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Research Article

PHYLOGENETIC RELATIONSHIPAND GENETIC DIVERSITY OF *BACILLUS THURINGIENSIS* ISOLATED FROM SOIL SAMPLES, JEDDAH, KSA

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A B S T R A C T

In the present study a total of 19 *Bacillus thuringiensis* were isolated from soil samples Jeddah, Saudi Arabia. The distinctive properties of 16s rRNA gene as universal phylogenetic marker was employed to carry out comprehensive diversity study of the bacterial isolates in order to reveal the evolutionary and phylogenetic relationships among these isolates. The DNA length and %GC content varied greatly between and within strains, and variation in GC-content of all studied sequences indicated no connection between sequence length and %GC content. Phylogenetic analysis showed that these isolates clustered in 5 clads. That is, it could be concluded that these observed variations in sequences and nucleotide of *Bacillus thuringiensis* strains are results of adaptation tosuch environment of Saudi Arabia. Further study for sequence analysis of salinity tolerance genes would be of great interest.

INTRODUCTION

Bacillus thuringiensis was discovered in 1901 when it was killing (Milner 1994). In the following years some scientists reported the existence of endospore within Bt spore, referred to as Cry toxin. Bt spores (cry toxin) becomes active by proteoletic enzymes in the alkaline gut juice (pH 8–10) when ingested by the susceptible insect and cause mortality, (Broderick, *et al* 2006; Bravo, *et al*., 2007). The Cyt toxin have a cytolytic to a wide range of invertebrate and vertebrate cells in vitro Ben-Dov, E. (2014); Palma, *et al*. (2014). BtCry proteins when deposited into soil and environment were degraded in soil within a few days, and had no effect on soil bacteria, actinomyces, fungi, protozoa, algae, nematodes, or earthworm (Alvarez-Alfageme, *et al*., 2010; Chakroun, *et al*., 2016). 16S rDNA and ERIC based on PCR method were tested for the effectiveness of the differentiation of *B. thuringiensis* and *B. cereus.* 16S rDNA-PCR showed no obvious difference between *B. cereus* and *B. thuringiensis* (Haitao, *et al*., 2011).

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Twenty isolates of *Bt*, which were collected from different geo-ecological regions of Ukraine, physiological and biochemical analyses were done with isolates and 16S rDNA nucleotide sequence analysis was also done by (Punina,, *et al.*, 2013). Identification of *Bacillus thuringiensis* by 16S rRNAwas done by (Osman, 2012)and screened strains for the presence of vip1 and vip2 genes by polymerase chain reaction (PCR) amplification, with only four strains producing the desired bands of Vip1 and Vip2. Cloning of the amplified fragments in pGEM-vector, then sequenced and analyzed. In the work of (Liu, *et al.,* 2017), The complete genome sequencing and bioinformatics analyses of *B. thuringiensis* strain BM-BT15426 was done and has a length of 5,246,329 bp and contains 5409 predicted genes with an average G + C content of 35.40%. They found that a total of 21 virulence factors and 9 antibiotic resistant genes were identified. Abdulrahman, *et al*., (2017) isolated native strains of *Bacillus thuringiensis*, they screened the isolates for the presence of parasporin–1 gene. DNA amplification using PCR based methodfor the gene-specific primers was used. Only four strain exhibited amplicons of a size analogous to that of the known parasporin–1 gene. Cloning was done of the gene in e pGEM-

vector and then were sequenced and analyzed. Bioinformatics analysis revealed that the nucleotide sequences obtained from the isolates were 99% homologous to the known DNA sequence of the parasporin gene (Blast software) and 98% homologous to the known amino acid sequence. Jin-Yu, *et al*., (2007). In their extensive studies for comparison *Bacillus thuringiensis* isolates by bioinformatics method they describe that function and the specificity determination regions, comprehensive evolutionary analyses of the *cry* gene family are rather rare and potential pressures driving the diversification of Cry proteins. Results also concluded that result in the functional divergence in Cry proteins. *Bacillus thuringiensis* isolates showed a great genetic diversity andcrystal shape heterogeneity (Cinar, *et al*., 2007)

In this study, *B. thuringensis* bacterial strains identified using 16s sequence analysis and bioinformaticstools was used for comparing multiple isolated marine samples was developed. Evolutionary andphylogenetic relationships among isolated strains using 16s rDNA sequences also studied.

MATERIALS AND METHODS

Sample collection and isolation of Bacillus thuringiensis

Five different soil samples were collected from different regions of Jeddah City.Isolation of *Bacillus thuringiensis* were done. 0.5g of soil samples was suspended in 10 ml of sterile distilled water, soil samples was homogenized vigorously with vortex. After mixing 1 ml of the supernatant was stored at 80^{o}C for some minutes to kill most non-spore-forming bacteria.100 µL of suspension spread over nutrient agar plates. The apparent colonies were spread on the entire surface of Nutrient agarplates with triplicate. After incubation at 30^{o}C for two days all colonies likebacillus colonies were chosen. The obtainedsingle colony re-purified by re-streaking over Nutrient agarplatesseveral times, stored in Nutrient agarslants at 4^{o}C, and long term storage in Nutrient agar broth with 30% glycerol at -80^{o}C (Masoud *et al*., 2015).

Extraction of DNA

Extraction of DNAs was carried out by I mL of each isolated strains was centrifuged to collect the pellet. 200µL of TES buffer (50mM EDTA, 100mM Tris pH8,and 10% SDS) and 20µL Lysozyme added to the bacterial pellet with gentle pipetting at 37^{o}Cfor 60 min., 20µL of proteinase Ksolution added to the mix incubated at 37^{o}C for 60 min., the pellet suspension transferred to ice path for 5 min. then 250 µL of Sodium acetate 4Madded. The mix was spin down for 5 min. at 10.000 rpm for 5 min. clear supernatant was transferred to a new tube and 250 µL of chloroform/isoamyle alcohol added to the supernatant in ice path for 5 min. centrifugation was done at 10.000 rpm for 5 min. the collected DNA pellet dried for 10 min in laminar flow cabinet. 50 µL of TE (10mM Tris pH8, 1mM EDTA) added to each tube. The DNA were detected by electrophoresis in 1.5% agarose gel.

PCR amplification and sequencing of 16S rDNA16S gene sequencing and analysis

The amplified PCR products of the 16S rRNA gene bacterial gene fragments were detected by electrophoresis in 1.5% agarose gel. The amplified fragments were purified and sequenced at MACROGEN sequencing company, Seoul, Korea using the automated sequencer ABI 3100 (AppliedBiosystems) with BigDye Terminator Kit v. 3.1 (Applied Biosystems). Primers 518 F (5' C CA GCAG CC GC GG TAATACG - 3') and 800 R (5'- TA CC AG GG TA TC TA AT CC -3') were used for sequencing. The sequences obtained were edited with the software Vector NTI Suite 9, and compared with the NCBI database through BLAST searches. In this comparison, sequences of type strains most closely related to the sequences of the isolates were searched. For the definition of operational taxonomic units (OTUs), a similarity limit of 97% was adopted.

Bioinformatics analysis

The evolutionary history was inferred using the Neighbor-Joining method by (Saitou, and Nei, 1987). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura, *et al*., 2004) and evolutionary analyses were conducted in MEGA X (Kumar.*et al.*, 2018).

RESULTS

Bacterial samples

Out of 19 Bacillusbacteria from soil samples near Jeddah were isolated on NA agar plates as pure culturein this study .The strains were identified by 16S rRNA sequence analysis at Macrogen Company Korea, with Macrogen universal primer as identified in materials and methods. The resultsin Table (1) showed isolated strains of bacillus bacteria

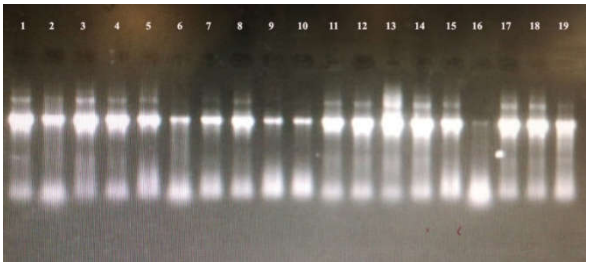

Fig 1 DNA pattern of 19 *Bacillus thuringiensis* isolates.

Bioinformatics analysis

Sequence Variations & GC-content

The isolated DNA of all 19 isolated strains are shown in fig (1), results shown that successful DNA isolation methods in all isolates strains. 16S rDNA were used to identify bacterial species of 19 isolates strains and one ATCC strain No (20), over nutrient agar media as shown in fig. 2.the 16s DNA bands are at 500 bp.

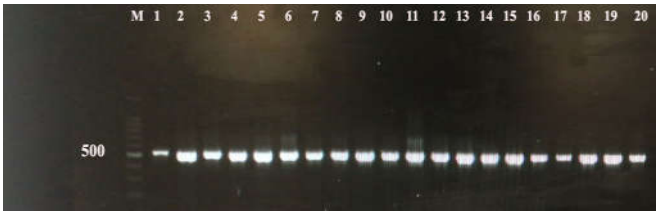

Fig 2 Agarose gel electrophoresis of 16s PCR products

Table 1 sequence length and GC content of all strains

Isolate	seq. length	GG content
BT.1	2014	0.559
BT.2	1596	0.43
BT.3	1593	0.519
BT.4	574	0.523
BT.5	1725	0.489
BT.6	1404	0.571
BT.7	2131	0.659
BT.8	1875	0.587
BT.9	1854	0.498
BT.10	1019	0.419
BT.11	1666	0.475

BT.12	1836	0.547
BT.13	1196	0.522
BT.14	1796	0.571
BT.15	1769	0.486
BT.16	1723	0.512
BT.17	3137	0.580
BT.18	510	0.527
BT.19	1644	0.583
Control ATCC 0461 L	505	0.497

BT strain

As shown in table (1) a description of the isolated species as well as their sequence length and the percentages of GC content of each isolate was done according to bioinformatics analysis of isolated strains. Graphical representations of sequence length and percentage of GC content are shown also in Figure (3). The all 19 isolated strains were identified as *Bacillus thuringensis*. DNA by comparing with NCBI. The length varied greatly between and within species, where the isolate BT 17 has the longest DNA sequence as illustrated in table (1)3137bp and BT 18 has the shortest sequence (510 bp), while the control ATCC strain was (505bp). For within species variation in the DNA Length showed variation noted in all strains.

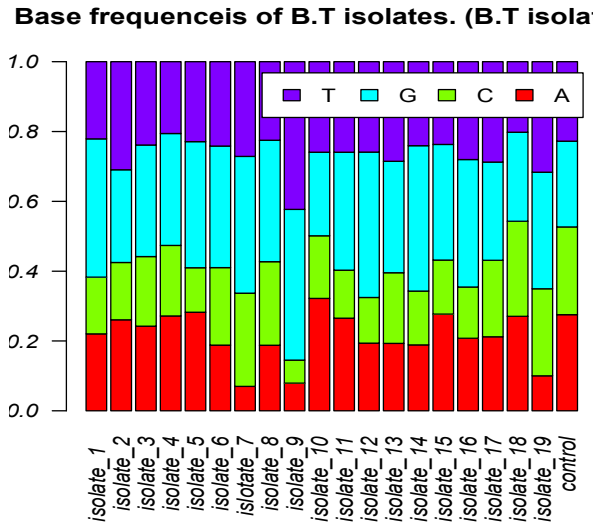

Fig 3 Base frequencies of *Bacillus thuriengensis* isolated strains and the control is ATCC

Base composition for the 19 and control strains is shown graphically in fig 3. Apart from *Bacillus thuringiensis*7, 19 and 9 which has the lowest and the largest percentage of Adenin. The noticeable variation in base composition were observed. These differ more pronounced when GC-content table (1) was considered. The GC-content of all studied sequences ranged from 0.43 for *Bacillus thuringiensis* 2 to 0.659 for *B.thuringiensis*7the sequence variation and GC content of each *Bacillus thuringiensis* strains indicate no connection between sequence length and %GC content. The plast results of all *Bacillus thuriengensis* strains are indicated in table (2).

Phylogenetic analysis

As shown from sequence analysis above Table (1,2) & Figure (3)) a considerable sequence variations were observed, as noticed in isolated strains. Such that, the final dataset comprised the 19 nucleotide sequences. The phylogenetic tree (Figure 4) was inferred using the Neighbor-Joining (NJ) method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test are shown on each node. The analysis involved 19 nucleotide sequences. All positions containing gaps and missing data were eliminated. This phylogenetic tree displayed clearly the resolved relationship of the 19 isolates. These 19 nucleotide sequences were clustered in 5 clades. Fairly longer branches lengths were observed among the rest of isolates, where it might be due to only partial sequences of 16s were isolated.

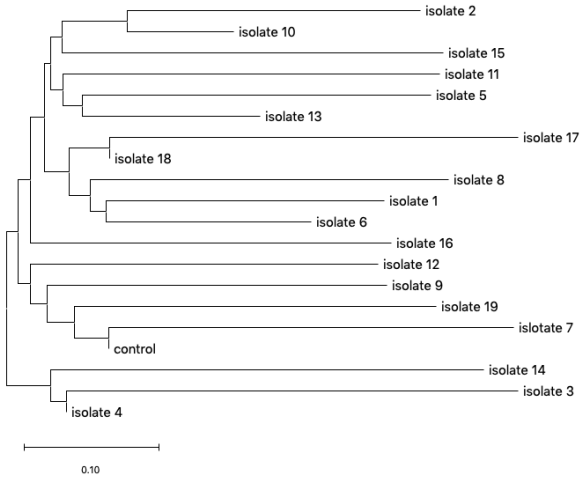

Fig 4 Neighbor- Joining phylogenic tree based on 16s gene sequencing of 19 isolated *Bacillus thuriengensis* strains

Table 2 Blast results of all isolates strains and the control ATCC strain

Isolate	Blast result	Query cover %	E-value	Per. Ident%
isolate1	*Bacillus thuringiensis* serovar*berliner*	23	0.0	99.2
isolate2	*Bacillus thuringiensis* serovar*berliner*	30	0.0	98.2
isolate3	*Bacillus thuringiensis* strain ATCC 10792, complete genome	30	0.0	89.1
isolate4	*Bacillus thuringiensis* strain ATCC 10792, complete genome	85	0.0	89.1
isolate5	*Bacillus thuringiensis* strain ATCC 10792, complete genome	27	0.0	98.8
isolate6	*Bacillus thuringiensis* serovar*berliner* ATCC 10792 chromosome, whole genome shotgun sequence	34	0.0	99
isolate7	*Bacillus thuringiensis* strain ATCC 10792, complete genome	22	0.0	98
isolate8	*Bacillus thuringiensis* strain ATCC 10792, complete genome	25	0.0	99
isolate9	*Bacillus thuringiensis*serovar*berliner* ATCC 10792 chromosome, whole genome shotgun sequence	25	0.0	99
isolate10	*Bacillus thuringiensis* serovar*berliner* ATCC 10792 chromosome, whole genome shotgun sequence	46	0.0	99
isolate11	*Bacillus thuringiensis* strain ATCC 10792, complete genome	29	0.0	98
isolate12	*Bacillus thuringiensis* strain ATCC 10792, complete genome	26	0.0	98
isolate13	*Bacillus thuringiensis* strain ATCC 10792, complete genome	40	0.0	98
isolate14	*Bacillus thuringiensis* strain ATCC 10792, complete genome	27	0.0	90
isolate15	*Bacillus thuringiensis* strain ATCC 10792, complete genome	26	0.0	99
isolate16	*Bacillus thuringiensis* strain ATCC 10792, complete genome	27	0.0	99
isolate17	*Bacillus thuringiensis* strain ATCC 10792, complete genome	15	0.0	99
isolate18	*Bacillus thuringiensis* strain ATCC 10792, complete genome	94	0.0	92
isolate19	*Bacillus thuringiensis* strain ATCC 10792, complete genome	29	0.0	96
control	*Bacillus thuringiensis* strain ATCC 10792, complete genome	81	0.0	86.5

DISCUSSION

Soil bacteria especially *B. thuringensis*its play a main role in biological control against different insects these bacterial strains are attracting attention as new biotechnological resources. Despite a hugemicrobial diversity, there is a

difference of laboratory isolation of this bacteria that are most abundant in the environment that severely limits development of bio-discovery research. However, a major limitationis culturing of certain strains have posed a difficulty. Once cultured, further studies can give information on the applications of these microbes. It is clear from this study that this approach provides a novel means of comparing multiple bacterial community structures using 16s sequences analysis. Bioinformatics programs analysis was used for comparing multiple bacterial communities and reveals the phylogenetic relationships among our isolates. Results indicated that these strains comprised one species (*B. thuriengensis)* these results are in agreement with Liu, *et al.,* 2017Randhawa,*et al*., 2011 Ibrahim, *et al.*, 2010.Graphical representations of sequence length varied greatly between and within species, some species shown longest DNA sequence and others shown shortest sequence, and pronounced differ in GC content between and when studied *B. thuringensis* strains. The percentage of GC content also studied; and we found that Variation in GC-content of all studied sequences which indicate no connection between sequence length and %GC content, these results are in agreement with Assaeedi, and Osman 2017;Sabir*et al*., 2013; andJin-Yu et al., 2007. Phylogenetic tree using Neighbor-Joining (NJ) method analysis of isolated marine bacterial strains shown sequence variations in most of studies strains and evolutionary distances were computed using the Maximum Composite Likelihood method, data represented that relationship of the most isolated strains and it was high percentage of bootstrap. However, fairly longer branches lengths were observed among the rest of isolates, where it might be due to only partial sequences of 16s were isolated; these data are in-agreement with Sabir *et al*., 2013; Osman, 2011.

Evolutionary relationships of taxa: The evolutionary history was inferred using the Neighbor-Joining method, the optimal tree with the sum of branch length = 4.5 is shown (Saitou, and Nei, 1987). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site (Tamura, *et al*., 2004). This analysis involved 20 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 3569 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar, *et al*., 2018)

CONCLUSION

In conclusion, results indicated that these observed variations in sequences and nucleotide of bacterial strains are results of adaptation to such harsh environment.

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