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Phylogenetic characteristics of novel *Bacillus weihenstephanensis* and *Pseudomonas* sp. to desert locust, *Schistocerca gregaria* Forskål (Orthoptera: Acrididae)

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Abstract

Thirty bacterial isolates were isolated from the gut contents of diseased/dead locust. Their pathogenicity was tested against 4th instar nymphs of desert locust, *Schistocerca gregaria* Forskål (Orthoptera: Acrididae). Two isolates, designated DL2 and DL6, out of thirty showed the highest insecticidal activities against locust nymphs in preliminary bracketing. They were bioassayed via leaf dip and per os techniques and toxicity was determined using SAS program. The insecticidal activity of DL6 was more than DL2, whereas LC_{50}'s values of 35×10^6 and 13×10^6 cfu's/ml were determined for DL2 and DL6, respectively, after 48 h of leaf-dip treatment. However, LD_{50}'s value of 53×10^6 and 26×10^6 cfu's/ml was determined for DL2 and DL6, respectively, after 24 h of per os treatment. The relative potencies of DL6 to DL2 were (2.6 and 2.03) folds in leaf-dip and per os treatments, respectively. Biochemical characterization was conducted, using GEN III MicroPlate™ Biolog identification system and confirmed with molecular identification via 16S rDNA gene sequencing. Nucleotide sequencing of each was submitted to a gene bank and an accession number was generated for each isolate. Obtained bacterial strains DL2 and DL6 were identified as *Bacillus weihenstephanensis* (KY630645) and *Pseudomonas* sp. (KY630649), with a similarity of 100 and 75% to *B. weihenstephanensis* strain PHCDB9 (NR_024697) and *Pseudomonas* sp. strain DSM11821 (KF417541), respectively. The tested strains proved their potential to be bio-pesticide agents involved in controlling desert locust nymphs.

Keywords: Desert locust, Entomopathogenic bacteria, Microbial control, Microbial identification, 16S rRNA genes sequencing, Biolog identification system, Bio-pesticides

Background

Locust swarms are considered to be a dramatic severe threat to sustainable food production globally (Lecoq, 2001). A lot of environmental concerns have been raised about the adverse impacts of the chemical pesticides used in controlling locusts. These concerns have drawn attention to the importance of developing biocontrol agents against locusts. Entomopathogenic bacteria such as *Bacillus* spp. possess antagonistic effects as pesticide either killer and/or antifungal agents (Mashtoly et al., 2009 and Mardanova et al., 2017). Many known Cry toxins have been produced from numerous *Bacillus thuringiensis* strains, efficiently initiating ion imbalance in brush border membrane vesicles (BBMVs) as a step in causing sepsis and then death (Bravo et al., 2013). Most of them are effective in alkaline pH gut conditions in lepidopteran insects. Some other *Bacillus* spp. such as *Bacillus cereus*, *B. subtilis*, *B. sphaericus*, and *B. weihenstephanensis* have the ability to produce antifungal compounds such as cyclic lipopeptides, polyketide synthases (PKS), or mycolytic enzymes like chitinases (Swiontek Brzezinska et al., 2014 and Aleti et al., 2015). Moreover,

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B. weihenstephanensis was reported as the only species to grow in psychrophilic conditions (Lechner, et al., 1998).

Most entomopathogenic bacteria are spore forming which count on Cry toxins to cause insect mortality. Some non-spore forming bacteria such as *Pseudomonas* spp. exhibited different virulence factors encoded in their excretions. Pathogenicity of *P. aeruginosa* Schroeder and *P. chlororaphis* (Gignard and Sauvageau) were investigated as efficient microbial agents in controlling some insect species including Acridoidea (Latchininsky et al., 2002). *P. entomophila* exhibited unique pathogenic characterization to lepidopteran, dipteran, and coleopteran insects (Vallet-Gely *et al.*, 2010). Usually, the insecticidal characteristics of non-spore forming bacteria to insects depend on the capability of the entomopathogen to enter, survive, persist in mid-gut physicochemical conditions and immune defenses, and excrete toxic substances that disrupt host physiology (Dieppois, et al. 2015).

Different reports indicate that the desert locust, *Schistocerca gregaria*, (Forskål) (Orthoptera: Acrididae) consumes approximately its own weight (2 g as adults) of fresh vegetation each day. Swarms often contain 50 million individuals per km^2 so that even a moderate swarm measuring 10 km^2 could consume about 1000 tons of fresh vegetation daily during migration (COPR (Centre of Overseas Pest Research), 1982). Periodic outbreaks of locusts are a threat to agricultural production in several countries (Szabo et al., 2003).

The present study aimed to isolate and investigate the toxicities of entomopathogenic bacteria from infected/dead locust nymphs. Phenotypic characterization and molecular identification were conducted for antagonistic isolates which may have the potential to be efficient biocontrol agents for desert locust.

Materials and methods

Insects

A culture of the desert locust, *S. gregaria*, was maintained in a laboratory at the Department of Locust and Grasshoppers Research, Plant Protection Research Institute, Agricultural Research Center, Giza, Egypt. The culture was examined daily for either dead or diseased insects, which were placed individually in separate sterile tubes. Diseased insects were lethargic and showed signs of bacterial infection such as a reddish or brown color on the thorax or abdomen along with flaccid bodies. Therefore, they were transferred to the Microbiological Resource Centre (MIRCEN) at the Faculty of Agriculture, Ain Shams University, Cairo, Egypt, for identification and bioassays.

Isolation of bacteria

Infected insects were dissected under sterile conditions. Individual swabs from the gut and dead locust paste (resulted from grinding the dead locusts in a sterilized phosphate buffer) were directly streaked on Tryptone Soy Agar Petri dishes (TSA) and incubated at 30 °C/72 h. Bacterial isolates were separated and picked up based on morphological characteristics such as colony shape and color. Streaking was repeated about 6 times for each isolate until pure colonies were obtained. Purified isolates were examined microscopically for parasporal inclusions and gram staining then subjected to preliminary bioassay.

Cultivation of bacterial isolates

One hundred milliliters of Tryptone Soy broth (TSB) in 250-ml baffled flasks was inoculated with individual purified bacterial isolate and incubated overnight at 30 °C with rotary agitation (150 rpm). Exponentially growing cells (~ 1×10^6 cells/ml) were harvested by centrifuging broth culture at 4000rpm/15 min/4 °C and the supernatants were discarded. Pellets of each isolate were washed 3 times by sterile phosphate buffer saline (PBS) and then suspended in 40 ml PBS. Absorbance of turbidity was measured at 600 nm for stock solution and its serial dilutions. A plate count of each isolate was carried out in Tryptone Soy Agar plates by inoculating 100 μl of each serial dilution per plate. All measures were conducted three times and the mean values were used to generate colony-forming units (cfu's) that correspond to the turbidity absorption at 600 nm after 48 h at 30 °C. Bacterial suspensions used in preliminary screening and bioassays were expressed as cfu's/ml.

Screening for bioactive bacterial isolates against locust

All isolates were subjected to preliminary bioassay for screening the bioactive isolates, using prepared stock solutions (~ 1×10^6 cells/ml). Fresh sticks and leaves of Egyptian clover were immersed in each bacterial suspension individually for 3 min, then allowed to dry under ambient temperature for about 10 min. Treated sticks and leaves of Egyptian clover were introduced as solo feed to five 4th instar nymphs of desert locust. Treated insects were placed individually in plastic boxes (30 × 18 cm). Daily inspection was continued up to 10 days and mortalities were recorded. Two bacterial isolates designated DL2 and DL6 proved an insecticidal activity to locust nymphs and were subjected to bioassay and molecular identification using 16S rRNA gene sequencing.

Morphological analysis

Pure colonies of the selected bacterial isolates were subjected to gram stain and examined for spores and crystals. One hundred milliliters of Tryptone Soy broth media were inoculated by one loop of each bacterial isolate and incubated at 30 °C with rotary agitation 150 rpm for 96 h. Fifty microliters of each suspension were

checked, by oil emersion × 100 lens of light microscope, for crystal protein and inclusion bodies.

Bioassay

Insecticidal activity of preliminary selected isolates was assessed for 4th instar nymphs of desert locust using leaf-dipping and per os techniques.

Leaf dipping technique

Serial dilutions of the selected bacterial isolates, Dl2 and DL6, were prepared in autoclaved phosphate buffer saline (1X PBS) under aseptic conditions. Colony-forming units per milliliter for all suspensions were adjusted (Table 1). Fresh stems and leaves of Egyptian clover were immersed entirely with gentle agitation in each bacterial suspension and in autoclaved 1X PBS, as control treatment for 3 min and then dried at ambient temperature for about 10 min. Stems and leaves treated with the same isolate were introduced as solo feed to three replicates of ten 4th instar nymphs individually and then kept in individual small wooden framed (30 cm in length, 18 cm in width, and 20 cm in height) cages equipped with wire gauze in front and top for easy access. Replicates of treatments and control were kept under the same rearing conditions (cages illuminated with 100 watts electric bulbs in winter and 75 watts electric bulbs in summer, 31 ± 2 °C and 50–70% R.H.) in the laboratory. Mortalities were examined daily and subjected to statistical probit analysis.

Per os technique

Serial dilutions of the two selected bacterial isolates, DL2 and DL6, were prepared in autoclaved phosphate buffer saline (1X PBS) under aseptic conditions. Cell suspensions were adjusted in (cfu's/ml) (Table 2). Two hundred microliters of each dilution were administered per os via 1-cm Hamilton syringe equipped with 27-gauge needle to ten 4th instar nymphs of *S. gregaria* and each of them was used as individual replicate. Control trial followed the same procedure, except using autoclaved 1X PBS instead of the bacterial suspension. Mortalities were recorded and subjected to statistical probit analysis.

Table 1 Preparation of different cell concentration (cfu's/ml) of the two selected isolates for leaf dipping bioassay technique

Strains	Stock suspension (cfu's/ml)	Dilutions (cfu's/ml)			
		1	2	3	4
DL2	29.8×10^6	14.9×10^6	7.4×10^6	3.7×10^6	1.5×10^6
DL6	131×10^6	65×10^6	32×10^6	16×10^6	8×10^6

Table 2 Preparation of different cell concentration (cfu's/ml) of the two selected isolates for per os bioassay technique

Strains	Stock suspension (cfu's/ml)	Dilutions (cfu's/ml)			
		1	2	3	4
DL2	59.7×10^6	29.8×10^6	14.9×10^6	7.4×10^6	3.7×10^6
DL6	295×10^6	147×10^6	73×10^6	36×10^6	18×10^6

Statistical analysis

Recorded mortalities of replicates in each concentration were pooled together and statistically analysed via PROC-PROBIT analysis system version 9.4 (SAS Institute 2012). LC_{50}'s values were compared and a failure of 95% CL to overlap was used as a measure of significant differences between treatments. In All cases, the likelihood ratio (L.R) chi-square goodness-of-fit values indicated that data adequately conformed to the probit model (Robertson and Preisler 1992).

Biochemical identification of bacterial isolates

Preliminary biochemical and phenotypic characterizations of the selected two bacterial isolates (DL2 and DL6) were conducted using the GEN III Biolog Microplates™ identification system at the Microbial Resources Centre (MIRCEN) (data not shown). Bacterial suspensions were prepared using the manufacturer's instructions and then inoculated into 96-well microplates, incorporated with redox tetrazolium dye, and incubated. Cellular respiration was resulted in changing colors due to the unique metabolic fingerprint of each bacterial strain. Microplates were read and compared to the extensive databases incorporated in OmniLog ID System.

16S rDNA gene sequencing for bacterial identification

Extraction of bacterial DNA

Pure individual colonies of DL2 and DL6 were picked up by a sterilized toothpick and suspended separately in 500 μl of sterilized phosphate buffer saline 1X (PBS 1x) in a 1500-μl centrifuge tube. Each tube was centrifuged at 10,000 rpm for 10 min using a micro-centrifuge. Supernatant was drawn off and the pellet was suspended in 500 μl of Genomic DNA Extraction Kit (Thermo Fisher Scientific Inc., Waltham, MA, USA). The suspension was incubated at 65 °C for 30 min and then heated to 100 °C for 10 min and finally cooled to 5 °C on ice and subjected to PCR.

Amplification of 16S rDNA

One set of universal primers (Integrated DNA Technologies, Inc., Mbiotech, Inc. South Korea) 27F (5′-AGA GTT TGA TCM TGG CTC AG-3′); 1492R (5′-TAC GGY TAC CTT GTT ACG ACT T-3′) was used to amplify the

targeted 16S rRNA gene. The final standard PCR mixture consisted of 45 μl of Platinum PCR Super Mix 1.1X (Invitrogen Corp., USA) and contains anti-TaqDNA polymerase antibody, Mg^{2+} dNTPs, and recombinant TaqDNA polymerase at concentrations sufficient to allow amplification during PCR. One microliter of one forward and one reverse primer and 3 μl of targeted bacterial DNA at the level of 1×10^8 templates were added to 45 μl of the standard PCR SuperMix in a final volume of 50 μl. PCR vials were placed in a C1000 Touch™ Thermal Cycler (Bio-Rad Laboratories, Inc.) which is fully programmable thermal cycler used for fast PCR reactions. The cycling protocol used consisted of initial denaturation at 95 °C for 3 min, followed by 45 denaturation cycles for 55 s at 94 °C, annealing at 55 °C for 40 s, and elongation at 72 °C for 90 s. Final elongation was performed at 72 °C for 600 s. Blanks consisted of all components of the reaction mixture except the substrate (DNA of the bacterial isolate). PCR products were subjected to 2% agarose gel electrophoreses stained with 5 μl of SYBR® safe DNA gel stain *10.000X concentrate in dimethyl sulfoxide (DMSO) (Invitrogen Corp, USA.) in running buffer (0.2 M Tris base, 0.1 M Sodium acetate, 0.01 M Na_2.EDTA, at pH 7.8). Digital images were taken by a Gel Doc™ EZ Gel Documentation System (Bio-Rad Laboratories, Inc.).

Purification of PCR product

Clear bands of bacterial DNA appeared with no background on agarose gel electrophoresis confirmed the purity of yielded DNA prior to amplification. PCR products were purified from unwanted primers and impurities such as salts, enzymes, and unincorporated nucleotides, using a QIAquick PCR purification kit (Qiagen), following the manufacturer's instructions via spin column technology optimized by uniquely designed silica membrane and three different functional buffers: binding buffer for binding of DNA in high-salt buffer, washing buffer for washing away all the impurities, and pure DNA was eluted with 30 μl of low-salt elution buffer (10 mM Tris Cl, pH 8.5) for high efficient recovery of DNA. The absorbance ratio of 260/280 of the purified DNA was determined, using a Smart Spec™ Plus spectrophotometer (Bio-Rad Laboratories, Inc.) and conformed the high purity of yielded DNA.

Gene sequencing and phylogenetic analysis

Purified bacterial DNA was diluted to 5 ng/μl of DNA with DNAase and RNAase free water and then each sample was divided into two samples. One set of universal primers (Integrated DNA Technologies, Inc., Mbiotech, Inc. South Korea) 518F (5′-CCA GCA GCC GCG GTA ATA CG-3′) and 800R (5′-TAC CAG GGT ATC TAA TCC-3′), was used for DNA sequencing. Sequencing of the purified DNA of approximately 1000–1100 bp was conducted via Macrogen sequencing service (Macrogen, Inc., Korea), using Big Dye terminator cycle sequencing kit and resolved on automated DNA sequencing systems model 3730XL and then analyzed by Sequence Scanner Version 1.0 © 2005 (Applied Biosystems, USA). 16S rRNA sequences of our isolates DL2 and DL6 were blasted and compared to other 16S rRNA sequences on the National Centre for Biotechnology Information (NCBI) database at https://www.ncbi.nlm.nih.gov/ (Altschul et al., 1990). Alignment of multiple sequences was examined against corresponding nucleotide sequences using the CLUSTAL W Program (Thompson et al., 1994) for proper matches with known species. Sequence analysis and phylogenetic tree were generated and constructed using the Molecular Evolutionary Genetics Analysis (MEGA) software version 7.0.

Results and discussion

Isolation of the entomopathogenic bacteria from the diseased and dead locusts resulted in thirty different purified bacterial strains, isolated from the gut and the dead locust paste (data not shown). The bioactivity of each bacterial isolate was tested via preliminary bracketing bioassay against 4th instar nymphs *S. gregaria*. Two bacterial isolates, designated DL2 and DL6, out of thirty showed significant antagonistic impacts to tested nymphs. The morphological examination of bioactive purified isolates via light microscope confirmed that DL2 isolate was a spore-forming, gram-positive bacteria, while DL6 was non-spore-forming, gram-negative bacteria. Neither DL2 nor DL6 had the ability to produce inclusion bodies or crystal protein.

Susceptibility of 4th instar nymphs of *S. gregaria* to the selected isolated bacteria

A preliminary bracketing bioassay revealed that 4th instar nymphs were highly susceptible and showed higher mortality rates to isolates DL2 and DL6 than any others. Moreover, leaf dipping bioassays showed that DL2 and DL6 were highly effective biocontrol agents, causing death to treated nymphs. After 48 h, data demonstrated that LC_{50}'s values of 35×10^6 and 13×10^6 cfu's/ml were determined for DL2 and DL6, respectively (Table 3). Per os treatment showed the same trend of results, where DL2 and DL6 were the most efficient isolates. Data in Table 4 showed LD_{50}'s values of 53×10^6 and 26×10^6 cfu's/ml for DL2 and DL6, respectively. Mean values of mortality rates in control treatments were 4.2 and 6.6% for leaf-dip and per os treatments, respectively.

Data in Table 3 indicated that isolate DL6 was more effective than DL2. Failure of overlap at 95% fiducial limits was considered as an evidence for significant differences between LC_{50} levels. The toxicity index values from the leaf-dip bioassay illustrated the relative potency between the most efficient isolate (DL6) as a standard

Table 3 Toxicity values of DL2 and DL6 isolates to 4th instar nymphs of *Schistocerca gregaria* using leaf dipping bioassay technique

Isolate	N	Slope (SE)	LC_{50}[ab]	χ^2 (df)[c]	95% FL	Toxicity index[d]
DL2	180	2.19 (0.35)	35×10^6b	1.65 (3)	25×10^6–45×10^6	37.14
DL6	180	1.19 (0.26)	13×10^6a	1.92 (3)	60×10^5–20×10^6	100

[a]LC_{50}'s reported in cfu's
[b]LC_{50}'s followed by the same letter are not significantly different based on overlap of their 95% fiducial limits ($P < 0.05$)
[c]L.R. chi-square goodness-of-fit values. Tabular values at $P = 0.05$ for 3 df = 7.81
[d]Toxicity index (Sun, 1950) = (LC_{50} of the most efficient compound (as Standard)/LC_{50} of the other tested compound) *100
N refers to total number of insects in treatment

and the other isolate DL2 (Sun, 1950). The potency of DL6 was approximately 2.6 folds than that of DL2 (Table 3). On the other hand, per os bioassay in Table 4 revealed a similar trend of efficacy, whereas DL6 had toxicity 2.03-folds of DL2. However, there was insignificant difference shown between DL2 and DL6 in per os bioassay, whereas an overlap occurred between the fiducial limits.

Although entomopathogenic bacteria are well known as the most efficient bio agents in controlling insects, *Pseudomonas aeruginosa* was the first bacterial isolate registered against the desert locusts (Ashrafi, et al., 1965). Obtained bacterial isolates may become promising entomopathogenic bacteria to control *S. gregaria* nymphs due to their fast killing rate.

Biochemical characterizations of selected bacteria using Biolog microbial identification system

Biolog MicroPlate™ pattern revealed that the most bioactive bacterial isolates, DL2 and DL6, to 4th instar nymphs of *S. gregaria* were preliminary identified as *B. weihenstephanensis* and *Pseudomonas* sp., respectively, according to their metabolic fingerprint.

Molecular identification of the most bioactive bacterial isolates using 16S rRNA sequencing

Images captured of agarose gel electrophoreses (Fig. 1) emphasized that the isolation process of DL2 and DL6 from the gut and paste of the infected/dead locusts was successfully conducted under aseptic and controlled conditions. The yielded bands were about 1000 to 1100 bp, for DL2 and DL6 isolates, respectively. DNA amplification and DNA sequencing were conducted using two sets of primers that were designed to complement the conserved regions of 16S ribosomal DNA of both DL2 and DL6 strains. The genera *Bacillus* and *Pseudomonas* were found to be the predominant identified bacteria, when sequences were blasted against nucleotide database using a nucleotide query algorithm.

The following partial 16S rRNA gene sequence is already submitted to NCBI under the accession number KY630645 and KY630649 for DL2 and DL6, respectively, as the following:

https://www.ncbi.nlm.nih.gov/nuccore/KY630645)
https://www.ncbi.nlm.nih.gov/nuccore/KY630649

Partial sequence of DL2, 409 bp

"GGAGCAGAGTGACAGGTGGTGCATGGTTGTCG
TCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCC
GCAACGAGCGCAACCCTTGATCTTAGTTGCCAT
CATTAAGTTGGGCACTCTAAGGTGACTGCCGGT
GACAAACCGGAGGAAGGTGGGGATGACGTCAAT
CATCATGCCCCTTATGACCTGGGCTACACACGTG
CTACAATGGACGGTACAAAGAGCTGCAAGACCG
CGAGGTGGAGCTAATCTCATAAAACCGCTCTCAG
TTCGGATTGTAGG
CTGCAACTCGCCTACATGAAGCTGGAATCGCTA
GTAATCGCGGATCAGCATGCCGCGGTGAATACG
TTCCCGGGCCTTGTACACACCGCCCGTCACAC
CACGAGAGTTTGTAACACCCGAAGTCGGTGG"

Partial sequence of DL6, 669 bp

"GGAGCTAACGCGTTAAGTTCACCGCCTGGGGA
GTACGGCCGCAAGACTGAACCTCAATGGATTTG
ACGGGGGCCCCCGCAACCGGGGAACTAGGTGGT
TTATTTCAAGCCACCCCGAAAACCCTTACCTCGC
CTTGCCTCGTCAAAAACTTTCCAAAAATGGATGG
TGGCCTTCGGAACCCCGAAACCAGGGCCTGCTTG

Table 4 Toxicity values of DL2 and DL6 isolates to 4th instar nymphs of *Schistocerca gregaria* using per os bioassay technique

Isolate	*N*	Slope (SE)	LD_{50}[ab]	χ^2 (df)[c]	95% FL	Toxicity index[d]
DL2	30	1.64 (0.77)	53×10^6a	0.80 (3)	68×10^2–12×10^8	49.05
DL6	30	1.64 (0.77)	26×10^6a	0.80(3)	33×10^2–59×10^6	100

[a]LD_{50}'s reported in cfu's
[b]LD_{50}'s followed by the same letter are not significantly different based on overlap of their 95% fiducial limits ($P < 0.05$)
[c]L.R. chi-square goodness-of-fit values. Tabular values at $P = 0.05$ for 3 df = 7.81
[d]Toxicity index (Sun, 1950) = (LC_{50} of the most efficient compound (as standard)/LD_{50} of the other tested compound) *100
N refers to total number of insects in treatment

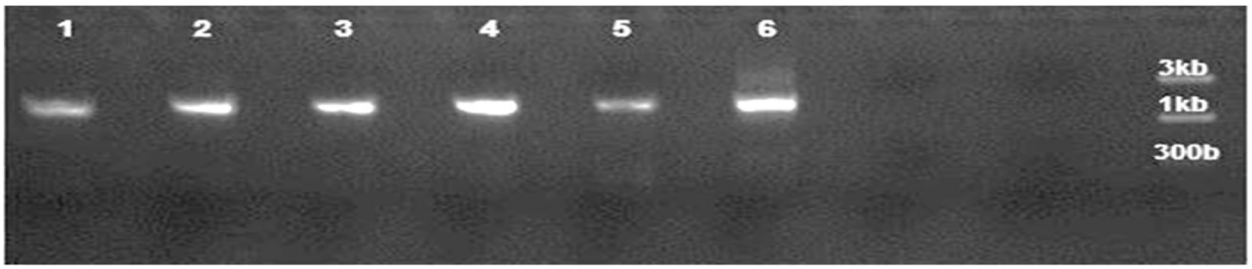

Fig. 1 Gel electrophoresis of 2% agarose gel illustrating the amplification of partial 16S rRNA genes of isolates DL2 and DL6 (lanes 2 and 6, respectively)

GTTGCCTCCACCTCTGGTCTGGAAAGGTGGGGTT
AAGTCCCCCCACCAACGCACCCCTTGCCCTTGTT
TGCCACCGCTCAAAGCCGGGGAATCTCAAGAGA
CAGCCCCTGAGACACTCCAGAAAAGGGGGGATC
ACCTCGTCTCCTCGGGCCCCTTAAGGACGGGGCT
ACACGCGCTCTACTGGGGTATGCACAGAGGAC
TGCACCCCCGCAAGCGAACGCACCTCCCAAAATC
CCTCTCACACCCGAGAGTAGACTCTGCAACTCTA
CTCTCTGAAATCTCAAACTCTCTTAATCACATAT
CAACGTTGCTGCGAAGACTATCTCCGCGGGTGTT
GTCCACGCCCGCCGTCCCACCGGGGGTAGTTTGT
TGCACCAGAAGCATGTAGCTTCTCCTTCCAGGAG
GCCGCTCGCCTCGGGATGCCCGACTACTGGGAA
GACCTCAAGAAGGGGCCCCCCAAAAAAAAAAA".

The isolated bacteria DL2 and DL6 were successfully identified as *B. weihenstephanensis* (KY6306 45) and *Pseudomonas* sp. (KY630649), with a very high similarity of 100% with *B. weihenstephanensis* strain PHCDB9 (NR_024697) and 75% with *Pseudomonas* sp. strain DSM11821 (KF417541), respectively.

The genus *Bacillus* has been considered as proteolytic bacteria (Visotto et al., 2009), enhancing toxicity agent (Mashtoly et al., 2010 & Mashtoly et al., 2011), and/or entomopathogenic bacteria (Sezen et al., 2005 and Mashtoly et al., 2009). Insecticidal characteristics of *Bacillus* spp. varied due to the ability in producing their entomopathogenic toxins. *Bacillus cereus* group includes six related species such as *B. cereus*, *B. anthracis*, *B. thuringiensis*, *B. mycoides*, *B. pseudomycoides*, and *B. weihenstephanensis* have been known to produce a lipase toxin namely phospholipase C (Lysenko 1972a, b) and the paralytic toxin sphingomyelinase C (Nishiwaki et al., 2004). *B. thuringiensis* has been considered the most attributed entomopathogenic bacteria in controlling insects. *B. weihenstephanensis* is known with a unique capability to survive in a psychrophilic condition (Soufiane and Cote, 2010). *B. thuringiensis* serovar *japonensis* and *B. cereus* have been successfully used as microbial control agents for the grubs of *Anomala orientalis, Cyclocephala borealis, Amphimallon solstitialis, Melolontha melolontha, Anomala dimidiata*, and *Holotrichia seticollis* (Guttmann and Ellar, 2000; Mashtoly, et al., 2010; Selvakumar et al., 2007 and Sushil et al., 2008).

In our previous research, two different strains of *B. cereus* causing significant mortality rates to the desert locust were identified (Reda et al., 2018) and both strains might be involved as integrated agents into locust management systems to protect the environment and wildlife from chemical use. *B. weihenstephanensis* and *B. thuringiensis*, in particular, and the crystal proteins of *B. thuringiensis* were found to be antagonist to *M. melolontha* larvae. *B. weihenstephanensis* showed 80% insecticidal activity, which proved its ability to be a significant biological control agent (Sezen et al., 2007).

The insecticidal effects of *P. fluorescens*, *P. aeruginosa*, and *P. chlororaphis* have been reported to different pests such as *M. melolontha* larvae (Coleoptera: Scarabaeidae), (Osborn *et al.*, 2002; Bucher, 1981; Sezen and Demirbag 1999). *Pseudomonas* sp. showed significant efficacy in controlling locust nymphs. Moreover, they displayed specific characteristic features of sepsis that appeared in thinning out of the internals and body skeletal, often with a specific rancid smell (Dieppois *et al.*, 2015). The mortality rate of *L. migratoria* infected with *Pseudomonas* sp. was approximately (30–50%) within 3 to 7 days after treatment (Lednev *et al.,* 2008). *P. entomophila* exhibited a unique characterization which demonstrated specific pathogenic properties to dipteran, lepidopteran, and coleopteran insects (Vodovar *et al.* 2005; Vallet-Gely *et al.* 2010).

Most entomopathogenic bacteria are spore forming counting on Cry toxins in causing insect mortality. However, there are non-spore-forming bacteria, such as *Pseudomonas* spp., that exhibit virulence factors encoded in their excretions. The insecticidal properties of non-spore-forming bacteria to insects often vary upon their capability to enter, persist, and survive in the gut physicochemical conditions and be protected from the gut immune defense system as well. Thus, the excretion of toxic substances may disrupt the host's physiology (Dieppois *et al.,* 2015). Obtained results indicate that the strains *B. weihenstephanensis* and *Pseudomonas* sp. have a significant impact as biocontrol agents, causing

nymphocidal activity to desert locusts. Further research is needed to investigate the whole characterization profile and host range of these isolates.

Attention has been drawn to the Biolog System™ (Biolog, Inc., Hayward, CA) as one of the most developed reliable technologies for rapid identifying and characterizing the environmental microbes (Al-Dhabaan and Bakhali, 2017). Carbon source utilization was the fundamental reaction that Biolog system counted on via exchange of the generated electrons during respirations through oxidizing colorimetric reaction. Such color and colorless pattern in 96-well microplates expressed the metabolic fingerprint of the tested microorganism (Miller and Rhoden, 1991). Evaluations of this biochemical identification tool resulted in precise, reproducible, and comprehensive bacterial characterizations (Chojniak *et al.,* 2015). Potential difficulties and overlaps in phenotypic identification of some bacteria could occur and 16S ribosomal DNA-based identification of unknown bacteria has been widely applied as a reliable accurate tool (Drancourt *et al.*, 2000). Universal identification methods based on general primer mixture was evaluated and validated for amplification of any bacterial DNA (Barghouthi, 2011). Sequenced 16S rDNA subjected to BLAST (Basic Local Alignment Search Tool) web-based program for alignment the obtained sequence to thousands of different sequences in the National Centre for Biotechnology Information (NCBI) at https://www.ncbi.nlm.nih.gov/ (Altschul *et al.*, 1990). It is of interest to note that 405 and 502 bp of the DL2 and DL6 sequences were highly aligned (100%) when they were tested with BLASTn, nucleotide alignment (bl2seq). This 100% identity indicated that PCR product was almost fully sequenced. Therefore, our bacterial isolates were identified as *B. weihenstephanensis* strain and *Pseudomonas* sp. strain for DL2 and DL6, respectively. Therefore, genotyping via 16S rRNA sequencing confirmed the phenotypic biochemical characterizations via Biolog system at the species level.

Phylogenetic analysis was inferred the inter-relationships between the identified bacterium and the existing relatives on Genbank based on 16S rRNA gene sequencing (Weisburg *et al.,* 1991). Phylogenetic tree for each isolate was derived from comparing sequences of 16S rRNA between obtained isolates and the existing sequences in the database, using a neighbor-joining method (NJ). The NJ phylogenetic tree was divided into 2 large clades. Isolates DL2 and DL6 were grouped with *B. weihenstephanensis* strain PHCDB9 (NR_024697) and *Pseudomonas* sp. strain DSM11821 (KF417541) with a high similarity of 100 and 75%, respectively (Figs. 2 and 3). Insecticidal characteristics of some *Pseudomonas* spp. such as *P. entomophila*, *P. fluorescens*, *P. Aeruginosa*, and *P. chlororaphis* have been investigated on coleopteran, dipteran, lepidopteran, and orthopteran pests (Osborn *et al.*, 2002, Vodovar *et al.* 2005, Lednev *et al.,* 2008 and Vallet-Gely *et al.* 2010). A unique sepsis characterization resulted from infected locusts with *Pseudomonas* sp. was displayed as fetid smell along with disruption of internal gut (Dieppois *et al.*, 2015). Therefore, *Pseudomonas* spp. may excrete enzymes or produce vegetative insecticidal protein, which interferes with receptors and disrupt the epithelial tissues in the mid-gut, therefore initiating septicaemia.

Although the insecticidal activity of *B. weihenstephanensis* has been little investigated, it showed mortalities of 80% (Sezen *et al.*, 2007) to *M. melolontha* larvae and 100% to desert locust nymphs, when subjected to a cell density of 29.8×10^6 and 59.7×10^6 for leaf-dip and per os bioassay. As production of inclusion bodies or crystal protein from the present tested isolate did not recognize, it is believed that it might have the ability of producing vegetative insecticidal protein or enteric toxins such as lipase toxin phospholipase and/or paralytic toxin sphingomyelinase that occurred in *Bacillus cereus* (Lysenko 1972a & b and Nishiwaki *et al.,* 2004).

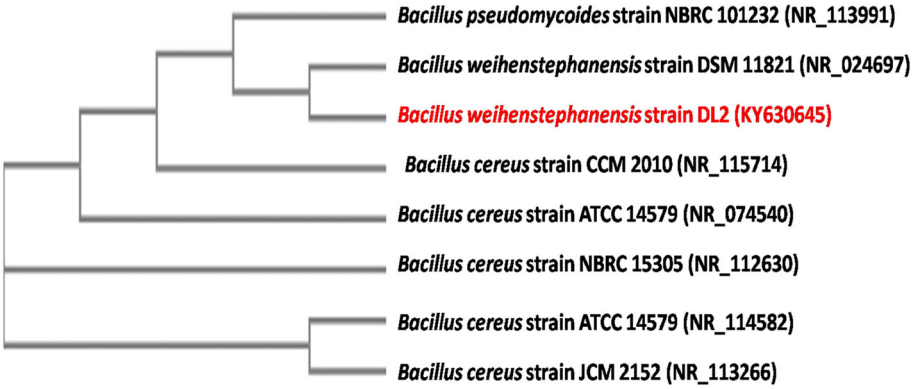

Fig. 2 Phylogenetic tree based on 16S rRNA gene sequences of DL2 bacterial strain and other *Bacillus* species on NCBI

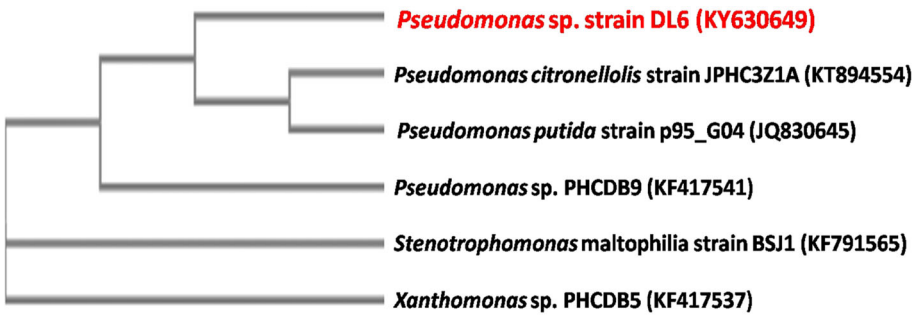

Fig. 3 Phylogenetic tree based on 16S rRNA gene sequences of DL6 bacterial strain and other closely related species on NCBI

Conclusion

Two isolated bacteria from infected locust *S. gregaria* nymphs in Egypt were molecularly identified as *Bacillus weihenstephanensis* and *Pseudomonas* sp. They proved the potential to be very efficient if they were formulated as biopesticide formulation and involved as a part of integrated locust management system to decrease the consuming quantities of chemical synthetic pesticides that are usually used to control locust. Further investigations are needed to characterize their mode of action and biochemical receptors involved in causing sepsis to desert locust nymphs and to test their potential against some other insect pests.

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Authors' contributions

TAM, MR, and AAM carried out the microbiology stuff such as isolation of the entomopathogenic bacteria from the dead/infected locusts, bacterial purification, and then preparation of the bacterial suspension in order to bioassay them. Also, they carried out the molecular identification of the promised isolates. MSE and TAM designed the experimental design, wrote the manuscript, and revised the final manuscript. AA and TAM shared the procedures of molecular identification and the sequence alignment. GMA participated with MR and TAM in the bioassay trials and statistical analysis. All authors read and approved the final manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article and the authors declare that they have no objection to the availability of data and materials.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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