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Nucleotide Variations and Base-pair Substitutions of 16S rRNA Gene in some Local *Bacillus thuringiensis* Isolates

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ABSTRACT

Three local *Bacillus thuringiensis* isolates; Sn-2, Ts-5 and As-3 were used in the study. They previously collected from the soil of three governorates; Sinai, Toshkey and Aswan and were characterized by their high toxicity to *Spodoptera littoralis* and *Culex pipiens*. Three primer pairs of the forward and reverse were designed from the very similar sequences within consensus regions of twelve GenBank *Bacillus thuringiensis* 16S rRNA gene sequences to amplify three fragments with 252, 984 and 604 bp from isolates; Sn-2, Ts-5 and As-3 respectively. Sequence alignment using the Blast search revealed 17 nucleotide positional differences with base-pair substitutions between Sn-2 isolate with 252 bp (GU062822), whereas the highest number (5) of positional differences was guanine (G) found in Sn-2 that changed to thymine (T) in all other GenBank isolates and strains based on the 16S rRNA gene similarity. Accession GU062823 with 984 bp (isolate Ts-5) showed a total number of 11 nucleotide positional differences. While, accession GU062824 with a fragment size 604 bp (isolate As-3). In addition, 10 nucleotide positional differences were obtained from the four nucleotide bases (C, G, T, A) to (…) and four from (…) to the four nucleotide bases. Phylogenetic analyses based on 16S rRNA gene sequences showed that each of the three local *B. thuringiensis* isolates (Sn-2, Ts-5 and As-3) formed a phylogenetically distinct group, separate from all other named isolates and species of *B. thuringiensis*. Such obtained results evidently indicated a large diversity with unique characteristics of the local Egyptian isolates from all the other isolates and strains established around the world.

Keywords: Accessions GU062822, GU062823 and GU062824, *Bacillus thuringiensis* isolates, 16S rRNA gene

INTRODUCTION

The 16S rRNA is part of the small (30S) ribosomal subunit which also contains 21 ribosomal proteins. This subunit is involved in the early steps of translation initiation and is the site of codon-anticodon interaction. In *Escherichia coli*, the structure around the region of the signature (bp 100 to 190) is involved in binding of the primary binding protein S20 (Stern *et al.* 1989). Analysis of 16S rRNA sequences is a simple, commonly used method for the identification of microorganisms (Amann *et al.* 1995). However, early studies performed with a limited number of isolates from the *Bacillus cereus* group revealed that the 16S rRNA sequences of species in this group had as high as a 99 to 100% similarity and, thus, suggested that rRNA sequences might not be useful for discrimination of members of that group. However, that study examined only five isolates from the *B. cereus* group. The *B. cereus* group contains seven closely related species: *Bacillus anthracis*, *B. cereus*, *B. thuringiensis*, *B. mycoides* (Turnbull 1999), *B. pseudomycoides*, *B. weihenstephanensis*, and *B. medusa*. To date, identification and discrimination of these species has been based on analysis of morphological, biochemical, and immunological characteristics.

Although conserved in sequence overall, the 16S rRNA actually exhibit great variation in some regions. These differences in 16S rRNA sequence provide the basis for the design of nucleic acid probes of various specificities, ranging from probes targeting all living organisms to group-specific and species-specific probes. Another advantage of using the rRNA as a target is the fact that these molecules are naturally amplified within the cell. In general, rRNA represents about 80% of total nucleic acids in microbial cells and, thus, is present in many hundreds of thousands of copies per cell. This natural amplification allows for direct detection of rRNA sequences without the need for intermediate amplification via PCR (Amann *et al.* 1995).

The 16S and 23S rRNA genes are currently considered the most useful molecules for the determination of prokaryotic phylogeny. Analysis of these rRNA sequences has resulted in a tremendous expansion in our knowledge of prokaryotic diversity and has demonstrated the limitations of the existing prokaryotic taxonomy, which is based primarily on the analysis of phenotypic traits (Ludwig and Klenk 2001). In order to determine if variations in rRNA sequence could be used for discrimination of the members of the *Bacillus cereus* group, Bavykin *et al.* (2004) analyzed 183 16S rRNA and 74 23S rRNA sequences for all species in the *B. cereus* group as well as 30 *gyrB* sequences for *B. cereus* group strains with published 16S rRNA sequences. Their results indicated that the three most common species of the *B. cereus* group, *B. cereus*, *Bacillus thuringiensis*, and *Bacillus mycoides* were each heterogeneous in all three gene sequences, while all analyzed strains of *Bacillus anthracis* were found to be homogeneous. They demonstrated that rRNA and *gyrB* sequences may be used for discriminating *B. anthracis* from other microorganisms in the *B. cereus* group. Strains having <3% difference between their 16S rRNA genes were considered the same species (Stackebrandt and Goebel 1994). However, differences between 16S rRNA genes for some *Bacillus* species, such as *B. anthracis*, *B. cereus*, and *B. thuringiensis*, are <1% (Thorne 1993). Such small differences (e.g., one base between sequences or partial matches at a single nucleotide position in the 16S rRNA gene) have not been used for species differentiation. Another study of Ash *et al.* (1991) clearly demonstrates that such small differences might be important for species identification. DNA-DNA hybridization and 16S

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Table 1 Forward and reverse primer sequences used for 16S rRNA gene amplification.

Forward and reverse primers	Primer sequences (5′-3′)	Expected size	Size after DNA sequencing
F	GCATGGTTCGAAATTGAAAG	540 bp	252 bp
R	CATTTCACCGCTACACATGG		
F	GCATGGTTCGAAATTGAAAG	1197 bp	984 bp
R	CCAGCTTCATGTAGGCGAGT		
F	*CCATGTGTAGCGGTGAAATG*	667 bp	604 bp
R	CCAGCTTCATGTAGGCGAGT		

rRNA sequencing studies have shown that these three *Bacillus* species are closely related and probably represent a single species. If the three were classified as a single species, 16S rRNA sequencing appears to have the potential to differentiate strains at the subspecies level. Sacchi *et al.* (2002) reported that in a bioterrorism event, a tool is needed to rapidly differentiate *B. anthracis* from other closely related spore-forming *Bacillus* species. During the recent outbreak of bioterrorism-associated anthrax, they sequenced the 16S rRNA genes from these species to evaluate the potential of 16S rRNA gene sequencing as a diagnostic tool. The authors found eight distinct 16S types among all 107 16S rRNA gene sequences that differed from each other at 1 to 8 positions (0.06% to 0.5%). All 86 *B. anthracis* had an identical 16S gene sequence, designated type 6; 16S type 10 was seen in all *B. thuringiensis* strains; six other 16S types were found among the 10 *B. cereus* strains. This report describes the first demonstration of an exclusive association of a distinct 16S sequence with *B. anthracis*. Consequently, they were able to rapidly identify suspected isolates and to detect the *B. anthracis* 16S rRNA gene directly from culture-negative clinical specimens from seven patients with laboratory-confirmed anthrax. The goal of this study was to establish the genetic diversity of some local *Bacillus thuringiensis* isolates based on 16S rRNA gene and evaluate the potential of sequencing isolates using housekeeping genes for rapidly identification of microbial strains.

MATERIALS AND METHODS

Three local *Bacillus thuringiensis* isolates; Sn-2, Ts-5 and As-3 previously collected from the soil of three governorates: Sinai, Toshkey and Aswan (Nariman *et al.* 2009) were grown at 30°C overnight in Luria broth medium containing 2% tryptone, 1% yeast extract, and 1% NaCl with shaking.

DNA extraction and PCR amplification of 16S rRNA gene

DNA was extracted from the three isolates following the method of Ben-Dov (1999). Genomic DNA was prepared from an exponential phase overnight in Luria-Bertani medium. Aliquots of 10 ml of bacterial culture were harvested by centrifugation at 12,000 rpm for 15 min and washed once in sterile distilled water. the pellets were resuspended in 400 µl of lysis buffer containing 2% glucose, 50 mM Tris-HCl (pH 8.0), 25 mM EDTA, 3 mg/ml lysozyme and 200 mg/ml RNase. The cell suspension was incubated for 1 h at 37°C. Further DNA extraction was performed as described by Sambrook *et al.* (1989).

Amplification was performed according to Xu and Côté (2003) in a thermal cycler, GeneAmp 9600 Perkin Elmer (Martinsburg, West Virginia, USA) in a total volume of 25 µl containing 50 ng DNA, 1 mM of each primer, 200 mM dNTP, 1.5 mM $MgCl_2$ and 0.5 U *Taq* DNA polymerase (Promega, Madison, USA). PCR was performed under the following conditions: 5 min at 95°C and then 40 cycles of 30 sec at 94°C, 1 min at 52°C and 1 min at 72°C and a final extension step at 72°C for 5 min. The PCR products were separated in a 1.2% agarose gel containing 0.5 mg/ml of ethidium bromide and were visualized using Gel Doc XR System (Bio-Rad Laboratories, Inc., Cali, USA).

Design of specific primers for 16S rRNA gene

Twelve different *Bacillus thuringiensis* 16S rRNA gene sequences of accessions AM292029, EU693501, EU702408, EU812752, EU862321, EU874887, EF685168, EU036759, EU273315, EU341308, EU723845 and EU744620 obtained from the NCBI GenBank were aligned using multalin FASTA format. Three primer pairs of the forward and reverse were designed from the very similar sequences within consensus regions and primers were picked using Primer 3 program as presented in **Table 1**.

16S rRNA gene purification, sequencing and analysis

PCR products of 252, 984 and 604 bp were purified with the QIA quick PCR Purification Kit (Qiagen GmbH, Germany) according to the manufacturer's instructions. DNA was eluted in 20 µl of sterile water. The 16S rRNA fragment was sequenced on an Applied Biosystems automatic sequencer (ABI PRISM® 1200 DNA Sequencer, Bioron GmbH, Germany).

Sequences were compared with sequences of representatives of the most related *Bacillus thuringiensis* strains deposited in GenBank and sequencing-genome databases by using the BLAST search (http://www.ncbi.nlm.nih.gov/blast). Analysis was performed using Geneious Pro 4.5.4 program. A phylogenetic tree was supported from 500 bootstrap replicates and a dendrogram was constructed using multiple alignment of the 16S rRNA from *B. thuringiensis* isolates and strains.

Nucleotide sequence accession numbers

The GenBank accession numbers for the partial nucleotide sequences of the 16S rRNA gene from *Bacillus thuringiensis* isolates Sn-2, Ts-5 and As-3 are GU062822, GU062823 and GU062824, respectively.

RESULTS

PCR amplification of 16S rRNA in *Bt* isolates

PCR amplifications of the three *B. thuringiensis* isolates Sn-2, Ts-5 and As-3 revealed three fragments with expected sizes of 540, 1197 and 667 bp that represented the 16S rRNA gene (**Fig. 1**).

Sequence analysis of PCR-amplified 16S rRNA of the local *Bacillus thuringiensis* isolates

A 252 bp nucleotide sequence of the partial 16S rRNA gene from Sn-2 isolate (accession no. GU062822) was aligned and compared in the GenBank using the BLAST search. A total of 99 16S ribosomal RNA gene partial sequences from different accessions of *B. thuringiensis* included 6 strains, 49 isolates, 21 serovars and 23 serotypes were identified (**Table 2**). Blast alignment revealed several accessions of *B. thuringiensis* scored 95% identity with GU062822, whereas total score and coverage percentage revealed 381 bits and 94%, respectively as shown in **Table 2**.

A 984 bp nucleotide sequence of Ts-5 isolate (accession no. GU062823) was aligned and compared in the GenBank using the BLAST search. A total of 98 16S ribosomal RNA gene partial sequences from different accessions of *B. thuringiensis* were obtained. Blast alignment showed several accessions of *B. thuringiensis* scored 97% identity with

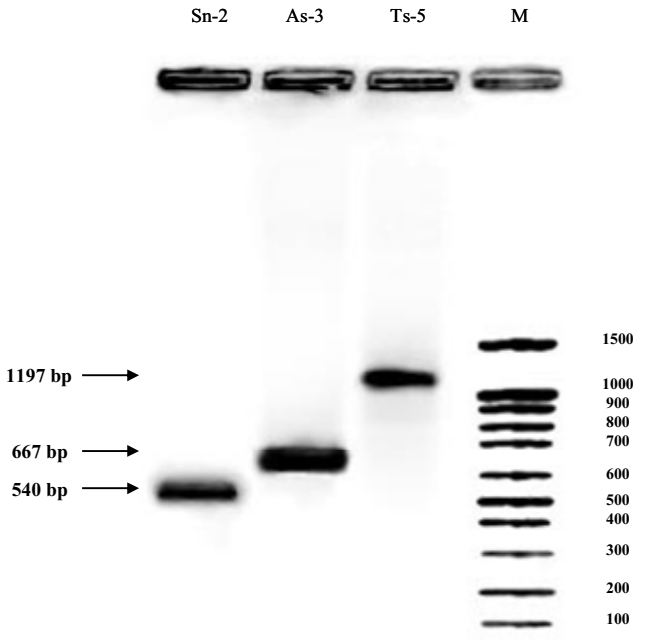


Fig. 1 PCR amplified products of 16S rRNA gene of the three *B. thuringiensis* isolates using three designed primers with expected sizes 1197, 667 and 540 bp.

GU062823, whereas total score was 1688 bits and coverage percentage showed 94%.

A 604 bp nucleotide sequence of the partial 16S rRNA gene from As-3 isolate (accession no. GU062824) was aligned and compared in the GenBank using the BLAST search that showed several accessions of *B. thuringiensis* scored 100% identity with GU062824, whereas total score was 1116 bits and coverage percentage showed 100%.

Sequence alignments in **Fig. 2**, **3**, **4** of the 16S rRNA gene of the *B. thuringiensis* isolates Sn-2, Ts-5 and As-3 (GU062822, GU062823 and GU062824, respectively) compared with *B. thuringiensis* GenBank strains, isolates, serovars and serotypes revealed positional differences in nucleotide sequences and base-pair substitutions between the three local isolates and the numerous isolates and strains.

The accession GU062822 with a fragment size 252 bp (isolate Sn-2) showed a total number of 17 nucleotide positional differences with base-pair substitutions, whereas the highest number (5) of positional differences was guanine (G) found in GU062822 that changed to thymine (T) in all other GenBank isolates and strains as shown in **Table 3**. A single base change or a mixed base (more than one nucleotide determined at a single position) is considered as a new 16S type. Three other nucleotide positional differences were obtained from C→T, T→A and T→G. Moreover, three of four nucleotide positional differences were obtained from (…) with no nucleotide base to T and one from (…) to G.

The accession GU062823 with a fragment size 984 bp (isolate Ts-5) showed a total number of 11 nucleotide positional differences with base-pair substitutions, whereas the highest number (4) of positional differences was guanine (G) found in GU062823 that changed to thymine (T) in all other GenBank isolates and strains as shown in Table (4). Two other nucleotide positional differences were obtained from T→A and T→C. Moreover, four and three of 11 nucleotide positional differences appeared; T→(…) and A→(…), respectively and from the other two nucleotides to (…).

It is interesting to note that, accession GU062824 with a fragment size 604 bp (isolate As-3) that showed 100% identity in Blast alignment search showed also high similarity without nucleotide positional differences or base-pair substitutions

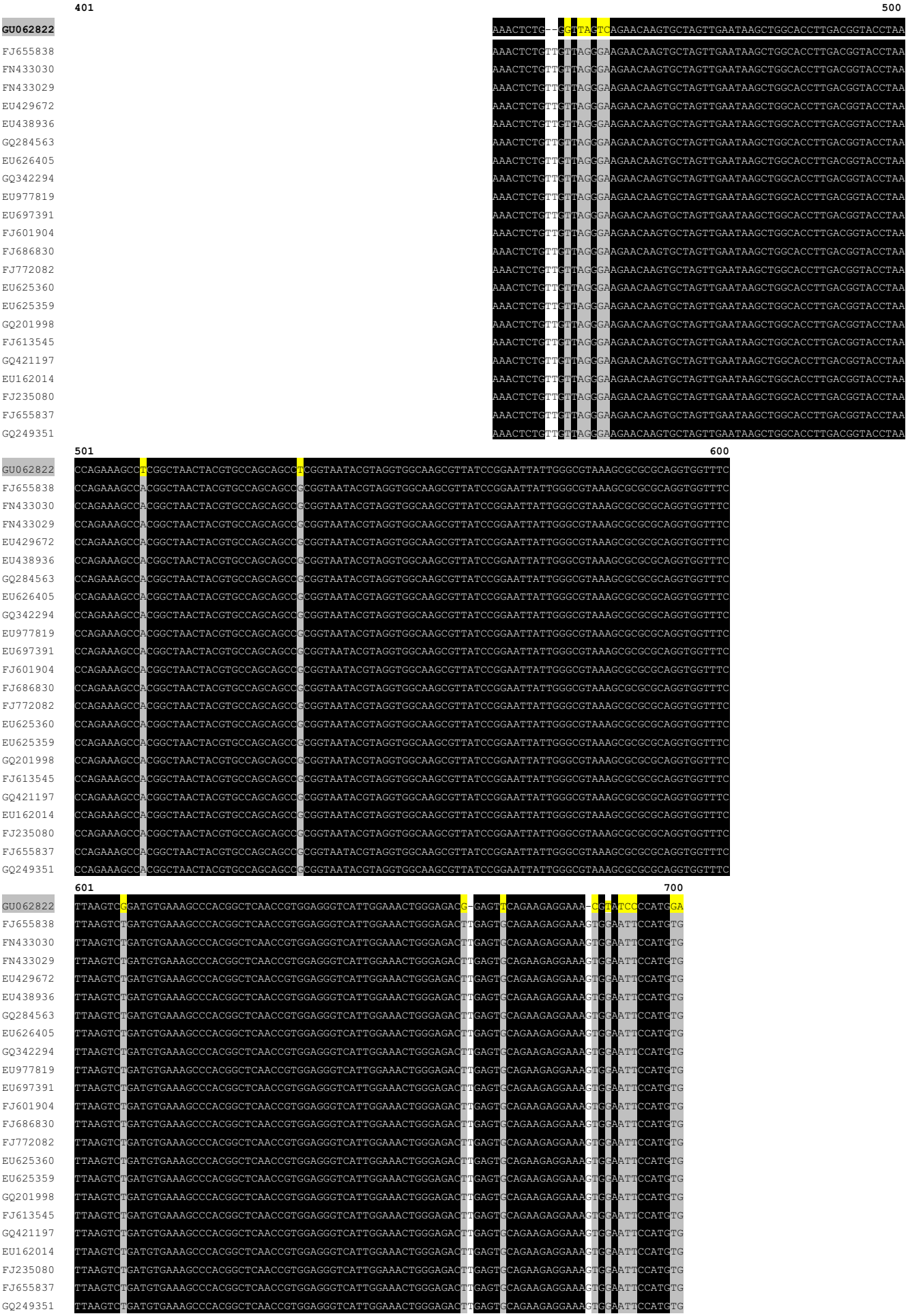
The phylogenetic tress represented the relationship between two of the three local *B. thuringiensis* isolates (Sn-2 and Ts-5) with their accession numbers (GU062822 and GU062823, respectively) and all described *B. thuringiensis* isolates, serovars, serotypes and related strains obtained from GenBank based on the 16S ribosomal RNA gene are shown in **Figs. 4**, **5**. The dendrograms divided all named GenBank isolates and strains into various main discrete clusters, whereas each of the three local isolates formed a phylogenetically distinct cluster, separate from all other named isolates and species. Such obtained results evidently indicated a large diversity with unique characteristics of the local *B. thuringiensis* Egyptian isolates from all the other isolates and strains established around the world.

DISCUSSION

Nucleotide partial sequence alignments of the three *B. thuringiensis* isolates (Sn-2 and Ts-5) with (GU062822 and GU062823, respectively) compared with strains, isolates, serovars and serotypes of *B. thuringiensis* GenBank revealed positional differences in nucleotide sequences and base-pair substitutions which scored total of 17 and 11 in Sn-2 and Ts-5 isolates, respectively (**Figs. 2, 3; Table 3**). Moreover, phylogenetic dendrogram trees based on 16S rRNA gene sequences using distance, parsimony and maximum-likelihood criteria showed that two of the three local isolates formed a phylogenetically distinct group, separate from all other named isolates and species (**Figs. 4, 5**). Such obtained results evidently indicate a large diversity with unique characteristics of the three local Egyptian isolates from all the other isolates and strains established around the world.

Foremost is the fact that 16S rRNA gene seems to behave as a molecular chronometer, as pointed out by Woese (1987). The degree of conservation is assumed to result from the importance of the 16S rRNA as a critical component of cell function. This is in contrast to the genes needed to make enzymes. Mutations in these genes can usually be tolerated more frequently since they may affect structures not as unique and essential as rRNA if a bacterium does not have the gene to make the enzymes needed to utilize lactose, it can use an alternative sugar or protein as an energy source. Although the absolute rate of change in the 16S rRNA gene sequence is not known, it does mark evolutionary distance and relatedness of organisms (Kimura 1980; Harmsen and Karch 2004). Problems in assigning a numerical value to this rate of change include the possibility that this rate of change of 16S rRNA gene may not be identical for all organisms (different taxonomic groups could have different rates of change), the rates could vary at times during evolution, and the rates could be different at different sites throughout the 16S rRNA gene. There are so-called "hot spots" which show larger numbers of mutations (Tortoli 2003); these areas are not the same for all species. 16S rRNA is also the target for several antimicrobial agents.

To investigate the frequency of heterogeneity among the multiple 16S rRNA genes within a single microorganism, Ueda *et al.* (1999) determined directly the 120 bp nucleotide sequences containing the hypervariable alpha region of the 16S rRNA gene from 475 *Streptomyces* strains. Display of the direct sequencing patterns revealed the existence of 136 heterogeneous loci among a total of 33 strains. The heterogeneous loci were detected only in the stem region designated helix 10. All of the substitutions conserved the relevant secondary structure. The 33 strains were divided into two groups: one group, including 22 strains, had less than two heterogeneous bases; the other group, including 11 strains, had five or more heterogeneous bases. The two groups were different in their combinations of heterogeneous bases. The former mainly contained transitional substitutions, and the latter was mainly composed of transversional substitutions, suggesting that at least two mechanisms, possibly misincorporation during DNA replication and horizontal gene transfer, cause rRNA heterogeneity. As such, mutations in the 16S rRNA gene can affect the susceptibility of the organism to these agents and the 16S rRNA gene


401
500
GU062822 AAACTCTG--GGTTAGTCAGAACAAGTGCTAGTTGAATAAGCTGGCACCTTGACGGTACCTAA
FJ655838 AAACTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCACCTTGACGGTACCTAA
FN433030 AAACTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCACCTTGACGGTACCTAA
FN433029 AAACTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCACCTTGACGGTACCTAA
EU429672 AAACTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCACCTTGACGGTACCTAA
EU438936 AAACTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCACCTTGACGGTACCTAA
GQ284563 AAACTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCACCTTGACGGTACCTAA
EU626405 AAACTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCACCTTGACGGTACCTAA
GQ342294 AAACTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCACCTTGACGGTACCTAA
EU977819 AAACTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCACCTTGACGGTACCTAA
EU697391 AAACTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCACCTTGACGGTACCTAA
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EU162014 AAACTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCACCTTGACGGTACCTAA
FJ235080 AAACTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCACCTTGACGGTACCTAA
FJ655837 AAACTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCACCTTGACGGTACCTAA
GQ249351 AAACTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCACCTTGACGGTACCTAA
501
600
GU062822 CCAGAAAGCCTCGGCTAACTACGTGCCAGCAGCCTCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGCAGGTGGTTTC
FJ655838 CCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGCAGGTGGTTTC
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EU438936 CCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGCAGGTGGTTTC
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EU977819 CCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGCAGGTGGTTTC
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EU162014 CCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGCAGGTGGTTTC
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601
700
GU062822 TTAAGTCGGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGAGAGG-GAGTTCAGAAGAGGAAA-CGTATCCCATGGA
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Fig. 2 (previous page) Sequence alignment of 252 bp of 16s rRNA gene in Sn-2 isolate (GU062822) compared with other *Bacillus thuringiensis* isolates and strains existed in NCBI GenBank. Conserved nucleotides between GU062822 and other sequences are boxed in black. Putative conserved between the different isolates with no identity with GU062822 are boxed in grey. The yellow box referred to the identity of all accessions except GU062822. Dashes correspond to gaps introduced to optimize the alignments.

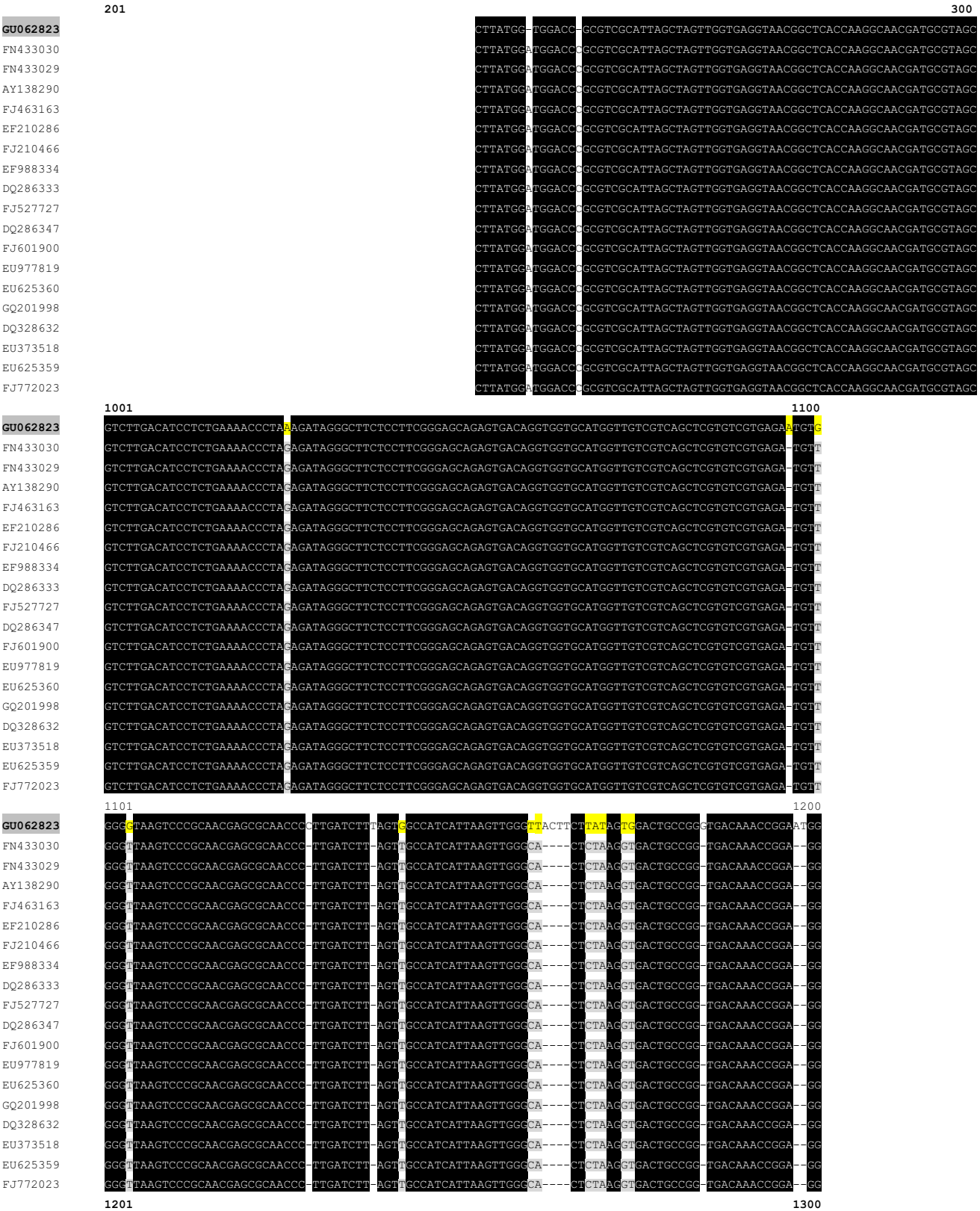

Fig. 3 Sequence alignment of 984 bp of 16s rRNA gene in Ts-5 isolate (GU062823) compared with other *Bacillus thuringiensis* isolates and strains existed in NCBI GenBank.

Fig 3 (cont.)

```
                1201                                                1300
GU062823        AAGGTGGGGGATGA
FN433030        AAGGTGGGG-ATGA
FN433029        AAGGTGGGG-ATGA
AY138290        AAGGTGGGG-ATGA
FJ463163        AAGGTGGGG-ATGA
EF210286        AAGGTGGGG-ATGA
FJ210466        AAGGTGGGG-ATGA
EF988334        AAGGTGGGG-ATGA
DQ286333        AAGGTGGGG-ATGA
FJ527727        AAGGTGGGG-ATGA
DQ286347        AAGGTGGGG-ATGA
FJ601900        AAGGTGGGG-ATGA
EU977819        AAGGTGGGG-ATGA
EU625360        AAGGTGGGG-ATGA
GQ201998        AAGGTGGGG-ATGA
DQ328632        AAGGTGGGG-ATGA
EU373518        AAGGTGGGG-ATGA
EU625359        AAGGTGGGG-ATGA
FJ772023        AAGGTGGGG-ATGA
```

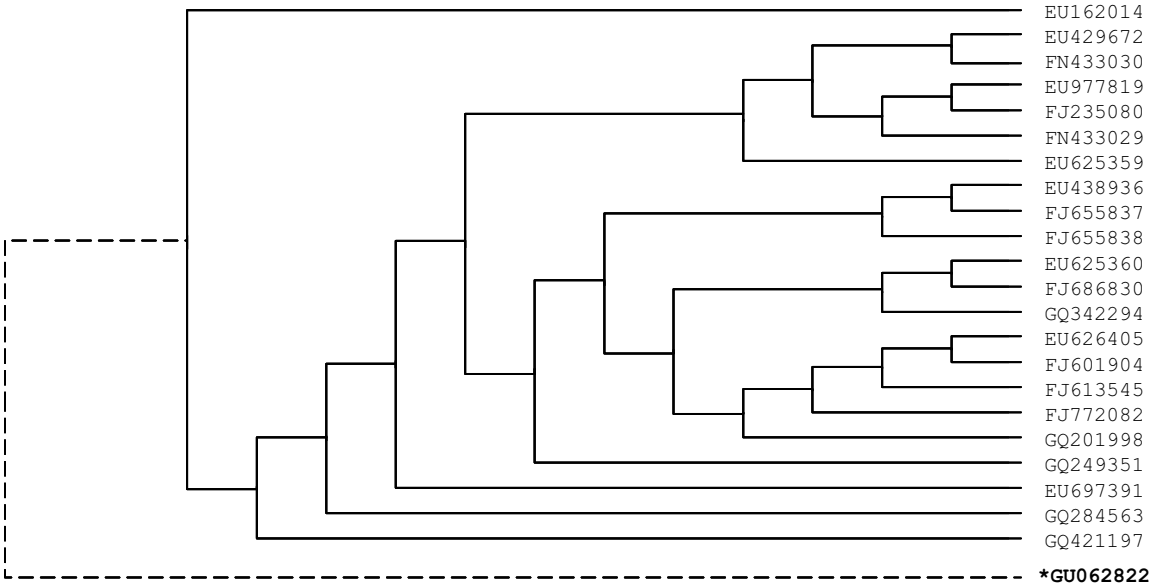

Fig. 4 Phylogenetic relationships between the local *B. thuringiensis* isolate Sn-2 (GU062822) and other GenBank related isolates and strains, based on the 252 bp of 16S ribosomal RNA gene.

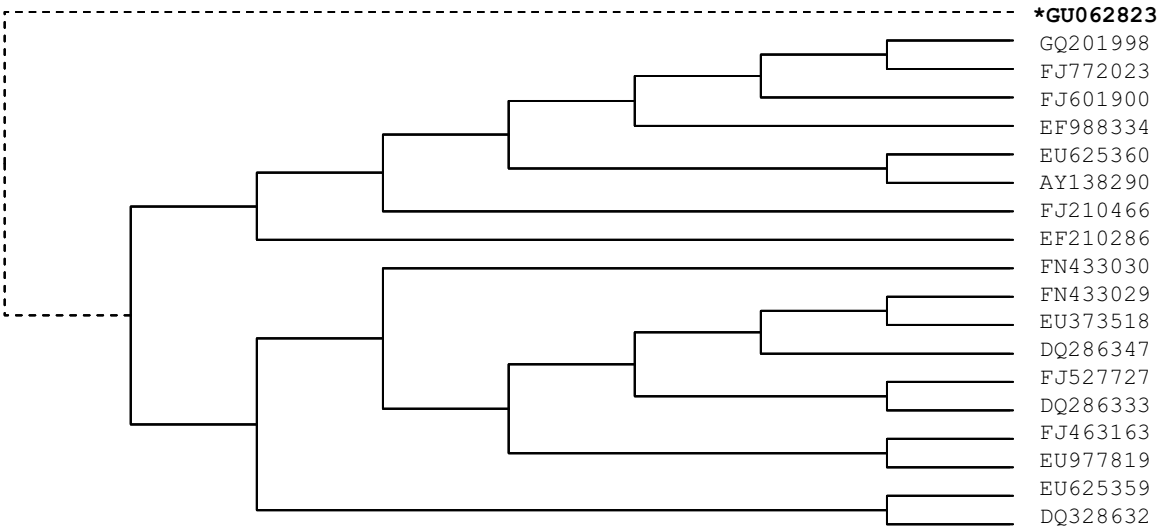

Fig. 5 Phylogenetic relationships between the local *B. thuringiensis* isolate Ts-5 (GU062823) and other GenBank related isolates and strains, based on the 984 bp of 16S ribosomal RNA gene.

Table 2 Blast search of 16S rRNA gene sequence identity between the three local *B. thuringiensis* isolates and GenBank sequences.

Accession	Isolate	Serovar	Serotype	Strain
FN433030	CCM11B			
FN433029	CCM15B			
FJ655838	S12			
FJ655837	S11			
GU003833	Clone C05			
GU003815	Clone A09			
GQ201998		kurstaki strain AR-10		
FJ772071		fukuokaensis strain IMER-B1-7		
EU429672		israelensis		
EU429671		tenebrionis		
EU429670		morrisoni		
EU429669		toumanoffi		
EU429668		thuringiensis		
EU429667		sotto		
EU429666		cameroun		
EU429665		berliner		
EU429664		ostriniae		
EU429663		kurstaki		
EU429662		galleriae		
EU429661		dendrolimus		
EU429660		colmeri		
FJ577369		kurstaki strain HD-1		
FJ358616		asturiensis strain IEBC-T53 001		
FJ358615		navarrensis strain IEBC-T50 001		
EF210303		azorensis strain BGSC 4CB1		
EU588682		kurstaki strain PTK2G		
EU438936		konkukian strain INBI-5		
DQ328632			H6a6c	
DQ328631			H61	
DQ328630			H59	
DQ328629			H58	
DQ328627			H10	
DQ286359			H4a4c	
DQ286358			H4a4b	
DQ286357			H140	
DQ286356			H69	
DQ286352			H57	
DQ286351			H55	
DQ286349			H48	
DQ286347			H44	
DQ286346			H40	
DQ286343			H37	
DQ286342			H36	
DQ286341			H35	
DQ286339			H32	
DQ286338			H29	
DQ286336			H27	
DQ286335			H26	
DQ286334			H25	
DQ286333			H24	
GQ505328				4-1-12
GQ421197				REG105
EU373518				FR1_3
EU939700				JS1
EU977819				1P03AC
EU977777				1P05SB
EU977773				1P04SC
EU977727				2P04AnC
EU977726				2P04AnB
EU977720				1P06AnC
EU977685				2P07AB
EU977651				1P06AA
EU977650				1P05AD
EU977649				1P05AA
EU977597				1P06MA
EU977592				1P04MB
GQ342294				CG-T1
GQ284563				AR1.2
GQ284562				AR1.5
GQ249351				BN2
GQ169100				BLDJ16
FJ932761				61436

Table 2 (Cont.)

Accession	Isolate	Serovar	Serotype	Strain
FJ772082				IMER-B3-6
FJ772063				IMER-B3-1
FJ772047				IMER-B3-20
FJ772044				IMER-B3-18
FJ772023				IMERB5-6
FJ772020				IMERB5-11
FJ772018				IMERB5-15
FJ686830				NB8
FJ601908				INRS12
FJ601907				INRS11
FJ601906				INRS10
FJ601904				INRS8
FJ601903				INRS7
FJ601900				INRS4
FJ601899				INRS3
FJ601897				INRS1
FJ613555				EIII-1
FJ613546				EI-18
FJ613545				EI-17
FJ613540				EI-12
FJ613538				EI-10
FJ577368				INTA77-10
FJ544327				810
FJ527727				Db4
FJ527725				ZJ3a
FJ357600				BBN2T-01d
AM293345				BAB-Bt2
FJ393313				TCCC11336
FJ232505				NH53O1
FJ236808				B4(1)
FJ235082				V3
FJ235080				V1
FJ210466				Zhou-1
EU162014				isolatePGOa6
EU697392				CMG861
EU697391				CMG857
EU697390				CMG854
EU723845				PRS-1
EU625360				isolateLDC-415
EU625359				isolateLDC-391
EU626405				biosZ2
EU647704				REG14
EU652060				me-9
EU624205				NJY1
EU168410				IBL01067
EU168406				IBL01056
EU168405				IBL01055
AB426479				NBRC101235
EU438933				INBI-2
EU414475				KR19-22

GU062822: 99 accessions, total score = 381, coverage (%)=94, identity (%) = 95
GU062823: 98 accessions, total score= 1688, coverage (%)=99, identity (%) = 97
GU062824, 96 accessions, total score= 1116, coverage (%)=100, identity (%) = 100

sequence can distinguish phenotypic resistance to antimicrobial agents (Pfister *et al.* 2003). However, these characteristics do not obviate or affect the use of 16S rRNA gene sequence for bacterial identification or assignment of close relationships at the genus and species level, as used in clinical microbiology. They can have a greater impact on the assignment of relationships of the deeper (more distantly related) branches (Garrity and Holt 2001). The sequence of the 16S rRNA gene has been widely used as a molecular clock to estimate relationships among bacteria (phylogeny) and more recently it has become important as a means to identify new isolates or an unknown bacterium to the genus or species level (Sacchi *et al.* 2002). The 16S rRNA gene sequence has been determined for a large number of strains. GenBank, the largest databank of nucleotide sequences, has over 20 million deposited sequences, of which over 90,000 are of 16S rRNA gene (Clarridge 2004). This means that there are many previously deposited sequences against which to compare the sequence of an unknown strain. Lastly, the 16S rRNA gene is universal in bacteria, and so relationships can be measured among all bacteria. In general, the comparison of the 16S rRNA gene sequences allows differentiation between organisms at the genus level across all major phyla of bacteria, in addition to classifying strains at multiple levels, including species and subspecies level. The occasional exceptions to the usefulness of 16S rRNA gene sequencing usually relate to more than one well-known species having the same or very similar sequences (Clarridge 2004).

Several reports support our findings and characterization of new bacterial isolates and strains using 16S rRNA gene sequence. For instance, a partial 16S rRNA gene sequencing analysis of Siderophoregenic *Bacillus* strain DET9 isolated from dairy waste shows that the isolate exhibited homology with *B. thuringiensis* and *B. weihenstephanensis*, whereas biochemical characterization revealed

Table 3 Positional differences and base pair substitutions in nucleotide sequences between the local *B. thuringiensis* isolates and numerous isolates and strains based on 16S rRNA gene similarity.

Accession	Isolate	Fragment size	Existing in our accessions as:	A	A	A	C	C	C	G	G	G	T	T	T	(No nucleotide bases)				C	G	T	A
																…	…	…	…				
			Changed in NCBI accessions to:	C	G	T	A	G	T	A	T	C	A	C	G	C	G	T	A	…	…	…	…
																				(No nucleotide bases)			
GU062822	Sn-2	252 bp	Change at nucleotide positions:		451		454		680		448		450		453		679	445					
					693				685		608		511		535			446					
									686		660		684		666			661					
											682												
											692												
Total number of nucleotide positions = (17)				**(2)**			**(4)**			**(5)**			**(6)**					**4**		**0**			
GU062823	Ts-5	984 bp	Change at nucleotide positions:		1026	1169					1100		1161	1160	1173	245			238	1129	1184	1138	1096
											1104		1170	1168						1063	1210	1064	1062
											1142											1065	1196
											1174											1197	
Total number of nucleotide positions = (11)				**(2)**			**(0)**			**(4)**			**(5)**					**2**		**11**			

its novelty. DET9 exhibited no mortality of fishes in a 60 day trial, when fishes (surfi tetra) were challenged up to 100 ppm cell concentration, with their daily diet (Patel *et al.* 2010). Moreover, Porwal *et al.* (2009) reported that Bacillus represents microbes of high economic, medical and biodefense importance. Bacillus strain identification based on 16S rRNA sequence analyses is invariably limited to species level. Secondly, certain discrepancies exist in the segregation of *Bacillus subtilis* strains. In the RDP/NCBI databases, out of a total of 2611 individual 16S rDNA sequences belonging to the 175 different species of the genus Bacillus, only 1586 have been identified up to species level. 16S rRNA sequences of *Bacillus anthracis* (153 strains), *B. cereus* (211 strains), *B. thuringiensis* (108 strains), *B. subtilis* (271 strains), *B. licheniformis* (131 strains), *B. pumilus* (83 strains), *B. megaterium* (47 strains), *B. sphaericus* (42 strains), *B. clausii* (39 strains) and *B. halodurans* (36 strains) were considered for generating species-specific framework and probes as tools for their rapid identification. Phylogenetic segregation of 1121, 16S rDNA sequences of 10 different *Bacillus* species in to 89 clusters enabled us to develop a phylogenetic frame work of 34 representative sequences. Using this phylogenetic framework, 305 out of 1025, 16S rDNA sequences presently classified as *Bacillus* sp. could be identified up to species level. This identification was supported by 20 to 30 nucleotides long signature sequences and *in silico* restriction enzyme analysis specific to the 10 *Bacillus* species. This integrated approach resulted in identifying around 30% of *Bacillus* sp. up to the species level and revealed that *B. subtilis* strains can be segregated into two phylogenetically distinct groups, such that one of them may be renamed. Based on the sequence difference in the variable region (V1) of 16S rRNA and in the *gyr*B gene between *B. cereus* and *B. thuringiensis*, PCR primers specific to these *Bacillus* spp. were designed (Chen and Tsen 2002). When these primers were used to discriminate *B. cereus* and *B. thuringiensis*, six of 82 *B. cereus* strains were identified as *B. thuringiensis* while 67 of 73 *B. thuringiensis* strains were identified as *B. cereus*. Sequence analysis of the primer annealing sites showed that there is no clear-cut difference in the V1 region of 16S rRNA, and in the *gyr*B gene, between *B. cereus* and *B. thuringiensis* strains. Although 16S rDNA based probes and *gyr*B gene based PCR primers have been suggested for the discrimination of *B. cereus* and *B. thuringiensis* strains, when a large number of *Bacillus* strains was tested, results showed that discrimination between *B. cereus* and *B. thuringiensis* is difficult. Therefore, to distinguish *B. thuringiensis* from *B. cereus*, a single feature, such as the presence of a parasporal crystal protein or *cry* genes may sometimes be reliable. Rampersad *et al.* (2003) isolated a novel bacterium during a screen for environmental isolates of *Bacillus thuringiensis* that possesses a novel filamentous structure. Nucleotide sequence from the isolate's 16S rRNA gene places the bacterium unambiguously within the *B. thuringiensis*/*B. cereus* group. Phase-contrast and electron microscopy indicate the presence of both a parasporal body and a long filament which are retained after sporulation. The filament is shown to consistently arise from the end of the exosporium and next to the parasporal body. Upon spore germination, the parasporal body/filament complex is retained on the cell wall of the resulting bacterium.

Soufiane and Côté (2009) investigated the capability of each of three genes, 16S rRNA, *gyr*B and *aro*E, to discriminate, first, among *Bacillus thuringiensis* H serotypes; second, among *B. thuringiensis* serovars from the same H serotype; and third, among B. thuringiensis strains from the same serovar. The 16S rRNA, *gyr*B and *aro*E genes were amplified from 21 *B. thuringiensis* H serotypes and their nucleotide sequences determined. Additional strains from four *B. cereus sensu lato* species were included for comparison purposes. These sequences were pair-wise compared and phylogenetic relationships were revealed. Each of the three genes under study could discriminate among *B. thuringiensis* H serotypes. The *gyrB* and *aroE* genes showed a discriminatory power among *B. thuringiensis* H serotypes up to nine-fold greater than that of the 16S rRNA gene. The *gyr*B gene was retained for subsequent analyses to discriminate *B. thuringiensis* serovars from the same H serotype and to discriminate strains from same serovar. A total of 42 *B. thuringiensis* strains, which encompassed 25 serovars from 12 H serotypes, were analyzed.

As previously reported by Nariman *et al.* (2009), the three new Egyptian isolates of *B. thuringiensis* Sn-2, Ts-5 and As-3 were highly toxic to 2nd instar larvae of cotton leafworm (*Spodoptera littoralis*) and northern house mosquito (*Culex pipiens*) larvae. Consequently, they are naturally found in soil may be correlated with the geographical origins of the isolates (three different governorates) and can be used as new commercial insecticidal bacteria, in view of their potential for the development of novel biopesticides.

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