**Isolation and Identification of *Bacillus megaterium* Bacteriophages via AFLP Technique**

1I. Elmaghraby, 2Francesco Carimi, 3A. Sharaf, 4E.M. Marei and 5A.M.M. Hammad  
1Central Laboratory of Organic Agriculture, Agricultural Research Center, Giza, 12619, Egypt  
2CNR-Institute of Biosciences and Bioresources (IBBR), UOS Palermo, 90129, Italy  
3Department of Genetic, Faculty of Agriculture, Ain Shams University, Cairo, Egypt  
4Department of Microbiology, Faculty of Agriculture, Ain Shams University, Cairo, Egypt  
5Department of Microbiology, Faculty of Agriculture, Minia University, Minia, 61111, Egypt  

Corresponding Author: A.M.M. Hammad, Department of Microbiology, Faculty of Agriculture, Minia University, Minia, 61111, Egypt

**ABSTRACT**

Ten bacteriophages specific for *Bacillus megaterium* were isolated from a clay loam soil sample collected from the Experimental Farm of Faculty of Agriculture, Minia University, Minia, Egypt. Four out of ten isolates were inactivated after exposure to 80°C for 10 min and three isolates were inactivated at 78°C for 10 min. Whereas, the other three phage isolates were inactivated at 82°C for 10 min. The isolated phages were found to be tolerant to wide range of pH 5-9. The longevity *in vitro* varied between the phage isolates. The highest longevity *in vitro* was recorded for four phage isolates (192 h). Electron micrographs of the isolated phages indicated that all phage isolates were of the head and tail types. Two different host specificities were observed for the ten phage isolates (two different populations). Six phage isolates (population 1) were found to be infectious to *B. megaterium* among the four species tested (i.e., *B. megaterium*, *B. circulans*, *B. polymexa* and *B. subtilis*). Whereas, the rest of the phage isolates (population 2) were found to be infectious to *B. megaterium* and *B. subtilis*. The dendrogram separated the 10 phage isolates into two main clusters (two populations) and then each cluster was separated into two sub clusters. Isolates that belonged to the same host range were grouped together. The percentage of variation was 9% among populations and 91% within populations. The five most remarkable isolates were submitted to the bacillus database and named BMC1, BMC2, BMC3, BMC4 and BMC5.

**Key words:** Bacteriophages, *Bacillus megaterium*, thermal inactivation point, longevity *in vitro*, host range, dendrogram, AFLP

**INTRODUCTION**

*Bacillus megaterium* has been an important industrial organism for decades. It produces penicillin amidase used to make synthetic penicillin, various amylases used in the baking industry and glucose dehydrogenase used in glucose blood tests. Further, it is used for the production of pyruvate, vitamin B12, drugs with fungicidal and antiviral properties, etc. (Vary *et al.*, 2007). It produces enzymes for modifying corticosteroids as well as several amino acid dehydrogenases.

*Bacillus megaterium* is known to produce poly-γ-glutamic acid. The accumulation of the polymer is greatly increased in a saline (2-10% NaCl) environment, in which the polymer comprises largely...
of L-glutamate (L-isomer content up to 95%) (Shimizu et al., 2007). At least one strain of \textit{B. megaterium} can be considered a halophile, as growth on up to 15% NaCl has been observed (Khan, 2011).

\textit{Bacillus megaterium} has been recognized as an endophyte and is a potential agent for the biocontrol of plant diseases. Nitrogen fixation has been demonstrated in some strains of \textit{B. megaterium} (Vos et al., 2009). In alkaline soils \textit{B. megaterium} plays an important role in supplying growing plants with available forms of phosphorus by producing organic acids and CO$_2$ which increase the soil acidity and convert the insoluble forms of phosphorus into soluble ones. Zayed (1998) and Hammad (1999) stated that presence of bacteriophages specific for \textit{Bacillus megaterium} had a depressive effect on the efficiency of these bacteria in dissolving phosphate.

Amplified fragment length polymorphism as such is a restriction-amplification method developed in the early 1990s (Vos et al., 1995). After an additional digestion of genomic DNA, by a combination of restriction enzymes, restriction site specific extensions are ligated to the multitude of DNA fragments. The attached linker contains site specific PCR priming sequences. When targeted by PCR, the combination of sites at the termini of an individual restriction fragment determines whether the fragment is amplified or not. Usually, due to the complexity of the restriction digest, one or two AFLP fingerprints suffice for obtaining a reliable genetic signature for a microbial strain. In principle, the method screens restriction site polymorphism and a clever extension of the primers used during AFLP also facilitates monitoring of DNA polymorphism in the restriction site neighbouring region. This has resulted in the establishment of reproducible and robust microbial typing strategies that do not only provide genetic epidemiological information, but which can also be used to identify new species, even within the \textit{M. tuberculosis} complex (Ahmed et al., 2003).

Since, presence of bacteriophages specific for \textit{Bacillus megaterium} had a depressive effect on the efficiency of these bacteria in dissolving phosphate, the objective of this study was to investigate the presence of the bacteriophages in the Egyptian soil. In addition, characteristics of these phages i.e., plaque morphology, particle size and morphology, thermal inactivation point and longevity in vitro as well as genetic similarities using AFLP technique were also studied.

**MATERIALS AND METHODS**

**Soil used:** A clay loam soil sample was collected from the surface 15 cm layer of the Experimental Farm of Faculty of Agriculture Minia University, Minia, Egypt. The collected soil was used for isolation of bacteriophages specific for \textit{Bacillus megaterium}.

**Bacteria used:** Four \textit{Bacillus} species (\textit{B. megaterium}, \textit{B. circulans}, \textit{B. polymexa} and \textit{B. subtilis}) were kindly supplied by Cairo MERCIN, Faculty of Agriculture, Ain Shams University, Cairo, Egypt.

**Isolation of bacteriophages:** The liquid enrichment technique was used to isolate the phages of \textit{Bacillus megaterium} as described by Barnet (1972). Twenty grams of the collected soil were incubated overnight with 40 mL of nutrient broth at 30-33°C. Five milliliter of chloroform were then added and the sample was shaken for 10 min followed by centrifugation at 4000 rpm to remove soil and bacteria. The supernatant was added to 10 mL of 24 h old liquid culture of \textit{Bacillus megaterium}. After multiplication of phages (24-30 h at 30-33°C), bacteria were killed by shaking with 5 mL of chloroform for 10 min, then the sample was clarified by centrifugation at 4000 rpm. The supernatant (phage lysate) was subjected to phage detection.
Detection of phages: Double layer agar plates (Adams, 1966) was used for phage detection as described by Hammad (1993). Plates were prepared by pouring a base layer of 20 mL of nutrient agar medium with 1.5% agar in petri dishes 10 cm in diameter. The basal layer was allowed to solidify. A mixture of 3 mL of melted yeast nutrient agar medium containing 0.7% agar and 300 μL of liquid culture of the indicator bacteria (B. megaterium) was poured in each plate. The phage lysate was spotted with a sterile micropipette on the upper layer after it had solidified. Plates were incubated at 30-33°C for 24-30 h and then examined for lysis of bacterial lawn at sites where drops had been applied. The lysed clear zones were picked and transferred separately into eppendorf tubes containing 1 mL of SM medium (Maniatis et al., 1982), which contains per litre 5.8 g NaCl, 2 g MgSO₄•7H₂O, 50 mL of 1 M Tris-HCl (pH 7.5) and 5 mL of 2% gelatin. Two hundreds microliter chloroform were added to each tube, then maintained at 4°C.

Purification of bacteriophage isolates: The single plaque isolation technique was used to obtain pure single isolates of bacteriophages as described by Kiraly et al. (1970).

Preparation of high titer phage suspension: Liquid enrichment technique was used as described by Franche (1987), to prepare high titer phage lysate.

Determination of thermal inactivation point: Eppendorf tubes each containing 1.5 mL of high titer phage suspension was prepared. Tubes were heated in water baths adjusted at 50, 55 and 60 upto 90°C for 10 min, Then cooled under tap water. Ten microliter from each tube were spotted over double agar layer plates containing the indicator bacteria (B. megaterium). After incubation for 24-30 h. At 30°C plates were inspected for lysis of bacterial lawn at the sites where spots had been applied (Marei, 2013; Hammad, 1993).

Longevity in vitro: Eppendorf tubes each containing 1.5 mL of high titer phage suspension were kept at room temperature. Samples were assayed qualitatively every 12 h. According to the method of Yoshida et al. (2006).

Host range assay: Double agar layer plates were prepared. Each of the four Bacillus strains (B. megaterium, B. circulans, B. polymexa and B. subtilis) was used as indicator host in individual plates. The surface of every plate was spotted with drops of each phage isolate. After incubation for 24-30 h at 30°C, plates were inspected for the lysed spots.

Electron microscopy of the phage isolates: Five milliliter of the high titer phage suspension were centrifuged at 30,000 rpm for 90 min at 4°C in a Bekman L7-35 ultra centrifuge. The pellet was gently resuspended in 0.5 mL of 0.2 M phosphate buffer pH 7.2. A drop of the resuspended pellet was placed on 200 mesh form var-coated grid and allowed to settle for 1 min. The excess liquid was removed with a filter paper wick. Grid was stained with 2% (w/v) phosphotungstic acid for 15 sec. The grid was air dried and examined in Jeol-Jem 1010 transmission electron microscope.

Extraction of bacteriophages DNA: The method described by Maniatis et al. (1982), with minor modification (Campos et al., 2003) was used. Fifteen milliliter of high titre phage suspension were incubated at 37°C for 30 min with DNase and RNase at final concentration of 1 μg mL⁻¹ each to get red of contaminating bacterial DNA and RNA.
Phage particles were precipitated by addition of NaCl and polyethylene glycol (PEG 6000) to final concentrations of 3 and 5% (wt/vol), respectively. The mixture was incubated on ice for 30 min and centrifuged at 12,000 rpm for 20 min.

The bacteriophage pellet was resuspended in 500 μL of 0.2 M ammonium acetate, 0.001 M Na-EDTA pH 7.8 and heated at 55°C for 20 min an equal volume of buffer saturated phenol was added and mixed by inverting the tube several times. The phases were separated by centrifugation at 13,000 rpm for 5 min at room temperature in microcentrifuge. The aqueous phase was transferred to a clean tube using 1 mL micropipette with disposable tip and extracted once again with chloroform. The aqueous phase was recovered as described above and transferred to a clean tube. The DNA was precipitated by adding 0.1 volume of 3 M sodium acetate pH 5.5 and equal vol. of cold ethanol followed by freezing for 7-8 h. The tube was thawed and DNA precipitate was collected by centrifugation at 13,000 rpm for 10 min at room temperature in microcentrifuge. The pellet was washed in 70% ethanol, air dried at room temperature, resuspended gently in approximately 30 μL of TE (10 mM tris-HCl, pH 8.0 and 1 mM EDTA) and stored at 4°C.

**AFLP analysis:** To obtain AFLP markers that have potential for genotyping, 27 selective primer pairs had been tested which are a combination of four IR Dye 800 labeled EcoRI primer with seven MseI primers as shown in the Table 4. The ten successful primers pairs had been used to analyze the ten bacteriophages isolates.

**Multiplexed AFLPs:** Infrared Dye Detection on Automated Sequencers had been used. The detection of AFLP fragments using infrared technology offers several advantages over conventional detection using autoradiography. In particular, the use of radioactivity is eliminated and gel images are available for analysis immediately after gel electrophoresis. The multiplexed, selective amplification procedure was designed to complement the two-dye detection capability of LI-COR automated sequencers. The ability to collect gel images in two channels and to load gels twice allowed to resolve and capture 384 lanes of AFLPs on a single 25 cm acrylamide gel.

The AFLP technique was performed essentially as described by Vos et al. (1995), except that adaptors and primers were designed according to Lan and Reeves (2000), which facilitates hot start PCR. Chromosomal DNA (0.1 μg) was used for digestion and ligation of adaptors, which were performed simultaneously. The ligation mix was then removed, diluted 1:10 and 1 μL was used for preamplification. Preamplification was done in a 20 μL volume with a PCR mix containing 0.2 μM of each dNTP, 4 μg μL−1 BSA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM MgCl2 and 0.5 U AmpliTaq Gold (PE Applied Biosystems) plus 0.3 μM preamplification primers. The cycling profile was 20 cycles of denaturation at 94°C for 15 sec except for 10 min for the first cycle, annealing at 56°C for 30 sec and extension at 72°C for 1 min. For final amplification 1 μL diluted product (1:10) from preamplification was mixed with 19 μL of PCR mix as for preamplification, but containing 0.3 μM unlabelled selective primer and 0.1 μM 33P labelled selective primer. Basic cycling parameters were denaturation at 94°C for 15 s except for 10 min for the first cycle, annealing at temperatures specified below for 30 sec and extension at 72°C for 1 min. The first 10 cycles were touchdown from 66-56°C decreasing by 1°C cycle and then 20 cycles at 56°C. The AFLP products were run on standard 6% polyacrylamide sequencing gels and visualized.

**Semi-automated scoring of AFLP images:** Infrared detection of labeled AFLP fragments on LI-COR automated sequencers produced clear, discrete banding patterns that were suitable for semi-automated scoring using dedicated scoring software. The PyElph software tool is entirely
implemented in Python which is a very popular programming language among the bioinformatics community. It provides a very friendly graphical user interface which was designed in six steps that gradually lead to the results. A strong point of the software is the visualization component for the processed data. The graphical user interface provides operations for image manipulation and highlights lanes, bands and band matching in the analyzed gel image. All the data and images generated in each step can be saved. The software has been tested on several DNA patterns obtained from experiments with different genetic markers.

The genetic diversity within each population analyzed was estimated by comparing the bands and allele frequency, number of alleles ($N_a$), number of effective alleles ($N_e$), Shannon’s Information Index ($I$) and gene diversity ($H_e$) using GENALEX 6.5 (Peakall and Smouse, 2012).

To determine the presence of significant genetic structuring among the host range populations, the genotype profiles were analyzed by molecular variance analysis (AMOVA) using ARLEQUIN v. 3.5.1.3 (Excoffier and Lischer, 2010). The variance components were calculated with 16,000 permutations.

The pairwise genetic distances for phylogenetic relationships among strains were estimated using Nei’s coefficient (Nei et al., 1983). Cluster analysis was carried out according to UPGMA (Unweighted Pair-Group Method with Arithmetical Averages) algorithm and a dendrogram was developed (Liu and Muse, 2005). A consensus tree was created in nexus format for viewing in tree view (Vos et al., 1995), supporting the nodes by bootstrap analysis (1,000 replicates). The genetic relationship among genotypes was also investigated by Principal Coordinate Analysis (PCoA) by using GENALEX 6.5 (Peakall and Smouse, 2012).

RESULTS

Bacteriophages of Bacillus megaterium: As shown in Fig. 1, the spot tests indicate that phages of $B$. megaterium are present in the collected soil sample, since lysis of the bacterial lawn was detected at the sites where drops had been applied.

Purification of bacteriophages: The single plaque isolation technique was used to purify and isolate single phage isolates. A typical plate containing single plaques is shown in Fig. 2. Ten single

Fig. 1: A bacterial lawn of Bacillus megaterium spotted with a drop of the prepared phage lysate and incubated for 24-30 h, at 30-33°C
Fig. 2: A double layer agar plate prepared with phage lysate of *Bacillus megaterium*. Single plaques of different morphologies can be clearly seen.

### Table 1: Thermal inactivation point, longevity *in vitro* and stability to different pH levels of the ten isolated phages

| LIV (h) | pH range | TIP (°C) | Phage isolates |
|---------|----------|----------|----------------|
| 144     | 5-8.4    | 80       | BMC1           |
| 144     | 5-8.4    | 80       | BMC2           |
| 168     | 5-9.0    | 80       | BMC3           |
| 168     | 5-9.0    | 80       | BMC4           |
| 192     | 5-9.2    | 82       | BMC5           |
| 192     | 5-9.2    | 82       | BMC6           |
| 144     | 5-8.0    | 82       | BMC7           |
| 144     | 5-8.2    | 78       | BMC8           |
| 192     | 5-8.4    | 78       | BMC9           |
| 192     | 5-8.4    | 78       | BMC10          |

LIV: Longevity *in vitro*, TIP: Thermal inactivation point

Plaques, each having different morphology were picked and kept as single phage isolates. The single plaques were found to be clear circular shape of 1-3 mm in diameters. These ten phage isolates were designated BMC1-BMC10.

**Characteristics of the isolated phages:** Different characteristics of the ten phage isolates (i.e., thermal inactivation point, longevity *in vitro* and stability to different pH levels) were studied. Data presented in Table 1 indicate that phage isolates BMC1, BMC2, BMC3 and BMC4 were inactivated after exposure to 80°C for 10 min. Moreover, the thermal inactivation point of phages BMC5, BMC6 and BMC7 was found to be 82°C. In addition, the thermal inactivation point of phages BMC8, BMC9 and BMC10 was found to be 78°C.

The isolated phages were found to be tolerant to wide range of pH. Phages BMC1, BMC2, BMC9 and BMC10 were tolerant to pH 5-8.4. In addition phages BMC3 and BMC4 were tolerant to pH 5-9. The longevity *in vitro* varied from one phage isolate to another. The phage isolates BMC1, BMC2, BMC7 and BMC8 were inactivated after 144 h of storage at room temperature. The highest longevity *in vitro* (192 h) was recorded for phage isolates BMC5, BMC6, BMC9 and BMC10.

**Host range of the phage isolates:** As shown in Table 2, two different host specificities were observed for the ten phage isolates (two different populations). Phage isolates BMC1, BMC2, BMC5, BMC6, BMC7 and BMC8 (population 1) were found to be infectious to *B. megaterium* among
the four species of *Bacillus*. Whereas, phages BMC3, BMC4, BMC9 and BMC10 (Population 2) were found to be infectious to *B. megaterium* and *B. subtilis*. Moreover, *B. circulans* and *B. polymexa* were resistant to all phage isolates under study, whereas, *B. megaterium* exhibited susceptibility to all phage isolates tested.

**Size and morphology of phage particles:** All phage isolates under study were examined by electron microscopy. Electron micrographs of the isolated phages are shown in Fig. 3 and the dimensions are recorded in Table 3. All phage isolates were of the head and tail types. Phages BMC2, BMC7, BMC9 and BMC10 appeared to have flexible tails, whereas, phages BMC1, BMC3, BMC4, BMC5, BMC6 and BMC8 appeared to have contractile tails.

**AFLP analysis:** Table 4 and Fig. 4, indicate that the ten AFLP primer combinations had been generated a total number of 524 clear and scorable fragments. With average (92.9%) polymorphism as shown in Table 5 and 6. The total number of fragments of each primer combinations ranged from 40 (M-CTG/E-ACG) to 63 (M-CTG/E-ACT). The percent polymorphism varied from
Fig. 4(a-c): AFLP gels profile for the ten isolates with, (a) M-CAA/E-AGC, (b) M-CTA/E-ACT and (c) M-CTC/E-ACT out of ten successfully primer combinations

Table 3: Dimensions* of bacteriophages (ten isolates) specific for Bacillus megaterium as determined from electron micrographs

| Phages | Head diameter (nm) | Width (nm) | Length (nm) |
|--------|--------------------|------------|-------------|
| BMC1   | 60                 | 15         | 140         |
| BMC2   | 54                 | 12         | 170         |
| BMC3   | 78                 | 18         | 132         |
| BMC4   | 82                 | 20         | 125         |
| BMC5   | 80                 | 17         | 163         |
| BMC6   | 82                 | 15         | 125         |
| BMC7   | 76                 | 15         | 138         |
| BMC8   | 85                 | 20         | 165         |
| BMC9   | 77                 | 18         | 125         |
| BMC10  | 79                 | 13         | 120         |

*Dimensions represent the average of four particles

85 (M-CAC/E-AGC) to 96% (M-CAC/E-AGC). Various statistical features like band frequency (p), No. of different alleles (Na), No. of effective alleles (Ne) = 1/(p^2+q^2), Shannon’s Information Index (I) = -1×(p×Ln (p)+q×Ln(q)) and diversity (He)= 1-(p^2+q^2) of a particular primer combination decides the powerfulness and discriminatory power for generating an excellent AFLP profile. The highest calculated p value (0.564) was shown by primer combination M-CTC/E-ACT followed by M-CTA/E-ACT (0.546) and M-CAA/E-AGC(0.524). On the other hand, lowest p value (0.333) was recorded for primer combination M-CAC/E-AGC. Number of different alleles (Na) varied from 1.18 (M-CTG/E-ACG) to 1.72 (M-CAT/E-ACG) with an average of 1.57. Number of effective alleles (Ne) varied from 1.35 (M-CTG/E-AGC) to 1.56 (M-CAC/E-AGC) with an average of 1.49.
Fig. 5: UPGMA tree based on 524 AFLP loci used to genotype 10 isolates of phages specific for *Bacillus megaterium* belonging to two different populations (host specificity) (Nei *et al*., 1983)

### Table 4: Successful AFLP primers combinations utilized in pre-amplification reactions

| Primers combinations | IRDye label | EcoRI primer selective nucleotides | MseI primer selective nucleotides |
|----------------------|-------------|-----------------------------------|----------------------------------|
| 1                    | 800         | AGC                               | CTG                              |
| 2                    | 800         | ACG                               | CTG                              |
| 3                    | 800         | ACT                               | CTG                              |
| 4                    | 800         | AGC                               | CAC                              |
| 5                    | 800         | ACG                               | CAT                              |
| 6                    | 800         | ACT                               | CAT                              |
| 7                    | 800         | AGC                               | CAA                              |
| 8                    | 800         | ACG                               | CTA                              |
| 9                    | 800         | ACT                               | CTA                              |
| 10                   | 800         | ACT                               | CTC                              |

*EcoRI adapter primer sequence without selective nucleotides was 5’GACTGCGTACCAATTC-3’, *MseI* adapter primer sequence without selective nucleotides was 5’GATGAGTCCTGAGTAA-3’

### Table 5: Genetic parameters for the strains genotype with AFLP, shared into two host range populations

| Parameters | Description |
|------------|-------------|
| p          | Band frequency and q = 1-p |
| N          | Samples size |
| Na         | No. of different alleles |
| Ne         | No. of effective alleles = 1/(p^2+q^2) |
| I          | Shannon’s information index = -1×(p×Ln (p)+q×Ln(q)) |
| He         | Diversity = 1-(p^2+q^2) |

### Table 6: Genetic identity and distance among the two host range population analyzed

| PopID | 1       | 2       |
|-------|---------|---------|
| 1     | 0.1703  | 0.8434  |
| 2     | -       | -       |

Nei’s genetic identity (above diagonal) and genetic distance (below diagonal) (Nei *et al*., 1983)

**Comparative class pattern analysis based on AFLP (phylogenetic tree):** The combined class pattern system was applied to obtain a better resolution. As shown in Fig. 5, the dendrogram separated the 10 isolates of phages into two main clusters and then each cluster was separated into two sub-clusters. Isolates that belonged to the same host range were grouped together.
Molecular analyses of variance (ANOVA): As shown in Fig. 6, two different populations were observed on the basis of the host range results. The percentage of variation is 9% among populations and 91% within populations.

DISCUSSION

Phages of *B. megaterium* were found to be common in the collected soil sample. Similarly, Hegazi *et al.* (1980), isolated phages of *B. mycoides, B. subtilis* and *B. cereus* from several soil samples collected from the Nile Valley. Zayed (1998) and Hammad (1999), isolated phages of *B. megaterium* from soils of Minia Governorate. Moreover, Abo-Sinna (2004), isolated phages of *B. subtilis* from soils of different Egyptian Governorates.

It is assumed that each plaque has originated from the progeny of a single phage particle (Kiraly *et al.*, 1970). In addition, plaque morphology and measurements are among the first criteria used to differentiate phages of various bacteria and it is commonly believed that the shape, size and outline of the plaques are characteristic of the phage strain (Hammad, 1989). Therefore, the single plaque isolation technique was used to purify and isolate single phage isolates. Ten single phage isolates were successfully isolated.

Characteristics of the ten phage isolates (i.e., thermal inactivation point, longevity *in vitro* and stability to different pH levels) were studied. The thermal inactivation points for the ten phage isolates ranged from 78-82°C. Similarly, Abo-Sinna (2004), reported that the thermal inactivation points for four phage isolates specific for *Bacillus subtilis* ranged between 50-80°C.

Moreover, the isolated phages were found to be tolerant to alkaline and acidic reactions. Elsharouny (2014), reported that phages specific for *Bacillus licheniformis* were found to be tolerant to wide pH range (pH 4-11).
Each of the ten phage isolates was tested against each of the four species of *Bacillus*. Two different host specificities were observed for the ten phage isolates. Phage isolates No. 1, 2, 5, 6, 7 and 8 were found to be infectious to *B. megaterium*. Whereas, phages No. 3, 4, 9 and 10 were infectious to *B. megaterium* and *B. subtilis*. The ability of a phage particle to lyse a bacterial strain is dependent upon the presence or absence of surface receptors for bacteriophage adsorption (Barnet, 1972; Mahy and Kangro, 1996).

The ten phage isolates were negatively stained and examined by electron microscopy. All phage isolates were of the head and tail type. Phages BMC2, BMC7, BMC9 and BMC10 appeared to have flexible tails, whereas, phages BMC1, BMC3, BMC4, BMC5, BMC6 and BMC8 appeared to have contractile tails. Similarly, Van Elsas and Penido (1982), isolated tailed phage of *Bacillus megaterium* with regular polyhedral head. Head and tail dimensions were 56.4 and 300 nm, respectively. Moreover, Marei (2013) found that the particles of a phage isolate specific for *B. subtilis* are tailed and have isometric head of 59 nm in diameter.

The dendrogram separated the 10 isolates of phages into two main clusters (two populations) and then each cluster was separated into two sub-clusters. Isolates that belonged to the same host range were grouped together. The percentage of variation is 9% among populations and 91% within populations.

This study proves that the AFLP can be extended into species with small genomes. This is especially appropriate because these species often occur in minute quantities and therefore are not amenable to techniques requiring large amounts of DNA, such as RFLP analysis. Furthermore, because no prior knowledge of the genome sequence is required, the technique can be applied to genotype and characterize new bacteriophage isolates (Vos et al., 1995).

**CONCLUSION**

Phages of *Bacillus megaterium* were found to be common in the soil of the Experimental Farm of Faculty of Agriculture, Minia University, Egypt. The phages were of head and tail type. The thermal inactivation point of the isolated phages ranged between 78-82°C. The phages were found to be tolerant to a wide range of pH 5-9.2 and their longevity *in vitro* ranged from 144-192 h. According to the host range of the isolated phages (10 phages), the phages were classified into two populations. The dendrogram separated the 10 isolates of phages into two main clusters (two populations) and then each cluster was separated into two sub-clusters. This indicates that the phage isolates which belong to the same sub cluster may have a high degree of similarity between their genetic backgrounds.

**ACKNOWLEDGMENTS**

The authors would like to thank IBBR, CNR-Palermo for providing the LI-COR instrument and AFLP reagents used in the study and to Dr. Francesco Mercati for his advice and assistant in the AFLP data analysis. Thanks also to Prof. Dr. Thomas Loyanchan for his critical revision of the manuscript.

**REFERENCES**

Abo-Sinna, A.S.M., 2004. Studies on some viruses occurred under wheat cultivations in some Egyptian soils. Ph.D. Thesis, Faculty of science, Al-Azhar University, Cairo, Egypt.

Adams, M.H., 1966. The Bacteriophages. Interscience Publishers Inc., New York, USA., pp: 447-461.
Ahmed, N., M. Alam, A.A. Majeed, S.A. Rahman, A. Cataldi, D. Cousins and S.E. Hasnain, 2003. Genome sequence based, comparative analysis of the fluorescent amplified fragment length polymorphisms (FAFLP) of tubercle bacilli from seals provides molecular evidence for a new species within the *Mycobacterium tuberculosis* complex. Infect. Genet. Evol., 2: 193-199.

Barnet, Y.M., 1972. Bacteriophages of *Rhizobium trifolii* L. morphology and host range. J. Gen. Virol., 15: 1-15.

Campos, J., E. Martinez, E. Suzarte, B.L. Rodriguez and K. Marrero et al., 2003.VGJφ, a novel filamentous phage of *Vibrio cholerae*, integrates into the same chromosomal site as CTXφ. J. Bacteriol., 185: 5685-5696.

Elsharouny, T.H.M., 2014. Studies on bacteriophages specific for phosphate dissolving bacteria in the soil. Ph.D. Thesis, Faculty of Agricultural, Minia University, University in Minya, Egypt.

Excoffier, L. and H.E.L. Lischer, 2010. Arlequin suite ver 3.5: A new series of programs to perform population genetics analyses under Linux and windows. Mol. Ecol. Resour., 10: 564-567.

Franche, C., 1987. Isolation and characterization of a temperate cyanophage for a tropical *Anabaena* strain. Arch. Microbiol., 148: 172-177.

Hammad, A.M.M., 1989. A comparative study of bacteriophage of *Rhizobium leguminosarum* in soils of Egypt and Scotland. Ph.D. Thesis, Faculty of Agricultural, Minia University, University in Minya, Egypt.

Hammad, A.M.M., 1993. Occurrence of bacteriophages of *Bradyrhizobium japonicum* in rhizosphere soil of soybean. Minia J. Agric. Res. Dev., 15: 609-624.

Hammad, A.M.M., 1999. Induction of bacteriophage-resistant mutants of nitrogen fixing and phosphate dissolving bacteria. Ann. Agric. Sci. Cairo, 44: 479-493.

Hegazi, N.A., M.A. Abou-El-Nasr, B.A. Othman and E.K. Allam, 1980. Ecological studies on bacteriophages specific for a number of soil bacteria with particular reference to *Azotobacter*. Proceedings of the 4th Conferene of Microbiology, December 24-28, 1980, Cairo, Egypt, pp: 283-302.

Khan, J.A., 2011. Biodegradation of Azo Dye by moderately halotolerant *Bacillus megaterium* and study of enzyme azoreductase involved in degradation. Adv. Biotechnol., 10: 21-27.

Kiraly, Z., Z. Klement, F. Solymosy and J. Voros, 1970. Methods in Plant Pathology with Special Reference to Breeding for Disease Resistance. 2nd Edn., Academia Kiado, Budapest, Hungary, pp: 183-192.

Lan, R. and P.R. Reeves, 2000. Unique adaptor design for AFLP fingerprinting. Biotechniques, 29: 745-750.

Liu, K. and S.V. Muse, 2005. Power Marker: An integrated analysis environment for genetic marker analysis. Bioinformatics, 21: 2128-2129.

Mahy, B.W.J. and H.O. Kangro, 1996. Virology Methods Manual. Academic Press, New York, USA., ISBN-13: 9780080543581, Pages: 374.

Maniatis, T., E.F. Fritsch and J. Sambrook, 1982. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, New York, Pages: 545.

Marei, E.M., 2013. Isolation and characterization of *Bacillus subtilis* phage from soil cultivated with liquorices root. Int. J. Microbiol. Res., 4: 43-49.

Nei, M., F. Tajima and Y. Tateno, 1983. Accuracy of estimated phylogenetic trees from molecular data: II. Gene frequency data. J. Mol. Evol., 19: 153-170.

Peakall, R. and P.E. Smouse, 2012. GenAlEx 6.5: Genetic analysis in Excel. Population genetic software for teaching and research-an update. Bioinformatics, 28: 2537-2539.
Shimizu, K., H. Nakamura and M. Ashiuchi, 2007. Salt-inducible bionylon polymer from *Bacillus megaterium*. Applied Environ. Microbiol., 73: 2378-2379.

Van Elsas, J.D. and E.G.C. Penido, 1982. Characterization of a new *Bacillus megaterium* bacteriophage, MJ-1, from tropical soil. Antonie Van Leeuwenhoek, 48: 365-371.

Vary, P.S., R. Biedendieck, T. Fuerch, F. Meinhardt, M. Rohde, W.D. Deckwer and D. Jahn, 2007. *Bacillus megaterium*-from simple soil bacterium to industrial protein production host. Applied Microbiol. Biotechnol., 76: 957-967.

Vos, P., R. Hogers, M. Bleeker, M. Reijans and T. van de Lee *et al.*, 1995. AFLP: A new technique for DNA fingerprinting. Nucl. Acids Res., 23: 4407-4414.

Vos, P., G. Garrity, D. Jones, N.R. Krieg and W. Ludwig *et al.*, 2009. Bergey’s Manual of Systematic Bacteriology: Volume 3, The Firmicutes. 2nd Edn., Springer, Berlin, Germany, ISBN-13: 978-0-387-68489-5.

Yoshida, T., Y. Takashima, Y. Tomaru, Y. Shirai, Y. Takao, S. Hiroishi and K. Nagasaki, 2006. Isolation and characterization of a cyanophage infecting the toxic cyanobacterium *Microcystis aeruginosa*. Applied Environ. Microbiol., 72: 1239-1247.

Zayed, G., 1998. Can the encapsulation system protect the useful bacteria against their bacteriophages? Plant Soil, 197: 1-7.