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ISOLATION AND CHARACTERIZATION OF *Bacillus thuringiensis* BACTERIOPHAGES USING ELECTRON MICROSCOPE AND RAPD-PCR

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ABSTRACT

Five bacteriophages have been isolated from soil able to propagate on *Bacillus thuringiensis* bacteria, designated A1, A2, A8, R3 and R8. Phages A2, A8, R3 and R8 produced small turbid plaques but phage A1 produced relatively large clear plaques. Transmission electron microscopy showed great differences in the morphology of these phages. Four phages have icosahedral heads with long tails (A1, A8, R3 and R8). Phage A2 has icosahedral head with tailless. The phages have a broad host range, where successfully forming plaques on *B. thuringiensis*(6 isolates) and *Bacillus cereus* bacteria. Five phages were able to mediate transduction between *B. thurimgiensis* and *B. cereus* with frequencies ranged from 1.32 to $1.8x10^{-9}$ for ampicilline resistance gene. The phages were able also to perform transduction between *B. thuringiensis* strains. Random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) was performed to produce unique and reproducible band patterns in the five different bacteriophages. Among using 20 RAPD primers, only seven produced polymorphic fragments with an average of 7.9 fragments per primer (ranging from approximately 135 to 1500 bp). The number of polymorphic fragments through each primer ranged from 6 to 14 fragments per primer. Primer OPD-07 produced the highest number of polymorphic fragments among the used primers while, primer OPC-08 produced the lowest number. The oligonucleotide OPD-07 presented the highest number of unique fragments (8) in all isolated bacteriophages while, OPC-08 and OPD-05 primers presented the lowest number (one fragment). All isolated bacteriophages except for R3 bacteriophage were distinguishable by unique RAPD markers. The highest number of unique fragments (11) after using all primers was detected in A8 bacteriophage followed by A1 bacteriophage which detected by 10 unique fragments. A2 bacteriophage was detected by two unique fragments; approximately 170bp with OPC-20 and 1200bp with OPD-05 and R8 bacteriophage was detected by two unique fragments with OPD-07; approximately 345bp and 570bp. The highest similarity value (0.828) was found between A2 and R8 bacteriophages and the lowest value (0.313) was found between A1 and R8 bacteriophages. This study demonstrates the effectiveness of RAPD as a technique for identifying characterization isolated bacteriophage from nature and may be useful in fingerprinting. Moreover, the phages isolated from soil in this study can be used as potential cloning vectors.

Keywords: Bacteriophages, *Bacillus thuringiensis*, electron microscopy, transduction, RAPD-PCR, dendrogram.

INTRODUCTION

Bacteriophages are natural viral attacking bacteria. Phages exist wherever bacteria occur, and share a common ecology with their respective hosts (Mclaughin *et al.,* 2006). Phages play an important role in the

population dynamics of bacteria resulting in their lysis and as well as in modifying bacterial phenotypes (Styriak *et al.*, 2005).

Bacillus thuringiensis strains have elicited a special interest because they produce a proteinaceous parasporal crystal that is toxic to susceptible insect larvae. Since this commercially important species has only limited means of genetic exchange, temperate phages could be important as genetic tools serving as mediators of generalized and specialized transduction (Reynolds *et al.,* 1988). Several phages that mediate generalized transduction in *B. thuringiensis* and one that converts spore-negative, crystal-negative cells to spore – positive, crystal – positive cells have been described (Perlak *et al.,* 1979). Transducing phages have been utilized to link genetic markers on the *B. thuringiensis* chromosome (Barsomin *et al.,* 1984) and to transfer plasmids among strains of *B. thuringiensis, B. cereus and B. anthracis* (Ruhfel *et al.,* 1984).

The randomly amplified polymorphic DNA (RAPD) assay, which detects nucleotide sequence polymorphisms by means of the polymerase chain reaction (PCR) and a single primer of arbitrary nucleotide sequence, is a useful method for generating molecular markers. RAPD assay was first described by two independent groups, Williams *et al.*, 1990 and Welsh and McClelland, 1990. The success of this method is due to the fact that no prior sequence information about the target is needed and a single short 10-mer oligonucleotide primer can be used in the reaction. The amplification happens at low stringency, allowing the primers to anneal to several locations on the two strands of the DNA. These primers detect polymorphisms in the absence of specific sequence information and the DNA sequence variations may work as genetic markers that can be used in genetic similarity and diversity studies. Among other DNA-based approaches, random PCR amplifications of DNA segments using short primers of arbitrary nucleotide sequence have been used to generate specific profiles or genomic fingerprints that are used to compare the genotypic diversity among (Guglielmotti *et al.*, 2006 and Maiti *et al.*, 2009). Randomly amplified polymorphic DNA (RAPD)-PCR using purified DNA has also been used to assess the genetic diversity of phages infecting *Pseudomonas aeruginosa* (Li *et al.*, 2010) and *Escherichia coli* (Dini and de Urraza, 2010) and vibriophages (Comeau *et al.*, 2006 and Shivu *et al.*, 2007).

This study amide to isolate *Bacillus thuringiensis* bacteriophages from soil and to characterize them by electron microscope and randomly amplified polymorphic DNA (RAPD)-PCR techniques.

MATERIALS AND METHODS

This study was carried out in Microbial and Molecular Genetics Lab., Genetics Dept., Fac. of Agric., Zagazig Univ. Egypt.

Bacterial strains:

All bacterial strains were obtained from stock Microbial Genetics Lab., Genetics Dept., Fac. Agric., Zagazig Univ. These bacteria were Bacillus *thuringiensis* (Bt) (Bt2, Bt3, Bt5, Bt7, Bt8, HD1), *Bacillus cereus*, *Bacillus*

spp., *Micrococcus* spp., *Rhizobium leguminosarum bv. vicieae* and *Pseudomonas aeruginosa* (PAO1).

Growth Media:

Yeast extract mannitol (YEM) agar and YEM broth media were used for Rhizobium. Nutrient agar (NA) and nutrient broth (NB) media were used for the other genera. Soft agar (0.7%W/V) agar was prepared in distilled water and kept at 45°C on water both. The antibiotics were added as sterilized solution after filtration through 0.2 µm filter membranes to the media after autoclaving. Phosphate buffer was prepared from 1/15M potassium phosphate (KH_2PO4) and 1/15M disodium phosphate (Na2 HPO4. 2H2O).

Isolation of bacteriophages:

Two different soil samples were used for enrichment cultures with NB medium and Bt3, Bt8, HD1 *B. thuringiensis* strains. Twenty five grams soil was added to 100 ml sterile distilled water and placed on magnetic stirrer for 15min. Added 45 ml soil suspension to 5ml 10x NB in a sterile flask and added 5ml of overnight Bt culture, mixed gently and incubated at 30°C for 24h. Ten ml of enrichment was decanted into a centrifuge tube and centrifuged at 5000 rpm for 30 min. The supernatant was filtered through 0.45µm membrane filter. In order to study the presence of antimicrobial agents in the supernatant, the sample were dotted on pre-solidified soft agar containing 200µL of overnight culture of each reference strain which had been overlaid pre-solidified NA. The plates were incubated over night at 30°C and monitored for formation of a growth inhibition zone (Shin *et al.,* 2011). Phage titers were usually examined by the double agar layer method on NA covered with 0.7% top agar (soft agar) having been inoculated with both host strain and diluted phage lysate (Styriak *et al.,* 2005). Bacteriophages were purified by three reisolations of single plaques (Landen *et al.,* 1981). The single plaque was transferred into 1 ml of phosphate buffer to which 1 drop of chlorophorm had been added. The mixture was held at room temperature for 1-2 h to allow the bacteriophage particles to diffuse out of the agar (Jamalludeen *et al.,* 2007).

Preparation of phage stock:

Phage stock was prepared as follows: NA plates were seeded with about 10^7 plaque – forming units (pfu) and a bout 10^7 colony – forming units (cfu) from cultures of late – exponential phase bacteria. Plates were incubated at 30°C overnight and then overlaid with 3 ml of dilution medium (Landen *et al.,* 1981). After standing at room temperature for 30 min., the phage suspension was recovered and centrifuged at 10000 rpm for 15min at 4°C. The supernatant usually contained 10^{10} to 10^{11}pfu/ml.

Great phage lysate production:

Selected bacteriophages were increased by inoculation of log phase broth cultures of the respective host and production of bacteriophage lysates in sufficient volume. Lysates were clarified by centrifugation (10000 rpm for 15 min at 4°C), decanted and filtered through 0.45 µm filters (Mclaughlin *et al.,* 2006).

Double agar layer (DAL) plaque method:

phages were detected by mixing 100µl of test sample with 100µl of fresh log – phase host culture into 5 ml of soft agar melted and held at 45°C

in a water bath. Test suspensions were mixed by vortexing and dispensed uniformly over the surface of 20 ml of hard NA in 96 mm diameter plates. Soft agar overlays were allowed to harden at room temperature and then plates were inverted and incubated overnight at 30°C. Plaques were counted or individually subcultured as appropriate to the enumeration or isolation protocol (Adams, 1959 and Mclaughlin *et al.*, 2006).

Electron microscopy of phages:

The phage titers determined and samples with a titer of >10^{10} pfu/ml were spreaded onto grids carbon coated Formvar support films (Prior *et al.*, 2007), negatively stained with phosphotungstic acid (both 2%, pH 7.2) (Styriak *et al.*, 2005) and examined in a transmission electron microscope. Photographs were taken at a magnification of X 60,000 – 150,000.

Determination of host specificity of bacteriophages:

The host range of each phage was determined by the spot test method (Raiski and Belyasova 2009) 20µl of lysate was spotted onto NA plates previously poured in a double layer, the 0.7% top agar layer having been inoculated with cultures of the strain tested (Styriak *et al.*, 2005).

Ability of phages to lysogenize strains:

The ability of phages to lysogenize many strains of *B. thuringiensis* has been investigated. Putative lysogens were isolated from the centers of turbid plaques (Amina and Amin 2006). After purification, it was assessed for phage production in order to confirm their identities. The lysogenic colonies were then checked for sensitivity to further infection by the same phage. Overnight cultures of the lysogenic strains and a wild – type strain were used to inoculate separate overlay agar plates. After overnight incubation at 30°C, the ability of phage lysate to lyse each strain was assessed (Kevin *et al.*, 1999).

Transduction experiments:

Recipient cells (*Bacillus cereus* and *B. thuringiensis* HDl) were grown at 30°C in NB medium for overnight. The bacteria were centrifuged for 5 min at 5000 rpm and resuspended in NB. Transduction was done by mixing 1.0 ml of culture and 1.0 of the phage suspension (propagated on the appropriate donor). After incubation at 30°C for 30 min, samples of the mixture (0.1ml) were plated on the appropriate medium. Controls for spontaneous reversion were run (Lecadet *et al.*, 1980). Plates were incubated at 30°C for 2-5 days before scoring for transductants. *Bacillus cereus* transductants were assayed for crystals production.

Genomic fingerprinting by RAPD analysis:

Phage DNA extraction:

A modification of the procedure of Prior *et al.*, 2007 was used for phage DNA **extraction** and purification. EDTA was added to purified phage at a 20 Mm final concentration. The phages were treated with proteinase K at 20 mg/ml and Sodium Dodecyl Sulphate (SDS) at 56°c for one hour to disrupt phage heads and digest phage proteins. An equal volume of phenol was added to extract proteins followed by an equal volume of chlorophorm: isoamyl alcohol (24:1 v/v) to remove remaining proteins and phenol. The supernatant containing phage DNA was precipitated by adding one-tenth volume of 3-M sodium acetate (ph 5.2) and two volumes of absolute alcohol

followed by overnight incubation at -20°c. DNA was collected by centrifugation at 14000 rpm in a microcentrifuge for 10 minutes and washed in 70% ethanol and centrifuged again at 14000 rpm for two minutes. After pellets had dried at room temperature, they were resuspended in 50 microliters of TE buffer (10 mM Tris and 1mM EDTA, ph 8.0). Five microliter samples were run on 7% agarose gels to determine purity and estimate the volume needed for RAPD-PCR.

Primers:

A set of 20 primers was analyzed and based on the accurate amplified bands profiles and the produced polymorphic patterns of DNA fingerprinting selected seven different primers were chosen (Table 1).

Table 1: Sequence and operon codes of the random primers used to detect variations in the isolated bacteriophages.

Primer codes	Sequence (5' to 3')
OPA-11	CAA TCG CCG T
OPB-11	GTA GAC CCG T
OPC-08	TGG ACC GGT G
OPC-20	ACT TCG CCA C
OPD-03	GTC GCC GTC A
OPD-05	TGA GCG GAC A
OPD-07	TTG GCA CGG G

Amplification reaction mixture:

The amplification conditions were based on Williams *et al.*, 1990 with some modifications. The reaction was prepared using 25µl per tube, containing 2µl DNA of each isolate (20 ng), 1unit of *Taq* DNA polymerase enzyme, 2µl 10X buffer, 2µl $MgCl_2$ (25 mM), 2µl $dNTP_s$ (2.5 mM of each), 2µl primer (10 pmol) and 14.8µl H_2O.

DNA amplification cycles:

The temperature cycling program used with a Perkin-Elmer Gene Amp PCR system (model 2400) was as follows: one cycle at 94°C for 5 min followed by 30 cycles consisting of one step of denaturation (94°C) for 1min, one step of annealing (35°C) for 1min, followed by one step of synthesis (72°C) for 2min and a final extension step consisting of 72°C for 7min and finally 4°C infinitive.

Band analysis:

The reaction products were analyzed by electrophoresis on 1.4% agarose gels, stained with ethidium bromide, and photographed under UV transilluminator by digital camera with UV filter adaptor. The synthetic DNA, ladder 100 bp (Pharmacia) was employed as molecular marker for bands molecular weight. Each amplified band profile was defined by the presence or absence of bands at particular positions on the gel. Profiles were considered different when at least one polymorphic band was identified. Fragments were scored as 1 if present or 0 if absent based on standard marker using GelAnalyzer 3 (Egygene) software. Pairwise combinations, genetic similarity and genetic distances were estimated following Lynch (1990 and 1991). The computer package SPSS was used to construct a dendrogram based on the

matrix of distance using Unweighted Pair Group Method with Arithmetic Averages (UPGMA) (Sneath and Sokal 1973).

RESULTS AND DISCUSSION

Isolation of bacteriophages:

Three isolates of *B. thuringiensis* (HD1, Bt3, and Bt8) have been used in this investigation to isolate the bacterio phages. After enrichment protocol and phage purification experiments, five phages were isolated, designated A1, A2, A8, R3 and R8. Four phages of the 5 formed morphologically distinct turbid plaques (A2, A8, R3 and R8). So, they are known as temperate phages (Reynolds *et al.,* 1988). The isolation of temperate bacteriophages from different natural environments has been reported (Girdreau *et al.,* 2000). A1 phage formed clear plaques, so it may be a virulent phage. Virulent phages cause bacterial host cell lysis and not only function to control bacterial population, but can be used as indicators of bacterial contamination (Tanj *et al.,* 2003) and as tools for identifying (typing) specific bacterial strains (Leclerc *et al.,* 2000).

Morphology of Isolated Phages:

Transmission electron microscopy showed great differences in the morphology particles of these five bacteriophages. A1, A8, R3 and R8 have a long tails and icosahedral heads. Phage A2 has icosahedral head with tailless (Fig 1). The dimensions of phages have also calculated in Table 2. So, a great diversity among *B. thuringiensis* phages can occur. These agree with others (Lecadet *et al.,* 1980, Landen *et al.,* 1981 and Reynolds *et al.,* 1988).

Table (2): Dimensions of phage particles.

Phage	Head diameter (nm)	Tail length (nm)
A1	91	190
A2	51	-
A8	56	188
R3	91	162
R8	95	196

Host range and lysogenize strain:

Many bacterial strains were used to investigate the host range and specificity of 5 bacteriophages. Tables 3 and 4 show that each phage was able to propagate successfully on all *B. thuringiensis* tested isolates and the pfu/ml ranged from $2.3x10^7$ to $9.68x10^{11}$. Upon using *B. cereus* pfu/ml ranged from 1.3 to $5.3x10^3$. Phage R3 only was able to lyse *Bacillus spp.* All phages didn't able to lyse the other tested bacterial genera.

The ability of five phages to lysogenize strains that propagated successfully on them was tested. Four phages were able to lysogenize these strains (A2, A8, R3, and R8) and the lysogenic isolates were stable. Phage A1 didn't lysogenize any of these isolates because, it produced clear plaques, so the plaques didn't contain a lysogen cells.

From these results and according to Shin *et al.*, 2011 it was clearly that the five phages have a broad host range because it lyse many isolates of Bt and beside strain *B. cereus*.

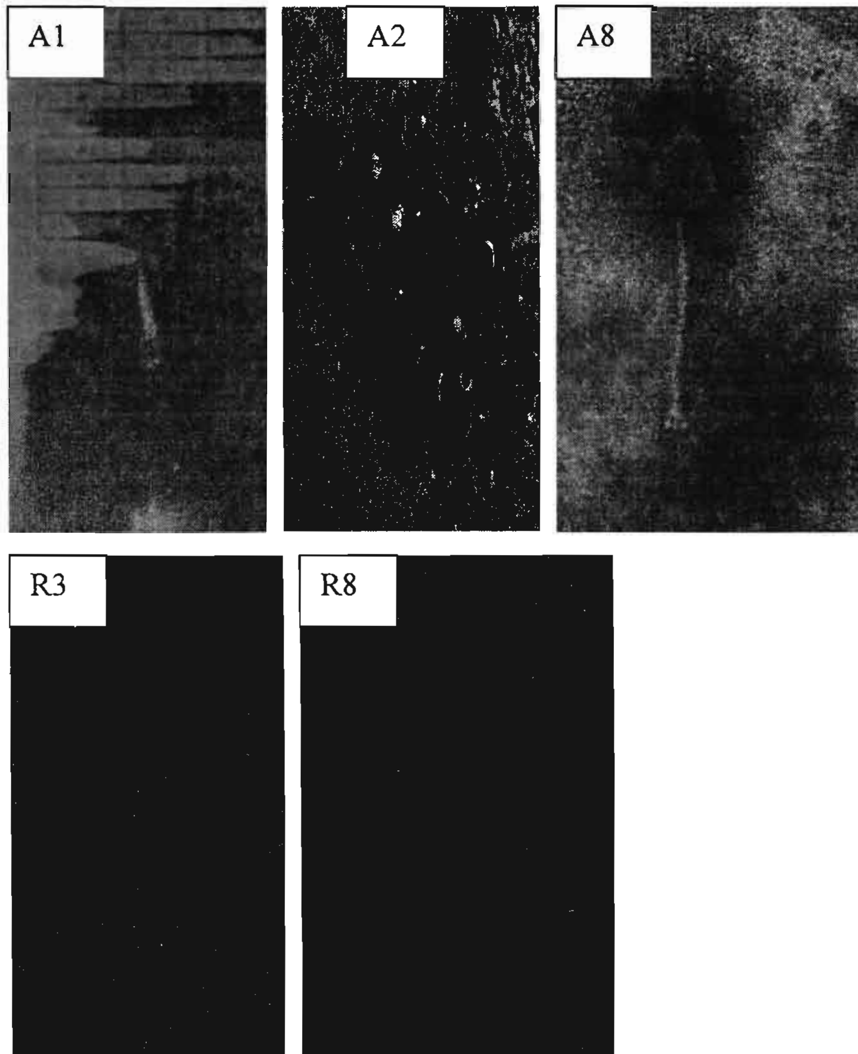

Fig. (1): Electron micrograph of five bacteriophages (A1, A2, A8, X 60,000, R3 X 100,000 and R8 150,000).

Table (3): Host range of the five phages.

Phages / Bacterial strain	A1	A2	A8	R3	R8
Bacillus thuringiensis					
Bt2	+	+	+	+	+
Bt3	+	+	+	+	+
Bt5	+	+	+	+	+
Bt7	+	+	+	+	+
Bt8	+	+	+	+	+
HD1	+	+	+	+	+
B. cereus	+	+	+	+	+
Bacillus spp.	-	-	-	+	-
Pseudomonas aeruginosa	-	-	-	-	-
Rhizobium leguminosarum	-	-	-	-	-
Micrococcus spp.	-	-	-	-	-

+ = lysis of test strain
- = no lysis of test strain

Table (4): Efficiency of plating (EOP).

Bacterial strain	Pfu/ml				
	A1	A2	A8	R3	R8
B. thuringiensis					
Bt2	$8.2x10^9$	$6.6x10^9$	$6.8x10^9$	$2.3x10^7$	$7.2x10^9$
Bt3	$8.7x10^{10}$	$7.9x10^{10}$	$5.6x10^9$	$3.3x10^8$	$2.6x10^{10}$
Bt5	$9.6x10^9$	$3.4x10^9$	$7.8x10^9$	$8.4x10^7$	$6.4x10^9$
Bt7	$6.8x10^9$	$5.2x10^9$	$2.3x10^9$	$2.4x10^7$	$9.1x10^8$
Bt8	$6.4x10^{11}$	$3.9x10^{10}$	$6.5x10^{10}$	$2.9x10^7$	$2.96x10^{11}$
HD1	$9.68x10^{11}$	$8.48x10^{11}$	$5.8x10^9$	$3.6x10^7$	$2.1x10^{10}$
Bacillus cereus	$5.3x10^3$	$3.6x10^3$	$2.4x10^3$	$1.3x10^3$	$3.4x10^3$
Bacillus spp.	NP	NP	NP	$1.6x10^3$	NP
P. aeruginosa	NP	NP	NP	NP	NP
R. leguminosarum	NP	NP	NP	NP	NP
Micrococcus spp.	NP	NP	NP	NP	NP

NP = no plaques.

Previous studies reported that phages fall into two categories, those with a limited host range (often only one strain) and those with a multistrains range (Eric *et al.,* 1978, Jensen *et al.,* 1998).

Transduction between *B. thuringiensis* and *B. cereus*:

The genetic markers of the antibiotics resistance profile of *B. thuringiensis* and *B. cereus* was done to determined donor and recipient bacteria for transduction experiments. Antibiotic resistance background of *B. thuringiensis* showed that all Bt isolates were resistant to penicillin and ampicillin but it was sensitive to chloramphencol, streptomycin and tetracycline (Table 5). From these results, tetracycline resistant *B. cereus* cells can be used as recipient, one strain of Bt was used as donor (Bt3) to transduce ampicilline resistance marker gene. Five phages were examined for the ability to mediate transduction. Five phages were propagated on Bt3, the supernatant was mixed with recipient suspension, after 30 min the diluted mixture was plated on selective media that allowed to transductants grow. Table (6) shows the ability of phages to transduce tetracycline resistant marker gene from Bt to *B. cereus*. Transduction frequency was very low it was ranged from 1.32 to $1.8x10^{-9}$. This may due to the low production of transducing particles and the low efficiency of plating of these phages on *B.*

cereus. *B. cereus* transductants were examined to determined crystal formation, no crystal formed; this indicated that the determinants of crystal formation did not transfer.

The previous studies showed that gene transfer between Bt and *B. cereus* can occur. Reynolds *et al.*, 1988 used five temperate phages from polylysogenic *B. thuringiensis* subsp *aizawai* to generalize transduction; *B. cereus* 12472 was used as recipient. Phage TP-17 was the only of five phages able to effect generalized transduction. Walter and Aronson (1991) were recorded that *B. thuringiensis* subsp *kurstaki* released transducing phage (TP21) was able to transduce three of seven markers into *B. cereus*.

Table (5): Antibiotics resistance profile of *B. thuringiensis* and *B. cereus* strains.

Antibiotics (µg/ml) / Strains	pen			str		amp	chl	tet
	100	500	1000	1000	5000	1000	100	100
Bt2	+	+	+	-	-	+	-	-
Bt3	+	+	+	-	-	+	-	-
Bt5	+	+	+	-	-	+	-	-
Bt7	+	+	+	-	-	+	-	-
Bt8	+	+	+	-	-	+	-	-
HD1	+	+	+	-	-	+	-	-
B. cereus	-		-	-	-	-	-	+

+ = resistance **- = senistive**
pen = pencillin **chl = chloramphenicol**
str = streptomycin **amp = ampicillin**
tet = tetracycline

Table (6): Phages mediated transduction between Bt and *B. cereus*.

Donor	Pfu/ml	Recipient	Selection marker	No. of transductants	Transduction frequency
Phages propagated on Bt3		B. cereus (tet^r)			
Lysate A1	$7.8x10^{10}$	B. cereus (tet^r)	Amp^r	$1.52x10^4$	$1.7x10^{-9}$
Lysate A2	$6.9x10^{10}$	B. cereus (tet^r)	Amp^r	$1.68x10^4$	$1.8x10^{-9}$
Lysate A8	$1.8x10^{10}$	B. cereus (tet^r)	Amp^r	$1.23x10^4$	$1.33x10^{-9}$
Lysate R3	$9.2x10^{10}$	B. cereus (tet^r)	Amp^r	$1.34x10^4$	$1.4x10^{-9}$
Lysate R8	$7.6x10^{10}$	B. cereus (tet^r)	Amp^r	$1.22x10^4$	$1.32x10^{-9}$

cfu/ml of recipient at zero time = $9.2x10^{12}$
Transduction frequency = No. of trancductants/no. of recipient bacterial cells (Lecadet *et al.*, 1980).

Conjugal transfer has been widely studied in *B. thuringiensis*, Yuan *et al.*, 2007 studied conjugation of *B. thuringiensis* subsp. *kurstaki* and three strains of *B. cereus*. Santos *et al.*, 2010 were studied conjugation between Bt and *B. cereus*. All these experiments found that there was a genetic exchange either within bacterial cells or between Bt and *B. cereus* can be performance.

Transduction between *B. thuringiensis* strains:

In this experiment, spontaneous antibiotics resistant mutant of Bt8 was isolated by spreading about 10^9 bacteria from an overnight culture on plates containing the appropriate antibiotic (Landen *et al.*, 1981) (1000 µg/ml) streptomycin, 100 µg/ml chloramphenicol and 100 µg/ml tetracycline). This mutant was used as donor (Bt8, str^r, chl^r, tet^r) with HD1 as recipient (HD1, str^s, chl^s, tet^s).

Numbers of transduction experiments were performed to select single, double and three markers. Table (7) shows that, five phages were able to transduce str^r, chl^r and tet^r genes at frequencies ranged between $2.9x10^{-10}$ to $2.5x10^{-7}$. Except phage A1 didn't transduce tet^r, and phage R8 didn't transduce str^r. Results of cotransduction show that four phages (A1, A2, A8, and R3) were able to transducer DNA fragments that carry both str^r, chl^r markers at frequencies $3.8x10^{-10}$ to $2.4x10^{-7}$. No transductants were detected for str^r tet^r. Phage A2 only was able to transducer chl^r tet^r ($5.1x10^{-9}$) and str^r chl^r tet^r ($5.4x10^{-9}$). It is clear that, five phages were able to transduce different antibiotic resistance gene. Thus, A1, A2, A8, R3 and R8 phages may be a generalized transducing phage. These results come agree with the results of Lecadet *et al.*, 1980, which indicated that phage CP-54 was a generalized transduction in *B. thuringiensis*. Landen *et al.*, 1981 found that phage ø63 which isolated from soil, was a generalized transduction in Bt.

Mapping of str^r, chl^r and tet^r genes:

From the results that showed in Table 6 it may determine the order of these genes. Cotransfer of str^r chl^r indicating a close linkage of these tow genes. The able not phages to transduce str^r, tet^r indicate that these genes are not locate beside each other. The ability of phage A2 to transduce chl^r tet^r indicate that, these tow genes are locate beside each other. From this description, the order of these three genes must be str chl tet or tet chl str.

From this study it may suggest that A1, A2, A8, R3 and R8 phages serve as interspecies and intraspecies transducing particles and may used for mapping of *B. thuringiensis* genes.

Table (7): Phages mediated transduction between *B. thuringiensis* strains using single, double and third-selection.

Selection	No. of transductants of A1 lysate	Transduction frequency	No. of transductants of A2 lysate	Transduction frequency	No. of transductants of A8 lysate	Transduction frequency	No. of transductants of R3 lysate	Transduction frequency	No. of transductants of R8 lysate	Transduction frequency
Str^r	$9.7x10^2$	$4.04x10^{-9}$	$3.47x10^4$	$1.45x10^{-7}$	$3.82x10^3$	$1.6x10^{-8}$	$7.0x10^1$	$2.9x10^{-10}$	NC	-
Chl^r	$3.65x10^3$	$1.52x10^{-8}$	$4.11x10^4$	$1.7x10^{-7}$	$4.73x10^4$	$1.97x10^{-7}$	$3.17x10^3$	$1.3x10^{-8}$	$3.26x10^3$	$1.4x10^{-8}$
Tet^r	NC	-	$2.96x10^4$	$1.2x10^{-7}$	$3.45x10^4$	$1.4x10^{-7}$	$3.59x10^4$	$1.5x10^{-7}$	$5.98x10^4$	$2.5x10^{-7}$
Str^r chl^r	$3.3x10^2$	$1.4x10^{-9}$	$5.79x10^4$	$2.4x10^{7}$	$3.19x10^3$	$1.33x10^{-8}$	$9.0x10^1$	$3.8x10^{-10}$	NC	-
Str^r tet^r	NC	-	NC	-	NC	-	NC	-	NC	-
Chl^r tet^r	NC	-	$1.23x10^3$	$5.1x10^{-9}$	NC	-	NC	-	NC	-
Str^r chl^r tet^r	NC	-	$1.3x10^2$	$5.4x10^{-10}$	NC	-	NC	-	NC	-

NC = no colonies
Cfu/ml of HD1 (str^r chl^r tet^r) recipient = $2.4x10^{11}$.

Genomic fingerprinting by RAPD-PCR analysis:

Among the 20 RAPD tested primers, only seven produced polymorphic bands between the five isolated bacteriophages and therefore chosen for this study. All Polymorphic PCR products were confirmed by repeating the reaction. Each of the seven primers used to analysis of individual isolate amplified different number of bands. The total number of amplified fragments,

number of polymorphic fragments and percentage of polymorphism obtained per each RAPD primer are shown in table 8 and figure 2 represents the patterns of the seven random primers with the five isolated bacteriophages. The total number of amplified RAPD fragments after using all primers was 55 fragments with an average of 7.9 fragments per primer (ranging from approximately 135 to 1500 bp) and 98 % was polymorphic. The number of polymorphic fragments through each primer ranged from 6 to 14 fragments per primer. Primer OPD-07 produced the highest number of polymorphic fragments among the used primers while, primer OPC-08 produced the lowest number of polymorphic fragments. This indicates that the primer OPD-07 was the highest among the tested primers in his ability to flank the DNA sequences of the five isolated bacteriophages.

Numbers of unique fragments which obtained from each RAPD primer per each isolated bacteriophage are summarized in table 9. The oligonucleotide OPD-07 gives the highest number of unique fragments (8) in all isolated bacteriophages while, OPC-08 and OPD-05 give the lowest number (one fragment). All isolated bacteriophages except for R3 bacteriophage were distinguished by unique RAPD markers. The highest number of unique fragments (11) after using all primers was detected in A8 bacteriophage followed by A1 bacteriophage (10 unique fragments). A2 bacteriophage was detected by two unique fragments; approximately 170bp with OPC-20 and 1200bp with OPD-05 and R8 bacteriophage was detected by two unique fragments with OPD-07; approximately 345bp and 570bp.

Table (8): Range of fragment sizes (bp), total number of fragments, polymorphic fragments and percentage of polymorphism obtained per each RAPD primer for all isolated bacteriophages.

Primers	Range of fragment sizes (bp)	Total No. of fragments	Polymorphic fragments	Polymorphism %
OPA-11	140-1100	10	10	100
OPB-11	260-1400	7	7	100
OPC-08	265-1100	7	6	86
OPC-20	165-940	7	7	100
OPD-03	255-1100	8	8	100
OPD-05	165-1200	8	8	100
OPD-07	135-1500	14	14	100
Total	135-1500	55	54	98
Average		7.9	7.7	

Table (9): Number of unique fragments obtained from each RAPD primer per each isolated bacteriophage.

	Unique fragments					Total
	A1	A2	A8	R3	R8	
OPA-11	2	0	2	0	0	4
OPB-11	2	0	1	0	0	3
OPC-08	0	0	1	0	0	1
OPC-20	2	1	2	0	0	5
OPD-03	2	0	1	0	0	3
OPD-05	0	1	0	0	0	1
OPD-07	2	0	4	0	2	8
Total	10	2	11	0	2	25

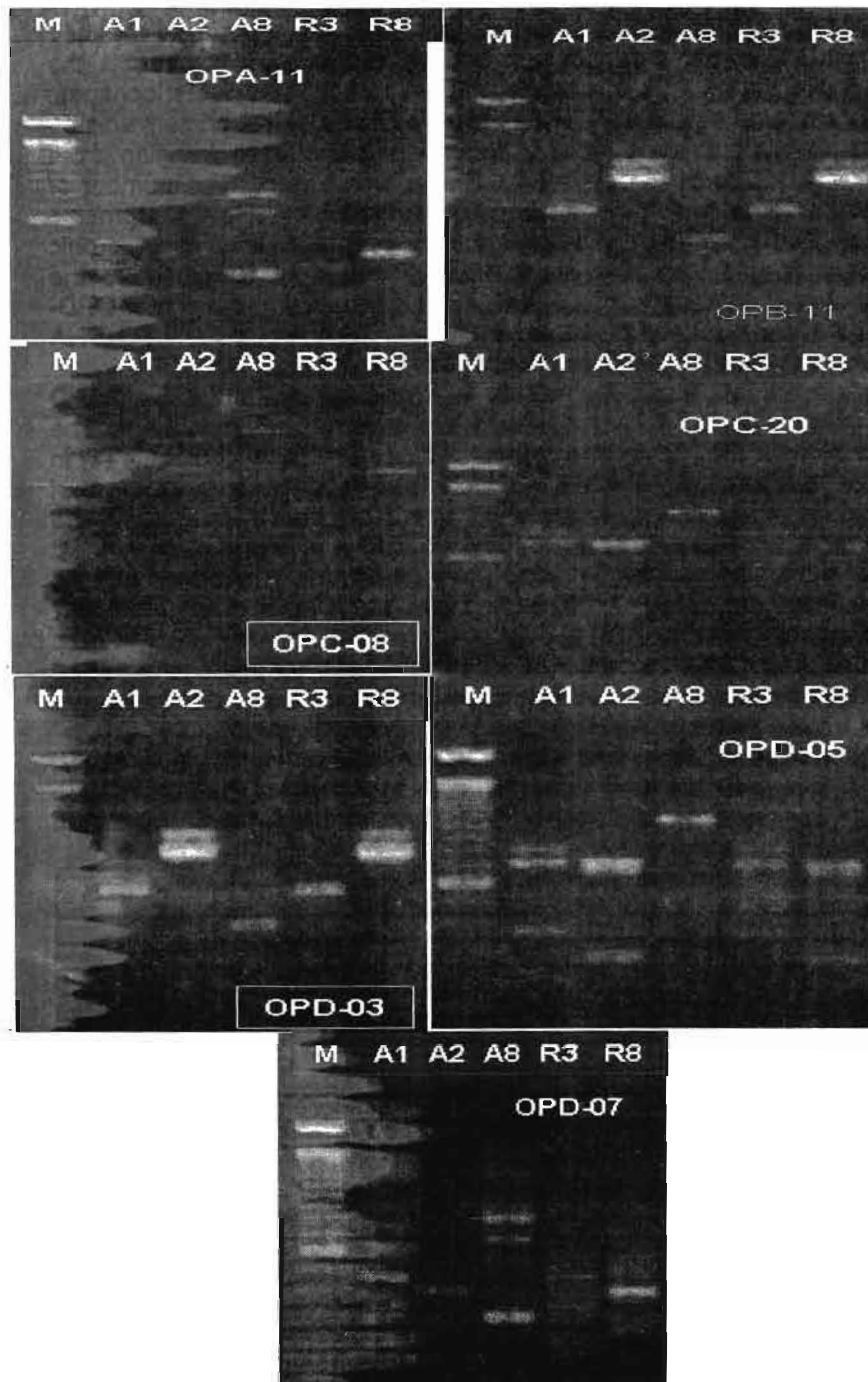

Fig. (2): RAPD band patterns obtained from the seven random primers with the five isolated bacteriophages.

Genetic identity and genetic distance values among all isolated bacteriophages based on fragment polymorphisms generated by RAPD-PCR are present in table (10). The highest similarity value (0.828) was found between A2 and R8 bacteriophages and the lowest value (0.313) was found between A1 and R8 bacteriophages. These small genetic identity values indicate that there is a large difference (dissimilarity) (0.687) among A1 and R8 bacteriophages. The dendrogram of genetic distances among all the isolated bacteriophages based on fragment polymorphisms generated by RAPD-PCR after using the primers is shown in (fig. 3). The dendrogram divited all isolated bacteriophages into two clusters. First cluster divided into two subclusters, first subcluster include A2 and R8 bacteriophages and second subcluster formed a separate subcluster with A3 bacteriophage. Second cluster include A1 and R3 bacteriophages.

Table (10): Measures of Genetic Identity (above diagonal) and Genetic distance (bellow diagonal) among isolated bacteriophages

Isolated bacteriophage	A1	A2	A8	R3	R8
A1		,328	,359	,734	,313
A2	,672		,438	,531	,828
A8	,641	,562		,438	,453
R3	,266	,469	,562		,516
R8	,687	,172	,547	,484	

```
Dendrogram using Average Linkage (Between Groups)

                    Rescaled Distance Cluster Combine

  C A S E      0         5        10        15        20        25
  Label    Num  +---------+---------+---------+---------+---------+

  A2        2
  R8        5
  A8        3
  A1        1
  R3        4
```

Fig. (3): The dendrogram of genetic distances among all isolated bacteriophages based on fragment polymorphisms generated by RAPD-PCR after using all primers.

Prior *et al.*, 2007 isolated bacteriophages of *Pseudomonas syringae* pv. tomato and characterized them using the restriction endonucleases EcoRI or HindIII and determined indicating three unique phage isolates with three different DNA fingerprint patterns. Youssef *et al.*, 2007 studied the relationship between some bacteriophages isolated from the environment propagating on *Pseudomonas* bacteria with RAPD-PCR. Li *et al.*, 2010 isolated seventeen virulent bacteriophages specific to *Pseudomonas*

aeruginosa strains by screening various environmental samples and grouped these isolated bacteriophages based on results obtained from restriction fragment analysis of phage genomes, random amplification of polymorphic DNA (RAPD) typing, morphology observations under transmission electron microscope, and host range analysis. Diana *et al.*, 2011 evaluated the randomly amplified polymorphic DNA (RAPD)-PCR technique to produce unique and reproducible band patterns from 26 different bacteriophages.

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عزل وتوصيف بكتريوفاجات متخصصة لبكتريـــا *Bacillus thuringiensis* باستخدام الميكروسكوب الالكترونى و الـــ RAPD-PCR

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تم عزل خمسة بكتريوفاجات متخصصة لبكتريا *B. thuringiensis* من التربة، هذه الفاجات سميت A1, A2, A8, R3, R8.الفاجات A2, A8, R3, R8 تنتج بليكس عكرة صغيرة الحجم، أما الفاج A1 فهو ينتج بليكس كبيرة نسبيا ورائقة، ولقد لوضح الميكروسكوب الالكترونى أن هناك اختلافات كبيرة فى الشكل المورفولوجى لجزيئات هذه الفاجات. لربعة منها وهى A1, A8, R3, R8 تمتلك رؤوس متعددة الأوجة مع نيل طويل، أما الفاج الخامس A2 فهو عبارة عن رلس متعددة الأوجه مع عدم وجود نيل. لوضحت دراسة المدى العوائلى لهذه الفاجات أنها تمتلك مدى عوائلى واسع حيث أنها استطاعت أن تتكاثر بنجاح على ٦ عزلات من بكتريا *B. thuringiensis* وأيضا على سلالة *B. cereus*.لوضحت دراسة قدرة هذه الفاجات على نقل المادة الوراثية بين نوعين مختلفين من جنس الـــ Bacillus أن هذه الفاجات استطاعت نقل جين

المقاومة للمضاد الحيوى أمبسيللين من بكتريا Bt إلى بكتريا *B. cereus*، وقد تراوح معدل النقل من ١.٣٢ إلى ١.٨×١٠^-٩. كما أستطاعت أن تقوم بإنجاز النقل بين سلالات بكتريا *B. thuringiensis*. أجريت تحليلات ال RAPD-PCR لانتاج أنماط فريدة من نوعها وقابلة للتكرارفى الخمسة بكتريوفاجات المختلفة ومن بين ٢٠ بادىء تم لختبارها فان سبعة بوادئ فقط أنتجت شظايا متعددة الأشكال بمتوسط ٧،٩ شظية لكل بادئ (يتراوح طولها تقريبا من ١٣٥ الى ١٥٠٠ زوج من القواعد) وعدد الشظايا المختلفة الأشكال لكل بادئ تراوحت مابين ٦ الى ١٤ شظية لكل بادئ ومن بين البوادئ المستخدمة أنتج البادئ OPD-07 أكبر عدد من الشظايا المختلفة الأشكال بينما البادئ OPC-08 أنتج أقل عدد وكذلك فان البادئ OPD-07 أنتج أكبر عدد من الشظايا الفريدة فى نوعها (٨) فى كل البكتريوفاجات بينما البادئان OPC-08 و OPD-05 أنتاجا أقل عدد (شظية واحدة) ونجد أن كل البكتريوفاجات المعزولة فيما عدا R3 تم تمييزها بواسمات فريدة من نوعها حيث تم تمييزالعزلة A8 بأكبر عدد (١١) بعد استخدام كل البوادئ يليها العزلة A1 تم تمييزها بعشرة شظايا فريدة بينما العزلة A2 تم تمييزها بشظيتين هما ١٧٠ زوج من القواعد مع البادئ OPC-20 و ١٢٠٠ زوج من القواعد مع البادئ OPD-05 وكذلك فان العزلة R8 تم تمييزها بشظيتين مع البادئ OPD-07 وهما ٣٤٥ و ٥٧٠ زوج من القواعد تقريبا وقد أثبتت هذه الدراسة فاعلية ال RAPD كتكنيك في توصيف البكتريوفاجات المعزولة من الطبيعة ويمكن أن تكون مفيدة في عمل البصمات الوراثية لها وبالاضافة لذلك فان الفاجات المعزولة من التربة فى هذه الدراسة يمكن استخدامها كنواقل فى تجارب الهندسة الوراثية.

قام بتحكيم البحث

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