CLONING AND SEQUENCING OF PHAGE RECEPTOR GENE FROM *BACILLUS THURINGIENSIS* STRAIN DI 29

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ABSTRACT

The entomopathogenic *Bacillus thuringiensis* (*Bt*) strains are highly sensitive to phage infection which lead to tremendous economic losses when used at industrial scale. The phage-sensitive local Bt strain, currently used at industrial scale to produce the first Egyptian biopesticide (AGERIN®), was subjected to strain improvement program and phage resistance mutants were derived from the wild-type (Wt) strain. The mutants showed resistant during phage-sensitivity assays either at solid or liquid media. Moreover, electron micrograph confirmed the inability of the phage to attach to the surface of the Bt mutants compared to the Wt (data not shown). Molecular analysis of the mutants showed a slightly different protein pattern than Wt and absence of the characteristic PCR product of the phage receptor gene. Further, the gene encodes for the phage receptor in the Wt was cloned and sequenced. Blast analysis of the nucleotide sequence of the cloned gene showed 98% similarity with similar genes from bacilli, especially *B.cereus* (a variant of Bt) confirming that it belongs to the same family of bacteria. The success of identification of the gene encoding for the phage receptor in Bt opens the door for deeper understanding of mechanism of phage sensitivity of industrial Bt strains and how to avoid economic losses due to phage infection.

INTRODUCTION

The gram-positive spore-forming bacterium *Bacillus thuringiensis* Berliner (*Bt*) is the most widely used biopesticide [**Perani et al., (1998) and Tamez-Guerra et al., (2004)**]. An important characteristic of *Bt* is the production of large, proteinaceous, crystalline inclusions (crystals) during sporulation containing δ-endotoxins or Cry proteins, which are responsible for the entomocidal properties of many *Bt* strains not only several insect orders, but also against other organisms such as Acari, Nemathelminthes, Platyhelminthes, and Sarcomastigorphora [**Tamez-Guerra et al., (2004)**].
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Indeed, almost all *Bacillus thuringiensis* are phage sensitive. The γ phage infection of bacillus was first reported in 1955 as a variant of phage W which was isolated in 1951 [**Davison et al., (2005)**].

Viruses must recognize and bind to the host cell in order to carry out the infection process. The initial recognition of the host cell, typically mediated by a cell surface receptor and leading to virus entry, is highly specific: proteins on the virion surface (receptor-binding proteins) specifically interact with molecules or molecular assemblies (receptors) exposed on the surface of a susceptible cell [**Gaidelyt et al., (2006)**]. There has been less work on bacterial phage receptors in gram-positive than in gram-negative bacteria. Viruses that infect gram-positive bacteria usually attach to the cell surface polysaccharides [**Davison et al., (2005) and Gaidelyt et al., (2006)**]. For example, phages; SP50 [**Davison et al., (2005)**] ϕ29, ϕ25 [**São-José et al., (2004) and Gaidelyt et al., (2006)**] and SP01 [**Gaidelyt et al., (2006)**] adsorb to the major and essential teichoic acid which described as the receptor in the cell wall of *Bacillus subtilis* [**São-José et al., (2004); Davison et al., (2005) and Gaidelyt et al., (2006)**], but other cell wall components are also involved in phage-bacterium interaction. In *Lactobacillus lactis*, saccharides are involved in initial phage binding. In some cases, this is followed by protein-dependent secondary binding [**Davison et al., (2005)**].

Cell wall in gram-positive peptidoglycan is covalently and noncovalently decorated with teichoic acids, polysaccharides, and proteins. Surface proteins typically carry two topogenic sequences, i.e., N-terminal signal peptides and C-terminal sorting signals. Sortases catalyze a transpeptidation reaction by first cleaving a surface protein substrate at the cell wall sorting signal. The resulting acyl enzyme

intermediates between sortases and their substrates are then resolved by the nucleophilic attack of amino groups, typically provided by the cell wall cross bridges of peptidoglycan precursors. The surface protein linked to peptidoglycan is then incorporated into the envelope and displayed on the microbial surface [**Navarre & Schneewind (1999) and Marraffini** *et al.*, **(2006)**]. A cell-wall-protein-anchoring mechanism has been described in *B. anthracis*, involving a polysaccharide, composed of galactose, *N*-acetylglucosamine, and *N*- acetylmannosamine [**Mesnage** *et al.*, **(2000)**].

Recent data for various gram-positive organisms suggest that there is a major sortase, SrtA, that generally anchors many LPXTG proteins, and that the other sortases have a more restricted subset of substrates, seemingly linked to a particular function and to specific LPXTG motif sequence [**Davison** *et al.*, **(2005)**].

Bacteriophage Bam35 infects gram-positive *Bacillus thuringiensis* cells. Bam35 morphology closely resembles that of bacteriophage PRD1, both have ~15-kbp-long linear double-stranded DNA genomes with 5'-covalently linked terminal proteins [**Strömsten** *et al.*, **(2003)**] and similar genome organizations (Ravantti *et al.*, 2003). Bam35, in contrast to PRD1, is a temperate phage [**Ackermann** *et al.*, **(1978)**] and can exist as a linear plasmid inside the host cell. The sequence of the Bam35 genome is similar to those of the *B. thuringiensis* phages GIL01 and GIL16 [**Strömsten** *et al.*, **(2003)**]. The Bam35 genome has no great sequence similarity to that of PRD1 [**Ravantti** *et al.*, **(2003) and Gaidelyt** *et al.*, **(2006)**].

Among the long-genome phages with Gram-positive hosts, phage 0305♦8–36, infective for *Bacillus thuringiensis*, has several unusual characteristics. These include plaque formation only in ultra-dilute gels and aggregation, as visualized by fluorescence microscopy. This phage has a 221-kb genome, as assessed by pulse-field gel analysis [**Thomas** *et al.*, **(2007)**].

Recently, the sequence of gram-positive bacteria exist more than one sortase has been suggested, and experimentally demonstrated in some [**Barnett** *et al.*, **(2004) and Bierne** *et al.*, **(2004)**]. It has been proposed that the sortases be divided into four classes, A to D, on basis of sequence [**Dramsi** *et al.*, **(2005)**]. Recent data for various gram-positive organisms suggest that there is a major sortase, SrtA, that generally anchors many LPXTG proteins, and that the other sortases have a more restricted subset of substrates, seemingly linked to a particular function and to specific

LPXTG motif sequence. For example, sortase B probably only anchors iron acquisition proteins [**Bierne** *et al.*, **(2004)**].

Sequencing of additional long-genome phages with Gram-positive hosts is vital for the determination of the extent of phage diversity. It is also critical for the addition of more members to phage protein families, which will lead to a clearer portrayal of the mechanisms by which phages evolve [**Serwer** *et al.*, **(2004)**]. Studies of phages with long genomes will also provide insight into why and how such long phage genomes exist [**Thomas** *et al.*, **(2007)**].

MATERIALS AND METHODS

Bacteria and growth conditions:

B. thuringiensis strains (wild-type and mutants) and *E. coli* strain XL1-Blue were grown in LB medium at 30°C with shacking at 250 rpm (containing g/L: peptone : 10, yeast extract: 5, and sodium chloride:10, and the pH was adjusted to 7.0), media were solidified by adding 15 g agar/L [**Osman** *et al.,* **(1999)**]. Sporulation and long term preservation (freeze dried) of the Bt strains was kept in T3 medium [**Travers et al.,** **(1987)**]. The concentration of ampicillin for clone selections was 100ug/ml. Long term preservation of *E. coli* was achieved by keeping the cells in 20% glycerol at -20°C.

Preparation of phage:

Sample of phage suspension was available at our laboratory and a 200 µl of phage suspension was used to inoculated 50 ml culture of actively growing of sensitive Bt (Wt) which was incubated and shacked (250 rpm) at 30°C for 3 days. After the expiry of the incubation period, the culture was allowed to stand for one hour and the supernatant fluid was collected (with syringe), after two cycles of low (3000 rpm for 30 min.) and high (13000 rpm for 20 min.) speed centrifugation then passed through Millipore filter (0.22 µm) to remove any bacteria from the preparation. Phage preparation (stock) was stored in LB broth containing 0.5% chloroform at 4°C until use. The phage stock was used in all subsequent experiments including sensitivity testes in solid or liquid media [**Adams (1959)**].

Phage sensitivity assay:

Phage sensitivity tests were carried out using the phages and the host Bt (Mutants and Wt). The bacterial strains were grown on LB

medium at 30 ° C for log-phase, then one drop of the phage suspention and 200ul aliquots of bacterial cultures were mixed in 3 ml LB semisolid medium (~45 ° C) and transferred to petri dishes containing LB agar layer and incubated at 30 °C for 12 h [**Tamez-Guerra** *et al.*, **(2004)**]. Phage sensitivity was detected visually by bacterial growth inhibition zones and reported as positive if growth inhibition plaques were observed.

Plasmid mini- preparation from *B. thuringiensis:*

Total plasmid DNAs were prepared according to [**Bron (1990)**]. The cell pellets from 5 ml culture were collected by centrifugation kept frozen at -20°C for overnight, thown at room temperature next morning and suspended in 200 µl of solution "A" (10 mM Tris-HCl pH 8.0, 10 mM EDTA pH 8.0, 50 mM NaCl and 20% (w/v) sucrose containing 2 mg/ml freshly prepared lysozyme) then incubated for 1 h at 37°C to lyses. Then 400 µl of solution "B" (0.2 M NaOH and 1% (w/v) SDS) was added to the cell lysate and kept on ice for 5 min. Next 300 µl of solution "C" (3M Potassium acetate pH 5.5) was added and left on ice for an extra 5 min. The supernatant (≈ 800 µl) was collected by centrifugation, then an equal volume of phenol chloroform iso-amyl solution was added and the upper aqueous phase containing plasmid DNA was removed to clean tube. One sixth volume of isopropanol was added to precipitate DNA at room temperature. The DNA pellet was collected by centrifuged and dried under vacuum. According to the size of DNA pellet, each was dissolved in the appropriate volume of TE-buffer and left to relax before subsequent uses.

Polymerase Chain Reaction (PCR):

Five primers were synthesized against phage receptor molecule they are srt1 (5´) and srt1 (3´) for sortases (SortA) gene, an anchored proteins in many gram positive cell wall bacteria) and B as (5´) and Bas (3´). Their oligonucleotide sequences are listed in the table below:

Primer No.	Primer name	n mol	Sequences
1	SortA (5´)	13.9	CATATGAATAAGCAAAGAATTTATAGTATA
3	srt1 (5´)	19.0	GGTATTGTATCGACTGTTCTTTATAAAGTT
4	srt1 (3´)	15.2	GGAATGATTATCGGACAAATTTTATTTACA
6	Bas (5´)	16.1	GGATCCGTCATGGGGACAAGTAGAAGCTGAAAC
7	Bas (3´)	20.5	AAGCTTAGCTGCTTTACGTCTCCATAATACATATGC

The *srtA* gene was amplified by the primers SortA (5ʹ) and srt1 (3ʹ), then with Bas (3ʹ) and srt1 (5ʹ) and srt1 (3ʹ). The gamR gene (*Gam*ma phage receptor) was sequenced by using the Bas (5ʹ) and Bas (3ʹ) oligonucleotides.

Generally, PCR was performed in a 25-μl reaction volume containing: 1.5μl DNA template, 12.5μl of 2X mastermix, 2μl of Primers, 8.5μl sd H_2O and an extra 0.5 u Taq DNA polymerase. A single denaturing step of 5 min at 94°C was performed and followed by 35 cycles of 1 min denaturation at 94°C, 45 sec annealing at 48°C and 3 min extension at 72°C. When necessary, an extra extension step of 7 min at 72°C was used after completion of the 35 cycles. The PCR product was analyzed by gel electrophoresis **[Wu *et al.*, (2004) and Reyes-Ramirez & Ibarra (2005)]**.

Cloning of the of the gene encodes for the receptor from wild-type Bt strain:

Total DNA from the Wt Bt strain was partially digested Sau3AI and the vector (pBluescript II SK+) was digested with BamHI both enzymes generate a complementary cohesive ends of DNA. The digested DNAs were ligated using T_4 DNA ligase at room temperature for overnight and the ligated mixture was used to transform competent *E. coli* cells using cold and heat shocks cycles **[Hanahan (1983)]**. Then 800 μl of LB medium was added the tube, and incubated at 37°C for 1 h to allow transformed cells to grow and divide. An appropriate fraction of culture (100 μl) was streaked onto surface of LB-agar plates containing the appropriate antibiotic (Ampicillin), IPTG and X-Gal as selectable markers. The plates were incubated at 37°C for 24 hours and white colonies were selected for subsequent experiments.

Selection of recombinant clones:

Recombinant plasmids were isolated from the transformed *E. coli* colonies according to **[Sambrook *et al.*, (1989)]**. The DNA inserts in the clones were released by BamH1 digestion to confirm the size of the insert. PCR analysis of the clones using the five primers was done to confirm the identity of the DNA insert, i.e. the gene encodes for the receptor of the phage.

DNA sequencing:

The dideoxynucleotide chain termination procedure originally developed by Sanger *et al.,* (1977), was employed for sequencing the double-stranded recombinant DNA plasmids obtained from the positive clones using the universal forward and the reverse primers T7 (forward) and T3 (reverse) at the autosequencing facility of Geospiza, Germany.

RESULTS

Phage sensitivity assay

The efficiency of the phage preparation was tested against a local Bt isolate supersensitive to it. Serial dilution of phage suspension, was mixed with the culture of a local Bt stain (#1') at ratio of 1:1 in a 3 ml LB semisolid medium (45°C) and poured onto surface of LB agar plate, incubated at 30°C for overnight, and the plaques were scored as in Fig. (1). No plaques appeared in the control plate and only appeared in the sensitive plates.

(A)   (B)
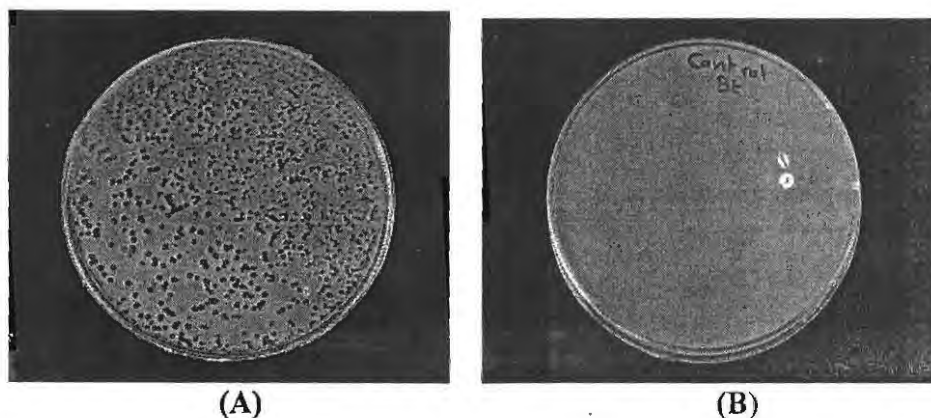
Fig. (1): Phage sensitivity assay showing plaque of sensitive Bt (WT) isolate (A) compared to the same bacterium without virus added (control) (B).

Moreover, the Wt (strain #3) and the phage resistant mutants (#1, 5) (mutant and trans-conjugant isolates respectively) were assayed for their phage sensitivity in both liquid and solid media. In liquid LB broth, wild-type strain, mutant and trans-conjugant isolates were allowed to grow for several hours before being challenged by purified virus preparation. The results were recorded by either turbidity and cell lysis (for liquid cultures) or appearance of plaques on solid media. The data showed good, homogenous growth of mutant (No.1) and trans-conjugant cells (No.5) in the presence phage in either solid or liquid media, but the wild-type strain (#3) lyzed in both liquid and solid media (Figs. 2 & 3).
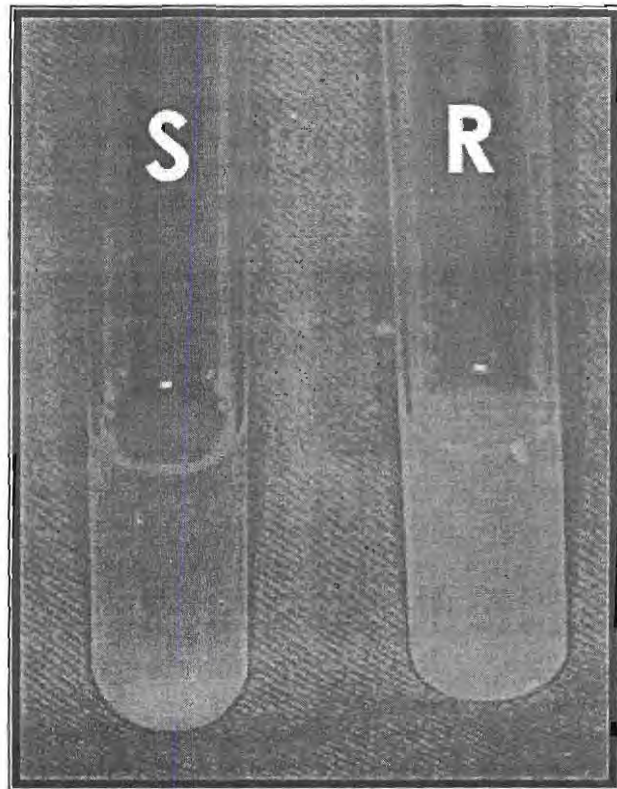
Fig. (2): Phage sensitivity assay in liquid medium. The Bt wild type (S) looks less turbid and cell debris appear in the bottom of the tube, and tube (R) mutant Bt which is highly turbid and cellular debris.
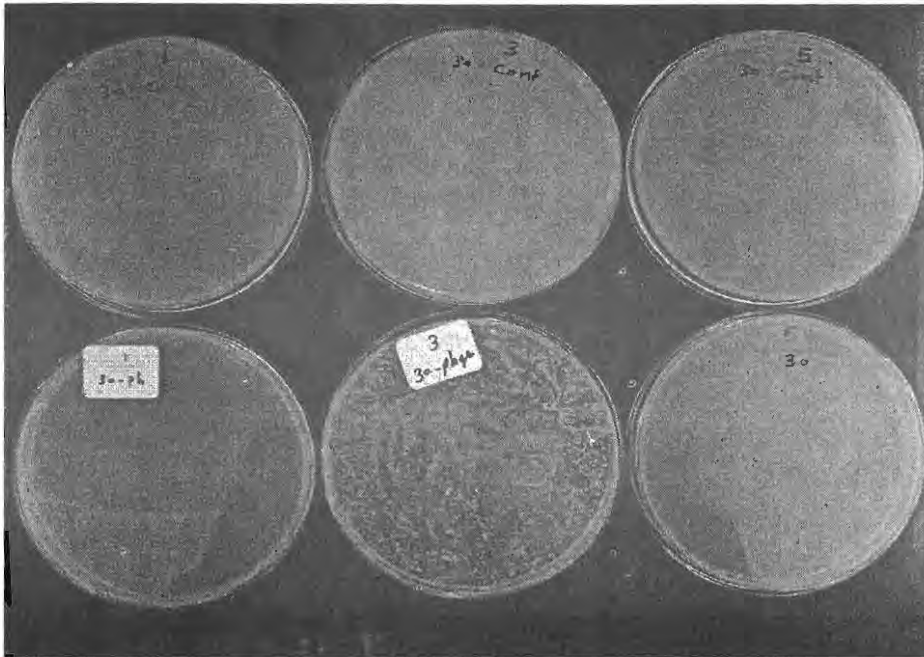
Fig. (3): Phage sensitivity assay on LB agar showing lysis only for the Bt (Wt) strain (#3).

Detection of *B. thuringiensis* receptor gene by PCR:

Polymerase chain reaction (PCR) was used to detect the presence or absence of receptor gene in B.t. isolates No. 1, 3, 5. Fig. (4) shows a 1.85 kb DNA fragment amplified by srt1 (5') and srt1 (3') (P3+P4 in photo) primers for SortA gene (sortases is anchored proteins in many gram positive cell wall bacteria), and 2.53 kb by Bas (5') and Bas (3') (P6+P7 in photo) for gamR gene (*Gam*ma phage receptor) as characteristic PCR products for the gene encodes for the receptor in the Wt strain no.3.

At the same time, when other sets of DNA primers designed to amplify sequences from this genes (Fig. 5) it amplified DNA fragment with different molecular sizes if the two primers SortA (5') with Bas (3') (P1+P7) were used. But negative product result with SortA (5') and srt1 (3') (P1+P4) primers.
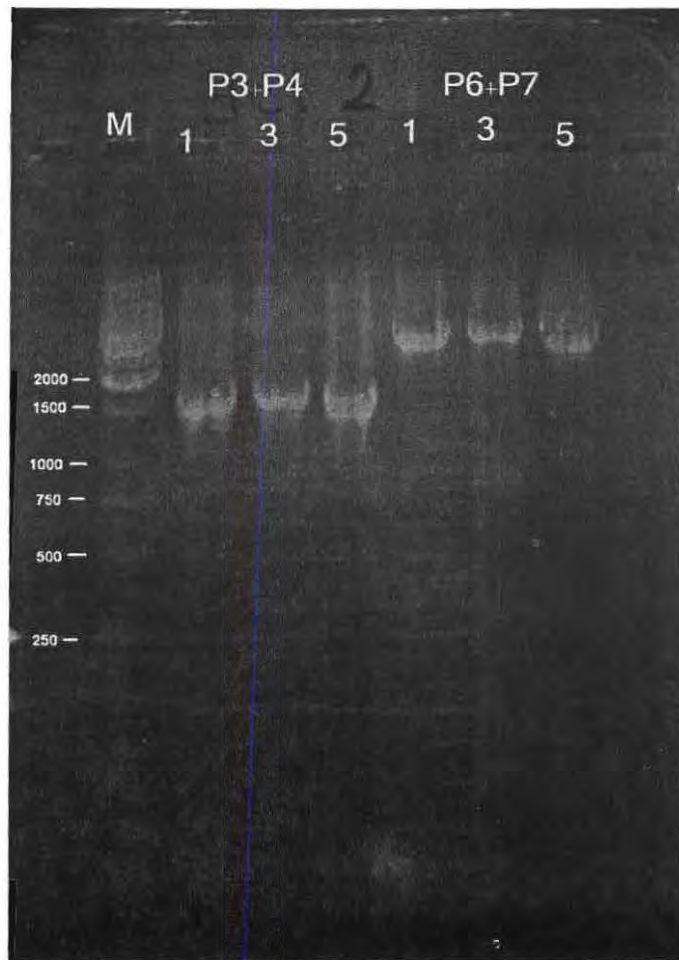
Fig. (4): Agarose gel electrophoresis of PCR products amplified from B.t. isolates with primers: P3+p4 and P6+P7, to detect gene encodes for the receptor gene in Bt strains 1, 3 and 5. The M lane is the DNA molecular weight marker.

Fig. (5): PCR product profile using the primers (P1+P7) and (P1+P4) for the Bt isolates 1, 3 and 5. M lane include DNA molecular weight marker.

Cloning of receptor gene from *B.t.*:

The purified recombinant plasmid DNA of the white colonies resulted from the cloning process when Wt B.t. strain (No.3) DNA was digested with Sau3AI, and ligated into the BamH1 site of the plasmid pBluescript SKII (Fig. 6). The selected white colonies were subjected to several screening procedures, as detailed below, to detect the recombinant clone harboring the specific gene encoding the receptor from the wild-type of *B.t.* strain: PCR analysis and BamH1 digestion (Figs 7 & 8).
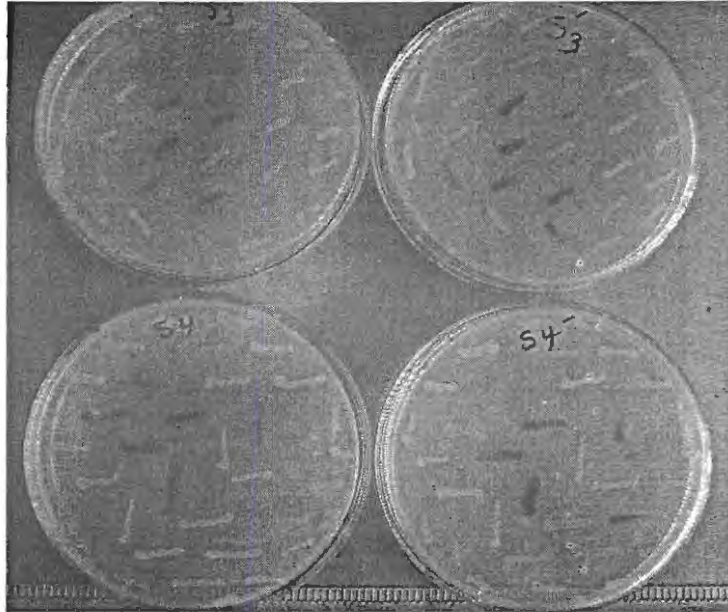
Fig. (6): Blue/white colonies, Blue colonies do not contain the recombinant DNA molecule while the white colonies are the ones suspected of containing the target gene.

PCR screening of recombinant clones: A PCR-based approach was used to identify specific clones containing the gene encoding the receptor. The two receptor-specific gene primers [Bas (5') and Bas (3')] were used for PCR reaction (Fig. 7). The data shown the colonies No. 16, 18, 21, 23, 26, 29, 31, 32, 33, 34, 35, 36, 37, 39 as the positive receptor gene. The colonies picked and tested by this technique.

Fig. (7): PCR product analysis of the clones using specific oligonucleotide receptor primers [Bas (5´) and Bas (3´)]. M lane contains DNA molecular weight marker.

BamHI- re-digestion of selected clones: In this screening method the plasmid DNA was prepared from *E. coli* clones by alkaline lysis and then digested with BamHI. A DNA fragment was released after and separated by agaroes gel electrophoresis as in Fig.8. This DNA fragment was large enough to encode the entire receptor protein present in this isolate of *B.t.*. Determination of the restriction site for the enzyme was necessary to construct subclones necessary for the nucleotide sequencing of the entire gene.

The real tests to substantiate the identity of the cloned DNA fragment as the gene encoding for the receptor are its ability to express itself. Therefore, the next level of screening was the gene expression in *E. coli*.
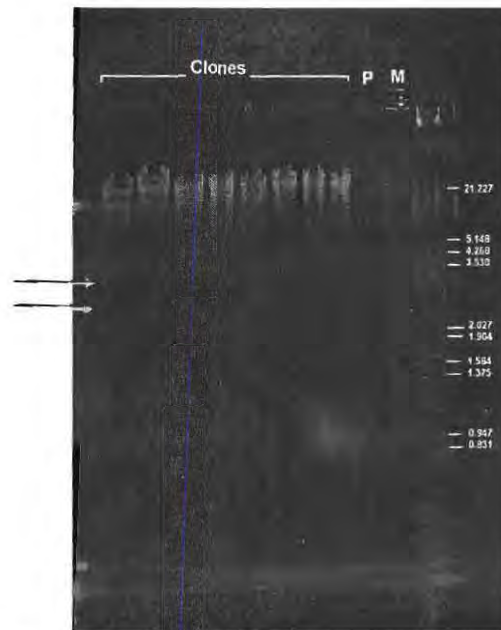
Fig. (8): Agaroes gel electrophoresis. *E. coli* clones and the plasmid digested with BamHI. The upper arrow indicate the plasmid without insert after digestion and the lower arrow shows the released insert of the receptor gene. M lane shows the DNA molecular weight marker.

Nucleotide sequencing of cloned gene: The complete nucleotide sequences of the coding region of receptor genes as well as its 3' and 5' flankin regions were determined by the dideoxynucleotide triphosphate chain-termination method originally developed by Sanger *et al.*, (1977) in the presence of different fluorescent dyes. Single colonies were

isolated from transformants and the plasmid DNAs from the appropriate clones were isolated and purified for sequencing. The sequencing reactions were done in the presence of T7 and T3 primers to obtain the complete nucleotide sequences from opposite directions. This strategy also generated overlapping fragments that facilitated the construction of the complete nucleotide sequence of the DNA insert. The nucleotide sequencing of the four genes were done automatically using the autosequencer in Germany and the data are shown in (Fig.9) for isolate No. 33 and fig.10 for isolate No. 39. The comparative sequence analysis between sequence 1 (length = 1262) and 2 (length = 889) bitscore and expect value are calculated based on the size of the nr database. The identities are 247 / 252 reach to (98 %).

5´GGGCGTCGAGGTCGACGGTATCGATAAGCTTGATATCGAATTC
CTGCAGCCCGGGGGATCCGCCCGGCTAGAGCGGCCGCCACCGCG
GTGGAGCTCCAGCTTTTGTTCCCTTTAGTGAGGGTTAATTGCGCG
CTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTAT
CCGCTCACAATTCCACACAACATACGAGCCGGAAGCATAAAGTG
TAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAATTGC
GTTGCGCTCACTGGCCGCTTTCCAGTCGGGAAACCTGTCGTGCCA
GCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGC
GTATTGGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTC
GGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGG
TAATACGGTTATCCACAGAATCAGGGGATAACGCAGGAAAGAAC
ATGTGAGCAAAAGGGCCAGCAAAAGGGCCAGGAACCGTAAAAA
GGGCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACG
AGCATCACAAAAATCGACGCTCAAGTCAGAGTGGCGAAACCCGA
CAGGACTATAAAGATACCAGGCGTTTCCCCTGGAAGCTACCTTCG
TGCGCTCTACTGTTCCGACCCTGCCGCTTACCGGATAACCTAGTC
CGCCTTATGCCGTCGGAAGCGTGGCTGCTTTGTCCGATAAGCTCG
ACCCCTGAGATTTTCCAATTCGGGGGTAAGTCGTCGCTCCAAGCT
GGGCTGGGGTTGCCGAAACCCCCGTTCAGCTGACCGTGGGCCTTT
TCGGTAACCTTTCTCCTGGACCCAACCGGAAAACCCTAAAATTCG
5´GCCCTTGGCAAAACACTGGTAAAGATTTCCAACCGGATTTGGGG
GGCCTCCAGGTCTGGAAAGTGGCCCACAGGCCCACTCCAAAACC
AATTGGGTGTGCTTGGTTTTGAACCTCTCCCCATCGAAAAGTATGT
TTGCCCCATACCTCGGGGTGGTGGGAGGGGGGCCGCGGTGGCG
GCGCTCTAGCCGGGCGGATCCCCCGGGCTGCAGGAATTCGATA
TCAAGCTTATCGATACCGTCGACCTCGAGGGGGGGCCCGGTAC
CCAATTCGCCCTATAGTGAGTCGTATTACGCGCGCTCACTGGC

CGTCGTTTTACAACGTCGTGACTGGGAAAACCCTGGCGTTACC
CAACTTAATCGCCTTGCAGCACATCCCCCTTTCGCCAGCTGGC
GTAATAGCGAAGAGGCCGCACCGATCGCCTTCCCACAGTGCG
CAGCTGAATGCGAATGGGAACGCGCCCCTGTAGGCGGCGCCC
ATTTAGGGCGCGCGGCGGGTGGGGGGGGTACCCGGCAGACGG
TGTGACCCGCTCTACACCTGTGCCACGCGCGCCCTAGGGCCCC
GCCTCCTTTTTCGCTTTTCTTTCCCTTTCCCTTTTCCCCCCCCGT
TCCGCCCGGTTTTTCCCCCGCAAAGTCTCTAAATCGGGGGGGGT
CTCCCCTTTGAGGTGCCCCAATTAAGGGGTTTTTCGGGCCCTCT
CCCCCCCCAAAAAAATTTTTTTATATAGGGGGGGGGGGGGGTTT
CCCCCCCGGGGGGGGGGGCCCCCCCCCCCAAAAAAAAAAAAA
AAAATTCCCCCCCCCCCCCGGGGGGGGGGGGGGCCCCCCCCCCT
TTTTATAAAAAAAAAAGAGGTTTTTTTTCAAAAAAAAAAAAA
AAAAACCCCCCCCCCCCCCCCTTTTTTTTTTTTTTTAAAAAAAG
GGGGGCCCCCCCCCCCCCCCCCCCCCCGCCAAAAAAAAAAAAA
AAAAAAAAAAAAAAAAAGCAGGAGAAAAAAAAGAAAAACCC
CCTCTCCTCCTCCATATGTCTTCTGCGGGGGGGGGGGGGAAGAGA
GGGGGGCCCCCCCCCCCCCCTTTTTTTTTTTTTTTTATTATAAAAA
AAAAAACAACGCGCCCCCCCCCCCCCCCCCAAAAAAGTTTAGA
ATAGGGTAAAAAACCAAAAAAAAAAAAAAAAAAAAAAAAAAAA
AAAAAAAAGGGGGGGCCCCCCCCCCCCCCCCCCCCCCCCTCTTTTT
TTTTTTTTTTTTTTTTTTTCTTTTCTCCCCCCCCCGCCCCCCCCCC
CCCCGGTTAAGAAAAAAGAGAAAAAAGGAAAAAAAGAAAAT
AGCCAGTAGAGGGGCAGGGGGGGAGGGTGGCGGCGCCATAAT
GGGGGGGGGGTTAGCAGGACGCGTTTCGCGCGAGGGGGGGGGAG
GAGGAGGACAGTATGTATTTGCCATTTTCTTCATTCTCTCCTCC
T 3'

Fig.(9): The nucleotide sequencing of cloned gene for phage receptor from the Bt wild-type isolate no.3.

DISCUSSION

Bacillus thuringiensis (Bt) is widely used as a biological insecticide, this mainly due to production of parasporal crystals during sporulation. These crystals have highly specific toxicity against certain insects especially within the orders *Lepidoptera*, *Diptera* and *Coleoptera* and also contribute a toxicity against nematodes. Bt also produces extracellular compounds such as phospholipases, proteases, <u>chitinases</u>

and other vegetative insecticidal proteins that may contribute to virulence. For this reason most of biochemical and genetic studies have focused on Bt.

Unfortunately Bt is highly <u>sensitive</u> to lysis by bacteriophages and consequently its commercial fermentation suffers mainly from bacteriophage infection which in-turn leads to great economic losses and make it less desirable in manufacturing process. This is a humble effort to improve this strain and make mutant(s) with increased resistance to bacteriophages. This point constituted the main aim (concern) of our search project. This was achieved by applying UV mutational wave lengthes of 254, 260, 280, and 315 nm. A resistant mutant was obtained and compared with wild type. It was found that the resistant mutant produces only spores and no crystals. This pushed our thinking to make a conjugation experiment in order to obtain a new progeny that carries both desirable characters, spore formation and crystal production.

Generally, two general approaches may lead to the development of successful biocontrol agents. First, one may search in the natural environment for organisms having the survival and antagonistic characteristics necessary to be promising candidates. Second, it is possible to alter the survival and/or efficacy of strains, either through improved methods of formulation or by genetic manipulation (Osman *et al.*, 2000) and the major objective of this work was set to genetically construct a Bt strain resistant and/or tolerant to bacteriophage infection. Reports had attributed bacterial phage resistance to either one of four mechanisms based on their mode of action: adsorption blocking of the phage on bacterial cell wall, phage DNA penetration blocking, restriction and modification (R/M) enzymes which degrade invading nucleic acid molecules and abortive infection (Abi) (Twomey *et al.*, 2000).

The Bt wild type was mutagenized by U.V. irradiation to produce a mutant capable of resisting phage infection. Mutated cells were tested for bacteriophage sensitivity, and a phage resistant mutant was selected, named (R) and subjected to further bacteriological and molecular characterization.

A simple bacteriological examination of the Bt phage resistant strain (R) was carried out by Gram and endospore stains to ensure the rod-shape and sporulating ability of the mutant strain; R. The phage-

resistant Bt mutant was a typically Gram +ve and sporulating rods similar to the Bt wild type strain. However, only Bt wild type strain produces the characteristic insecticidal crystal proteins (ICPs) along with spore production, while the phage-resistant mutant has lost this most important character (i.e. production of ICP). This suggested that there may be a correlation between bacteriophage resistance and insecticidal crystal protein production. Molecular characterization of Bt wild-type and mutant strains (R) included both protein banding patterns (resultant of gene expression) of both vegetative and sporulated cells, plasmid profile, and the detection of the genes encode for the production of the insecticidal crystal proteins (ICPs). The careful analysis of protein banding patterns of specially sporulated cultures showed an almost 100% identity in the protein bands between the Bt wild type and the mutated Bt phage-resistant R strain, except the protein band representing the ICP or the Bt toxin was lost from the R strain, confirming the absence of crystals formation by this strain; R. Plasmid profiles data were not conclusive, while the PCR detection of genes encode for ICP was missing from the R strain.

The gene encodes for the receptor on the surface of the Wt Bt strain no.3 was cloned using traditional cloning procedure and sequencing using the automatic sequencing facility of the Geopiza, Germany. The analysis of the sequencing data revealed the resemblance of the cloned gene to that of the *B.cereus* with a matching identity of 98% suggesting the belonging of the cloned gene to the proteins of the family Bacillaceae.

However, further work in needed to establish the phage resistance of all Bt strains used at industrial, scale to produce Bt-based biopesticides.

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إستنساخ وتحديد التسلسل النيوكلوتيدي للجين المسئول عن مستقبل الفاج لبكتريا الباسيلس ثُرينجينسس سلالةDI29

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بكتريا باسيلس ثرينجينسيس الممرضة للحشرات لها قابلية شديدة للإصابة بالفيروسات مما يتسبب فى خسائر إقتصادية كبيرة عند إستخدامها على النطاق الصناعى . ولذلك فالسلالة المستخدمة الان في مصر لإنتاج المبيد الحيوى اجيرين(AGERIN®) قد تم إخضاعها لبرنامج تحسين وراثي لإنتاج سلالة منها مقاومة للعدوى بالفيروسات . (حيث لوحظ أن هذه الطفرات أظهرت مقاومة للفاجات من خلال اختبار الحساسية في البيئات الصلبة أو السائلة . علاوه على ذلك ، أكد الميكروسكوب الالكترونى عدم قدرة الفاج وعجزه عن الإلتصاق بالسطح للسلالة المطفره مقارنة بالسلالة البرية . التحليل الجزيئي للطفرات أظهرت بعض الإختلافات الطفيفة في البروتينات وغياب ناتج التخليل التسلسلي لأنزيم البوليمريز PCR لجين المستقبل الفاجي . كذلك تم استنساخ الجين المسئول عن المستقبل الفاجي وكذلك تحديد التسلسل النيوكليوتيدي حيث وصلت نسبة التطابق الى ٩٨% مع الجين المماثل في بكتريا الباسيلس سيريس (البديل الوراثي) حيث انهما تنتميان للعائله ذاتها من البكتريا. ان هذا النجاح في تعريف وتحديد هذا الجين فتح الباب لفهم أعمق لآلية الحساسية للفاج في بكتريا Bt الصناعية وكيفية تفادي الخسائر الاقتصادية الناجمة عن العدوى بالفيروسات .