA base composition *Proc. Natl. Acad. Sci. U.S. A.,* **1962,** 48, pp. 582-592
18. Varial de Melo L.H.; Francine Y.O.R.; Marcia S.V.; Patrícia, M.G.; Gabriel M.A.; Camila P.B.; Pablo B.A.; Elizabeth T.A.R.; Carolina N.R.; Stefan S.; Paulo M.F.B. S.; Jordano P.A.; José I.B., Diversity and biotechnological potential of endophytic Bacillus species originating from the stem apoplast fluid of sugarcane plants. *Appl. Soil Ecol.* **2021**, *166*, ISSN 0929-1393,
19. Victor, H.G.D.; Priscila da, S.F.C.G.; Allan, C.A.M.; Bianca, C.A.C.; August E.W.; Bruce B.; Rodrigo S.M.; Rosane, S., Evaluation of 16S rRNA hypervariable regions for bioweapon species detection by massively parallel sequencing. *Inter. J. Microb.* **2020,** ID 8865520.
20. Wulff, E.G.; Mguni, C.M.; Mansfeld-Giese, K.; Fels, J.; Lübeck, M.; Hockenhull, J. Biochemical and molecular characterization of *Bacillus amyloliquefaciens*, *B. subtilis* and *B. pumilus* isolates with distinct antagonistic potential against *Xanthomonas campestris* pv. campestris. *Plant Pathol.* **2002**, *51*, 574-584.