(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
10 January 2013 (10.01.2013)

WIPO | PCT

(10) International Publication Number
WO 2013/005176 A1

(51) International Patent Classification:
A01N 63/00 (2006.01) *A01P 7/04* (2006.01)

(21) International Application Number:
PCT/IB2012/053426

(22) International Filing Date:
5 July 2012 (05.07.2012)

(25) Filing Language: Turkish

(26) Publication Language: English

(30) Priority Data:
201 1/06664 5 July 201 1 (05.07.201 1) TR

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK,

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(54) Title: NOVEL BACTERIAL STRAINS FOR BIOLOGICAL CONTROL OF MOSQUITOES

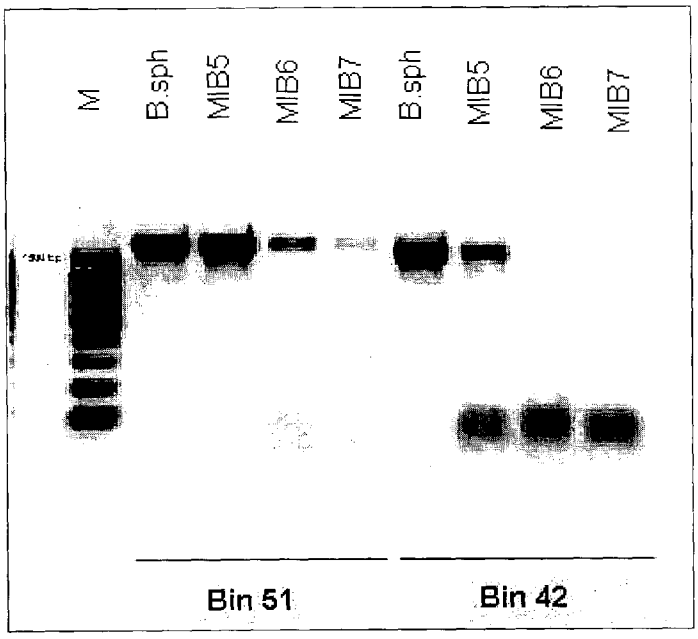

Figure 1

(57) Abstract: The present invention relates to novel bacteria strains that can be used in biological control against mosquito larvae *(Culex* spp.). The protein obtained from a novel *B.sphaericus* spp. isolates with the invention is used as larvicide, the step of isolating the protein at product obtaining stage is eliminated. By means of the invention, thee bacterial strains (MIB 5,6,7) investigated for biological control of mosquitoes are effective in both polluted and fresh water.

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EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*

NOVEL BACTERIAL STRAINS FOR BIOLOGICAL CONTROL OF MOSQUITOES

Field of the Invention

The present invention relates to novel bacteria strains that can be used in biological control against mosquito larvae *(Culex* spp.).

Background of the Invention

Mosquitoes are vectors of many diseases such as Mosquito-borne arboviruses, malaria, filariasis and Japan encephalitis. Generally mosquito control does with chemical pesticides more than biopesticides in the world. These chemical pesticides are known as dichlorodiphenyltrichloro ethane (DDT), gammaxane, malathion, chlordane and organophosphates . All of them have high toxic range for human health and environment. 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 *Bacillus sphaericus* are being widely used. Mosquitocidal bacteria are environmentally friendly alternatives to chemical pesticides for controlling water mosquitoes.

Bacillus thrungiensis subs. *israilensis* (Bti) is the most extensively used mosquito larvicidal bacteria in the world. Bti produces crystal glycoprotein (protoxin) coded by different genes such as Cry4A, Cry4B, CrylOA, Cryl lA and CrylA during sporulation. Bti Cry toxins have been widely used in the control of broad range of mosquito and blackfly species as well as nematodes, mite and protozoa. Another potential microbial pesticide insecticide, *Bacillus 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 carrying a single Bin (binary) toxin gene have been reported in many countries

European Patent document no EP0349769, an application known in the state of the art, discloses *Bacillus sphaericus* bacteria genetically engineered with toxin producing genes taken from *Bacillus thuringiensis* var. *israelensis* (B.t.i.) bacteria and transferred to *Bacillus sphaericus* strains. The genetically modified (GM) *Bacillus sphaericus* strains produced are capable of producing B.t.i. toxins in effective amounts and can control against mosquito larvae and black flies effectively.

European Patent document no EP0454485, an application known in the state of the art, discloses using insect killing toxins obtained from *Bacillus thuringiensis* or *Bacillus sphaericus* bacteria against pests living in water such as mosquito larvae. The spores of these bacteria kill some insect larvae feeding on these spores. The spores are digested in intestines of the larvae and release their toxins and neutralize the larvae. The known applications in the technique disclose taking toxins of the bacteria to apply on larvae for biological control against mosquito. Taking the toxins of the bacteria requires extra labor and cost. That is, protein isolation step is performed in these applications.

Even though, many commercial products are introduced to the market, development resistance in mosquito populations to some known biological control products are 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.

Summary of the Invention

The objective of the present invention is to provide novel bacterial strains that can be used as larvicide in biological control.

A further objective of the present invention is to provide novel bacterial strains for biological control wherein the toxin protein isolation step in product obtaining state is eliminated.

Another objective of the present invention is to provide novel bacterial strains for biological control which are effective in both polluted and fresh water.

Detailed Description of the Invention

"Novel Bacterial Strains for Biological Control of Mosquitoes" developed to fulfill the objective of the present invention is illustrated in the accompanying figures wherein,

Figure 1 Bin 51 and bin 42 toxin genes PCR amplified by primers B.sph: *B.sphaericus,* MBI5, MBI6 and MBI7

Figure 2 Mtx 1 and Mtx 2 toxin genes PCR amplified by primers
B.sph: *B.sphaericus,* MBI5, MBI6 and MBI7

Figure 3 is the PCR bands of MBI5, MBI6 and MBI7 bacterial strains in gel imaging system (BIORAD) after electrophoresis in 1% agarose gel with ethidium bromide (NC: Negative Control).

Figure 4 Neighbour-joining tree: the phylogenetic relationships among the *Bacillus sphaericus-Yike* strains.

Figure 5 is the scanning electron microscope image of *B. sphaericus* bacterial cells.

Figure 6 is the scanning electron microscope image of MBI5 bacterial strains.

Figure 7 is the scanning electron microscope image of MBI6 bacterial strains.

Figure 8 is the scanning electron microscope image of MBI7 bacterial strains.

Figure 9 is the 16 rDNA sequence of MBI5 bacterial strain.

Figure 10 is the 16S rDNA sequence of MBI6 bacterial strain.

Figure 11 is the 16S rDNA sequence of MBI7 bacterial strain.

In the inventive biological control against the mosquito larvae, the strains of *B. sphaericus* species are applied against the mosquito larvae. Deposit number is taken for the inventive strains from United States Department of Agriculture

Research, Education and Economics Agricultural Research Service on January 28, 2009. The deposit numbers of sub strains belonging to *B. sphaericus* species and named MBI5, MBI6, MBI6 are respectively registered as NRRL B-50199, NRRL B-50200 and NRRL B-50201 .

In laboratory experiments carried out against mosquito larvae *(Culex* spp.), it has been found out that the larvicide effects of *B.sphaericus* MIB5,6,7 strains and presence of Bin genes are the same as in the commercial strains of *B. sphaericus.* Furthermore, it has been observed that in experiments against the mosquito larvae the inventive bacteria strains show faster effect in a higher ratio than the known *B.sphaericus* strains. In the applications of the previous technique, an extra process is performed in order to obtain protein from the isolates. By means of the invention, the protein isolation step in obtaining product stage is eliminated. It is observed that newly found bacteria strains (MBI5, MBI6, MBI7) are effective when they are given to the medium in which the larvae are present directly without performing protein isolation. At the same time *Bacillus sphaericus* strains show high larvicide effect both in polluted and fresh water. Various experimental studies have been carried out in order to test the effectiveness of the invention.

Experimental Studies

Single colonies of newly isolated bacterial strains and Bti 4Q4, Bti ATCC 35646, *B. sphaericus* and were cultivated on to NYSM (Nutrient Yeast Salt Medium) agar and incubated for 48h at 30°C. Bacterial growth of each strain was harvested and resuspended in 10 ml of distilled water. Absorbance was adjusted to 0.2 with water and then 1 ml of suspension was added to 100 ml of fresh water/polluted

water in 250ml flasks containing 100 larvae (at the stage of 3 or 4th instar) of *Culex* spp. The inoculated flasks were maintained on laboratory bench and observed for 48h at room temperature. In order to determine larvicidal bacterial strains, which were capable of killing 90% of larvae, positive and negative control flasks treated with reference strains ,and sterile water, respectively, were kept the same condition as inoculating ones. After toxicity test, three strains of *B.sphaericus* (MBI 5, 6, 7) were selected as high toxic mosquitocidal bacteria and used for further studies. According to the bioassay test results MBI 5,6,7 have potential to be toxic to larvae of *Culex* spp. Investigation of larvacidal features of three bacteria were done in fresh and polluted water that contained 100 larvae (see Table 1). Bti ATCC 35646,. Bti 4Q4 and commercial *B.sphaericus* were used as positive control.

Table 1: The effectiveness of MBI 5, MBI 6, MBI 7 strains and *B.sphaericus,* Bti ATCC 35646 and Bti 4Q4 bacteria against *Culex* spp. larvae in polluted and fresh water.

Bacteria name	*Culex* spp. Live larvae number (500ml water/100 live larvae)		
	Polluted water		Fresh water
	24h	48h	24h
B.sphaericus (500µL)	10	4	0
MBI 5	6	4	0

(500µL)			
MBI 6 (500µL)	7	3	0
MBI7 (500µL)	9	2	0
BtiATCC35646 (500µL)	20	20	16
Bti 4Q4 (500µL)	34	32	24

According to the test results, it was found that MBI 5, MBI 6, MBI 7 strains are more effective in polluted water in 24 hours relative to the known *B.sphaericus*, Bti ATCC 35646 and Bti 4Q4 bacteria. The effectiveness percentage of MBI 5, MBI 6, MBI 7 strains were determined as 94%, %93 and %91, respectively. In tests performed in fresh water, it was observed that MBI 5, MBI 6, MBI 7 strains and *B.sphaericus* bacteria have 100% success by killing all existing healthy larvae in 24 hours. On the other hand, it was found out that Bti ATCC 35646 and Bti 4Q4 bacteria are effective against larvae in ratio of 84% and 76% in fresh water, respectively (Table 1).

Diagnostic studies

Phenotypic diagnostic studies

All of the methods provided to understand cell properties of three new strains of bacillus. MBI5, MBI6 and MBI7 were an aerobic, Gram-positive bacteria According to electron microscope images of MBI5, MBI6, MBI7 and *B.sphaericus*, they are rod-shaped bacteria (Figure-6, Figure-7, and Figure-8) and similar with *B.sphaericus* (Figure-5).

They were also growth 20-35°C, and the optimum growth temperatures were 27-30°C. Growth at 50°C and 4°C were not observed on nutrient agar. The physiological characteristics of MBI5, MBI6 and MBI7 were summarized and selective characteristics with related model as *B.sphaericus* were compared (Table 2).

Table 2: Phenotypic characteristics of strains MBI5, MBI6, MBI7 compared with commercial *B.sphaericus.*

Characteristics	MBI5	MBI6	MBI7	*B.sphaericus*
Gram staining	+	+	+	+
Oxidase	-	-	-	-
Catalase	-	-	-	-
Capsule Staining	+	+	+	+
Endospor Staining	+	+	+	+
Hemolysis	+	+	+	+
Anaerobic test	-	-	-	-
Penicilline	+	+	+	+

(+, positive; -, negative)

Fatty acid profile analysis

Each MBI strains were characterized as unique and novel in terms of BIOLOG, FAME profiles and 16S rRNA sequencing data.

The cellular fatty acid profiles of MBI 5, 6, 7 and *B.sphaericus* were listed in Table 3. The major cellular fatty acids in MBI5 included iso-pentadecanoic acid ($C_{15:0}$ iso, 45,00%) and $C_{16:0}$ iso, 12,65%. Minor amounts of the iso-branched fatty

acids $C_{i4;0}$ iso (0.60%), C]6:0 (1.72%), $C_{,7:}$, iso ω1Oc (1,43%). The major cellular fatty acids in MBI6 included iso-pentadecanoic acid ($C_{i5:0}$ iso, 44,99%) and C|6:0 iso, 15,24%. Minor amounts of the fatty acids $C_{i6:0}$ (0.78%), $C_{17:1}$ iso colOc (1,40%). The major cellular fatty acids in MBI7 included iso-pentadecanoic acid ($C_{15:0}$ iso, 45,84%) and $C_{i15:0}$ anteiso, 13,13%. Minor amounts of the iso-branched fatty acids C|4:0 iso (0.68%), $C_{i8:1}$ iso co9c (1,03%). Consequently, significant similarities in fatty acids profiles were found between *B.sphaericus* and MBl group. All of the groups MBl and *B.sphaericus* were identified with MIDI as *Bacillus-sphaericus*- GC subgroup E.

Table 3: Cellular fatty acid composition of MBl 5, 6, 7 and *B.sphaericus*

Numerical Names of the Fatty acids (Peak names)	Percentage % MBI 5	Percentage % MBI 6	Percentage % MBI 7	Percentage % *B.sphaericiis*
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 30H	-	-	-	1,05
16:1 w7c alkol	9,93	12,38	9,55	6,80
16:iso	12,65	15,24	8,14	5,48
16:1 w i l e	3,31	2,04	3,31	5.62
16:0	1,72	0,78	1,78	1,64
17:1 iso wlOc	1,43	1,40	2,35	4,92
Sum In Feature 4	1,65	1,72	2,32	2,58
17:0 iso	6,1 1	4,67	5,69	10,86
17:0 anteiso	4,70	3,19	4,67	4,45
18:1 w9c	-	-	1,03	-
Summed Feature 4	1,65	1,72	2,32	2,58

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Sequence analysis with nucleic acid based 16S-rDNA PCR amplification

DNA extraction from bacterial strains:

Total genomic DNA from bacterial strains was extracted according to methodology described by Jimenez with some modifications. The pure strains were

cultured in Nutrient Agar (NA) solid medium 16-20 hours at 27C and one single colony contaminated into 10ml Nutrient Broth (NB) at 27C for 3-4 hours until the absorbances up to 1 at 660nm. The bacterial cells were collected from media afer lOmin at 2000g centrifugation. The cells were suspended with lml of Tris-EDTA buffer(10mM Tris Base, IraM EDTA, 0.05% Tween 20, pH 9.0) and transfered into 2ml microcentrifuge tube, centrifuged at 14000g for 2min, supernatant discarded from the tube and added lml of Tris-EDTA buffer and repeated application for 3 times. Finally 300µ1 of Tris-EDTA buffer added and boiled at 94C for 30 min in water bath. Centrifuged at 14000g for 2min and 200µ1 DNA was collected from supernatant and stored at -20 for further PCR applications.

PCR amplification and purification of 16S rRNA:

16S rRNA genes of the bacterial DNA isolates (MBI 5, MBI 6, MBI 7 and *Bacillus sphaericus* serotype H for control) amplified by the PCR (BIORAD, İtaly) using purified DNA and primers 27f and 1492r (Lane, 1991). PCR amplifications was caried out in total volume of 50ul reaction mixture containing 0.2 mM of 27f and 1492r primers for total 16S, 1 U of *pfu* DNA polymerase (Fermentas, USA), 0.2mM of each deoxynucleoside triphosphate (dNTP), 1 mM MgS04, lOmM Tris and 50ng template DNA. PCR conditions were as follows : preamplification 94°C for 5 min : denaturation at 94°C for 30s : annealing at 55°C for 40s : elongation at 72°C for 2min repeated 34 cycles and then post amplification for final extention 10 min at 72°C.

We designed specific two new primers for *Bacillus sphaericus* like members of Bacillaceae family. We amplified 550bp of 16S rRNA gene fragments of the bacterial DNA isolates (MBI 5, MBI 6, MBI 7 and *Bacillus sphaericus* serotype H for control) by the PCR (BIORAD, İtaly) using purified DNA and primers FAMl and FAM2. PCR amplifications was caried out in total volume of 50ul reaction mixture containing 0.2 mM of FAMl and FAM2 primers for 550bp of 16S, 1 U of *pfu* DNA polymerase (Fermentas, USA), 0.2mM of each deoxynucleoside triphosphate (dNTP), 1 mM MgS04, lOmM Tris and 50ng template DNA. PCR conditions were as follows : preamplification 94°C for 5 min : denaturation at

94°C for 30s : annealing at 51°C for 40s : elongation at 72°C for 45sec repeated 34 cycles and then post amplification for final extention 10 min at 72°C.

The amplified DNA products was detected by using Biorad image analysing system (BIORAD, İtaly) after electrophoresis of PCR amplicons in a 1% agarose gel stained with ethidium bromide.

16S rRNA gene sequencing and phylogenetic analysis

Pure amplification products were sequenced with a Prism ABI 3100 Genetic Analyzer 16 caillaries, dideoxy terminator cycle sequencing kit (Applied Biosystems). The protocols used were due to manufacturers recommendations. 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 (Lane, 1991; Nakamura, 1996). The clustal w program (Higgins *et al.,* 1992) was used to align the 16S DNA sequences generated with sequences of *Bacillus sphaericus* like members from GenBank NCBI (Larsen *et al.,* 1993). The sequences of 16s rDNA genes were obtained (Figure-9, Figure- 10, Figure- 11).

Genetic distance was computed by using Kimura's two-parameter model (Kimura, 1980) and used for neighbour-joining analysis. Phylogenetic trees were constructed using neighbour-joining and maximum-parsimony methods provided by CLC Genomics Workbench_2_l_l both methods produced trees with similar topologies. Nucleotide sequences generated in this study have been deposited with GenBank under the accession numbers.

Another study was Neighbour-joining tree analysis that is based on 1450 nucleotide sequences. 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 *Bacillus sphaericus,* as shown by the high bootstrap value (Figure-4).

Determination of Toxin Genes

Toxin genes investigated according to methodology described by Nishiwaki et al., PCR of toxin genes of the bacterial DNA isolates *(MBI 5, MBI 6, MBI 7* and *Bacillus sphaericus serotype H* for control) possesses has done for the genes encoding the mosquitocidal binary toxin (51 and 42 kDa), Mtx1, and Mtx2. PCR was constructed according to the following conditions: preamplification 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 MgS04, 0.2 mM of each deoxynucleoside triphosphate (dNTP), 20 ng of template DNA, and 5 pmol of each primer in total volume of 50ul reaction mixture.

The amplified DNA products was detected by using Biorad image analysing system (BIORAD, İtaly) after electrophoresis of PCR amplicons in a 1% agarose gel stained with ethidium bromide (Figure- 1, Figure-2).

The PCR amplification of Bin and Mtx toxin genes of MBI 5, 6, 7 and commercial *B. Sphaericus* have done. Figure 1 reveals that *B. sphaericus*, MBI 5, MBI 6 and MBI 7 have Bin 5 1 and Bin 42 toxins. At the same time, MBI 5, MBI 6 and MBI 7 have not Mtx land Mtx 2 toxins (Figure-2). In addition, commercial *B. sphaericus* has both Bin and Mtx toxins.

References

1. Luis Jimenez, , Stacey Smalls ve Raymond Ignar, "Use of PCR analysis for detecting low levels of bacteria and mold contamination in pharmaceutical samples", USA Journal of Microbiological Methods Volume 41, Issue 3, August 2000, Pages 259-265.

2. David J. Lane,* Susan M. Barns, Dale A. Pelletier, ve William G. Weisburg, "16S Ribosomal DNA Amplification for Phylogenetic Study". Journal Of Bacteriology, Vol. 173, No. 2, Jan. 1991, p. 697-703.

3. A. de Lillol, F. P. Ashleyl, R. M. Palmerl, M. A. Munsonl, L. Kyriacoul, A. J. Weightman2, W. G. Wadel, "Novel subgingival bacterial phylotypes detected using multiple universal polymerase chain reaction primer sets", Oral Microbiology Immunology 2006: 2 1: 61-68.

4. Hisashi Nishiwaki, Kenta Nakashima, Chiharu Ishida, Tadayuki Kawamura, and Kazuhiko Matsuda*, "Cloning, Functional Characterization, and Mode of Action of a Novel Insecticidal Pore-Forming Toxin, Sphaericolysin, Produced by *Bacillus sphaericus",* Applied And Environmental Microbiology, May 2007, p. 3404-341 1.

5. Takeo Maruyama and Motoo Kimura, "Genetic variability and effective population size when local extinction and recolonization, of subpopulations are frequent", Proc. Natl. Acad. Sci. USA, Vol. 77, No. 11, November 1980, pp. 6710-6714.

6. Desmond G.Higgins, Alan J.BIeasbyl and Rainer Fuchs, "CLUSTAL V: improved software for multiple sequence alignment", Vol.8, no.2., 1992, Pages 189-191.

CLAIMS

1. MBI 5, MBI 6, MBI 7 bacterial strains which are sub strains of *Bacillus sphaericus* bacteria and which are used in biological control.

2. A MBI 5 bacteria strain for biological control according to claim 1, which is deposited with NRRL B-50199 number.

3. A MBI 6 bacteria strain for biological control according to claim 1, which is deposited with NRRL B-50200 number.

4. **A** MBI 7 bacteria strain for biological control according to claim 1, which is deposited with NRRL B-50201 number.

5. Bacterial strains for biological control according to claim 1 to 4, which are effective against mosquito larvae.

6. Bacteria strains for biological fight according to claim 5, which are effective when they are given directly to the medium in which the larvae are present without isolating protein.

7. Bacterial strains for biological control according to claim 6, which shows high larvicide effect in fresh and polluted water.

8. Bacteria strains for biological contol according to claim 7, which are an aerobic, Gram-positive, rod-shaped bacteria

9. Bacterial strains for biological control according to claim 8, which have 99% closeness with *Bacillus* sp. ZYM and *Bacillus* sp. BD-95.

10. Bacterial strains for biological control according to claim 9, which includes Bin 51 and Bin 42 toxin genes.

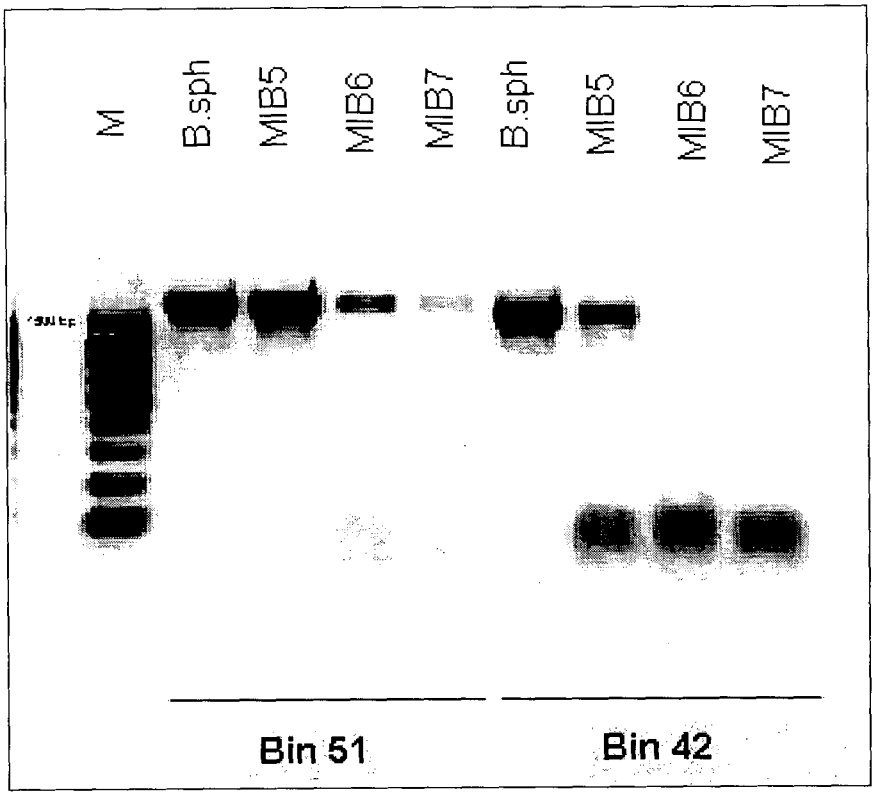


Figure 1

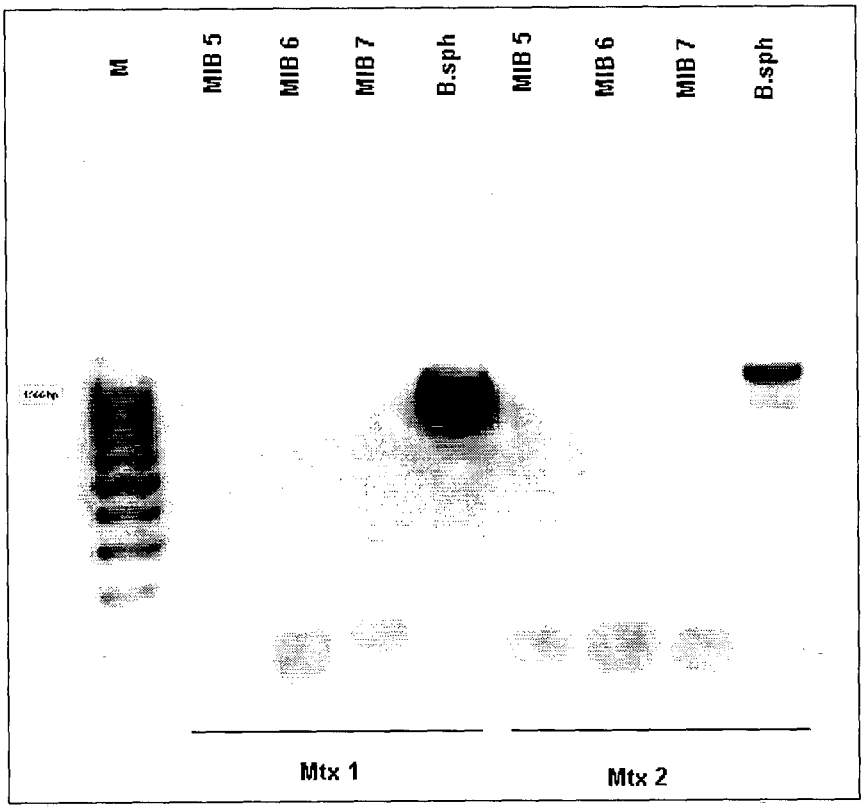


Figure 2

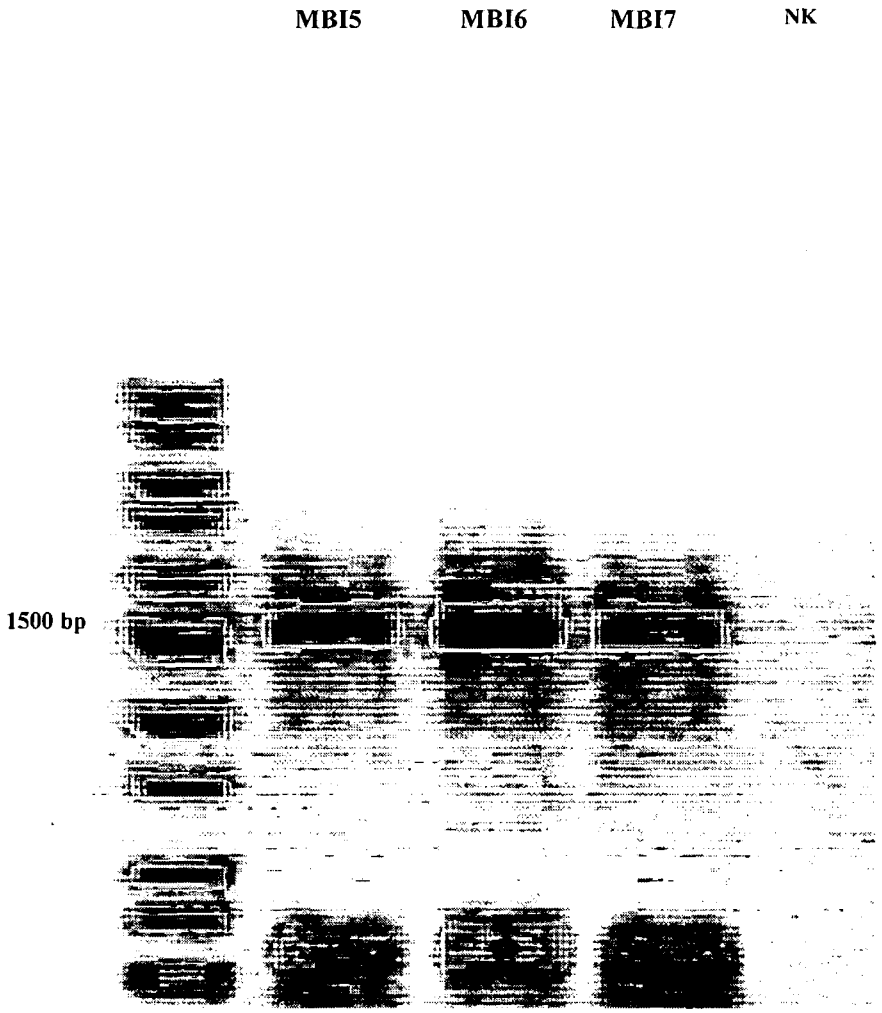
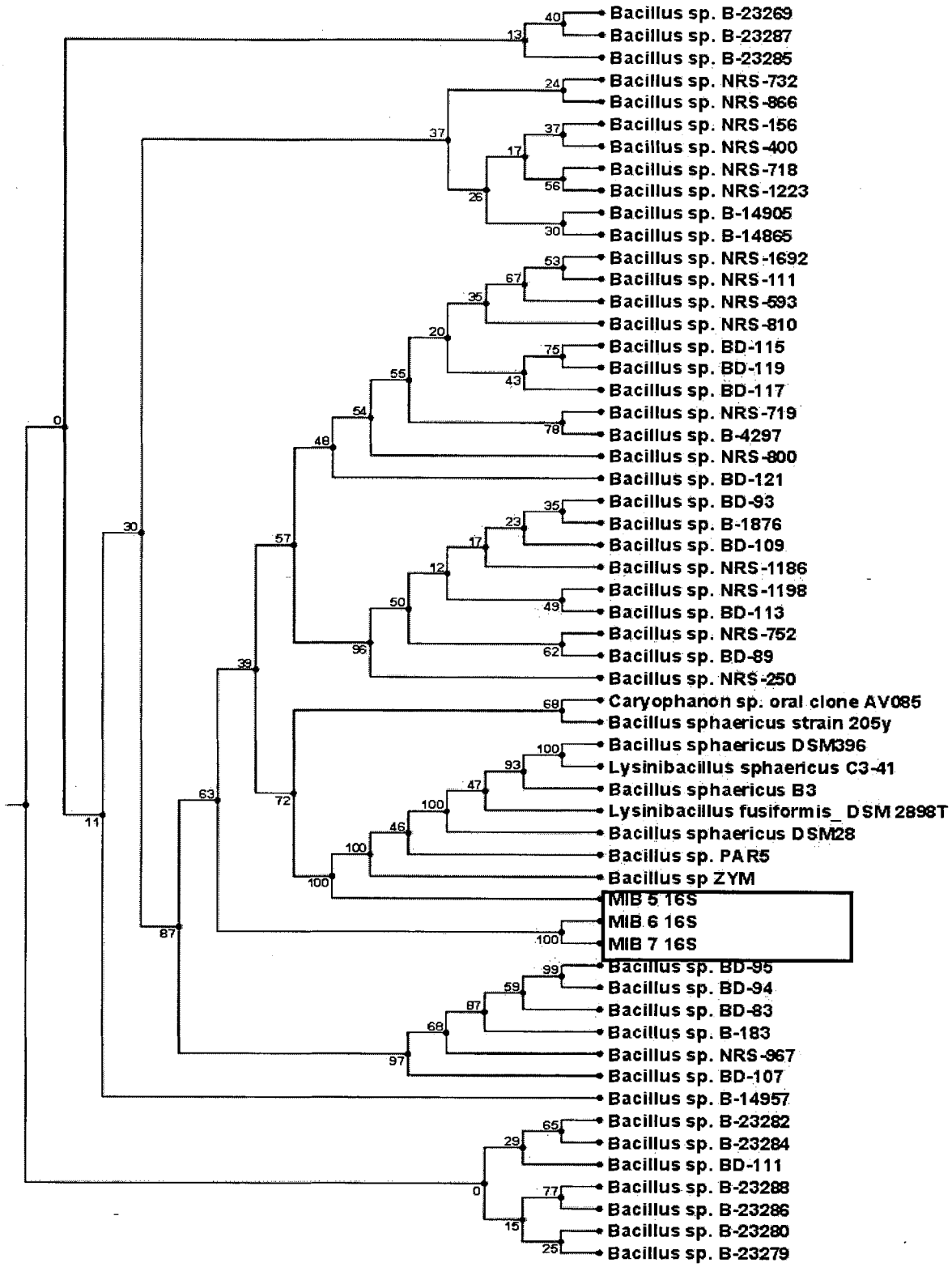


Figure 3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Bacillus sp. B-23285
Bacillus sp. NRS-732
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Bacillus sp. NRS-718
Bacillus sp. NRS-1223
Bacillus sp. B-14905
Bacillus sp. B-14865
Bacillus sp. NRS-1692
Bacillus sp. NRS-111
Bacillus sp. NRS-593
Bacillus sp. NRS-810
Bacillus sp. BD-115
Bacillus sp. BD-119
Bacillus sp. BD-117
Bacillus sp. NRS-719
Bacillus sp. B-4297
Bacillus sp. NRS-800
Bacillus sp. BD-121
Bacillus sp. BD-93
Bacillus sp. B-1876
Bacillus sp. BD-109
Bacillus sp. NRS-1186
Bacillus sp. NRS-1198
Bacillus sp. BD-113
Bacillus sp. NRS-752
Bacillus sp. BD-89
Bacillus sp. NRS-250
Caryophanon sp. oral clone AV085
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Lysinibacillus fusiformis_ DSM 2898T
Bacillus sphaericus DSM28
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Bacillus sp ZYM
MIB 5 16S
MIB 6 16S
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Bacillus sp. NRS-967
Bacillus sp. BD-107
Bacillus sp. B-14957
Bacillus sp. B-23282
Bacillus sp. B-23284
Bacillus sp. BD-111
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Bacillus sp. B-23286
Bacillus sp. B-23280
Bacillus sp. B-23279

Figure 4

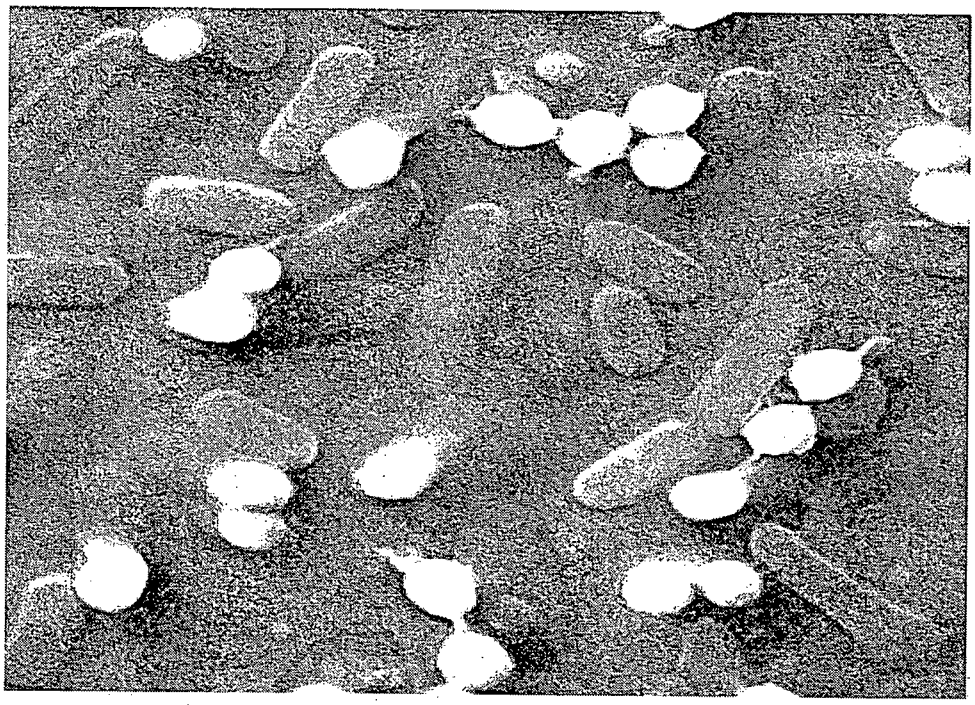

Figure 5

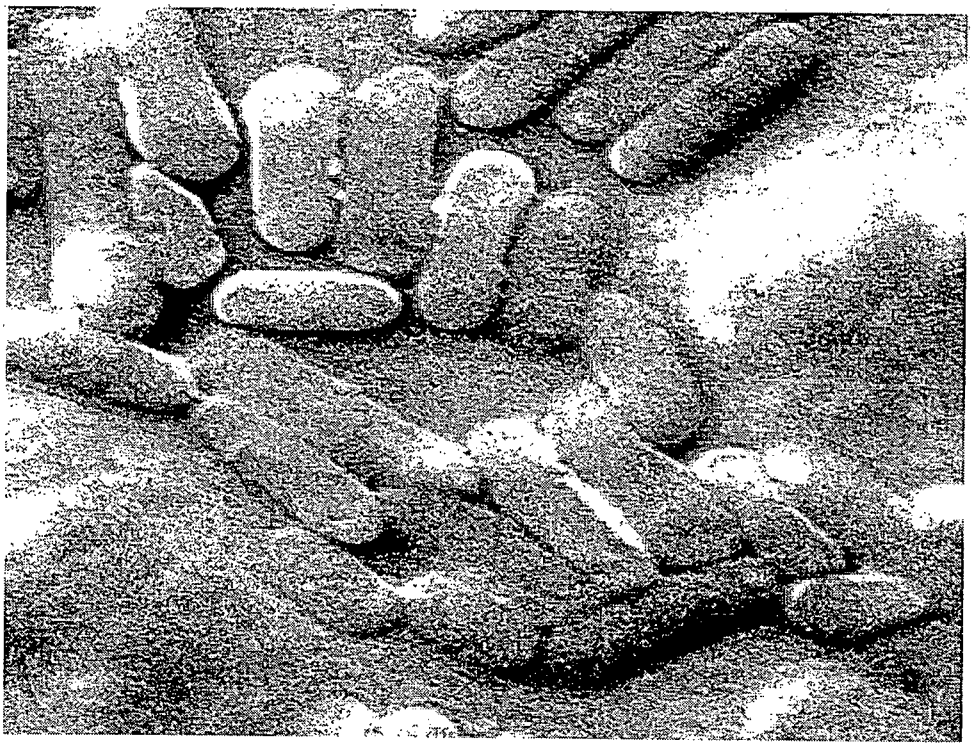

Figure 6

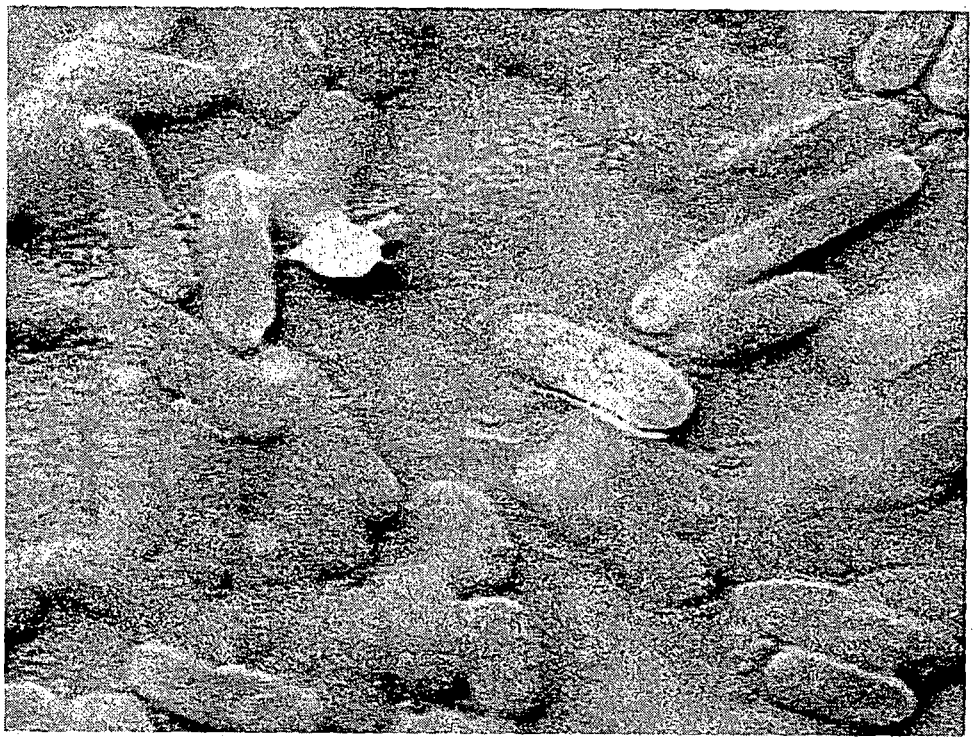

Figure 7

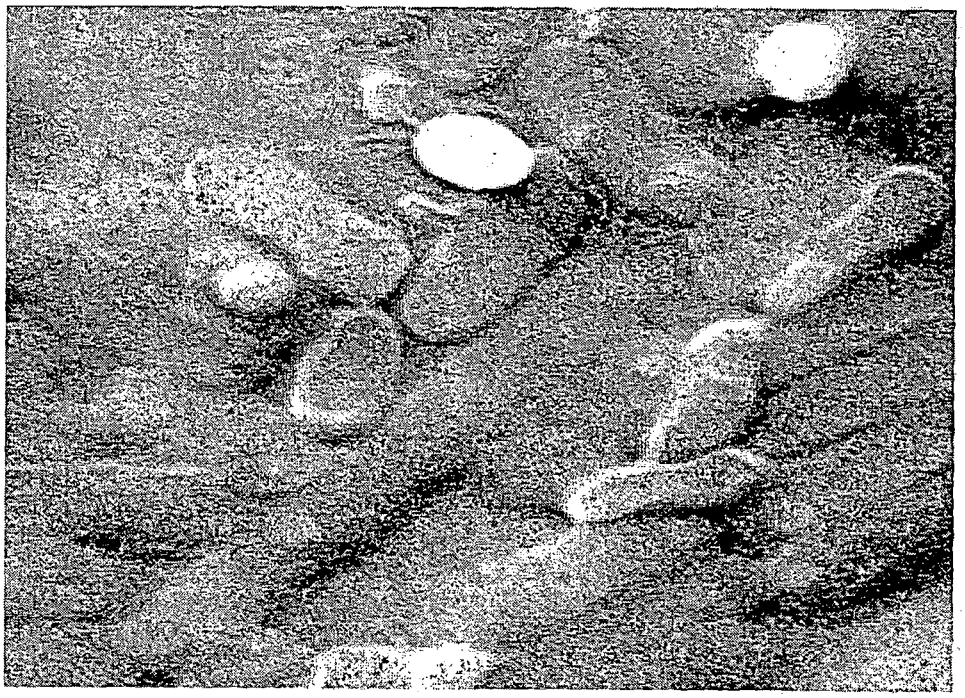

Figure 8

MBI 5

ACGCTGGCGGCGTGCCTATACATGCAGTCGAGCGAACAGAGAAGGAGCTTGCTCCTTCG
ACGTTAGCGGCGGACGGGTGAGTAACACGTGGGCAACCTACCTTATAGTTTGGGATAAC
TCCGGGAAACCGGGGCTAATAC
CGAATAATCTGTTTCACCTCATGGTGAAACACTGAAAGACGGTTTCGGCTGTCGCTATA
GGATGGGCCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGAT
GCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTC
CTACGGGAGGCAGCAGTAGGGAATCTTCCACAATG
GGCGAAAGCCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGATTTCGGTTCGTAAAAC
TCTGTTGTAAGGGAAGAACAAGTACAGTAGTAACTGGCTGTACCTTGACGGTACCTTAT
TAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGT
TGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGT
GGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTG
GGAGACTTGAGTGCAGAAGAGGATAGTGGAATTCCAAGTGTAGCGGTGAAATGCGTAG
AGATTTGGAGGAACACCAGTGGCGAAGGCGACTATCTGGTCTGTAACTGACACTGAGGC
GCGAAAGCGTGGGGAGCAAACAGGATTAGATACC
CTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGT
GCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCA
AAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACG
CGAAGAACCTTACCAGGTCTTGACATCCCGTTGA
CCACTGTAGAGATATAGTTTCCCCTTCGGGGGCAACGGTGACAGGTGGTGCATGGTTGT
CGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCT
TAGTTGCCATCATTTAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAG
GTGGGGATGACGTCAAATCATCATGCCCCTTAT
GACCTGGGCTACACACGTGCTACAATGGACGATACAAACGGTTGCCAACTCGCGAGAG
GGAGCTAATCCGATAAAGTCGTTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATG
AAGCCGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCT
TGTACACACCGCCCGTCACACCACGAGAGTTTGT
AACACCCGAAGTCGGTGAGGTAACCTTTGGAGCCAGCCGCCGAAGGTGGATAGATGAT

Figure 9

MBI 6

TGCAAGTCGAGCGAACAGAGAAGGAGCTTGCTCCTTCGACGTTAGCGGCGGACGGG
TGAGTAACACGTGGGCAACCTACCTTATAGTTTGGGATAACTCCGGGAAACCGGGG
CTAATACCGAATAATCTGTTTCACCTCATGGTGAAACACTGAAAGACGGTTTCGGCT
GTCGCTATAGGATGGGCCCGCGGCGCATTAGCTAGTTGGTG
AGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCA
CACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTC
CACAATGGGCGAAAGCCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGATTTCGGT
TCGTAAAACTCTGTTGTAAGGGAAGAACAAGTACAGTAGTAA
CTGGCTGTACCTTGACGGTACCTTATTAGAAAGCCACGGCTAACTACGTGCCAGCAG
CCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGC
GCAGGTGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATT
GGAAACTGGGAGACTTGAGTGCAGAAGAGGATAGTGGAAT
TCCAAGTGTAGCGGTGAAATGCGTAGAGATTTGGAGGAACACCAGTGGCGAAGGCG
ACTATCTGGTCTGTAACTGACACTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATT
AGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCCG
CCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGG
GGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGG
TGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCC
CGTTGACCACTGTAGAGATATAGTTTCCCCTTCGGGGGCAACGGTGACAGGTGGTGC
ATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAG
TCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCATCATTTAGTTGGGCACTCTAA
GGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCC
CCTTATGACCTGGGCTACACACGTGCTACAATGGACGATACAAACGGTTGCCAACTC
GCGAGAGGGAGCTAATCCGATAAAGTCGTTCTCAGTTCGG
ATTGTAGCTGCAACTCGCCTACATGAAGCCGGAATCGCTAGTAATCGCGATCAGCAT
GCCGCGGTGAATACGTTCCCGGGCCTTGTA

Figure 10

MBI 7

AGGAGCTTGCTCCTTCGACGTTAGCGGCGGACGGGTGAGTAACACGTGGGCAACCTA
CCTTATAGTTTGG
GATAACTCCGGGAAACCGGGGCTAATACCGAATAATCTGTTTCACCTCATGGTGAAAC
ACTGAAAGACGGTTTCGGCTGTCGCTATAGGATGGGCCCGCGGCGCATTAGCTAGTTG
GTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGC
CACACTGGGACTGAGACACGGCCCAGACTCCTACGGG
AGGCAGCAGTAGGGAATCTTCCACAATGGGCGAAAGCCTGATGGAGCAACGCCGCGT
GAGTGAAGAAGGATTTCGGTTCGTAAAACTCTGTTGTAAGGGAAGAACAAGTACAGT
AGTAACTGGCTGTACCTTGACGGTACCTTATTAGAAAGCCACGGCTAACTACGTGCCA
GCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGG
AATTATTGGGCGTAAAGCGCGCGCAGGTGGTTTCTTAAGTCTGATGTGAAAGCCCACG
GCTCAACCGTGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGATAGTG
GAATTCCAAGTGTAGCGGTGAAATGCGTAGAGATTTGGAGGAACACCAGTGGCGAAG
GCGACTATCTGGTCTGTAACTGACACTGAGGCGCGAAA
GCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTG
CTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCCGC
CTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAG
CGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGA
ACCTACCAGGTCTGACTTCCCGTT

Figure 11

INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2012/053426

A. CLASSIFICATION OF SUBJECT MATTER
INV. A01N63/00 A01P7/04
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal , BIOSIS, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	J.-F. Charles ET AL: "BACILLUS SPHAERICUS TOXINS: Molecular Biology and Mode of Action", Annu. Rev. Entornol , 1 January 1996 (1996-01-01) , pages 451-472 , XP55044393 , Retrieved from the Internet: URL: http://www.annualreviews.Org/doi /pdf/l 0.1146/annurev.en.41.010196.002315 [retrieved on 2012-11-15] page 452 , line 33 - line 34 page 455 , 1ine 1 - 1ine 6 page 457 , 1ine 3 - 1ine 7 tables 1, 2 ----- -/- ·	1-10

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
20 November 2012	03/12/2012

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Habermann , Jbrg

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ALEX W. SMITH ET AL: "Crystallization of the mosquito-larvicidal binary toxin produced by Bacillus sphaericus", ACTA CRYSTALLOGRAPHICA SECTION D BIOLOGICAL CRYSTALLOGRAPHY, vol. 60, no. 5, 1 May 2004 (2004-05-01), pages 952-953, XP55044631, ISSN: 0907-4449, DOI: 10.1107/S0907444904006535 page 952, column 1, line 2 - line 20	1-10
X	MULLIGAN III F S ET AL: "Laboratory and Field Evaluation of Bacillus sphaericus as a Mosquito Control Agent", JOURNAL OF ECONOMIC ENTOMOLOGY, ENTOMOLOGICAL SOCIETY OF AMERICA, LANDHAM, MD, US, vol. 71, no. 5, 1 October 1978 (1978-10-01), pages 774-777, XP001471073, ISSN: 0022-0493 page 774, column 2, line 32 - line 42 page 775, column 1, line 20 - line 33 page 775, column 2, line 12 - line 15 page 776, column 1, line 19 - line 42	1-10
X	HIRE R S ET AL: "Purification and characterization of mosquitocidal Bacillus sphaericus BinA protein", JOURNAL OF INVERTEBRATE PATHOLOGY, SAN DIEGO, CA, US, vol. 101, no. 2, 1 June 2009 (2009-06-01), pages 106-111, XP026185489, ISSN: 0022-2011, DOI: 10.1016/J.JIP.2009.03.005 [retrieved on 2009-04-05] abstract	1-10
X	PARK H W ET AL: "Properties and applied use of the mosquitocidal bacterium, Bacillus sphaericus", JOURNAL OF ASIA PACIFIC ENTOMOLOGY, KOREAN SOCIETY OF APPLIED ENTOMOLOGY, SUWON, KR, vol. 13, no. 3, 1 September 2010 (2010-09-01), pages 159-168, XP027079648, ISSN: 1226-8615, DOI: 10.1016/J.ASPEN.2010.03.002 [retrieved on 2010-03-10] page 160, column 2, line 2 - line 14	1-10

-/--

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SILVA-FILHA ET AL: "Culex quinquefasciatus field populations subjected to treatment with Bacillus sphaericus did not display high resistance levels", BIOLOGICAL CONTROL, SAN DIEGO, CA, US, vol. 44, no. 2, 11 October 2007 (2007-10-11), pages 227-234, XP022399860, ISSN: 1049-9644, DOI: 10.1016/J.BIOCONTROL.2007.10.002 page 232, column 2, line 47 - line 55	1-10
X	PALY K P ET AL: "Utility of Bacillus sphaericus in controlling Culex tritaeniorhynchus breeding", FEMS MICROBIOLOGY LETTERS, NO LONGER PUBLISHED BY ELSEVIER, vol. 45, no. 6, 1 December 1987 (1987-12-01), pages 313-318, XP023916803, ISSN: 0378-1097, DOI: 10.1111/J.1574-6968.1987.TB02407.X [retrieved on 1987-12-01] page 313, column 2, line 1 - page 314, column 2, line 24 page 317, column 2, line 45 - page 318, column 1, line 6	1-10
X	US 3 420 933 A (CORDS HELMUTH ET AL) 7 January 1969 (1969-01-07) column 8, line 3 - line 9 claim 1	1-10
T	MÜLige Yazici ET AL: "A novel formulation of bacillus sphaericus strains as mosquito pathogen", , 2 December 2011 (2011-12-02), pages 1-3, XP055044828, 2. Biyosidal Kongresi Bildiri Özetleri, Bildirileri ve Sunumlari Retrieved from the Internet: URL: http://www.turkiyeselcom/uvkb.org/2-biyosidal-kongresi-bildiri-ozetleri-bildirileri-ve-sunumlari/1304-sivri sinekle-mucadelede-kullanilmak-uzere-bacillus-sphaericus-suslarindan-gelistirile.html [retrieved on 2012-11-20]	

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/IB2012/053426

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 3420933 A	07-01-1969	NONE	