

DIVERSITY OF *BACILLUS* GENOTYPES IN SOIL SAMPLES FROM EL-OMAYED BIOSPHERE RESERVE IN EGYPT

Eman A. H. Mohamed¹, Mikiko Abe², Khaled M. Ghanem¹, Yasser R. Abdel-Fattah³, Yasuyoshi Nakagawa⁴ and Ehab R. El-Helow^{1*}

¹Department of Botany, Faculty of Science, University of Alexandria, Alexandria, Egypt;

²Department of Chemistry and BioScience, Faculty of Science, Kagoshima University, Kagoshima 890-0065, Japan; ³Department of Bioprocess Development, Genetic Engineering and Biotechnology Research Institute, Alexandria, Egypt; ⁴Biological Resource Center, National Institute of Technology and Evaluation, Kisarazu 2920818, Japan

*Corresponding author, e-mail: elhelow@link.net

Summary

Sequencing of the 16S rDNA hypervariable region was applied to determine the presence and composition of *Bacillus* species in 40 soil samples randomly collected from different habitats in El-Omayed biosphere reserve, Egypt. Although purified cultures showed 18 different phenotypes that were morphologically distinct on a sporulation medium plate, only 4 different nucleotide sequences designated Seq A, B, C and D were revealed. Computational analysis of DNA sequence data suggested that 17 of these isolates are closely related members of the *Bacillus cereus/thuringiensis* group (Seq B, C and D) and one isolate is belonging to the *Bacillus subtilis* group (Seq A). Further phenotypic investigations confirmed the diversity of the 17 novel *Bacillus cereus/thuringiensis* isolates and indicated that the new *Bacillus subtilis* group isolate is a *Bacillus amyloliquefaciens* strain. A simple phenotypic discrimination key that can be applied for distinguishing between such closely related *Bacillus cereus/thuringiensis* members is presented.

Key words: *Bacillus amyloliquefaciens*; *Bacillus cereus*; *Bacillus thuringiensis*; spore formers; 16S rDNA

Introduction

Soil is the main reservoir of the genus *Bacillus* [26]. Members of this genus are used for the synthesis of a very wide range of important medical, agricultural, pharmaceutical and other industrial products. These include a variety of antibiotics, enzymes, amino acids and sugars [11].

Sequencing of the 16S rDNA hypervariable region (approx 275 bp close to the 5' end region) is a rapid and reliable way for *Bacillus* classification and basically informative at species level [4]. Nevertheless, full sequencing of

the 16S rDNA gene is sometimes useful for more detailed classification within some *Bacillus* groups. On the other hand, closely related taxa are often extremely similar in their 16S rDNA sequences [13]. For instance, some members of the *Bacillus cereus* group (*B. anthracis*, *B. cereus* and *B. thuringiensis*) have high levels of 16S rDNA sequence similarity (>99 %) [21].

However, some phenotypic characters such as β -hemolytic activity can be used to distinguish between *B. cereus* and *B. thuringiensis* on the one hand and *B. anthracis* on

the other hand [5, 14, 20].

The main objective of the present work is to explore the presence and composition of *Bacillus* species in representative soil samples collected from EI-Omayed biosphere reserve in

Egypt. Eighteen different spore forming bacterial strains were isolated, characterized and identified at the molecular level. A discrimination key for distinguishing between closely related individual members is presented.

Materials and Methods

Bacterial isolation. The study area, EI-Omayed biosphere reserve, is a part of the northwestern Mediterranean coastal zone of Egypt [8]. Forty soil samples were collected from five natural habitat types, including coastal dunes, inland ridges with skeletal shallow soils, saline marshy depressions, non-saline depressions and inland plateau. In all cases, soil samples were taken from 2 to 5 cm layers. A gram of each well-mixed sample was suspended in 50 ml of 2 x SG sporulation medium [23], then incubated at 37 °C under shaking conditions (200 rpm) for 48 h. Vegetative cells were killed by adding chloroform (1 % v/v), vortexing and keeping for 24 h at room temperature. Spores were germinated by plating 0.1 ml of each spore suspension with and without dilutions, into solidified 2 x SG medium. Isolated colonies were further purified by streaking on agar plates of the same medium.

Reference strains. *B. cereus* 4F1 and *B. thuringiensis* 4A1 were obtained from *Bacillus* Genetic Stock Center (BGSC), Ohio State University, Columbus, OH, U.S.A. *B. amyloliquefaciens* 23350 was obtained from American Type Culture Collection (ATCC) and *B. subtilis* 3007 was obtained from the Institute for Fermentation, Osaka (IFO), Japan.

Phenotypic analyses. Pure cultures were examined for motility, Gram reaction, spore mor-

phology, Voges- Prauskauer test, citrate utilization, nitrate reduction, β -hemolytic activity, catalase and oxidase expression and production of acids from D-glucose, L-arabinose, D-xylose and D-mannitol [5, 2, 24]. The expression of some other enzymes such as β -glucosidase and N-acetyl- β -glucosaminidase was also examined in addition to the ability to degrade Tween 80, gelatin, casein, chitin, starch, pectin xylan and mannan [9, 18]. Fatty acid profiles were determined using the method described by Sasser [22].

DNA preparation and PCR amplification. Bacterial cells were obtained from late-exponential cultures shaken in LB medium at 37 °C. Genomic DNA of *Bacillus* strains was prepared for PCR amplification [25] and the primers used for PCR and sequencing are shown in Table 1. The primers 16S-1 and 16S-2 were used to amplify a fragment of about 270 bp that includes the 16S rDNA hypervariant region [27]. F9 and R1510 primers were used to fully amplify the 16S rDNA gene. F9, F515, F785, F1099, R1510, R1115, R802 and R536 were separately used for DNA sequencing. The PCR reaction mixture contained 200 μ M of each dNTP, 0.5 μ M primers, 10 mM tris-HCl pH 8.3, 1.5 mM MgCl₂, 50 mM KCl, 2.5 U Taq polymerase and about 100 ng of template DNA. For 16S-1 and 16S-2, amplicons were obtained with a PCR

Table 1. Primers specific for 16S rDNA.

Primer	Sequence (5' to 3')
16S-1 ¹	TGGCTCAGAACGAACGCTGGCGGC
16S-2 ¹	CCCACTGCTGCCTCCCGTAGGAGT
F9 ²	GAGTTTGATCCTGGCTCAG
F515 ²	GTGCCAGCAGCCGCGGT
F785 ²	GGATTAGATACCCTGGTAGTC
F1099 ²	GCAACGAGCGCAACCC
R1510 ²	GGCTACCTTGTTACGA
R1115 ²	AGGGTTGCGCTCGTTG
R802 ²	TACCAGGGTATCTAATCC
R536 ²	GTATTACCGCGGCTGCTG

Note: ¹ According [27]; ² Department of Biochemistry and Bioscience, Faculty of Science, Kagoshima University, Japan.

cycling program of 94 °C for 5 min, 30 cycles at 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s. At the end, the reaction was incubated at 72 °C for 7 min. For F9 and R1510, the cycling program was 95 °C for 3 min, 30 cycles at 95 °C for 30 s, 55 °C for 15 s and 72 °C for 1 min and at the end, the reaction was incubated at 72 °C for 5 min. Amplicons were visualized by electrophoretic separation on 2 % agarose gels stained with ethidium bromide.

DNA sequencing and data analysis. PCR fragments were purified by QIAquick PCR purification reagents (Qiagen), labeled with Big Dye

Terminator Cycle Sequencing Kit and DNA sequences were obtained using ABI PRISM 310 sequencer (Perkin Elmer, Applied Biosystem, USA). Homology search was performed against DDBJ (DNA Data Base Japan) using Blast program. Multisequence alignment and molecular phylogeny analyses were performed using ClustalW (a distance-based analysis program). The resulted tree topology was evaluated using bootstrap analysis of the neighbor-joining method based on 1000 resamplings [3]. All sequences have been deposited in the Gen-bank and their accession numbers are given in Table 2.

Table 2. Accession numbers of the novel *Bacillus* soil isolates.

Isolate number	Sequence type	Accession number
EM44	16S rDNA partial sequence	DQ389737
EM62	16S rDNA partial sequence	DQ389738
EM1	16S rDNA partial sequence	DQ389739
EM3	16S rDNA partial sequence	DQ389740
EM4	16S rDNA partial sequence	DQ389741
EM15	16S rDNA partial sequence	DQ389742
EM25	16S rDNA partial sequence	DQ389743
EM33	16S rDNA partial sequence	DQ389744
EM35	16S rDNA partial sequence	DQ389745
EM37	16S rDNA partial sequence	DQ389746
EM42	16S rDNA partial sequence	DQ389747
EM46	16S rDNA partial sequence	DQ389748
EM50	16S rDNA partial sequence	DQ389749
EM52	16S rDNA partial sequence	DQ389750
EM58	16S rDNA partial sequence	DQ389751
EM60	16S rDNA partial sequence	DQ389752
EM63	16S rDNA partial sequence	DQ389753
EM29	16S rDNA partial sequence	DQ389754
EM44	16S rDNA full sequence	DQ389755

Results and Discussion

A number of spore-forming bacteria isolated from 40 different soil samples showed 18 different phenotypes that were morphologically distinct on sporulation medium plates. Colony morphology of the isolate EM44 was very unique because of its wrinkled configuration. All isolates were further purified for molecular and phenotypic characterization. Preliminary results showed that all of the iso-

lates are Gram positive bacilli and form oval spores that do not cause mother cell swelling.

Sequencing of the hypervariable region (275 bp) of 16S rDNA [4] in the 18 soil isolates resulted in only 4 different nucleotide sequences designated Seq A, B, C and D. Based on these sequences and their comparative computational analyses, phylogenetic positions of the experimental isolates are shown in Fig. 1.

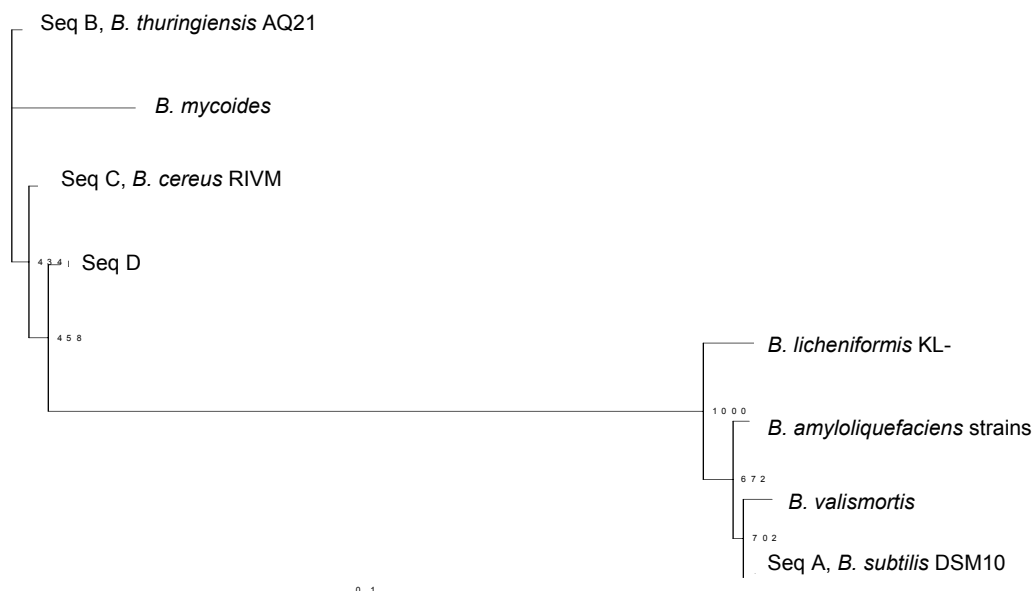


Fig. 1. A phylogenetic tree based on partial sequencing of the 16S rDNA gene. The tree was constructed by neighbor-joining method using ClustalW software. The scale indicates substitutions per site. The numbers at the nodes indicate the frequency of occurrence in 1000 bootstrapped trees. App.: Seq A, isolate EM44; Seq B, isolate EM62; Seq C, isolates EM1, EM3, EM4, EM15, EM25, EM33, EM35, EM37, EM42, EM46, EM50, EM52, EM58, EM60 and EM63 and Seq D, isolate EM29.

These results suggested that the isolate EM44, which carries Seq A, is belonging to the *B. subtilis* group and that each of the other 17 isolates, which comprise Seq B, C or D is likely a member of the *Bacillus cereus/thuringiensis* group.

For further characterization, the 18 isolates together with representative reference members of the genus *Bacillus* (*B. cereus*, *B. thuringiensis*, *B. subtilis* and *B. amyloliquefaciens*) were examined with respect to some biochemical and nutritional features. All strains expressed catalase, chitinase and caseinase. Besides, all of them were able to ferment D-glucose producing stable acidic end products (positive methyl red test) but not neutral ones (negative Voges- Prauskauer reaction). On the other hand, the utilization of pectin, xylan and mannan; fermentation of D-xylose and expression of β -hemolytic activity appeared to be powerful features in differentiation between examined *B. cereus* and *B. subtilis* groups (Table 3).

Members of the *B. cereus* group, especially *B. anthracis*, *B. cereus* and *B. thuringiensis*, are so closely related to each other that they are treated by some authors as only one species [10, 19, 7, 17]. Likewise, the novel 17 morphologically distinct *B. cereus/thuringiensis* isolates were grouped into only three clusters based on the hypervariant region of the 16S rDNA. According to the results presented in Table 3,

a discrimination key is suggested for complete resolution of these closely related isolates. The ability to degrade Tween 80 can divide these isolates into degraders and non-degraders. Then, citrate utilization can subdivide each category into 2 subgroups. Sequential tests including nitrate reduction, mannitol utilization, gelatin hydrolyses, N-acetyl- β -glucosaminidase expression, β -glucosidase activity and starch degradation subdivide them further until reaching a full discrimination between the 17 isolates.

The entire new *B. cereus* strains' group exhibited chitinase and β -hemolytic activities. In addition, the majority of them were motile. These results rule out for all isolates the possibility of being *B. anthracis* [5]. However, further investigations are required for these new isolates that may comprise a variety of *B. thuringiensis* genotypes, used as highly specialized and powerful biological agents for controlling insect pests [1, 16].

Generally, the *B. subtilis* group includes a number of industrially important species which are used in a wide range of industrial applications [2, 6, 15]. Our results suggest a possible industrial importance of the isolate EM44. This strain has the ability to produce a considerable range of extracellular enzymes such as α -amylase, gelatinase, xylanase, mannanase, pectinase and caseinase.

Table 3. Phenotypic characteristics of the *Bacillus* soil isolates.

Isolate or strain	Mot	Cit	Nit	β -Hem	Oxd	L-Ara	D-Man	D-Xyl	β -Glu	N-Amn	T-80	Gel	Str	P, X, M
EM1	+	+	-	+	+	-	-	-	+	-	-	+	+	-
EM3	+	+	+	+	+	-	-	-	-	+	+	+	+	-
EM4	-	-	-	+	-	-	-	-	+	-	-	-	+	-
EM15	+	-	+	+	-	-	-	-	+	-	-	+	+	-
EM25	+	-	+	+	+	+	+	-	-	-	+	+	-	-
EM29	+	-	+	+	+	-	-	-	-	-	+	-	+	-
EM33	+	-	+	+	+	-	-	-	-	-	-	+	+	
EM35	+	+	+	+	-	+	+	-	-	+	+	-	+	-
EM37	+	+	+	+	+	+	+	-	-	-	-	+	+	-
EM42	+	-	-	+	+	-	-	-	-	+	-	+	+	-
EM44	+	+	+	-	-	+	+	+	+	-	+	+	+	+
EM46	+	-	-	+	+	+	+	-	-	-	+	-	+	-
EM50	+	-	+	+	-	-	-	-	-	-	-	+	-	-
EM52	-	-	+	+	+	-	-	-	-	-	+	+	+	-
EM58	+	+	+	+	-	-	-	-	-	-	+	+	+	-
EM60	+	-	-	+	+	-	-	-	-	-	+	+	+	-
EM62	-	-	-	+	+	-	-	-	-	-	-	+	+	-
EM63	+	+	+	+	+	-	-	-	-	-	-	+	+	-
Ba 23350	+	+	-	-	-	+	+	+	+	-	+	+	+	+
Bs 3007	+	-	+	-	+	+	+	+	+	-	+	+	+	+
Bc 4F1	+	+	-	+	-	-	-	-	-	+	-	+	-	-
Bt 4A1	+	-	+	+	-	-	-	-	-	-	+	+	+	-

Legend: motility (Mot), citrate utilization (Cit), nitrate reduction (Nit), β -hemolysis (β -Hem), oxidase production (Oxd), acid production from L-arabinose, D-mannitol and D-xylose, respectively (L-Ara, D-Man and D-Xyl), β -glucosidase production (β -Glu), N-acetyl- β -glucosaminidase productuin (N-Amn), utilization of Tween 80 (T-80), gelatin hydrolysis (Gel), starch degradation (Str), degradation of pectin, xylan and mannan, respectively (P, X, M); *B. amyloliquefaciens* (Ba), *B. subtilis* (Bs), *B. cereus* (Bc); *B. thuringiensis* (Bt).

Because of its promising applicable features, isolate EM44 was subjected to further analyses to determine the exact species to which it belongs within the *B. subtilis* group. Full sequencing of the 16S rDNA gene of the isolate EM44 and its computer database alignment recorded a similarity of 99% to a *B. amyloliquefaciens* reference strain (Fig 2.). For further verification whole cell fatty acid composition of

isolate EM44, along with reference strains of *B. amyloliquefaciens* and *B. subtilis* (Table 4), was analyzed. The results revealed that the levels of the most dominant fatty acids in the cells of isolate EM44 (15:0 Iso and 15:0 Anteiso) are closer to *B. amyloliquefaciens* rather than to *B. subtilis*. Moreover, this novel *Bacillus* strain was clearly characterized by over-expression of α -amylase on starch plates (data not shown).

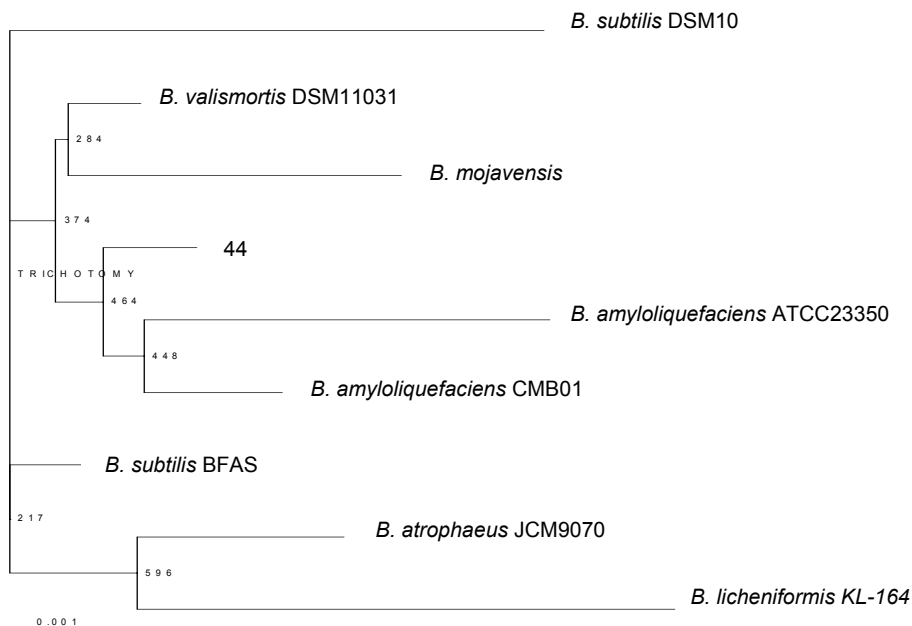


Fig. 2. Phylogenetic position of isolate EM44 based on full sequencing of 16S rDNA. The tree constructed by neighbor-joining method using ClustalW software. The scale indicates substitutions per site. The numbers at the nodes indicate the frequency of occurrence in 1000 bootstrapped trees.

Table 4. Fatty acid composition analysis of isolate EM44 and two reference strains. Values are shown as percentages.

Fatty acid	Bacterium		
	<i>B. amyloliquefaciens</i>	<i>B. subtilis</i>	EM44
11:0 Iso 3OH	-	0.11	-
12:00	-	-	0.34
13:0 Iso	0.15	0.67	0.25
13:0 Anteiso	-	0.16	0.12
14:0 Iso	2.7	1.68	1.57
14:00	0.43	1.51	0.95
15:0 Iso	16.24	31.37	20.01
15:0 Anteiso	33.57	27.28	33.82
15:00	0.27	0.32	0.22
15:0 Iso 3OH	-	-	0.42
15:0 2OH	-	-	0.35
16:0 Iso	10.01	4.33	4.37
16:00	5.99	9.03	10.73
17:0 Iso	16.1	16.69	14.81
17:0 Anteiso	13.39	6.55	11.31
18:00	0.63	0.3	0.72

References

1. Baum, J. A., T. B. Johnson, B. C. Carlton, 1999. *Bacillus thuringiensis* natural and recombinant bioinsecticide products. In: *Methods in Biotechnology*, Hall, F. R., J. J. Mean (Eds), Vol 5, Totowa, NJ: Humana Press, Inc., 189–209.
2. Carr, J. G., 1983. *J. Appl. Bacteriol.*, **55**, 383-402.
3. Felsenstein, J., 1985. *Evolution*, **39**, 783-791.
4. Goto, K., T. Omura, Y. Hara, Y. Sadaie, 2000. *J. Gen. Appl. Microbiol.*, **46**, 1-8.
5. Guttman, D. M., D. J. Ellar, 2000. *FEMS Micro-*

- biol. Lett.*, **188**, 7-13.
6. Hara, T., S. Veda, 1982. *Agricul. Biol. Chem.*, **29**, 45-72.
 7. Helgason, E., O. A. Okstad, H. A. Johansen, A. Fouet, M. Mock, 2000. *Appl. Environ. Microbiol.*, **66**, 2627-2630.
 8. Ismail, H. A., S. M. H. Abd El-Rahman, F. H. Abdel-Kader, 1986. *Cah. Orstom., ser. Pedol.*, **XXII**, 63-76.
 9. Ivanova, E. P., V. V. Mikhail, V. I. Sventashiv, O. I. Nedashkovskayan, N. M. Gorshkova, V. V. Mikhailov, N. Yumoto, Y. Shigeri, T. Taguchi, S. Yoshikawa, 1999. *Internatl. Microbiol.*, **2**, 267-271.
 10. Jensen, B. G., N. Fisker, T. Sparso, L. Andrup, 2005. *Internatl. J. Food Microbiol.*, **104**, 113-120.
 11. Joung, K. B., J.-C. Cote, 2002. *J. Appl. Microbiol.*, **92**, 97-108.
 12. Koneman, E. W., S. D. Allen, W. M. Janda, P. C. Schreckenberger, W. C. Winn, 1992. *Diagnostic Microbiology*, 4th edn, Philadelphia, Pa: J. B. Lippincott Co.
 13. La-Duc, M. T., M. Satomi, N. Agata, K. Venkateswaran, 2004. *J. Microbiol. Meth.*, **56**, 383-394.
 14. Mabuchi, N., I. Hashizume, Y. Araki, 2000. *Can. J. Microbiol.*, **46**, 370-375.
 15. Parry, J. M., P. C. B. Turnbull, J. R. Gibson, 1983. *A Color Atlas of Bacillus Species*, Ipswich: Wolfe Medical Publications Ltd.
 16. Pena, G., J. Miranda-Rios, G. de la Riva, L. Pardo-Lopez, M. Soberno, A. Bravo, 2006. *Appl. Environ. Microbiol.*, **72** (1), 353-360.
 17. Peter, T. C. B., P. J. Jackson, K. Hill, P. Kein, A.-B. Kolsto, D. J. Beecher, 2002. Long standing taxonomic enigmas within the *Bacillus cereus* group are on the verge of being resolved by far-reaching molecular developments: Forecasts on the possible outcome by an ad hoc team. In: *Applications and Systematics of Bacillus and Relatives*, Berkeley, R., M. Heyndrickx, N. Logan, P. De Vas (Eds), Oxford: Blackwell Publishing, 23-36.
 18. Pirttijarvi, T. S. M., M. A. Andersson, M. S. Salikinoja-Salonen, 2000. *Internatl. J. Food Microbiol.*, **60**, 231-239.
 19. Rasko, D. A., M. R. Altherr, C. S. Han, J. Ravel, 2005. *FEMS Microbiol. Rev.*, **29**, 303-329.
 20. Rhodenhamel, E. J., S. M. Harmon, 2001. *Bacillus cereus*. In: *Bacteriological Analytical Manual*, Belay, N., D. B. Shah, R. Bennett (Eds), 8th edn, USA: FDA, Center of Food Safety and Applied Nutrition.
 21. Sacchi, C. T., A. M. Whitney, L. W. Mayer, R. Morey, A. Steigerwalt, A. Boras, R. S. Weyant, T. Popovic, 2002. *Emerg. Infect. Dis.*, **8**, 1117-1123.
 22. Sasser, M., 1990. Identification of bacteria through fatty acid analysis. In: *Methods in Phytobacteriology*, Klement, Z., K. Rudolph, D. C. Sands (Eds), Budapest: Akademiai, Kiado, 900-911.
 23. Schaeffer, P., 1969. *Bacteriol. Rev.*, **33**, 201-204.
 24. Sneath, P. A., N. S. Mair, M. E. Sharpe, 1986. *Bergey's manual of systematic bacteriology*, vol. 2, Baltimore, London, Los Angeles, Sidney: William and Wilkins.
 25. Wang, S., S. Wu, G. Thottappilly, R. D. Locy, N. K. Singh, 2001. *J. Biosci. Bioengin.*, **92**, 59-66.
 26. Watanabe, K., K. Hayano, 1993. *Can. J. Microbiol.*, **39**, 674-680.
 27. Young, J. P. W., H. L. Downer, B. D. Eardly, 1991. *J. Bacteriol.*, **173**, 2271-2277.

РАЗНООБРАЗИЕ НА ГЕНОТИПОВЕ *VACILLUS* В ПОЧВЕНИ ПРОБИ ОТ БИОСФЕРНИЯ РЕЗЕРВАТ EL-ОМАЙЕД В ЕГИПЕТ

Еман А. Х. Мохамед¹, Микико Абе², Калед М. Джанем¹, Ясер Р. Абдел-Фатах³,
Ясуйоши Накагава⁴, Ехаб Р. Ел-Хелюу^{1*}

Резюме

Приложено е секвениране на хипервариантната област на 16S рДНК за определяне присъствието и състава на вида *Bacillus* в 40 почвени проби, събрани случайно от различни местообитания в биосферния резерват El-Omayed, Египет. Въпреки че пречистените култури показват 18 различни фенотипа, които се разграничават морфологично на среда за спорулация, са открити само 4 различни нуклеотидни последователности, означени като Seq A, B, C и D. Компютърният анализ на данните от ДНК секвенирането предполага, че 17 от тези изолати са тясно родствени членове на групата *Bacillus cereus/thuringiensis* (Seq B, C и D), а един изолат принадлежи към групата *Bacillus subtilis* (Seq A). Понататъшните фенотипни изследвания потвърждават разнообразието на 17-те нови изолати *Bacillus cereus/thuringiensis* и показват, че новият изолат от групата *Bacillus subtilis* е щам на *Bacillus amyloliquefaciens*. Представен е един прост фенотипно дискриминативен ключ, който може да се приложи за разграничаване между тясно родствени членове на групата *Bacillus cereus/thuringiensis*.