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ISOLATION AND MOLECULAR CHARACTERIZATION OF THREE VIRULENT ACTINOPHAGES SPECIFIC FOR *Streptomyces flavovirens*

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Abstract- Three lytic actinophages specific for *Streptomyces flavovirens* were isolated from Egyptian soil and designated Sf1, Sf2 and Sf3. Phage isolates Sf1, Sf2 and Sf3 produced clear circular single plaques of 2, 1 and 3 mm in diameters, respectively. Thermal inactivation point, dilution end point and longevity *in vitro* of phages Sf1, Sf2 and Sf3 were found to be 86°C, 82°C and 76°C; 10^{-5}, 10^{-3} and 10^{-5}; 7, 6 and 7 days, respectively. The isolated phages were found to be stable at different pH levels ranging from 5 to 13.

Particle size and morphology of each phage isolate were examined by transmission electron microscopy. The three isolated phages were of head and tail type. They varied in their head diameters and tail lengths.

Using RAPD-PCR assay, only seven primers succeeded to generate polymorphic DNA products. Genetic distance value between phages Sf1 and Sf2 was 0.0, phages Sf1 and Sf3 was 1.0 and phages Sf2 and Sf3 was 0.89.

Keywords- Actinophages, RAPD-PCR, *Streptomyces flavovirens*, Transmission electron microscope

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Introduction

Actinomycetes are widely used in biotechnology for production of bioactive compounds. Although actinophages are widely used for typing *Streptomyces* in taxonomic studies [6], some of actinophages can attack antibiotic producing strains resulting in serious problems in pharmaceutical industry [14].

Most actinophages have been isolated from soil in which they are widespread. Methods for the direct isolation of actinophages from soil usually yield extremely low titers [1,32].

The morphology of isolated strains of *Streptomyces* phages have been examined by electron microscopy. These phages have polyhedral heads and non-contractile tails [8], isometric heads and long, apparently non-contractile tails [23], *Streptomyces* phages showed that the phages heads were of an icosahedral form, but some of the phages had a very long tail and another type had a very short tail [3].

The random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) markers, being one of the DNA-based markers, enable random sections of DNA to be rapidly reproduced using short polynucleotide primers. The polymerase chain reaction (PCR)-based RAPD assay was developed by [33,34]. RAPD-PCR has been used to generate specific profiles or genomic fingerprints which are used to compare the genotypic diversity, for example, bacterial isolates [17,24]. RAPD-PCR using purified DNA has been also used to assess the genetic diversity of vibriophages [7,29], phages infecting *E. coli* [11] and *Pseudomonas aeruginosa* [22]. Using this assay, genomic fingerprints from different phages infecting *Bacillus subtilis, Escherichia coli Lactobacillus casei, Lactococcus lactis*, *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Streptococcus thermophilus* bacterial strains were distinct and showed variation in number of bands, fragment size and intensity [13].

The objective of this study is to investigate the presence of actinophages specific for *S. flavovirens* in the Egyptian soils. In addition, characteristics of these phages i.e. plaque morphology, particle size and morphology; thermal inactivation point, dilution end point and longevity *in vitro* as well as genetic similarities were also studied.

Materials and Methods

Source of *Streptomyces* species

Three *Streptomyces* species (*S. parvulus, S. flavovirens and S. corchorusii*) were used in this study. These isolates were obtained from Microbiology Department, Faculty of Agriculture, Ain Shams University, Cairo, Egypt.

Isolation of Lytic Actinophages

A soil sample (fifty grams) was collected 5 cm depth of soil surface from the Horticulture Farm, Faculty of Agriculture, Ain Shams University. The soil sample was collected in sterilized plastic bag and kept at 4°C until use. Actinophages were isolated from the soil sam-

ple as described by [28].

Actinophages Assay

The quantitative assay of the lytic Actinophages in the prepared phage suspension was carried out using plaque assay technique as described by [16]. Phages were distinguished according to the differences in plaque size and morphology. The single plaques isolation (SPI) technique was used to purify the phage isolates.

Determination of Actinophage Stability

Thermal inactivation point (TIP) of each phage isolate was determined as described by [21]; dilution end point (DEP) of actinophage isolates were determined by plaque assay technique according to the method of [16]; and longevity *in vitro* (LIV) of actinophage isolates were determined by spot test technique according to the method of [2]. The effect of different pH values on stability of actinophages were determined by spot test technique according to [12].

Electron Microscopy of Actinophages

The high titer phage suspension of each phage isolate was prepared using liquid culture enrichment technique. Five ml of the high titer phage suspension of each phage was ultracentrifuged at 30.000 rpm for 90 min. at 4°C in a Beckman L7-35 ultracentrifuge. 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 formvar-coated grids and allowed to settle for 1 min. The excess liquid was removed with a filter paper wick. Grids were stained with 2% (W/V) phosphotungstic acid for 15 seconds. The grids were air dried and examined in Jeol-Jem 1010 transmission electron microscope.

Extraction of Actinophages DNA

DNA extraction and purification techniques were carried out as described by [9]. Ten ml of high titer phage suspension were incubated at 37°C for 30 min. with DNase and RNase at a final concentration of 1µg/ml each to get rid of contaminating streptomyces DNA and RNA.

Actinophages were pelleted by centrifugation at 30.000 rpm for 90 min at 4°C in a Beckman L7-35 ultracentrifuge. The actinophage pellet was resuspended in one ml of extraction buffer (100 mM Tris-HCl pH 8.0, 50 mM EDTA and 0.5 M NaCl) then 0.2 ml 20% SDS was added. The mixture was incubated at 65°C in a water-bath for 20 min. One ml of a mixture containing phenol, chloroform and isoamyl alcohol (25: 24: 1) was added. Centrifugation was performed at 10.000 rpm for 10 min at 4°C. The supernatant of each sample was transferred separately into another sterilized tube and then 1 ml of a mixture containing chloroform and isoamyl (24: 1) was added, then centrifugation was carried out at 10.000 rpm for 10 min at 4°C. The supernatant of each sample was transferred into a sterilized tube, then 1 ml of isopropanol was added and the tubes were kept overnight in a freezer, then centrifuged. The DNA-containing pellets were resuspended in 1 ml ethanol then centrifugation was performed at 10000 rpm for 2 min at 4°C. The DNA pellets were resuspended in 80 µl TE buffer (10 mM Tris-HCl pH 8.0 and 1 mM EDTA). DNA concentration was then subjected to further resolving on 1% agarose gel electrophoresis.

RAPD-PCR Analysis

The PCR amplification was performed in a 25 µl reaction volume containing the following: 2.5 µl of dNTPs (2.5 mM), 1.5µl of Mg Cl_2 (25 mM), 2.5 µl of 10x buffer, 2.0 µl of primer (2.5 µM), 2.0 µl of template DNA (50 ng/µl), 0.3 µl of Taq polymerase (5 U/µl) and 14.7 µl of sterile double distilled water. The reaction mixtures were overloaded with a drop of light mineral oil per sample. Amplification was carried out in Techne TC-512 PCR System. The reaction was subjected to one cycle at 95 ºC for 5 min, followed by 35 cycles at 94 ºC for 30 sec, 37 ºC for 30 sec and 72 ºC for 30 sec, then a final cycle of 72 ºC for 12 min. PCR products were run at 100 V for one hr. on 1.4 % agarose gels to detect polymorphism between phage isolates under study. After electrophoresis, the RAPD patterns were visualized with UV transilluminator. RAPD markers were scored from the gels as DNA fragments present or absent in all lanes. Gels were photographed using a Polaroid camera.

Twenty-one 10-mer random DNA oligonucleotide primers were independently used in the PCR reaction. Only seven primers [Table -1] able to produce polymorphic DNA bands. These primers were synthesized by (Operan biotechnologies, Inc.,Germany).

Table 1- Names and nucleotide sequences of the used primers

	Name	Sequence
1	OP- AX16	5´ GTC TGT GCG G 3`
2	OP-C02	5´ ACG GCG TAT G 3`
3	OP-E03	5´ CCA GAT GCA C 3`
4	OP-G05	5` TGG TGG ACC A 3`
5	OP-I17	5` TGG TGG ACC A 3`
6	OP-L 12	5` TGG TGG ACC A 3`
7	OP-M01	5` GTT GGT GGC T 3`

Statistical Analysis

The DNA bands generated by each primer were counted and their molecular sizes were compared with those of the DNA markers (1500-1000-900-800-700-600-500-400-300-200-100bp). The bands scored from DNA profiles generated by each primer were pooled together. Then the presence or absence of each DNA band was treated as a binary character in a data matrix (coded 1 and 0, respectively) to construct dendrogram tree and to calculate genetic similarity among the studied three phage isolates. Similarity coefficients were calculated according to [10] as implemented in the computer program SPSS-10.

Results

Biological Characters of *Streptomyces flavovirens* Phages

Isolation and Qualitative Assay of Virulent Phages

Three virulent phages specific for *Streptomyces flavovirens* were isolated from a soil sample collected from the Horticulture Farm, Faculty of Agriculture, Ain Shams University. The phages were isolated using Single Plaque Isolation Technique, depending upon plaque size and morphology. The isolated phages were designated Sf1, Sf2 and Sf3.

These phages were not infectious to *S. parvulus* and *S. corchorusii.* As shown in [Fig-1] the single plaques formed by phages Sf1, Sf2 and Sf3 were found to be clear circular shape of 2, 1 and 3 mm in diameter, respectively.

Determination of Stability of Phages

The phage isolates of *S. flavovirens* varied in their thermal inactivation points (TIP), dilution end points (DEP) and their longevity *in vitro*. The three isolated phages were found to be stable at different pH levels ranging from 5 to 13. The recorded values of thermal inactivation points (TIP) were 86°C, 82°C and 76°C; dilution end points (DEP) were 10^{-5}, 10^{-3} and 10^{-5} and the longevity *in vitro* were 7, 6 and 7 days for phage isolates Sf1, Sf2 and Sf3, respectively.

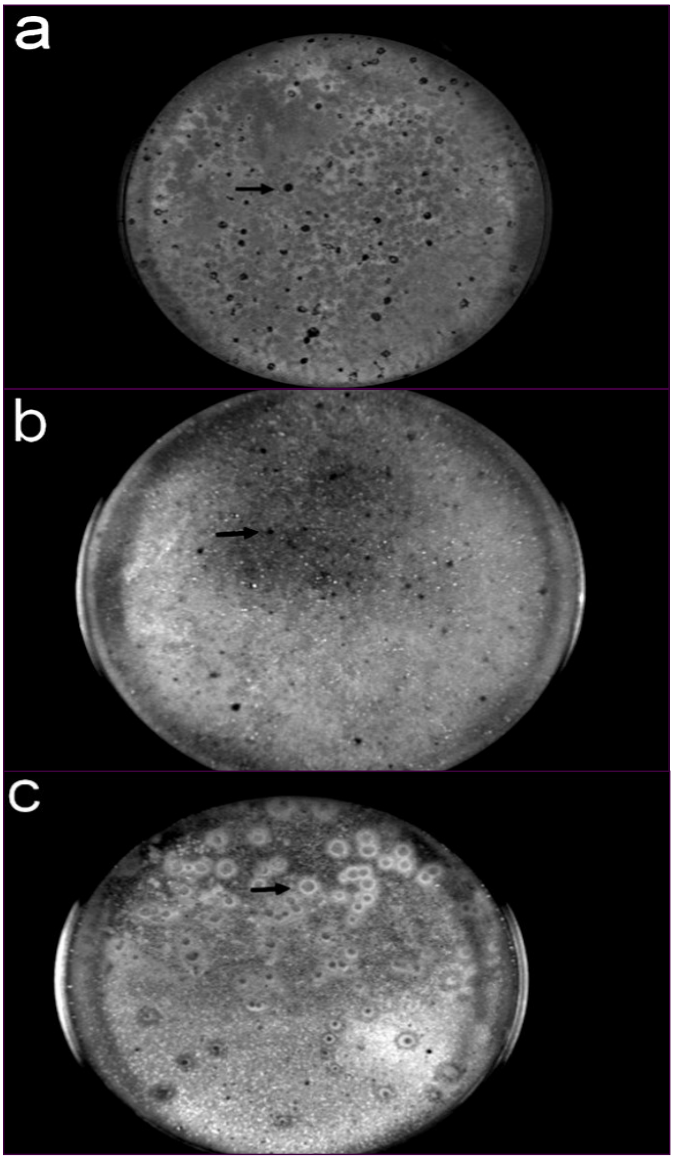

Fig. 1- Single Plaques produced by the three lytic actinophages of *S. flavovirens*, (a) phage Sf1, (b) phage Sf2 and (c) phage Sf3. The single plaques are indicated by arrows.

Morphological Characters of *S. flavovirens* Phages

As shown in [Fig-2], electron micrographs of purified virulent phages specific for *S. flavovirens*, showed tadpole shaped particles. Phages had isometric particles with long contractile tail. The head diameters of the three *S. flavovirens* phages were 70, 60 and 80 nm and their tail dimensions were 170 x 14, 180 x 20 and 200 x 20 nm for phages Sf1, Sf2 and Sf3, respectively.

Molecular Analysis of *S. flavovirens* Phages

RAPD Assay

The random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) was used to determine the polymorphic DNA products and similarities of genetic distances of the three *S. flavovirens* lytic phages under study. Twenty one 10-mer arbitrary oligonucleotide primers were used to establish RAPD-PCR fingerprints of these three *S. flavovirens* phages. Only seven primers succeeded to generate reproducible polymorphic DNA products. Each of the seven primers displayed a strong and distinct amplified bands profile for each of the studied isolates. The number and size of the amplified products varied considerably from primer to another. The total amplified products were 80 DNA bands, generated by OP-AX16, OP-C02, OP-E03, OP-G05, OP-L17, OP-L12 and OP-M01 primers. Of these bands, 44 bands were found to be unique or polymorphic bands, while 36 bands were monomorphic [Fig-3]. The size of the amplified fragments ranged from 160 to 1940 bp.

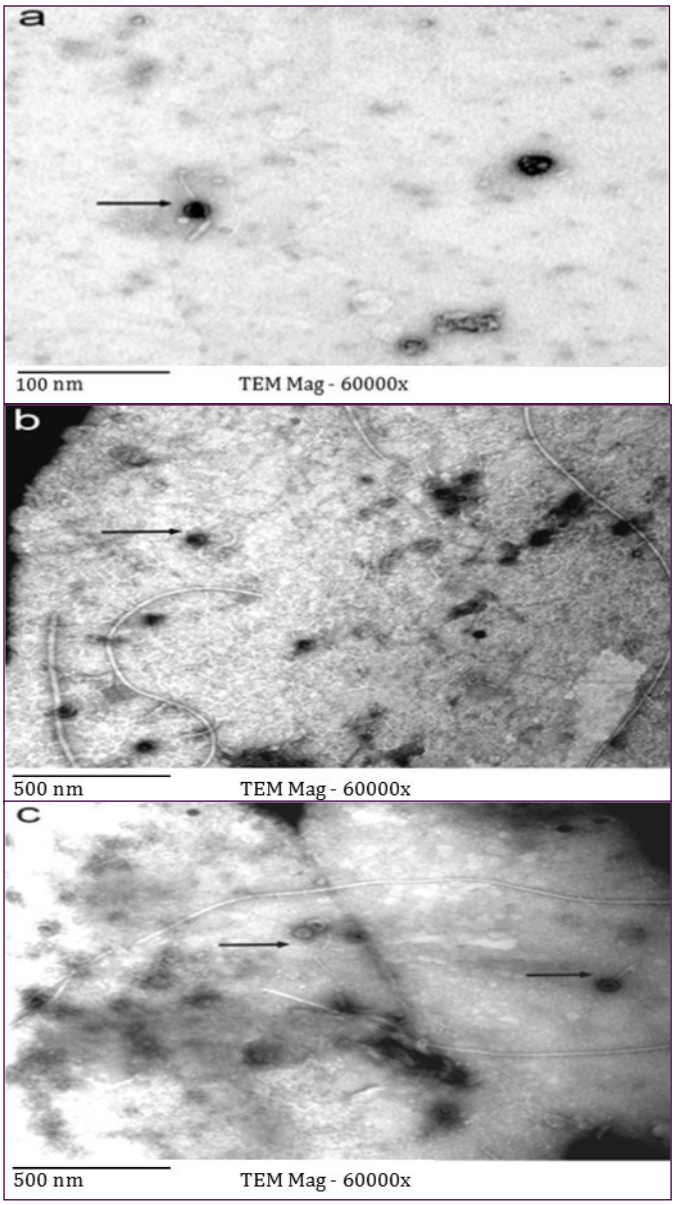

Fig. 2- Electron micrographs of the lytic actinophages; (a) phage Sf1, (b) phage Sf2 and (c) phage Sf3 specific for *S. flavovirens* , negatively stained with (2% w/v) phosphotengestic acid. Phage particles are indicated by arrows.

For OP-AX16 primer, ten DNA bands were appeared, their sizes ranged from 245 to 1540 bp. Three DNA bands with sizes of 900, 1000 and 1160 bp were polymorphic, since they were observed in two and disappeared in one of the used phage isolates. Two DNA bands were considered as unique bands, the first (1540 bp) was detected only in Sf1 phage isolate and the second (245 bp) was appeared only in Sf2 phage isolate. The remaining five bands with sizes of 370, 480, 530, 660 and 800 bp were monomorphic bands while they were presented in all the three phage isolates.

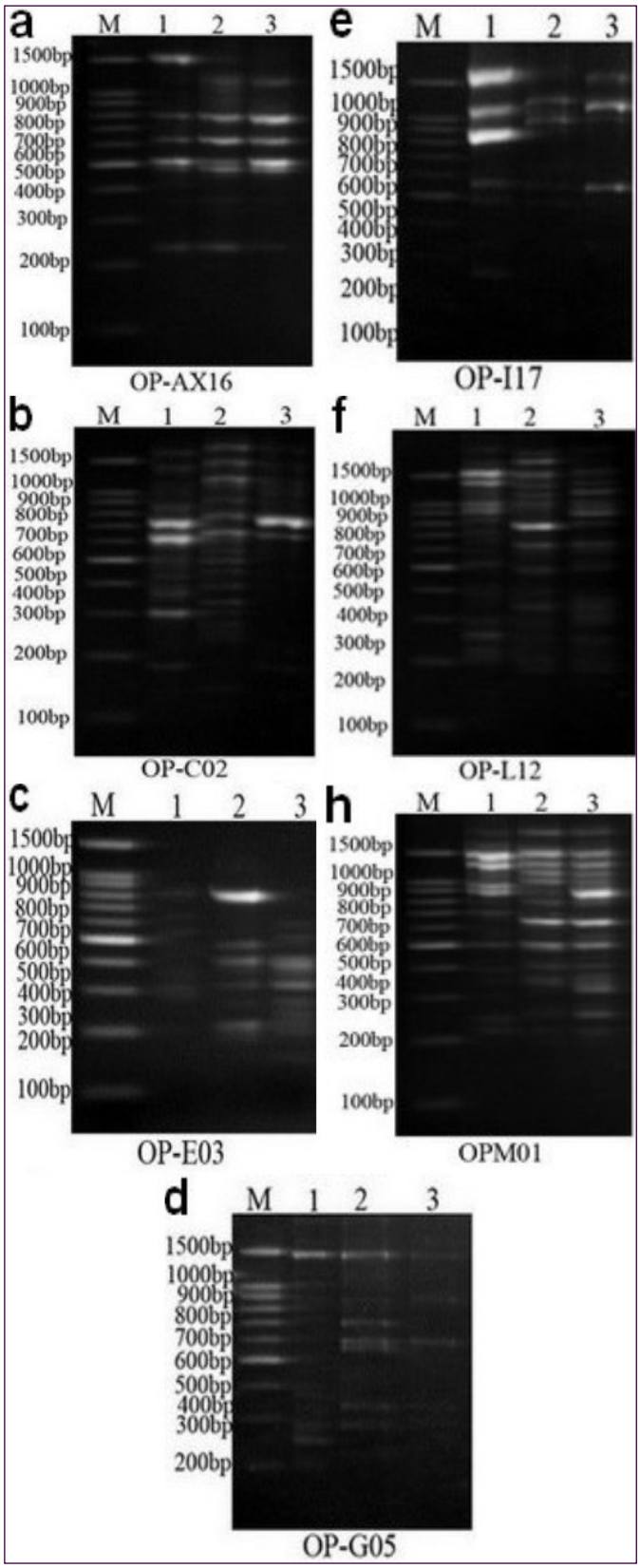

Fig. 3- RAPD patterns with primers(a) OP-AX16 , (b) OP-C02, (c) OP-E03, (d) OP-G05, (e) OP-I17, (f) OP-L12 and (h) OP-M01. Lane M : DNA ladder; lane 1: phage Sf1; lane 2: phage Sf2 and lane3: phage Sf3.

According to OP-C02 primer, thirteen DNA bands ranged in size between 180 and 1690 bp were appeared, out of them four bands were polymorphic and five were monomorphic. Only four bands with sizes of 180, 300, 400 and 590 bp were unique bands, three of them (180, 300and 590 bp) were appeared in Sf2 phage isolate but one band with size of 400 bp was detected in phage isolate Sf1.

Regarding the DNA primer OP-E03, only nine DNA bands were observed, their sizes ranged between 160 and 830 bp. Five DNA bands were polymorphic and two were monomorphic. Two DNA unique bands (160 and 265 bp) were presented, both DNA bands were found only in the phage isolate Sf3.

For OP-G05 primer, ten bands were recorded, the size of these bands ranged from 210 to 1540 bp. Out of them four bands were polymorphic and five were monomorphic. Only one band with size of 600 bp proved to be unique band, it was appeared only in the phage isolate Sf2.

Also, OP-I17 primer amplified only eight DNA bands with molecular size ranged between 250 and 1590bp. Four bands were polymorphic and three were monomorphic bands. The band of 825 bp proved to be the only unique band generated by this primer and appeared only in the Sf1 phage isolate.

Moreover, primers OP-L12 and OP-M01 generated the highest number of bands (15 bands for each). The size of the bands generated with the OP-L12 primer ranged between 190 and 1940 bp. Five bands were polymorphic and eight bands proved to be monomorphic. Only two bands with sizes of 420 and 1750 bp were unique bands and appeared only in the phage isolate Sf2. On the other side, OP-M01 primer generated bands with sizes ranged between 220 and 1860 bp. Five polymorphic and eight monomorphic DNA bands were recorded. Only two DNA bands with sizes of 290 and 690 bp proved to be unique, presented in phage isolate Sf1. On the basis of the obtained results, 80 amplified DNA bands were generated after the application of the used seven RAPD primers. Out of these bands, 30 were polymorphic and 14 were unique polymorphic bands representing a polymorphism precentage of 55 %. The other 36 bands proved to be monomorphic, since they were appeared in all phage isolates.

Data presented in [Table-2] showed that 14 unique DNA bands were recorded using the seven primers.

Table 2-The unique amplified DNA bands produced by the used primers.

Primers	No. of unique bands	Band size (bp)	Sf1 phage	Sf2 phage	Sf3 phage
OP-AX16	2	1540	1	0	0
		245	0	1	0
OP-C02	4	590	0	1	0
		400	1	0	0
		300	0	1	0
		180	0	1	0
OP-E03	2	265	0	0	1
		160	0	0	1
OP-G05	1	600	0	1	0
OP-I17	1	825	1	0	0
OP-L12	2	1750	0	1	0
		420	0	1	0
OP-M01	2	690	1	0	0
		220	1	0	0

Genetic distances relationship among the three phage strains were studied by Dendogram based on their similarity indices data of RAPD analysis [Fig-4] and [Table-3]. Genetic distance value between phage 1Sf and 2Sf was 0.0 and phage 1Sf and 3Sf was 1.0 while the genetic distance value between phage 2Sf and 3Sf was 0.89.

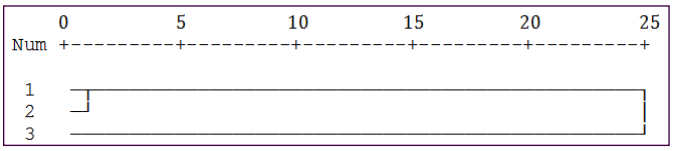

Fig. 4- Dendrogram for the relationships of genetic distances among three phage strains based on similarity indices data of RAPD analysis.

Table 3- Similarity indices among three phage strains based on RAPD analysis

	Sf1	Sf2
Sf2	0	
Sf3	1	0.89

Discussion

Three actinophage isolates specific for *S. flavovirens* were isolated from Egyptian soil and designated Sf1, Sf2 and Sf3. In this study, the observed plaque sizes and morphologies of phages Sf1, Sf2 and Sf3 were found to be similar to those isolated by [3,21,23]. The plaque characters may be affected by some factors such as phage isolate, age of indicator host and presence of host debris as reported by [16,15]. The thermal inactivation point of *S. flavovirens* phages Sf1, Sf2 and Sf3 were found to be 86°C, 82°C and 76°C; dilution end point were 10^{-5}, 10^{-3} and 10^{-5} as well as longevity *in vitro* for the three phages were estimated to be 7, 6, and 7 days, respectively.

The three isolated phages of *S. flavovirens* were stable at pH ranging from 5 to 13. These results are relatively in agreement with those obtained [21], but the stability of some actinophages was recorded at pH ranging from 5.5 to 9.0 and rapidly inactivated at pH 4 and below. The rapid inactivation of phages at the low pH levels may be due to the acid denaturation of protein [30]. Moreover, the adsorption of phages attacking a variety of hosts was influenced by pH value including *Escherichia coli* [27] and certain *Salmonella* species [31]. In addition, [29] reported that actinophages were inactivated at pH higher than 9.0.

The electron micrographs of the three phage isolates (Sf1, Sf2 and Sf3) indicated that, all phages had isometric heads and contractile tails, but they varied in their head diameters and length of tails. These results are in agreement with those of [3,8,19,21,23,26].

RAPD analysis has been used to differentiate between closely related six *Leuconostoc fallax* bacteriophages isolated from industrial sauerkraut fermentation [5]. Moreover, this technique has been used to make fingerprint of 10 isolated phages against *Escherchia coli* (ETEC) [18]. In this study, RAPD analysis of phage DNA provided a simple and reproducible method for differentiation of three virulent actinophage isolates specific for *S. flavovirens*. Data showed that the 44 bands are polymorphic (14 unique and 30 polymorphic), while 36 bands are monomorphic**.** The size of amplified fragments ranged from 160 bp to 1940 bp.

From the molecular genetic point of view, the unique DNA bands are considered of special interest, while they identify special characters for specific phage isolates.

For Sf1 phage isolate, a total of five unique DNA bands was found, these bands were generated by the application of four RAPD primers. The size of these DNA fragments ranged between 220 bp by OP-M01 primer and 1540 bp by the RAPD primer OP-AX16. These fragments may carry gene(s) which are responsible for the thermal inactivation point and longivity *in vitro*.

Regarding Sf2 phage isolate, seven unique DNA bands were recorded, their sizes ranged from 180bp, generated by OP-C02 primer to 1750 bp by primer OP-L12. Also, some or all of these seven DNA fragments may carry gene(s) responsible for the thermal inactivation point and longivity *in vitro* of Sf2 phage.

Finally, for the phage isolate Sf3, only two unique DNA bands were recorded. Both DNA fragments were generated by the same RAPD primer (OP-E03) and their sizes ranged between 160 and 265 bp. One or both DNA fragments may contain gene(s) which are responsible for the thermal inactivation point and longivity *in vitro* of Sf3 phage.

Genetic distance value percentage was studied between phage Sf1and Sf3 showed maximum genetic distance estimates (100%), genetic distance between phage Sf2 and Sf3 was 89.0%, while genetic distance percentage between phage Sf1 and Sf2 was 0.0% *i.e.* no genetic distance was found. This method has been used extensively for microbial characterization and differentiation between bacteriophage isolates [5,20,25]. The simplicity and applicability of the RAPD technique have captivated many scientists' interests. Perhaps the main reason for the success of RAPD analysis is the gain of a large number of genetic markers that require small amounts of DNA without the requirement for cloning [4]. To the best of our knowledge, this is the first report on isolation and characterization of three lytic phages specific to *S. flavovirens* from Egyptian soil.

Conclusion

Generally, on the basis of the obtained results it can be concluded that, the Egyptian soils were found to contain lytic actinophages specific for *Streptomyces flavovirens*. Three lytic actinophages were successfully isolated and designated Sf1, Sf2 and Sf3. The isolated phages were of head and tail type. Thermal inactivation point of the isolated phages ranged between 76°C and 86°C ; dilution end points ranged between 10^{-3} and 10^{-5} and their longevity *in vitro* were 6 to 7 days. Genetic distance value between phages Sf1 and Sf2 was 0.0, phages Sf1 and Sf3 was 1.0 and phages Sf2 and Sf3 was 0.89.

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References

[1] Ackermann H.W., Berthiaume L. and Jones L.A. (1985) *Inter. Virology,* 23, 121-130.

[2] Ackermann H.W. and Dubow M.S. (1987) *Viruses of Prokaryotes,* CRC Press, Boca Raton.

[3] Anne J., Wohlleben W., Burkardt H.J., Springer R. and Puhler A. (1984) *J. Gen. Microbiol.,* 130, 2639-2649.

[4] Bardakci F. (2001) *Turk. J. Biol.,* 25,185-196.

[5] Barrangou R., Yoon S.S., Breidt F.Jr., Fleming H.P. and Klaenhammer T.R. (2002) *Appl. Environ. Microbiol*., 68(11), 5452-5458.

[6] Bradley S.G. and Ritzi D. (1967) *J. Gen. Virol.,* 1, 285-290.

[7] Comeau A.M., Chan A.M. and Suttle C.A. (2006) *Environ. Microbiol.,* 8, 1164-1176.

[8] Coyette J. and Clairem C. (1967) *J. Gen. Virol.,* 1, 13-18.

[9] Dellaporta S.L., Wood J. and Hicks J.B. (1983) *Plant Mol. Biol.,*

1, 19-21.

[10]Dice L.R. (1945) *Ecology,* 26, 297-302.

[11]Dini C. and Urraza P.J. (2010) *J. App. Microbiol.,* 109, 873-887.

[12]Flowers T.H. and Williams S.T. (1977) *Microbios,* 18, 223-228.

[13]Gutiérrez D., Martín-Platero A.M., Rodríguez A., Martínez-Bueno M., García P. and Martínez B. (2011) *FEMS Microbiol. Lett.,* 322(1), 90-97.

[14]Hamedi J., Nahvinia H. and Amoozegar M. (2006) 11*th International Symposium on Microbial Ecology, Vienna, Austria.*

[15]Hammad A.M. (1989) *A Comparative Study of Bacteriophage of Rhizobium leguminosarum in Soils of Egypt and Scotland*, Ph.D. Thesis, Fac. Agric., Minia. Univ.

[16]Hopwood D.A., Bibb M.J., Chater K.F., Janssen G.R., Malpartida F. and Smith C.P. (1985) *Regulation of Gene Expression*, Cambridge University Press, Cambridge, 251-276.

[17]Johansson M.L., Quednau M., Molin G. and Ahrné S. (1995) *Lett. Appl. Microbiol.,* 21, 155-159.

[18]Jothikumar N., Reddy C.G., Sundari R.B. and Saigopal D.V.R. (2000) *J. Environ. Monit*., 2, 372-374.

[19]Kolstad R.A. and Bradley S.G. (1966) *J. Bacteriol.,* 91(3), 1372-1373.

[20]Kumari S., Harjai K. and Chhibber S. (2009) *Am. J. Biomed. Sci.,* 1(2), 91-102.

[21]Lawrence H.M., Merivuori H., Sands J.A. and Pidcock K.A., (1986) *Appl. Environ. Microbiol.,* 52(4), 631-636.

[22]Li L., Yang H., Lin S. and Jia S. (2010) *Can. J. Microbiol.,* 56, 925-933.

[23]Luis A.D., Hardisson C. and Rosario Rodicio M. (1989) *J. Gen. Microbiol.,* 135, 1847-1856.

[24]Maiti B., Shekar M., Khushiramani R., Karunasagar I. and Karunasagar I. (2009) *J. Genet.,* 88, 273-279.

[25]Mileham A.J. (1997) *Molecular Biotechnology*, 8(2),139-145.

[26]Painter B.G. and Bradley S.G. (1965) *J. Bacteriol.* 89, 240-244.

[27]Puck T.T. and Tolmach L.J. (1954) *Arch. Biochem.,* 51, 229-245.

[28]Shirley L. and Williams S.T. (1982) *J. Gen. Microbiol*., 128, 2063-2071.

[29]Shivu M.M., Rajeeva B.C., Girisha S.K., Karunasagar I., Krohne G. and Karunasagar I. (2007) *Environ. Microbiol.,* 9, 322-331.

[30]Sykes I.K., Lanning S. and Williams S.T. (1981) *J. Gen. Microbiol*., 122, 271-280.

[31]Takeda K. and Uetake H. (1973) *Virology,* 52,148-159.

[32]Tan J.S.H. and Reanney D.C. (1976) *Soil Biology and Biochemistry*, 8, 145-150.

[33]Welsh J. and McClelland M. (1990) *Nucl. Acids Res*., 18, 7213-7218.

[34]Williams J.G.K., Kubelik A.R., Livak K.J., Rafalski J.A. and Tingey S.V. (1990) *Nucl. Acids Res.,* 18, 6531-6535.