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Heterogeneity within the 16S rRNA gene of *Bacillus subtilis* isolates differing with antagonistic activity against *Cochliobolus sativus* pathogen

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Abstract

Bacillus subtilis, a gram-positive bacterium, has attracted considerable interest in both academic and industrial research. However, despite its substantial importance, there is a lack of appropriate and adequate information regarding the genetic diversity of its isolates. In this work, the nucleotide sequences of the 16S ribosomal RNA (16S rRNA) of 19 *B. subtilis* isolates differing in antagonistic activity were examined. The results indicated that 16S rRNA gene had sequence variations at 14 positions in the 514 bp including insertions, deletions and SNPs. 16S rRNA data clearly divided isolates of *B. subtilis* into two major clades. Clade 1 contained major sub-clusters consisting of 14 isolates, whereas the remaining 5 isolates were placed in clade 2. On the other hand, *B. subtilis* had significant antagonistic activities against the barley fungal pathogen *Cochliobolus sativus*, and 16S rRNA data did not result in any clusters/clades specific to antagonism. The molecular parameter used in this study provides crucial information about the genetic heterogeneity among16S rRNA gene of *B. subtilis* isolates which can have a profound effect on the classification of taxa.

Keywords: *Bacillus subtilis*, 16S rRNA, heterogeneity, antagonism, *Cochliobolus sativus*

1. Introduction

Bacillus subtilis, a gram-positive bacterium, is a noticeable diverse bacterial species that is capable of growth under various environments [1]. The *B. subtilis* genome has evolved with the certain function of enabling survival within its natural environment. However, the availability of a big number of defined reporter gene mutants in *B. subtilis* provides an unparalleled resource for studying functional genes in a soil bacterium [2,3]. Previous works have reported a number of sequence variations in different *B. subtilis* strains [4,5]. However, while a large information is reported about *B. subtilis* at the molecular level, relatively little is known about its genetic variation.

The 16S rRNA gene has been widely used for the taxonomic classification of bacteria by detection of sequence differences in the hypervariable regions of this gene, since it has specific properties, including its ubiquitous distribution, relative stability [6] and mosaic structure [7]. However, variations in 16S rRNA gene sequences often can be detected within the genome of a single strain, due to the presence of single nucleotide polymorphisms (SNPs) or small insertions or deletions (indels) among the multiple 16S rRNA copies. Such polymorphisms have been documented in different genera and species [8].

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 16S rRNA gene sequences have become powerful tools in the taxonomic classification of microorganisms with the increasing use of PCR technology [10].

However, despite its substantial importance, a limiting factor for taxonomic and diversity characterization of bacteria, there is a lack of

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appropriate and adequate information regarding 16S rRNA structure of *B. subtilis* isolates. The primary objective of the current research was to investigate the variables in the 16S rRNA sequence of ribosomal DNA of *B. subtilis* isolates collected from different regions of Syria, and their relationship with antagonism activity.

2. Materials and Method

2.1. B. subtilis isolates

Soil samples were randomly collected from different regions of Syria (Table 1). Nineteen *B. subtilis* isolates were screened among 525 isolates on nutrient broth (NB) culture [11], the colonies were identified according to Wulff et al. (2002) [12]. A pure culture of each *B. subtilis* isolate was first grown on NB and incubated at 37 °C for 24 h (Table 1).

2.2. In vitro estimation of antagonism

The antagonism of *B. subtilis* isolates was screened on the bases of fungal growth inhibition as described by Harba et al. (2020) [13]. The soil-borne fungus *Cochliobolus sativus*, the causal agent of barley common root rot was used for this purpose. Briefly, *B. subtilis* isolate Pt4 was streaked as thick bands on four opposite edges on the NA plates, and mycelium disc (5 mm in diameter) of an actively growing culture of *C. sativus* fungus was placed onto the center of above NA plates. The Petri dishes were incubated 21 ± 1 °C in the dark for 24 hours [14]. Mycelia discs on NA medium without bacteria was maintained as control. The antifungal activity (the distance between the edge of antagonistic bacterial growth and the edge of *C. sativus* mycelium) was measured according to Rabindran and Vidhyasekaran (1996) [15]. The experiment was conducted in triplicate.

2.3. 16S rRNA gene sequencing

The 16S rRNA was amplified using universal oligonucleotide primers BacF (5'-GTGCCTAATACATGCAAGTC-3') and BcaR (5'-CTTTACGCCCAATAATTCC-3'), which produced amplicons of approximately 545 bp [16]. The amplification was performed in 50 µL of reaction mixture containing Taq polymerase (5U/µL, Fermentas), 1x reaction buffer (TrisKCl-$MgCl_2$), 2 mM $MgCl_2$, 0.2 mM dNTP, 1 µM of each primer, and 2 µL (50-100 ng) of purified genomic DNA. PCR steps were 95 °C for 5 min, 95 °C for 1 min, annealing for 1 min at 54 °C, an extension at 72 °C for 90 s, and a final step of extension was at 72 °C for 10 min. PCR amplification was performed in a total of 30 serial cycles. PCR products were electrophoresed on a 1.5 % agarose gel and visualized under UV light after staining with ethidium bromide. Prior to sequencing, PCR products were purified with a commercial QIAgen gel extraction kit (28704). Sequences of 16S rRNA were determined using ABI 310 sequencer (Perkin-Elmer Applied Biosystems) and compared with known sequences using the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov).

Table 1 . *Bacillus subtilis* used in the study

Isolates no.	Accession number	Location		Morphology
B. subtilis SY118C	MT159362	040.54°E	34.29°N	Dry surface with irregular edges
B. subtilis SY130D	MT159364	040.58°E	34.24°N	Dry surface have tree edges
B. subtilis SY134D	MT159368	040.54°E	34.28°N	Dry surface with irregular edges
B. subtilis SY190E	MT159374	039.17°E	39.20°N	Crimped surface with almost-regular edges
B. subtilis SY168C	MT159373	037.56°E	36.30°N	Smooth surface with smooth edges
B. subtilis SY139D	MT159370	040.27°E	35.01°N	Dry surface with irregular edges
B. subtilis SY124B	MT159363	040.39°E	35.53°N	Smooth surface, polished, smooth edges
B. subtilis Sy41B	MT159356	038.44°E	35.29°N	Dry surface with irregular edges
B. subtilis SY133D	MT159366	040.53°E	34.43°N	Dry surface with irregular edges
B. subtilis SY132E	MT159365	040.43°E	34.33°N	Dry surface with irregular edges
B. subtilis SY35A	MT159355	039.17°E	36.39°N	Dry surface with irregular edges
B. subtilis SY151C	MT159371	039.46°E	35.37°N	Dry surface with irregular edges
B. subtilis SY44A	MT159357	039.21°E	35.52°N	Dry surface and wrinkled
B. subtilis SY116C	MT159361	040.42°E	35.37°N	Dry surface with irregular edges
B. subtilis SY132C	MT159367	040.42°E	34.48°N	Dry surface, sticky with irregular edges
B. subtilis SY160C	MT159372	038.50°E	35.29°N	Crimped surface with smooth ends
B. subtilis SY135D	MT159369	040.27°E	35.01°N	White crinkled surface
B. subtilis SY73B	MT159359	036.46°E	36.39°N	Dry surface with irregular edges
B. subtilis SY60A	MT159358	038.21°E	35.58°N	Wrinkled surface with smooth edges

Table 2. Sequence differences in 16S RNA among *B. subtilis* collections examined in this study and their antagonistic activity against *C. sativus* .

		Base position													
Isolates no.	*Antifungal activity	50	54	57	59	101	163	204	279	391	398	402	406	410	439
B. subtilis SY118C	+++	G	G	G	T	A	C	T	C	G	A	A	T	T	A
B. subtilis SY130D	+	G	G	G	T	A	C	T	C	G	A	C	C	T	A
B. subtilis SY134D	+++	G	G	G	T	A	C	T	C	G	A	C	C	T	A
B. subtilis SY190E	++	G	G	G	T	A	C	T	C	G	A	C	C	T	A
B. subtilis SY168C	+++	G	G	G	T	A	C	T	C	G	A	C	C	T	A
B. subtilis SY139D	+++	-	-	C	G	G	A	C	C	A	G	T	G	T	A
B. subtilis SY124B	+++	-	-	C	G	G	A	C	G	G	A	T	G	C	A
B. subtilis Sy41B	+++	-	-	C	G	G	A	C	G	G	A	T	G	C	A
B. subtilis SY133D	+++	-	-	C	G	G	A	C	G	A	G	T	-	T	C
B. subtilis SY132E	+++	A	-	C	G	G	A	C	G	A	G	T	G	T	A
B. subtilis SY35A	+++	A	-	C	G	G	A	C	G	A	G	T	G	T	A
B. subtilis SY151C	+++	-	-	C	G	G	A	C	G	A	G	T	G	T	A
B. subtilis SY44A	++	-	-	C	G	G	A	C	G	A	G	T	G	T	A
B. subtilis SY116C	+++	A	-	C	G	G	A	C	G	A	G	T	G	T	A
B. subtilis SY132C	+++	-	-	C	G	G	A	C	G	A	G	T	G	T	A
B. subtilis SY160C	++	-	-	C	G	G	A	C	G	A	G	T	G	T	A
B. subtilis SY135D	+++	-	-	C	G	G	A	C	G	A	G	T	G	T	A
B. subtilis SY73B	++	-	-	C	G	G	A	C	G	A	G	T	G	T	A
B. subtilis SY60A	++	-	-	C	G	G	A	C	G	A	G	T	G	T	A

Only positions where differences occurred are shown; all other positions are identical

Dashes represent alignment gaps

Weak inhibition: + (Fungal growth was slightly inhibited by bacteria)

Average inhibition: ++ (Loosely arranged mycelial growth over the bacterial zone)

Strong inhibition: +++ (Fungal growth was completely inhibited before the bacterial zone)

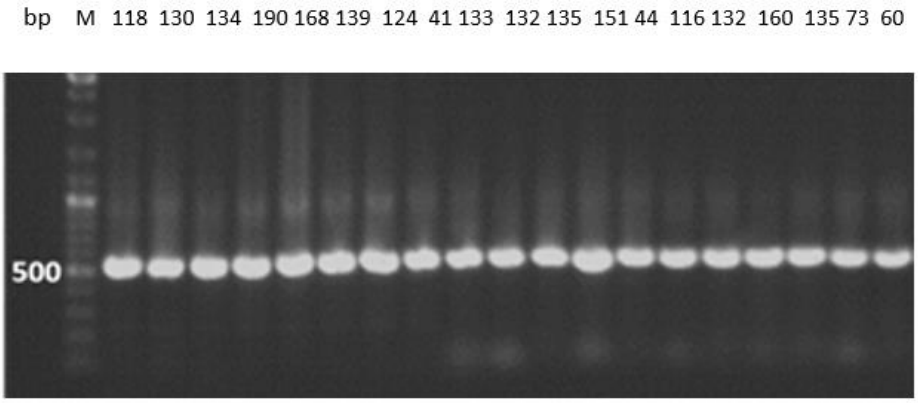

Figure 1. Agarose gel electrophoresis of 16S rRNA of 19 *B. subtilis* isolates used in the study. M represents the 100-bp DNA marker (*Hin*fI; MBI Fermentas, York, UK)

2.4. DNA sequence assembly and alignment

Sequence were aligned for the 19 *B. subtilis* isolates using the program Vector NTI [17], and the alignment was inspected and adjusted manually where necessary. A phylogenetic tree was generated by performing distance matrix analysis using the NT system. The experiments were performed in triplicate.

3. Results and Discussion

In the present investigation, previously collected *B. subtilis* isolates from Syrian soils [11] were used to address levels of genetic variation in 16s rRNA regions in a natural bacterial population. Data showed that Bac primers yielded single DNA fragments of ~545 bp, presented in 19 *B. subtilis* isolates (Figure 1). Based on 16S rRNA gene sequencing *B. subtilis* isolates displayed similarities ≥98 % to their closely related type strains. The nucleotide sequences were previously deposited in GenBank under accession numbers MT159352 to MT159391 (Table 1).

A total alignment of 545 bases including gaps was obtained and used in the comparisons among isolates. The results indicate that the sequences of 16S rRNA regions in different isolates are not

identical and sequence variations were found at 14 positions in the 545 bp that were sequenced (Table 2). The distribution of different mutation types (transitions [A↔G and C↔T], transversions [purine↔pyrimidine] and insertions/deletions) in multiple differing 16S rRNA gene copies are shown in Table 2.

The variability in 16S rRNA region may have arisen through point mutations, gene flow and/or recombination. Here we were not able to determine which, if any, of these particular mechanisms was responsible for the degree of the observed genetic diversity. However, Schmidt (1997) [18] reported that a part of heterogeneity might be attributed to the fact that rRNA genes are often organized as a part of a multigene family, with the copy number ranging from 1 to 15. The sequence polymorphism within this family has been documented to be dependent on the frequency of molecular interaction mechanisms such as gene conversion [19].

However, it has been suggested that mutation in *B. subtilis* is in linkage with the transcription-associated stationary-phase mutagenesis, which is in relation with the high expression level of the target gene and Mfd, the transcription repair coupling factor [20]. Our results which utilized sequence variability within 16S rRNA region among *B. subtilis* agree with results of similar studies conducted with other bacterial species [10,21].

The phylogenetic tree based on *16S rRNA* sequences split *B. subtilis* isolates into two major clades. Clade 1 contained major sub-clusters consisting of 14 isolates, whereas the remaining 5 isolates were placed in clade 2 (Figure 2). These isolates had a significant (P<0.05) antagonistic activity against the *C. sativus* (Figure 3), and no any 16S rRNA clusters/clades were specific to antagonism patterns. However, with some exceptions, for instance, the two isolates *B. subtilis SY139D* and *B. subtilis SY124B* placed under same sub-cluster and had similar complete inhibition effect against *C. sativus*. In addition, the two isolates *B. subtilis SY60A* and *B. subtilis SY73B* placed under the same sub-cluster and had similar average inhibitions against this pathogen (Figure 2). However, the high inhibitory capacity of some *B. subtilis* isolates found in this work might be attributed to the ability of this bacterium to produce a wide variety of antifungal compounds and antibiotics belonging to the family of iturins and subtilins [22].

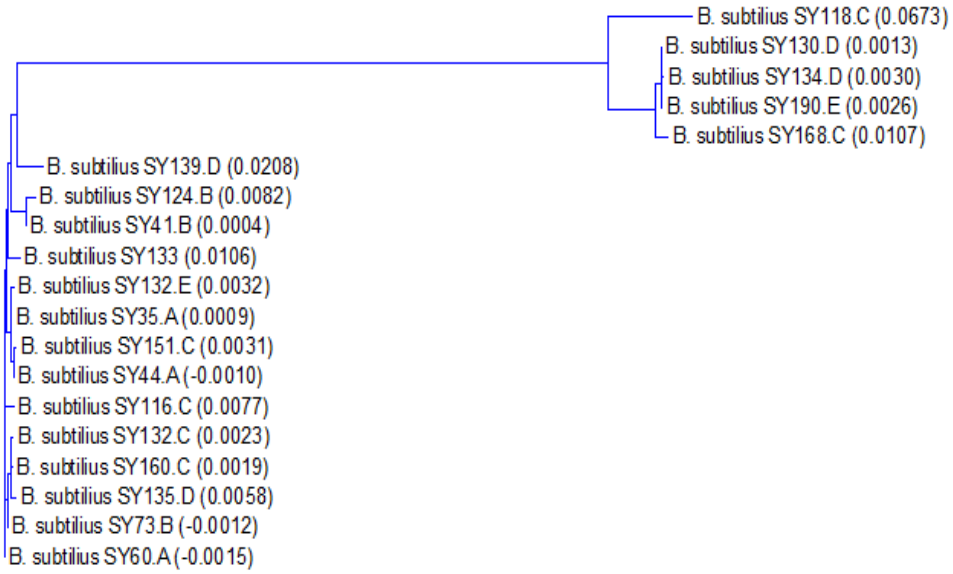

Figure 2. Phylogenetic tree showing the relationship among 16S rRNA gene sequence data detected among *B. subtilis*. Sequence similarity are given in parentheses

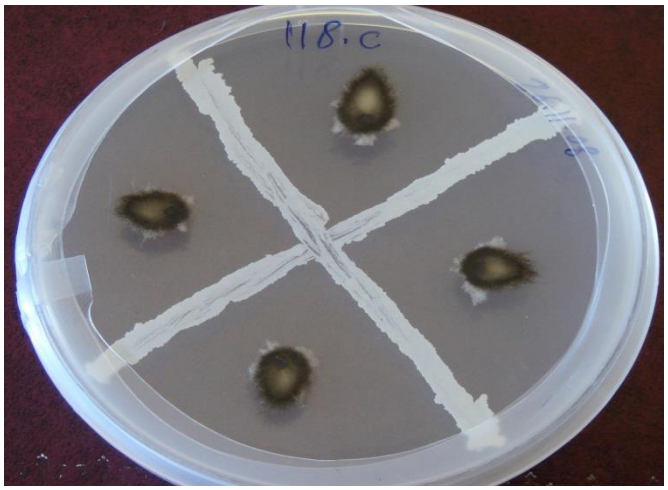

Figure 3. *Bacillus subtilis* SY118c showing antagonistic activity against Pt4 *C. sativus* isolate in the NA culture plate assay

4. Conclusion

Taken together, this work clearly showed the presence of sequence heterogeneity in 16s rRNA gene among *B. subtilis* isolates, and 16S rRNA data did not result in any clusters/clades specific to antagonism. The results presented here may provide important information concerning the application of 16S rRNA sequences for phylogenetic analyses and the genetic diversity among *B. subtilis* isolates.

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Compliance with Ethics Requirements. Authors declare that they respect the journal's ethics requirements. Authors declare that they have no conflict of interest.

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