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Molecular Screening of Chitinase Gene in *Bacillus* spp.

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Abstract: In a molecular screening program to select a potent chitinase producing bacteria, a locally isolated *Bacillus* isolate 66 (ac: DQ524821) recovered from dead *Biomphalaria alexandrina* snails, was shown to secret the enzyme upon testing the activity in YNB-Agar and broth media, containing chitin. For PCR screening of chitinase gene, reference strains: *B. thuringiensis tenebrionis* (tt4); *B. thuringiensis israelensis* (NRRL HD-522); *B. thuringiensis Berliner* ACCC 10061 along with the local isolate 66 were tested using designed set of degenerative primers. The same size PCR fragment (310 bp) was obtained upon testing these different templates. Furthermore, the fragment related to isolate 66 was sequenced and a phylogenetic relationship was performed where, it showed 96% similarity to chtinase gene of *B. thuringiensis* A1 Hakam (protein-id: ABK86590). The sequence results was submitted into GenBank under accession number EU734811.

Key words: chitinase, PCR, *Bacillus* sp., phylogeny

INTRODUCTION

Chitin, an insoluble linear β-1,4-linked polymer of Nacetylglucosamine (GlcNAc), is one of the most abundant polysaccharides in nature with numerous industrial, medical and commercial uses. It is a common constituent of insect exoskeletons, shells of crustaceans, fungal and some algal cell walls[1,2]. Chitinases (EC 3.2.1.14) are a glycosyl hydrolases that catalyze the hydrolytic degradation of chitin, and is found in a wide variety of organisms including bacteria, fungi, invertebrates, plants and animals[3], but serve a variety of functions in nature including morphogenesis, defense, nutrition and pathogenesis[4]. Chitinases that produced by different micro-organisms have received increased attention due to their wide range of biotechnological applications, especially in the production of chitooligosaccharides and N-acetyl D-glucosamine[5], biocontrol of pathogen and pests[6,8], preparation of sphaeroplasts and protoplasts from yeast and fungal species[9,10], and bioconversion of chitin waste to single cell protein[11].

One of the most important insecticidal microbes used in biological control is *Bacillus thuringiensis* (*Bt*), Insecticidal proteins, mainly Cry (crystal) proteins, play the leading role in controlling of insect pests. Some factors cause limitations in usage of Bt in biological control such as, susceptibility of important pests to only a few toxins[12], development of resistance to Cry proteins, slow mode of action, and the requirement of ingestion, along with some other limitations, This leads to search for new approaches to improve the conventional use of *B. thuringiensis*[13].

It has been reported that chitinases are widely distributed in *Bt* strains and that some of the chitinase-producing strains can enhance the insecticidal activity of *Bt*[14]. Furthermore, chitinases from *B. thuringiensis serovar kurstaki* HD-1(G) culture supernatants increase the insecticidal activity against *Plutella xylostella* when mixed with the spore-crystal complex[15].

The chitinase gene from tobacco (Nicotiana tabacum) endochitinase and the cry1Ac gene from *Bacillus thuringiensis* were cloned into the vector β pHT315 and designated as pHUAccB5plasmid to improve the insecticidal activity in acrystalliferous *B. thuringiensis*[16].

In this paper we report the production of chitinase enzyme from a previously locally isolated *Bacillus thuringiensis* 66 in comparison with reference strains related to the same species. Also, molecular screening of chitinase gene in these different strains was performed using a degenerative primers designed according to Multialignment between different bacillus species. Moreover, the amplified band of the local isolate resulted from PCR reaction was sequenced to verify its relatedness to chitinase gene. This work is considered an initial step in the way to improve the insecticidal activity of *B. thuringiensis* through its interaction with chitinases.

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MATERIALS AND METHODS

Bacterial Strain and Growth Conditions: Four *B. thuringiensis* strains were used in this study *B. thuringiensis tenebrionis* (tt4) strain was kindly supplied by Prof. Priest, FG, Heriot University, England). *B. thuringiensis israelensis* (NRRL HD-522) was kindly supplied by Prof. Nakamura, LK, United State Department of Agriculture. *B. thuringiensis Berliner* ACCC 10061 was bought from China Center of Industrial Culture Collection, CICC and a local isolate *B. thuringiensis* isolate (designated 66) (ac: DQ 524821, DQ 524822) was recovered from dead *Biomphalaria alexandrina* snails in Egypt.

Growth and Enzyme Detection: A preliminary test for production of chitinase enzyme by tested strains was carried out by cultivation of bacterial stains on Yeast-Nitrogen Base (YNB) medium (Difco Laboratories, Detroit, Mich) containing 0.2% (wt/v) chitin and 0.5% yeast extract as described by Watanabe *et al.*[1] and incubated at 30°C for up to 8 days. The ability of tested isolates to hydrolyze chitin substrate was detected qualitatively by formation of clearing zone during the culturing period with 0.1% Congo red solution[1].

Preparation of Crude Enzyme from Culture Fluid of Tested Strains: The cells of tested strains were grown in LB broth medium for 18 h at 30°C as the preculture. A portion of the preculture was inoculated at 30°C for up to 8 days with constant shaking (200rpm) using (YNB) medium (Difco Laboratories, Detroit, Mich.) containing 0.2% (wt/v) chitin; 0.5% yeast extract. During the culturing period, samples were collected from each flask and used for determining chitinase activity.

Enzyme Assay: Quantitative estimation of chitinase activity was measured using colloidal chitin as the assay substrate. One unit of chitinase activity was defined as the amount of enzyme which produces 1µ,mol of reducing sugar per 1hr at 37ºC. The reducing sugar released during the reaction, using colloidal chitin as substrate was measured, according to Somogyi-Nelson method[17].

DNA Isolation, Manipulation and Pcr Amplification: The genomic DNA was performed according to Ausubel *et al.,*[18]. Preparation of genomic DNA and separation of fragments by agarose gel electrophoresis were performed as described by Sambrook *et al.,*[19]. Polymerase Chain Reaction (PCR) techniques were performed with Taq polymerase (Fermentas) and the amplified fragment was cut and purified from agarose gel using Sephaglas™ BandPrep Kit.

Primer Design and PCR: Polymerase chain reaction (PCR) technique was performed for amplification of a conserved region of chitinase gene as partial sequences. A set of primers designed based on sequencing alignment of chitinase producing Bacilli (ac: D10594; D63702; AB04193; EF103273; AF275724; AF510723; EF040226; M57601; AF265220 and AB110081). The conserved region of aligned nucleotide sequences used to design degenerate 20-mer oligonucleotides forward and reverse PCR primers F:5' TTCA(T/C)GTTCAACACTACAA3'; R: 5'CATTAGGCCGCGGA(A/G)TG3', respectively. PCR was performed in 50-µl reactions containing 50 ng of template DNA, 10 pmoles of each primer, 200 µM of each dNTP, standard buffer (supplied with the *Taq*) containing 1.5 mM $MgCl_2$, and 1 U of *Taq* DNA polymerase, in thermal cycler, as follows: initial denaturing step 94 ºC for 3 min., then (30 cycles) comprised a denaturing step at 94ºC for 1 min, annealing at 50 ºC for 1 min and extension at 72 ºC for 1.5 min. followed by final extension 72 ºC for 7 min. with forward and reverse primers.

DNA Sequencing and Sequence Analysis: Nucleotide sequencing was performed using an ABI PRISM 310 (Perkin-Elmer Foster City, CA) at the DNA Sequencing Facility, VACSERA, Cairo, Egypt. PCR- forward and reverse specific primers were used to obtain the nucleotide sequences of both DNA strands of PCR fragment. The obtained sequence (259 bp) has been submitted, deposited into GenBank under accession number (ac: EU734811). Deduced amino acid sequences were analyzed using the BLASTX family program.

Phylogenetic Analysis: Muli aligment between the target sequence and the closely related (id: ABK86590; AAK69033; AAS42658; ABY44663; ATT60545; AAT32966; AAT55875; AAU16783; AAP27590; ABM05818; AAP47142; BAB16890 and AAP10651) was performed with CLUSTAL W. On the basis of the resulting multiple sequence alignments a phylogenetic tree was calculated for each protein by applying the maximum-likelihood method implemented in the Tree-Puzzle software, version 5.2.

Multiple sequence alignment and molecular phylogeny were performed using BioEdit[20]. The phylogenetic tree was displayed using the TREEVIEW program[21] subsequently.

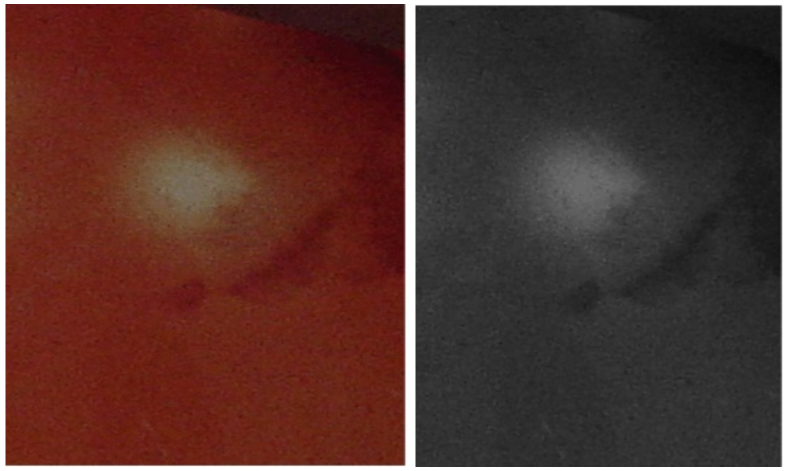

Fig. 1: Clearing zones of colloidal chitin formed by chitinases excreted by tested bacterium (local isolate 66) upon growing in YNB- agar plate containing chitin (0.2%). After 11 days incubation the plate stained with 0.1% Congo red solution.

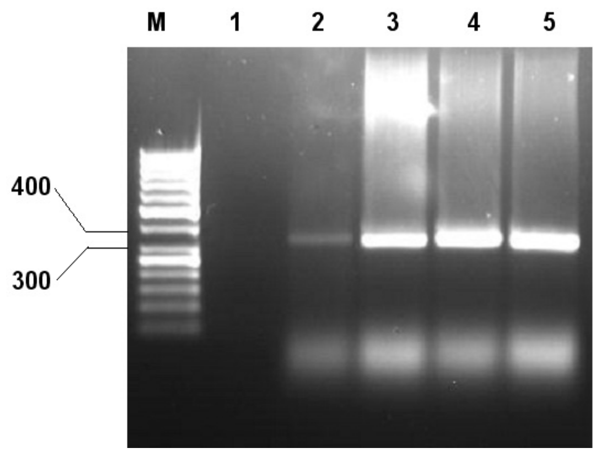

Fig. 2: Agarose gel electrophoresis analysis of PCR products.
M: 50bp DNA marker; Lane 1: Negative control; Lane 2-5: local isolate 66; *B. thuringiensis tenebrionis* (tt4); *B. thuringiensis israelensis* (NRRL HD-522); *B. thuringiensis Berliner* ACCC 10061 respectively.

RESULTS AND DISCUSSION

Selection of Potent Chitinase Producing Local Bacteria: In order to test qualitatively the capability of a local isolate and some reference type strains of thuringiensis species for chitinase production. A preliminary test was performed by growing the tested bacteria in YNB agar plate containing chitin. The activity was detected through floating the plate with congo red solution, where the appearance of clear zone is an indication of chitinase activity. All tested bacteria produced this zone, but the local isolate showed a high potency (Fig. 1).

The local potent chitinase producing isolate, as detected by chitin agar plate method, was the previously isolated *Bacillus thuringiensis* 66 and reported by Salem *et al.*[22,24].

Furthermore, the levels of enzyme production by a selected local isolate was performed using YNB-chitin containing broth medium as described in material and method. A 1% of 12hr old preculture cell in LB was used to inoculate the tested medium, then allowed to grow in shaking (200rpm) at 30ºC for up to 8 days. The chitinase production by the investigated strain was detected in the supernatant and reached to 0.66 (U/ml). Results in accordance to Zhang *et al.*[25], where the exochitinase from *Stenotrophomonas maltophilia* C3 was produced after 8-days incubation.

Molecular Screening and Phylogeny: Generally, due to relatively low levels and long incubation time which required to express this gene by native strains, we

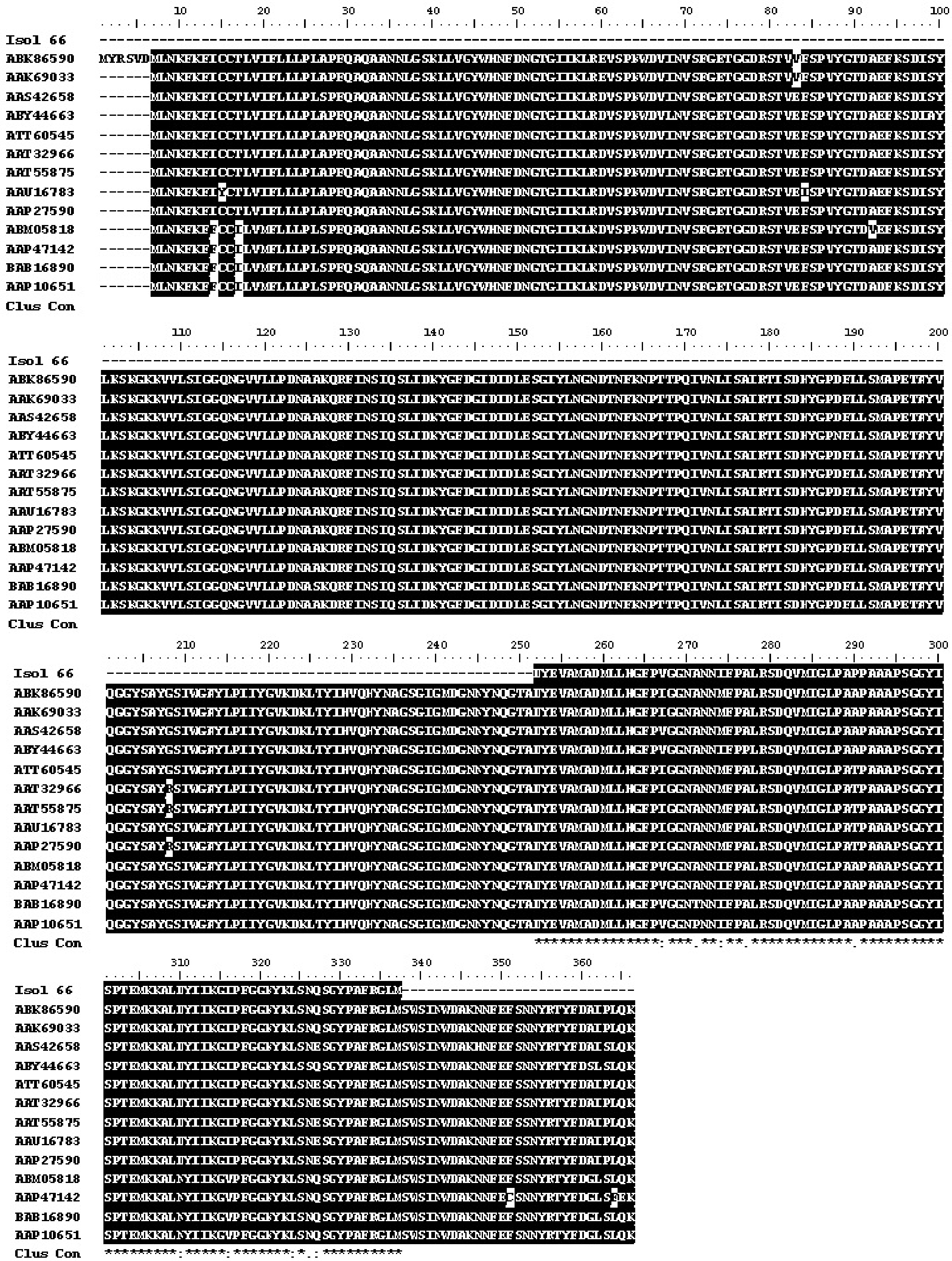

Fig. 3: Aligment of amino acid sequences of chitinase gene from various species (ac: ABK86590; AAK69033; AAS42658; ABY44663; ATT60545; AAT32966; AAT55875; AAU16783; AAP27590; ABM05818; AAP47142; BAB16890 and AAP10651) compared to partial chitinase sequence of isolate 66 (ac: EU734811). The alignment was performed with CLUSTAL W as described under material and methods.

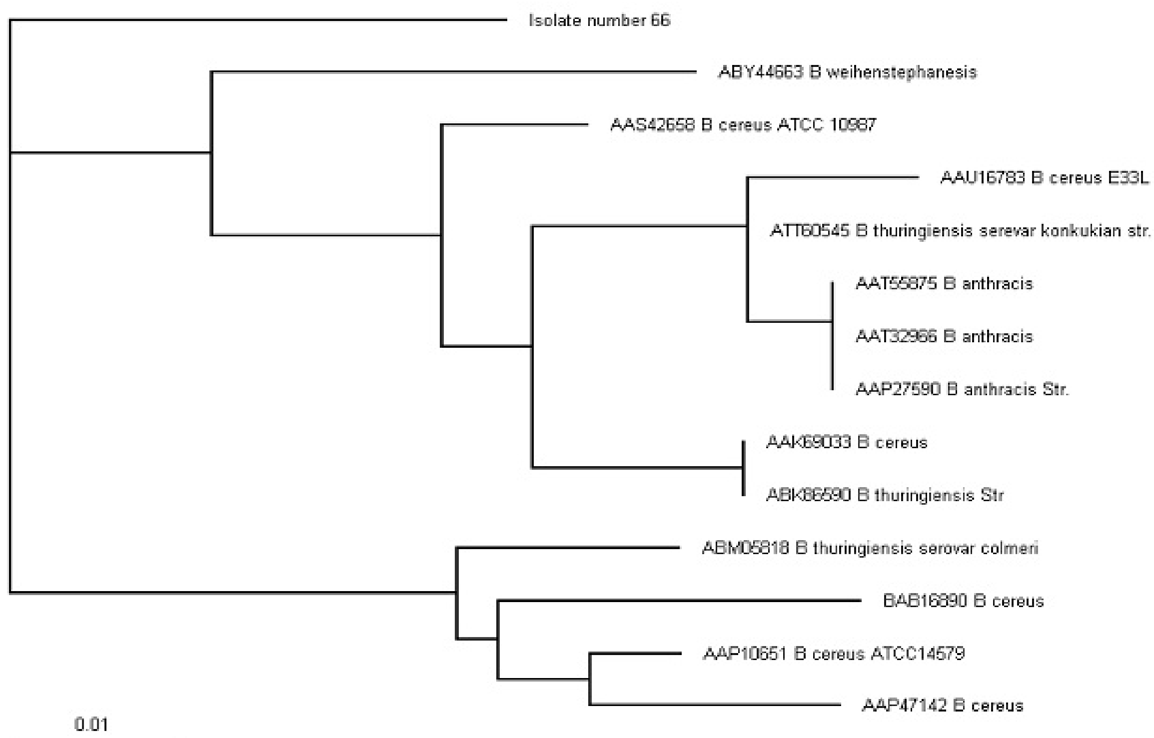

Fig. 4: Phylogeneyic tree of the partial sequence of chitinase (local isolate 66, ac: EU734811) with respect to closely related sequences available in GenBank.

directed to clone and overexpress it. Currently many genes have been cloned, expressed, fused with another protein to enhance the toxicity[16,26,27]. Therefore, initially in a program for molecular screening of chitinase producing strain, PCR primer was deigned according to alignment data of chitinase nucleotide sequences of different species of bacilli as described previously. The primer was used to amplify a conserved region of the gene (310bp). The PCR reaction was carried out on genomic DNA of a number of some local and reference type strains related to *Bacillus thuringiensis,* where the tested type strains as well the local isolate showed amplification fragment with an expected molecular size (310 nucleotides) (Fig. 2). On sequencing the PCR fragment of local isolate, around 96% similarity to chitinase, from *B. thuringiensis* A1 Hakam (protein-id: ABK86590) was measured. Multialignment between amino acid sequences of conserved region obtained by PCR of *Bacillus thuringiensis* 66 (ac: EU734811) and the closely related sequences (id: ABK86590; AAK69033; AAS42658; ABY44663; ATT60545; AAT32966; AAT55875; AAU16783; AAP27590; ABM05818; AAP47142; BAB16890 and AAP10651) showed minor differences in aa (Fig. 3). Also, a phylogenetic tree was displayed and presented in Fig. 4 .

Conclusion: The present study addressed the ability of the local isolate *Bacillus* sp. isolate 66 (ac: DQ524821) to produce the chitinase enzyme. Sequence similarity of amplified fragment resulted from PCR upon using a primer set designed to amplify a conserved region (310 bps) of chitinase gene available in the GeneBank, proved its relatedbness to this gene. It is worthwhile to express this gene under a defined promoter using another set of primer to amplify the full length sequence gene.

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