BIOTECH STUDIES

RESEARCH PAPER

Isolation, identification and characterization of three new strains of *Bacillus sphaericus* as mosquito pathogen

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Abstract

In the present study, three entomopathogenic strains of *Bacillus sphaericus* (MBI5, MBI6, and MBI7) were isolated from greenhouse pest and larval habitat of Istanbul, Turkey. All of the MBI groups tested in laboratory bioassays were able to kill larvae, but not pupae and adults of *Culex* spp. in water. The most efficient larvicidal activity of the strains was found in the presence of two toxin genes, *binA* and *binB* for MBI5, MBI6, and MBI7 strains. Each *B. sphaericus* strains identified in this study were characterized as unique and novel in terms of fatty acid methyl ester (FAME) profiles and 16S rRNA sequencing data. The results of this study suggest that the three strains of *B. sphaericus* may be new sources of potential biocontrol agent of mosquito.

Introduction

Mosquitoes are vectors of many diseases such as mosquito-borne arboviruses, malaria, filariasis and Japan encephalitis. Generally, the control of mosquitoes has been carried out more by chemical pesticides than biopesticides in the world. These chemical pesticides are known as dichlorodiphenyltrichloro ethane (DDT), gammaxene, malathion, chlordane, and organophosphates (van den Berg, 2009). All of them have high toxic range for both human health and environment (Casida & Quistad, 1998; Costa, 2006; Didia et al., 1975; Hurlbert et al., 1972). Compared to chemical pesticide, microbial insecticides are often species specific and do not contaminate environment, therefore, safe to non-target organisms in the nature. Among various microbial pesticides, *Bacillus thrungiensis* and *B. sphaericus* are being widely used.

Mosquitocidal bacteria are environmentally friendly alternatives to chemical pesticides for controlling water mosquitoes (Federici et al., 2006). *B. thrungiensis* subs. *israilensis* (*Bti*) is the most extensively used mosquito larvicidal bacteria in the world. *Bti* produces different type of insecticidal proteins during its sporulation, which are known as Cry or Cyt toxins (Schnepf et al., 1998; Soberón et al., 2007). Cry toxins have been widely used in the control of broad range of mosquito and blackfly species as well as nematodes, mite, and protozoa (Marroquin et al., 2000; Pinto et al., 2003; Schnepf et al., 1998). Another potential microbial insecticide, *B. sphaericus*, is known to be effective against *Culex* spp. and *Anopheles* spp. species and has better residual activity in polluted waters by production of binary toxin (Bin) and mosquitocidal toxins (Mtx). Mosquito resistance to some of *B. sphaericus* strains have been reported in many countries (Berry, 2012; Broadwell & Baumann, 1986; Park et al., 2010; Rao et

al., 1995; Silva-Filha et al., 1995; Wirth et al., 2007; Yuan et al., 2000; Lacey et al., 2015). Therefore, there is always great need and force for the scientists to search for new natural mosquitocidal bacterial strains, which can be used for development of new strains for development of new commercial microbial insecticide.

In the present study, three new strains of *B. sphaericus* (MBI5, MBI6, MBI7) isolated from larval habitat and some greenhouse pests have been identified and characterized in terms of phenotypic features as well as larvicidal activity in laboratory conditions.

Materials and Methods

All the *B. sphaericus* strains were isolated (as described below) from unhealthy mosquito larvae and aphid samples, which were collected from the district of Istanbul, Turkey. *Bti* ATCC 35646, *Bti* 4Q4, and *B. sphaericus* serotype H were obtained from the culture collection unit in Department of Genetics and Bioengineering at Yeditepe University, Istanbul, Turkey.

Isolations of *B. sphaericus* strains from mosquito larvae and aphid samples

Fifty-three *Culex* spp. larvae samples were collected from natural habitat in Istanbul, Turkey. In addition, 18 samples of unhealthy aphids were taken from tomato plants in grown greenhouse. Each of the unhealthy larvae and aphid samples were crushed in sterile water and then plated on nutrient agar (NA) media for isolation of the microorganisms. All inoculated plates were incubated at 27°C for 4 days, and then individual bacterial colonies grown on the plates were selected and purified on NA. Totally, 157 and 22 bacterial strains were isolated from mosquito unhealthy larvae and aphid samples, respectively. *B. thrungiensis* ATCC 35646, *B. thrungiensis* 4Q4 and *B. sphaericus* serotype H were also used as reference strains in this study. All bacterial strains were stored in 15% glycerol at -80°C for further studies.

Bioassays

Single colonies of newly isolated bacterial strains and reference strains (*Bti* 4Q4, *Bti* ATCC 35646, *B. sphaericus* serotype H) were cultivated on nutrient yeast salt mineral agar (NYSM) containing 5 g glucose (bacteriological), 5 g peptone, 5 g NaCl, 3 g beef extract, 5 g yeast extract, 203 mg $MgCl_2$, 10 mg $MnCl_2$ and 103 mg CaCl and incubated for 48 h at 30°C. Each strain was harvested and re-suspended in 10 mL of distilled water. Absorbance was adjusted to 0.2 with distilled water and then 0.5 mL of suspension was added to 500 mL of fresh water/polluted water in 1000 mL flasks containing 100 larvae (at the stage of 3rd instar) of *Culex* spp. The larvae were taken with polluted water, which has rich organic matter, from natural habitat in Istanbul, Turkey. The inoculated flasks were maintained on laboratory bench and observed for 48 h at room temperature. The

positive and negative control flasks (treated with reference strains and sterile water, respectively) were kept at the same conditions to determine larvicidal bacterial strains capable of killing 90% of larvae. The numbers of alive larvae were counted, and the percentage viability was reported from the average of four replicates.

Morphological, physiological and biochemical characterizations of isolates

Bacterial strains selected as larvicidal in bioassay test were identified based on morphological biochemical, physiological features according to Bergey's Manual of Systematic Bacteriology (Sneath et al., 1986). Phenotypic characteristics of the strains include cell and colony shape on NA, while spore formation on NYSM media. Biochemical tests are composed of gram reaction, oxidase, catalase, capsule staining. Physiological characteristics of natural strains were determined in terms of growth ability at different conditions. To do the hemolysis test, *Bti* ATCC 35646, *Bti* 4q4, *B. sphaericus* serotype H and MBI group were cultivated onto Sheep Blood Agar. They were incubated for 24 h in CO_2 incubator. Moreover, all bacteria were grown on 20 mL nutrient agar at 25°C for 48 h for anaerobic test. On the other hand, MBI group and *B. sphaericus* serotype H were grown aerobically on 20 mL nutrient agar with penicillin disk at 25°C during 48 h for penicillin susceptibility test. To perform temperature test, high (50°C) and low (4°C) temperature were chosen to incubate bacteria in order to understand growth range.

Extraction and analysis of fatty acid methyl ester (FAME) profiles

Extraction and analysis of FAME from whole cell fatty acids of bacterial strains were performed according to the method described by the manufacturer's manual (Sherlock Microbial Identification System version 4.0, MIDI, Inc., Newark, DE, USA) (Miller & Berger, 1985). FAMEs were separated by gas chromatography (HP6890, Hewlett Packard, Palo Alto, CA, USA) with a fused-silica capillary column (25 m×0.2 mm) with cross-linked 5% phenyl methyl silicone. FAME profiles of the strains were identified by the commercial TSBA6 database with Microbial Identification System (MIDI, Inc., Newark, Delaware, USA) software (MIS version no: 6:0). The cellular concentrations of the fatty acids for each strain were determined and strains were identified at species level.

DNA extraction from bacterial strains

Total genomic DNA from bacterial strains was extracted according to methodology described by Jimenez et al. (2000) with some modifications.

PCR amplification and purification of 16S rRNA

16S rRNA genes of the bacterial DNA isolates (TABLMBI5, MBI6, MBI7 and *B. sphaericus* serotype H

for control) amplified by the PCR (Bio-Rad, Italy) using purified DNA and primers 27f and 1492r (Weisburg et al., 1991). PCR amplifications were carried out in total volume of 50 µL reaction mixture containing 0.2 mM of 27f and 1492r primers for total 16S, 1 U of *pfu* DNA polymerase (Fermentas, USA), 0.2 mM of each deoxynucleoside triphosphate (dNTP), 1 mM MgSO$_4$, 10 mM Tris and 50 ng template DNA. PCR conditions were as follows: pre-amplification 94°C for 5 min; denaturation at 94°C for 30 s; annealing at 55°C for 40 s; elongation at 72°C for 2 min repeated 34 cycles and then post amplification for final extension 10 min at 72°C.

We designed specific two new primers for *B. sphaericus* like members of Bacillaceae family. We amplified 550 bp of 16S rRNA gene fragments of the DNA from bacterial isolates (MBI5, MBI6, MBI7, and *B. sphaericus* serotype H for control) by the PCR (Bio-Rad, Italy) using purified DNA and primers FAM1 and FAM2. PCR amplifications were carried out in total volume of 50 µL reaction mixture containing 0.2 mM of FAM1 and FAM2 primers for 550 bp of 16S, 1 U of *pfu* DNA polymerase (Fermentas, USA), 0.2 mM of each deoxynucleoside triphosphate (dNTP), 1 mM MgSO$_4$, 10 mM Tris and 50 ng template DNA. PCR conditions were as follows: pre-amplification 94°C for 5 min; denaturation at 94°C for 30 s; annealing at 51°C for 40 s; elongation at 72°C for 45 s repeated 34 cycles and then post amplification for final extension 10 min at 72°C. All the primer sequences are given in Table 1.

The amplified DNA products were detected by using Bio-Rad image analyzing system (Bio-Rad, Italy) after electrophoresis of PCR amplicons in a 1% agarose gel stained with ethidium bromide.

Table 1. Primers used for PCR amplification of 16S rRNA of MBI group and toxin gene sequencing

16S universal	27F	AGAGTTTGATCCTGGCTCAG
primers	1492R	CGGCTACCTTGTTACGAC
(1500 bp)	FAM1	CTCTGTTGTAAGGGAAGAAC
16S (550 bp)	FAM2	CCATGCACCACCTGTCACCG
	51 kDa F	CGCTAAATACTACTCCTACAAGCC
	51 kDa R	GGATACGATTGTATACCTGCC
	42 kDa F	CCCACAGAAGGAAAGTACATTCGC
Toxin Gene	42 kDa R	CCTAGTAAAGGTTCACTTGC
Primers	Mtx1 F	CAAGCTGCTTCACTTACATG
	Mtx1 R	GTCCAGTTACATCTTGAGCC
	Mtx2 F	GGAGACTAATTGAATTTTCGGTTTCC
	Mtx2 R	GCGATGCTGGGCTATGTTCGTTGTTA

16S rRNA gene sequencing and phylogenetic analysis

Pure amplification products were sequenced with Prism ABI 3100 Genetic Analyzer 16 capillary, dideoxy terminator cycle sequencing kit (Applied Biosystems). The manufacturers' recommendations were followed for sequencing. Sequences were determined with an automated DNA sequencer (model: Prism ABI 3100; Applied Biosystems). Both strands were sequenced using the primers 27f, 1492r, FAM1 and FAM2 (Roberts et al., 1996). The Clustal Omega program (Higgins et al., 1992) was used to align the 16S DNA sequences

generated with sequences of *B. sphaericus* like members from GenBank NCBI (Larsen et al., 1993). Genetic distance was computed by using Kimura's two-parameter model (Maruyama & Kimura, 1980) and used for neighbor-joining analysis. Phylogenetic trees were constructed using neighbor-joining and maximum-parsimony methods provided by CLC Genomics Workbench_2_1_1 both methods produced trees with similar topologies.

Determination of toxin genes

Toxin genes investigated according to methodology described by Nishiwaki et al. (Nishiwaki et al., 2007). PCR amplification was performed of the toxin genes using MBI5, MBI6, MBI7, and *B. sphaericus* serotype H for control as template to find the mosquitocidal binary toxin genes, *mtx1*, and *mtx2*. Toxin gene primers are given in Table 1. PCR was constructed according to the following conditions: pre-amplification 94°C for 2 min followed by 30 cycles of denaturation at 94°C for 15 s, annealing at 55°C for 30 s, and elongation at 72°C for 1 min 30 s. The master mix consisted of 1 U of TSG polymerase (Biobasic, Canada), 1 mM MgSO$_4$, 0.2 mM of each deoxynucleoside triphosphate (dNTP), 20 ng of template DNA, and 5 pmol of each primer in total volume of 50 µL reaction mixture. The amplified DNA products was detected by using Bio-Rad image analysing system (Bio-Rad, Italy) after electrophoresis of PCR amplicons in a 1% agarose gel stained with ethidium bromide.

Scanning Electron Microscope

The bacterial samples (MBI5, MBI6, MBI7, and *B. sphaericus* serotype H) were grown axenically and aerobically for 24 h at 27°C on 20 mL nutrient agar. The bacteria were collected with sterile plastic inoculating loops solid culture plate. The collected samples were added into 1 mL deionized water, vortexed, and centrifuged for 5 min at 7500 rpm. The supernatant was discarded. This procedure was repeated three times. Finally, the washed bacteria were diluted 20 times with sterile water. Then, 5 µL of the bacteria solution was spotted and dried on a scanning electron microscope (SEM) specimen stub (Carl Zeiss, Germany). The accelerating voltage was in the range of 5 to 10 kV.

Results and Discussion

Bioassay results

Investigation of the larvicidal features of three bacteria were carried out in fresh and polluted water that contained 100 larvae (Table 2). *Bti* ATCC 35646, *Bti* 4Q4 and *B. sphaericus* serotype H were used as positive control. The *Bti* were less effective on 3rd instar larvae with respect of strains of *B. sphaericus*. The MBI group were re-isolated from dead mosquito larvae and re-identified after the bioassay treatments (Figure 1). Based on the larvicidal activity of three strains (MBI5,

MBI6, MBI7) were selected and characterized. Bioassay test results clearly show that MBI5, MBI6, and MBI7 have a potential to be toxic for larvae of *Culex* spp.

Table 2. Bioassay results of MBI5, MBI6, MBI7, *B. sphaericus* serotype H, *Bti* ATCC 35646 and *Bti* 4Q4 on of *Culex* spp. larvae in polluted/fresh water

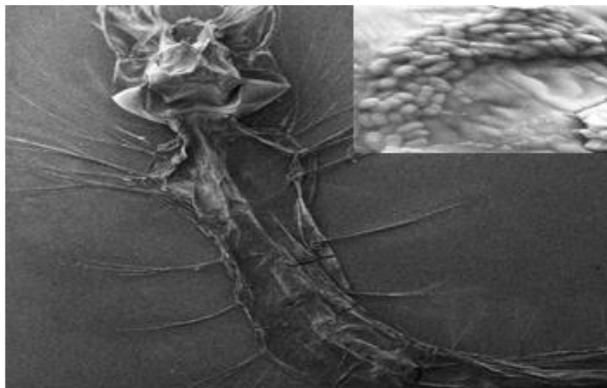| Bacteria Name	The number of alive larvae of *Culex* spp. (Total number : 100 in 500 mL)		
	Polluted water		Fresh water
	24h	48h	24h
B. sphaericus serotype H (0.5 mL)	10	4	0
MBI5 (0.5 mL)	6	4	0
MBI6 (0.5 mL)	7	3	0
MBI7 (0.5 mL)	9	2	0
*Bti*ATCC35646 (0.5 mL)	20	20	16
Bti 4Q4 (0.5 mL)	34	32	24

Figure 1. Scanning electron microscope image of dead mosquito larvae.

Morphological, physiological, and biochemical characterizations of newly isolated strains

The physiological characteristics of MBI5, MBI6, and MBI7 were summarized and selective characteristics with related model as *B. sphaericus* were compared in Table 3. According to the results; the new strains of *Bacillus* spp. MBI5, MBI6, and MBI7 were aerobic, gram-positive, spore-forming, capsulated and rod-shaped bacteria (Figure 2). Also, they have penicillin susceptibility. The optimum growth temperatures were 27-30°C. There is no growth at 4°C and 50°C on NA. All of the tested strains were oxidase and catalase negative. However, they were hemolysis positive.

Table 3. Phenotypic characteristics of strains MBI5, MBI6, MBI7 compared with *B. sphaericus* serotype H

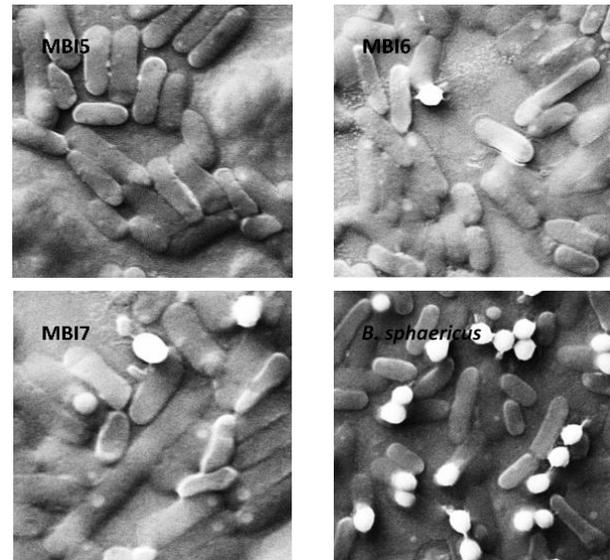| Characteristics	MBI5	MBI6	MBI7	*B. sphaericus* serotyp*e H*
Gram reaction	+	+	+	+
Oxidase	-	-	-	-
Catalase	-	-	-	-
Capsule staining	+	+	+	+
Endospore staining	+	+	+	+
Hemolysis	+	+	+	+
Anaerobic test	-	-	-	-
Penicillin	+	+	+	+

+, positive; -, negative; (+)

Figure 2. Scanning electron microscope images of MBI5, MBI6, MBI7 and *B. sphaericus* serotype H.

FAMEs analysis

The cellular fatty acid profiles of MBI group (MBI5, MBI6, and MBI7) and *B. sphaericus* serotype H were listed in Table 4. The major cellular fatty acids in MBI5 included iso-pentadecanoic acid ($C_{15:0}$ iso, 45%) and $C_{16:0}$ iso, 12.65%, minor amounts of the iso-branched fatty acids $C_{14:0}$ iso (0.60%), $C_{16:0}$ (1.72%), $C_{17:1}$ iso ω10c (1.43%). The major cellular fatty acids in MBI6 included iso-pentadecanoic acid ($C_{15:0}$ iso, 44.99%), and $C_{16:0}$ iso, 15.24%. Minor amounts of the fatty acids $C_{16:0}$ (0.78%), $C_{17:1}$ iso ω10c (1.40%). The major cellular fatty acids in MBI7 included iso-pentadecanoic acid ($C_{15:0}$ iso, 45.84%), and $C_{15:0}$ anteiso, 13.13%. Minor amounts of the iso-branched fatty acids $C_{14:0}$ iso (0.68%), $C_{18:1}$ iso ω9c (1.03%). The peak of 14:0 iso 3-OH^{-} was found only *B. sphaericus* serotype H. The bacteria of MBI group do not exist this peak. Consequently, significant similarities in fatty acids profiles were found between *B. sphaericus* serotype H and MBI group. All of the groups MBI and *B. sphaericus* serotype H were identified with MIDI as *B. sphaericus* GC subgroup E.

Table 4. Cellular fatty acid composition of MBI5, MBI6, MBI7, and *B. sphaericus* serotype H

Fatty acids	Fatty acid concentration (%)			
	MBI5	MBI6	MBI7	*B. sphaericus* serotype H
14:0 iso	2.02	4.38	1.51	1.26
14:0	0.60	-	0.68	0.85
15:0 iso	45.00	44.99	45.84	46.61
15:0 anteiso	10.87	9.22	13.13	7.89
14:0 iso 3OH	-	-	-	1.05
16:1 ω7c alcohol	9.93	12.38	9.55	6.80
16:iso	12.65	15.24	8.14	5.48
16:1 ω11c	3.31	2.04	3.31	5.62
16:0	1.72	0.78	1.78	1.64
17:1 iso ω10c	1.43	1.40	2.35	4.92
Sum In Feature 4	1.65	1.72	2.32	2.58
17:0 iso	6.11	4.67	5.69	10.86

PCR of 16S rRNA amplification

We carried out PCR amplification of *bin* and *mtx* toxin genes of MBI group and *B. sphaericus* serotype H. Our analyses revealed that *B. sphaericus* serotype H, MBI5, MBI6, and MBI7 all had the *binB* gene (Figure 3). Besides, *binA* toxin gene is present in *B. sphaericus* serotype H, MBI5, and MBI6, except MBI7. At the same time, MBI5, MBI6, and MBI7 did not have *mtx1* and *mtx2* toxins. In addition**,** commercial *B. sphaericus* serotype H has both *bin* and *mtx* toxin genes (Figure 4).
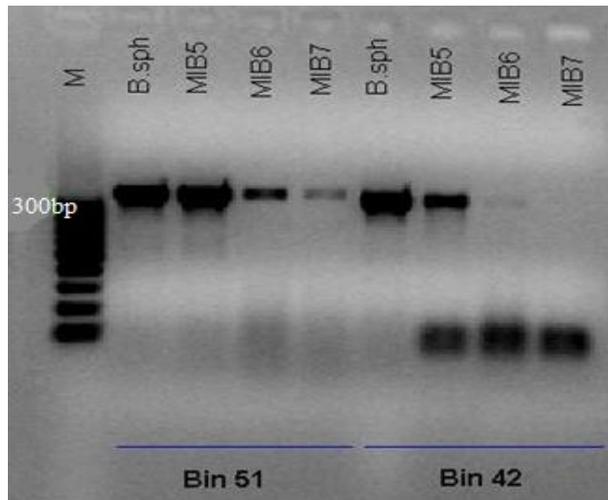

Figure 3. PCR amplicon of *bin 51* and *bin 42* toxin genes of B.sph: *B. sphaericus* serotype H, MBI5, MBI6, and MBI7.
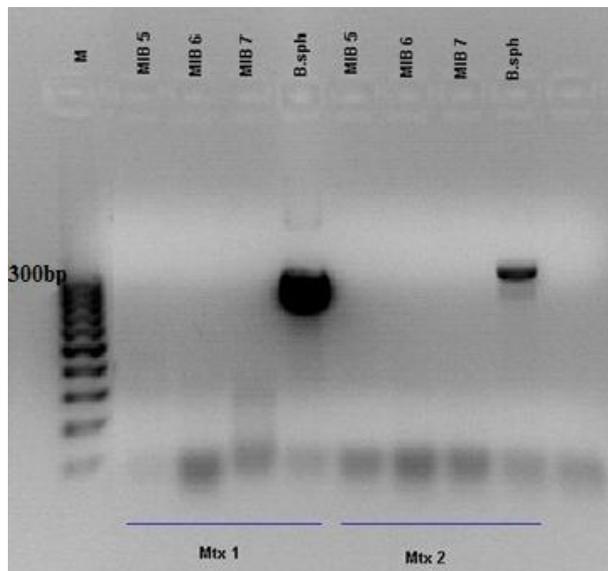

Figure 4. PCR amplicon of *mtx 1* and *mtx 2* toxin genes of B.sph: *B. sphaericus* serotype H, MBI5, MBI6, and MBI7.

Another study was neighbour-joining tree analysis that is based on nucleotide sequences of MBI group from 16S rRNA gene sequencing data (Figure S1). Confidence limits estimated from bootstrap analyses (100 replications) appear at the nodes. A maximum-parsimony tree generated from the sequence data exhibited similar topology to this tree. In the phylogenetic tree; MBI5, MBI6, and MBI7 clearly belonged to the strains of *B. sphaericus*, as shown by the

high bootstrap value (Figure 5). Moreover, the MBI group was located at different nodes within the *B. sphaericus*-like strains in the phylogenetic tree (Figure 5).

Generally, bacteria that are used for biological control of mosquitoes have effects on varied mosquitoes. This variety derives from specificity of bacteria toxins and their host range. *B. sphaericus* is more active against *Anopheles* and *Culex* spp. and less active against *Aedes* spp**.,** on the contrary *B. thrungiensis* subsp*. israilensis* is more active against *Aedes* and *Culex* spp., whileless active against *Anopheles* spp. In addition, *B. sphaericus* has ability of larvacidein polluted aquatic environments. But *B. thrungiensis* subsp*. israilensis* has lost its activity due to organic components of polluted aquatic environments (Suryadi et al., 2016; Wirth et al., 2010).

These bioassays compromise the determination of the lifecycle of the *Culex* spp. larvae after the bioinsecticide bacteria were inoculated in their natural habitats. Here, we have observed their life cycle at the laboratory condition, which is explained at the experimental section. MBI5, MBI6, MBI7, *B. sphaericus* serotype H, *Bti* ATCC 35646, and *Bti* 4Q4 were examined on larvae of *Culex* spp.. Each bacterium was inoculated in 0.5 mL using flasks. In fresh/polluted water, experiment results showed that MBI5, MBI6, MBI7, and *B. sphaericus* were effective as a biological control agent of larvae of *Culex* spp. Due to loss of activities of *B. thrungiensis* subsp. *israilensis* ATCC 35646 and 4Q4, the observed number of died larvae were decreased as compared to *B. sphaericus* (Table 2). Eventually MBI5, MBI6, MBI7, and *B. sphaericus* serotype H had mosquito pathogen ability in both environments (Table 2). In addition, MBI groups were more effective in fresh water for 24 hours. These results demonstrated that MBI5, MBI6, and MBI7 have potential to be used as bacterial insecticides because of their high toxicity on larvae. After this study, we identified mosquito pathogen bacteria through widely used techniques that are based on morphology, FAME, and PCR.

According to FAME analysis of MBI5, MBI6, MBI7, and *B. sphaericus,* they have similar results due to their fatty acid compositions**.** Due to the fact that, the MIDI library is not sensitive between sub strains of bacteria. According to our findings, MBI5, MBI6, MBI7, and *B. sphaericus* serotype H are the same bacteria, which are named as *B. sphaericus* GC subgroup E. Our analyses revealed the fatty acid structure differences between MBI5, MBI6, MBI7, and *B. sphaericus* serotype H (Table 4). However, the cell and colony morphology, and the physiological properties of MBI5, MBI6, and MBI7 were similar to *B. sphaericus* serotype H. Therefore, we performed 16S rRNA gene sequencing of MBI5, 6 and 7 for obtaining detailed information about their exact classification under the genus *Bacillus*. The 16S rRNA gene sequencing of MBI5, MBI6, and MBI7 showed that they are new members of *Bacillus* genus.
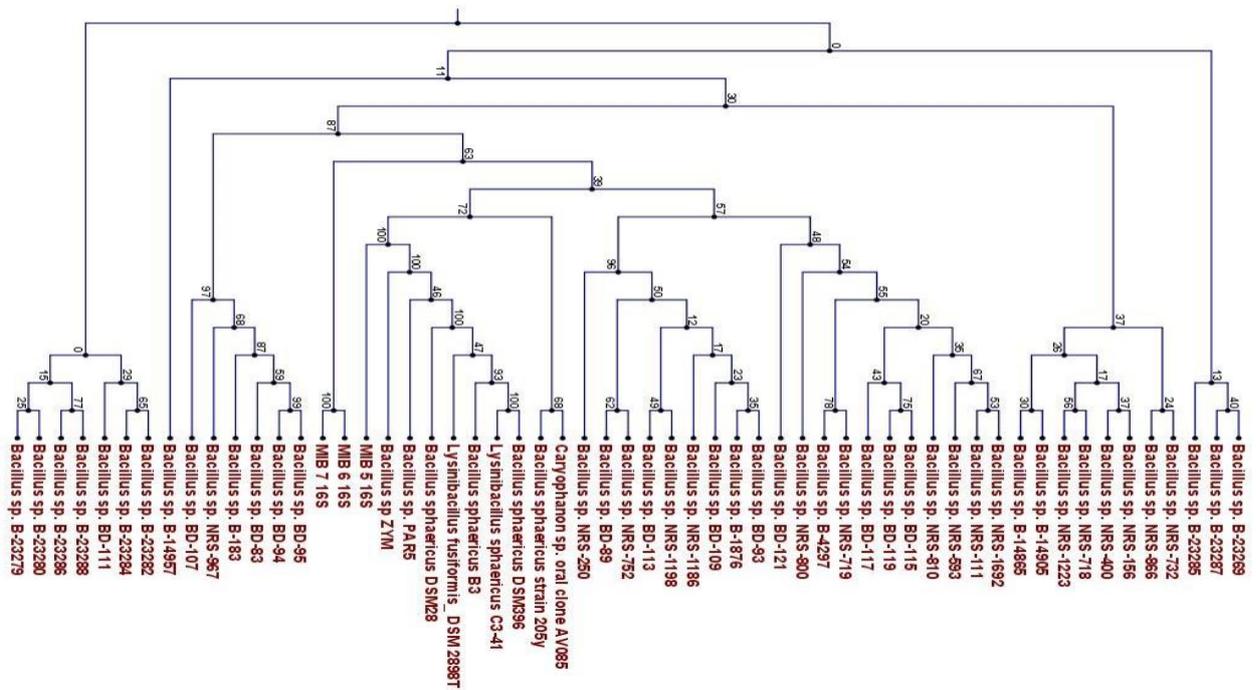
Figure 5. Neighbour-joining tree: the phylogenetic relationships among the *B. sphaericus*-like strains.

The *B. sphaericus* produces proteins that are toxic to mosquito larvae and the mosquito pathogen strains are divided into two groups depending on their toxicity. Highly active strains of *B. sphaericus* produce the crystalline binary toxin Bin during sporulation which is extremely toxic to *Culex* species. Bin is composed of BinA (41.9 kDa) and BinB (51.4 kDa). BinB binds to a specific receptor in *Culex pipiens* on the mosquito larvae midgut. Whereas BinA binds to a receptor only present on BinB. Both BinA and BinB are responsible for insecticidal activity (Park et al., 2010).

On the contrary, the low-activity-showing strains of *B. sphaericus* lacked Bin. Insecticidal activity of these strains is provided by proteins known as Mtx toxins (mosquitocidal toxins such as Mtx1 and Mtx2). Mtx1 and Mtx2 increase the toxicity of low activity strains of *B. sphaericus*. Several Mtx toxins exist in *B. sphaericus* strains which have both high and low insecticidal activities (Wirth et al., 2007). The three new isolated bacteria have bio insecticidal activity on *Culex* spp. larvae.

We have done PCR amplification of *bin* and *mtx* toxin genes of MBI5, MBI6, MBI7, and commercial *B. sphaericus* serotype H has both of *Bin* and *Mtx* toxin genes (Figure 3 and Figure 4). However, MBI5 and MBI6 have *BinA* and *BinB* toxin genes but not *Mtx*. Furthermore, MBI7 has only *BinB* toxin gene. The data of the amplicon of toxin genes has demonstrated that the bacteria of the MBI group are different from the point of toxin genes in comparison with *B. sphaericus* serotype H.

Conclusion

According to FAME, phenotypic characteristics and PCR-based 16S rRNA gene sequencing results, MBI5, MBI6, and MBI7 have been identified as new *B. sphaericus* like strains. Therefore, MBI5, MBI6, and MBI7 were deposited into the Agricultural Research Service (ARS) Patent Culture Collection (USDA) as NRRL B-50199, NRRL B-50200, NRRL B-50201, respectively.

Bioassay applications have proved that the three newly isolated bacteria are more effective in a short time than commercial *B. sphaericus* on the larvae of *Culex* spp. This study demonstrates a new isolated *B. sphaericus* like strains can be used as a mosquito pathogen against to *Culex* spp..

Acknowledgments

Additional Information

Supplementary data accompanies this paper at http://biotechstudies.org/uploads/BIO-84_Supp1.pdf.

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