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Characterization of Some Induced Phages Specific for Two Bacteria Isolated from Potato Naturally Infected with Soft Rot Disease

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ABSTRACT

Background: U.V irradiation was used for indication of temperate phages of *Serratia marcescens*. The RAPD-PCR was used for quick typing detection of phage isolates from different bacteria.
Aims: This study aims to isolate and identify some pathogenic bacteria from potato naturally infected with soft rot disease. In addition, the temperate phages were induced by U.V irradiation from the isolated pathogenic bacteria.
Materials and Methods: U.V irradiation was used to isolate temperate phages from the pathogenic bacteria. Biological and molecular experiments were used to characterize and identify the induced phage isolates.
Results: Two bacterial isolates were isolated from a potato tuber showing soft rot symptoms. These two isolates were identified as *Serratia marcescens ss marcescens* and *Entrobacter cloacae ss dissolvens.* Both of the isolated bacteria were found to be lysogenic bacteria. Two temperate phages designated SmI and EdI were induced from *Serratia marcescens ss marcescens* and *Entrobacter cloacae ss dissolvens,* respectively, using U.V irradiation. Electron micrographs of the two induced phages indicated that the particles of both phages have hexagonal icosahedral heads and contractile tails. The two induced phages were found to have a narrow host range. In RAPD-PCR, sixteen bands were unique polymorphism DNA products with six primer used. The size of all the amplified fragments ranged from 288 to 1588 bp.
Conclusion: The results of this study indicated that *Serratia marcescens ss marcescens* and *Entrobacter cloacae ss dissolvens* were found to contain prophages. The temperate phages were induced by U.V irradiation from *Serratia marcescens ss marcescens* and *Entrobacter cloacae ss dissolvens* and designated SmI and EdI, respectively.
KEY WORDS: Soft rot bacteria, Temperate phages, U.V irradiation, cytopathic effect, Biological properties and RAPD-PCR.

INTRODUCTION

Bacterial diseases cause significant loss of plant productions during cultivation and storage. Some members of family *Enterobacteriaceae*, such as *Dickeya dadantii* sp. **[1],** formerly known as *Erwinia chrysanthemi* **[2],** and *Pectobacterium chrysanthemi,* cause soft rot disease in a wide range of crop plants. Infection of seed tubers by pectinolytic *Erwinia* species can lead to the development of various symptoms during vegetative growth of potato crops, including non- emergence of plants, chlorosis, wilting and typical blackleg **[3].** Bell pepper is affected by several bacterial diseases such as *Xanthomonas spp*. **[4]** and particularly by soft rotting bacteria such as *Ralstonia, Pectobacterium and Dyckeya*. The disease is becoming a serious problem and causing reductions in yield and fruit quality **[5].**

Also, **[6]** mentioned that *serratia marcescens* infected bell pepper and caused soft rot disease. However, isolation and identification of two isolates belonging to genus *Enterobacter mori sp.* from root mulberry pathogenic bacteria by **[7]**, these bacteria associated with bacterial caused wilt symptoms and the plants eventually became defoliated. U.V irradiation was for induce the *Serratia marcescens* to produce bacteriocins and phages **[8]**. Also, the *serratia* phage η was originally characterized as a temperate *siphovirus* possessing hypermodified guanine residues in its DNA **[9]**.

Also**, [10]** isolated tailed phages different bacteria belong to the *Enterobacteriaceae*. These phages were 32 lytic and 24 temperate and described to these fully sequenced genomic for all isolates, that have similarity percentage over >50% of the genomes within each cluster, but substantially less sequence similarity between clusters.

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The use of RAPD-PCR for quick typing, study the detection and genetic diversity of phage isolates from different bacteria**[11].**

The present investigation aims to isolate and identify some pathogenic bacteria that responsible for potato soft rot disease. In addition, induction and characterization of temperate phages from the isolated bacteria were also carried out.

MATERIALS AND METHODS

1- Bacteria used:

a- Soft rot bacteria were isolated from naturally infected potato tubers showing soft rot symptoms and yellow ooze obtained from El-Mansoura-Egypt as described by **[12,13]**.

A sample of ten grams of soft tissue and ooze was suspended in 100 ml distilled sterilized water. A loop full of the prepared suspension was streaked on a plate containing D3 medium PH 7 according to **[12,13]**, and incubated at 28-30ºC for 48 hrs. Two single colonies showing different morphologies were transferred from the plate into test tubes containing slant surfaces of nutrient agar medium. The slant tubes were incubated for 48 hrs at 28-30ºC, then kept at 4ºC. The isolated bacteria were identified by the **[14].**

b- *Erwinia Carotovora* sub sp. *Carotovara* ATCC 1063 was kindly supplied by Cairo MERCIN, Faculty of Agriculture, Ain Shams University, Cairo, Egypt.

2- Induction of temperate phages from soft rot bacterial strains:

Temperate phages were induced by U.V from the isolated soft rot bacteria according to **[8]**. For phage detection agar double layer plates were prepared and incubated at 28-30 ºC for 48 hrs as described by **[15]**. Plates were exposed to U.V irradiation (240 nm) at distance of 60 cm for 5 -30 mins., ascending 5 mins. Three ml of nutrient broth were placed in each plate followed by incubation at 28-30 for 48 hrs. The nutrient broth in each plate was collected and centrifuged at 5000 rpm for 15 min. the phages were assayed qualitatively and quantitatively.

3- Stock of high titer phages suspension:

Liquid enrichment technique was used to prepare high titer suspensions of the induced phages, according to **[16].**

4- Host Range Assay:

The host range of each induced phage isolate was determined using the spot test. Each of the phage isolates was tested against three soft rot bacterial strains as indicator host in individual plates. After incubation for 24-48 hrs at 28-30°C, plates were inspected for the lysed spots.

5- Effect of some physical and chemical factors on the induced phage:

a- Physical factors

Stability of the induced phages to different concentration of hydrogen ions ranging from pH 4-12 was studied. Effect of different concentrations of sodium chloride solutions (2, 4, 6 and 8 M) on the induced phages also studied. The thermal inactivation points (TIP) of the induced phages were determined as described by **[17].** The dilution end points (DEP) and longevity *in vitro* (LIV) of the induced phages were determined according to **[18].**

b- *Chemical factors

Formaldehyde and Chloroform

Formaldehyde was added at rate of 1.5, 2.5, 3.5 and 4.5 % and chloroform was added at rate of 0.1,0.2 ,0.3,… up to 1 % to Eppendorf tubes each containing 1 ml of each induced phage suspension (10^6 pfu/ml). Eppendorf tubes were incubated for 1 hr. (in case of formaldehyde) and 24 hrs (in case of chloroform) at 30°C. Tubes were centrifuged at 6000 rpm for 15 mins. Phages in the above layers were assayed qualitatively by spot test. After incubation for 24-48 hrs at 28-30°C, plates were inspected for the lysed spots.

Ethanol, Sodium dodecyl sulfate (SDS) and Ascorbic acid

One ml of each induced phage suspension (10^6 pfu/ml) was added to 9 ml of ethanol with different concentration ranging from 30 -70 %, 4 ml of 0.1, 0.2, 0.3, 0.4,.. upto 1% of SDS and 9 ml of 0.04, 0.08, 0.12, 0.16 and 0.2% of ascorbic acid. The mixture was incubated for 24 hrs, (in case of ethanol and SDS) and 1 hr (in case of ascorbic acid) at 30°C. Samples were assayed qualitatively by spot test for each treatment. After incubation for 24-48 hrs at 28-30°C, plates were inspected for the lysed spots.

*All chemicals were used obtained from Gmehourea company, Egypt.

6-Electron microscopy:

a- Negative staining

High titer suspensions of the induced phages were prepared using the liquid culture method for phage propagation. Phages were precipitated using Backman L 7-35 ultracentrifuge at 30.000 rpm for 1.5 hrs at 4°C. The pellets were gently suspended in 0.2 M phosphate buffer saline (pH 7.2). A drop of the purified virus was placed on coated grid and stained with 2% (w/v) phosphotungstic acid. The grid was examined in Jeol-Jem 1010 transmission electron microscope.

b-Cytopathic effect of induced phages on soft root bacterial cells

The pellet of lysed cells which treated with induced phages were fixed and ultra thin section was done examined in Jeol-Jem 1010 transmission electron microscope according to **[19].**

7-DNA Extraction and RAPD-PCR of induced Phages:

DNA of both induced phage isolates was extracted and purified as described by **[20].**

8- Determination of DNA concentration:

DNA concentration was determined estimated using (Infinite®200 PRO multimode microplate readers, Agriculture genetic engineering research institute) at 260 nm and 280 nm absorbance.

9- Random Amplified Polymorphic DNA (RAPD-PCR) Analysis:

In order to obtain clear reproducible amplification products, different preliminary experiments were carried out in which number of factors were optimized. These factors included PCR temperature cycle profile and concentration of each of the template DNA, primer, MgCl2 and Taq polymerase. A total of ten random DNA oligonucleotide primers were independently used according to **[21]** in the PCR reaction. PCR amplification was performed using ten random 10 mer arbitrary primers, synthesized by operon biotechnologies, Inc. Germany tables (1 and 2) with the following show the used primers and their sequences.

Table (1): List of the primer names matched and their nucleotide sequences used in the study

No	Name of primers	Sequences
1	OPAN – 12	5¯AACGGCGGTC3¯
2	OPAM – 07	5¯ AACCGCGGCA3¯
3	OPT – 20	5¯AAAGTGCGGG3¯
4	OPF – 17	5¯ AACCCGGGAA3¯
5	OPT – 08	5¯ AACGGCGACA3¯

Table (2): List of the primer names non-matched and their nucleotide sequences used in the study

No	Name of primers	Sequences
1	OPAD – 16	5¯AACGGGCGTC3¯
2	OPAS – 16	5¯AACCCTTCCC3¯
3	OPAA – 14	5¯ AACGGGCCAA3¯
4	OPC – 11	5¯ AAAGCTGCGC3¯
5	OPS – 14	5¯ AAAGGGGTCC3¯

The PCR was carried out in Techne TC-512 PCR System. The cycle condition of PCR according by **[21].** PCR products were separated at 100 V for one hour on 1.0 % agarose gels after that were visualized with UV trans-illuminator and detected for presence or absence of bands **[21]** to determine the polymorphism bands between phage isolates under study. Gels were photographed using a Polaroid camera. The DNA bands existed molecular sizes for each induced phage isolates were compared with those of the DNA markers and analyzed by Totallab120 program.

RESULTS

Isolation and identification of soft rot bacteria:

Two isolates of soft rot bacteria were isolated from soft tissue and ooze of infected tubers on plates containing D3 agar medium. The two isolates were indentified by Biolog method. These isolates were found to be *Serratia marcescens ss marcescens* and *Entrobacter cloacae ss dissolvens*.

Induced phages of *Serratia marcescens ss marcescens* and *Entrobacter cloacae ss dissolvens*:

The spot tests showed that two induced phages were successfully isolated from *Serratia marcescens ss marcescens* and *Entrobacter cloacae ss dissolvens* after the induction by U.V irradiation (240 nm) in figure (1 A & B). These two induced phages of *Serratia marcescens ss marcescens* and *Entrobacter cloacae ss dissolvens* were designated SmI and EdI, respectively.

On the other hand, no prophage was detected inside *Erwinia Carotovora* sub sp. *Carotovara* ATCC 1063.

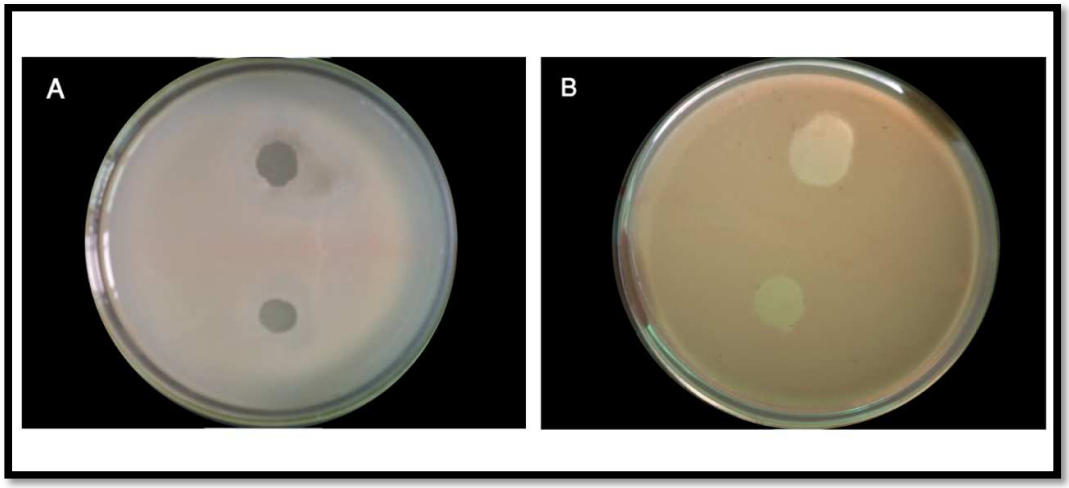

Figure (1): Spot test of induced phages specific to *Serratia marcescens ss marcescens* (SmI-A and *Entrobacter cloacae ss dissolvens* (EdI- B). Lysis of the bacterial lawn can be clearly seen (SmI-A).

Purification of induced phages:

The single plaque isolation technique was used to purify and isolate single phage isolates. A typical plate containing single plaques is shown in figure (2 A & B). One single plaque, for each bacterial isolate (*Serratia marcescens ss marcescens* and *Entrobacter cloacae ss dissolvens*) was picked and kept as single phage isolate. The single plaques were found to be clear circular shape of 2 and 3 mm in diameters for SmI and EdI phages, respectively.

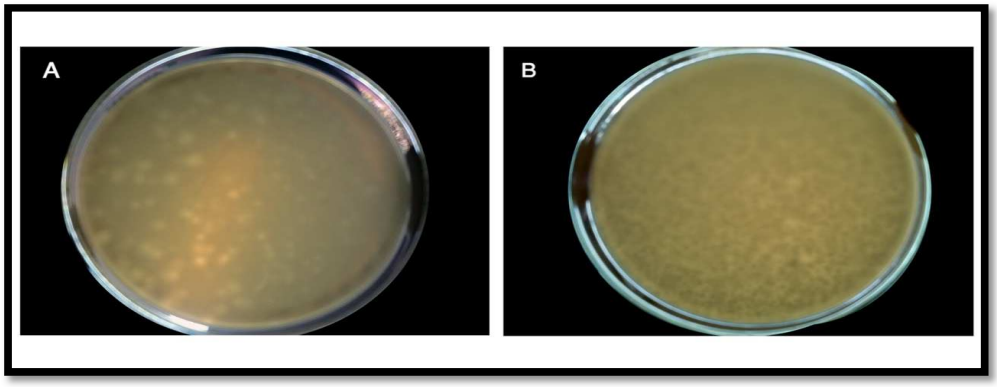

Figure (2): Double layer agar plates prepared with induced phages lysate of *Serratia marcescens ss marcescens* (SmI- A) and *Entrobacter cloacae ss dissolvens* (EdI- B).

Host range of the induced phage isolates:

The induced phage SmI was infectious only to *Serratia marcescens ss marcescens* among the three bacterial strains tested. Moreover, phage EdI was found to be infectious only to *Entrobacter cloacae ss dissolvens* in table (3).

On the other hand, *Erwinia Carotovora* sub sp. *Carotovara* ATCC 1063 exhibited resistance to both induced phages. These results indicate that the two induced phages have a narrow host ranges.

Table (3): Host range of induced phage SmI and EdI isolates

Soft root bacterial strains	Induced phage (SmI)	Induced phage (EdI)
Serratia marcescens ss marcescens	+	-
Entrobacter cloacae ss dissolvens	-	+
***Erwinia Carotovora* sub sp. *Carotovara* ATCC 1063**	-	-

+ = Lysis - = No lysis

Characterization of the induced phages:

Data presented in table (4), indicate that the thermal inactivation point (TIP) of the induced phages SmI and EdI were found to be 66°C and 54°C, respectively.

Moreover, the dilution end points (DEP) were estimated to be 10^{-5} and 10^{-4} for SmI and EdI phages, respectively. In addition, the longevity *in vitro* (LIV) for SmI was 8 days, and 4 days for EdI.

The induced phages were found to be tolerant to wide range of pH 6 to12 for SmI and pH 8 to10 for EdI for 24 hrs. and stable in NaCl concentration of 2 to 8 M and 2 to 6 M after 1 hr incubation for SmI and EdI, respectively.

Also, data presented in table (4), showed that induced phage isolates SmI and EdI were found to be stable after treatment with formaldehyde concentrations (1.5 – 3.5 % for SmI and 1.5 – 2.5 % for EdI after 1 hrs incubation).

Moreover, the induced SmI and EdI phages were inhabited at 0.8 % and 0.5 % chloroform after incubation for 24 hrs., respectively. Ethanol inactivated SmI and EdI at concentrations of 70 % and 40 % after incubation for 24 hrs., respectively. The induced phages were found to be tolerant to wide range of SDS at concentration of 0.1-0.9% and 0.1-0.5% after incubation for 24 hrs, and ascorbic acid at 0.04- 0.08 % and 0.04 % after 1 hrs incubation for SmI and EdI, respectively.

Table (4): Physical and chemical stability of induced phage isolates

Induced phages	TIP/ °C	LIV/ Days	DEP	PH range	Osmotic chock/ range	Formaldehyde/ range	Chloroform / %	Ethanol/ %	SDS/ range	ascorbic acid/ range
SmI	66°c	8	10^{-5}	6to12	2 to 8 M	1.5 – 3.5 %	0.8 %	70 %	0.1-0.9%	0.04- 0.08 %
EdI	54°C	4	10^{-4}	8to10	2 to 6 M	1.5 – 2.5 %	0.5 %	40 %	0.1-0.5%,	0.04 %

Morphological characters of the induced Phage isolates:

As shown in figure (3 A & B) the induced phages have head and tail. The SmI and EdI phages were found to have hexagonal icosahedral heads of 60 and 80 nm in diameter and contractile tails of 160.0 and 180.0 nm in length, respectively.

On the basis of the particle morphology of both phages, they seem to be belonging to family *Myoviridae*.

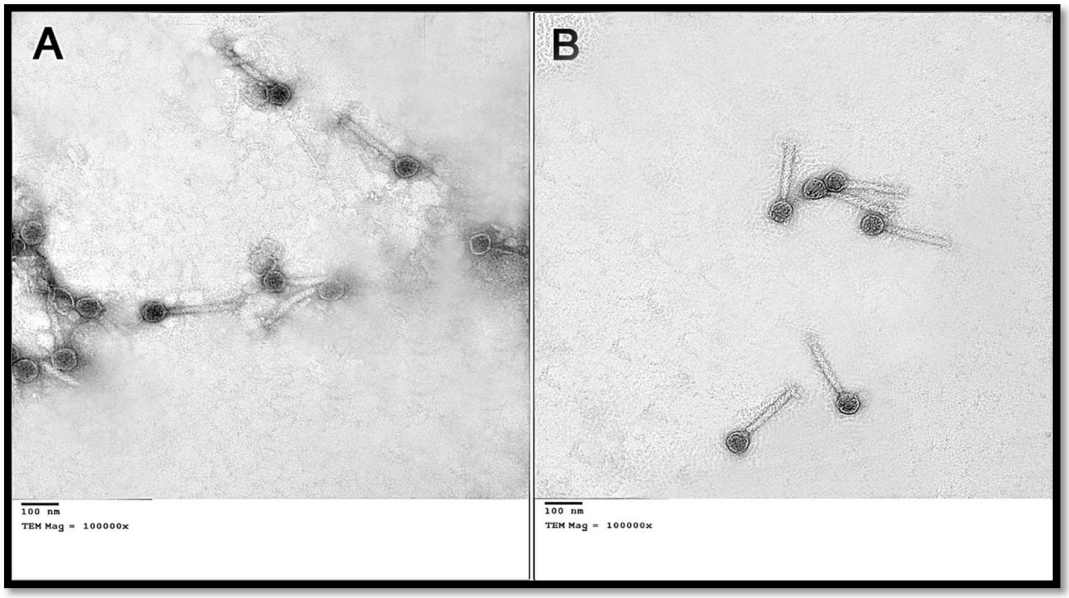

Figure (3): Electron micrograph of the induced phage SmI (A) and EdI (B) isolates. Negatively stained with (2%) phosphotengestic acid at pH 6.8.

Cytopathic effects of the induced phage (SmI) infected *Serratia marcescens ss marcescens* cells:

The ultrathin sections of healthy *Serratia marcescens ss marcescens* cells (figure 4 A) show that the cells have normal cell wall thickness, normal size, cytoplasm and cytoplasmic membrane and semi circular shape, when compared with infected cell (figure 4 B). Whereas, infected cells of *Serratia marcescens ss marcescens* with induced phage **(**SmI**)** showed highly thickening and irregular shape of the cell wall, coagulation of both the cytoplasm and cytoplasmic membrane, The virus particles appeared inside the infected cell (figure 5 A & B). In the late stage of infection, more cytoplasmic coagulation, lysis of the cell wall and release of the induced phage particles were also observed (figure 5C).

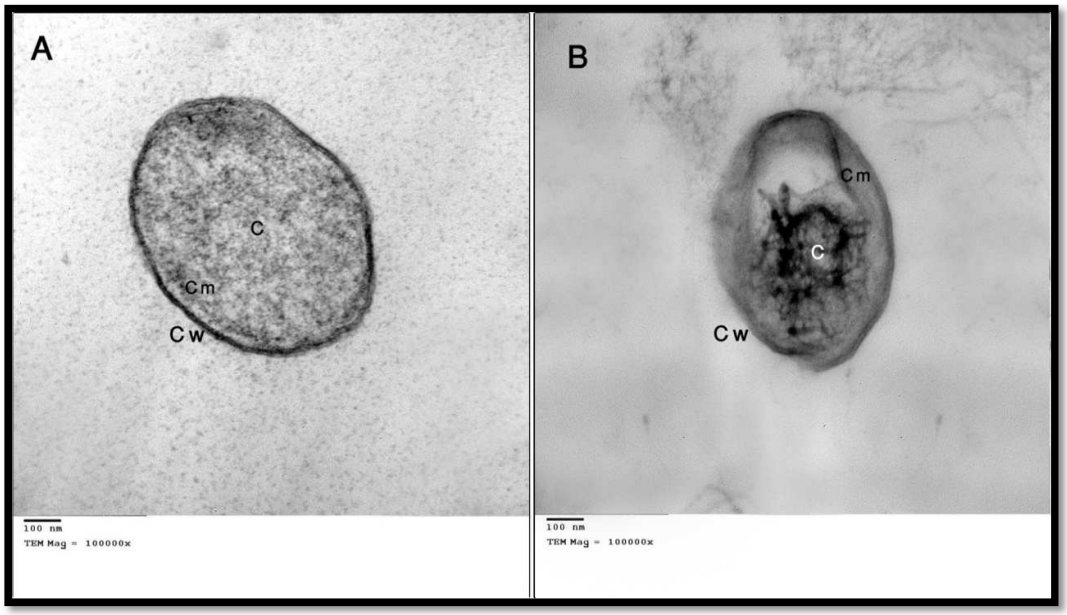

Figure (4): (A) Healthy cells of *Serratia marcescens ss marcescens* and **(B)** Infected cells.
CW= Cell wall, Cm = Cytoplasmic membrane, C= Cytoplasm.

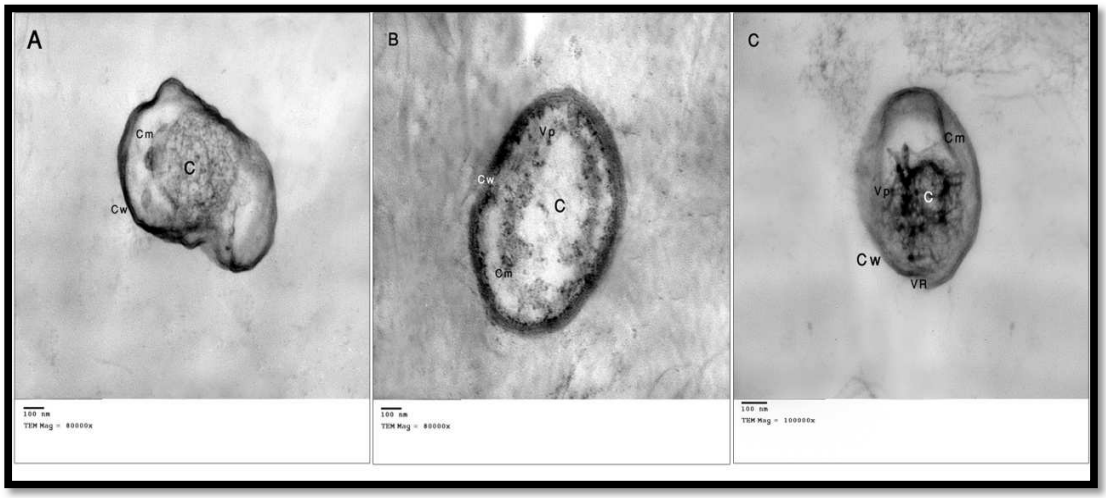

Figure (5): Electron micrographs of *Serratia marcescens ss marcescens* infected cells with induced phage (SmI) showing thickness and irregular shape of the cell wall, coagulation of cytoplasm and wavy cytoplasmic membrane and virus particles were appeared (A, B & C). The virus particles and lysis of cell wall to emerge the virus particles showed in (C).
CW= Cell wall, Cm = Cytoplasmic membrane, C= Cytoplasm, VP= Viral particles, VR= Virus release.

DNA purity and concentration of induced phages genome:

The purity and concentration of DNA isolated from the SmI and EdI induced phages isolates were determined by the absorbance at 260 and 280 nm. The DNA concentration of SmI and EdI induced

phages were 11.3 and 18.8 ng/µl, respectively. The ratio of A_{260}/A_{280} and A_{280}/A_{260} as a measure of DNA purity were found to be 0.9, 1.02, and 1.01, 0.90 for SmI and EdI induced phages isolates, respectively.

The Random Amplified Polymorphic DNA (RAPD-PCR) of induced Phages:

The RAPD-PCR was used to study the genetic diversity and distances of DNA products of the two induced phages from *Serratia marcescens ss marcescens* (SmI) and *Entrobacter cloacae ss dissolvens* (EdI) under study. Only five primers succeeded to generate reproducible polymorphic DNA products with different size with each primer. The total amplified products were 16 DNA bands, generated by OPAN – 12, OPAM – 07, OPT – 20, OPF – 17, and OPT – 08 primers. Sixteen bands were found to be unique polymorphic bands for two induced phages isolates SmI and EdI (figure 6). The size of the amplified fragments were ranged from 288 to 1588 bp.

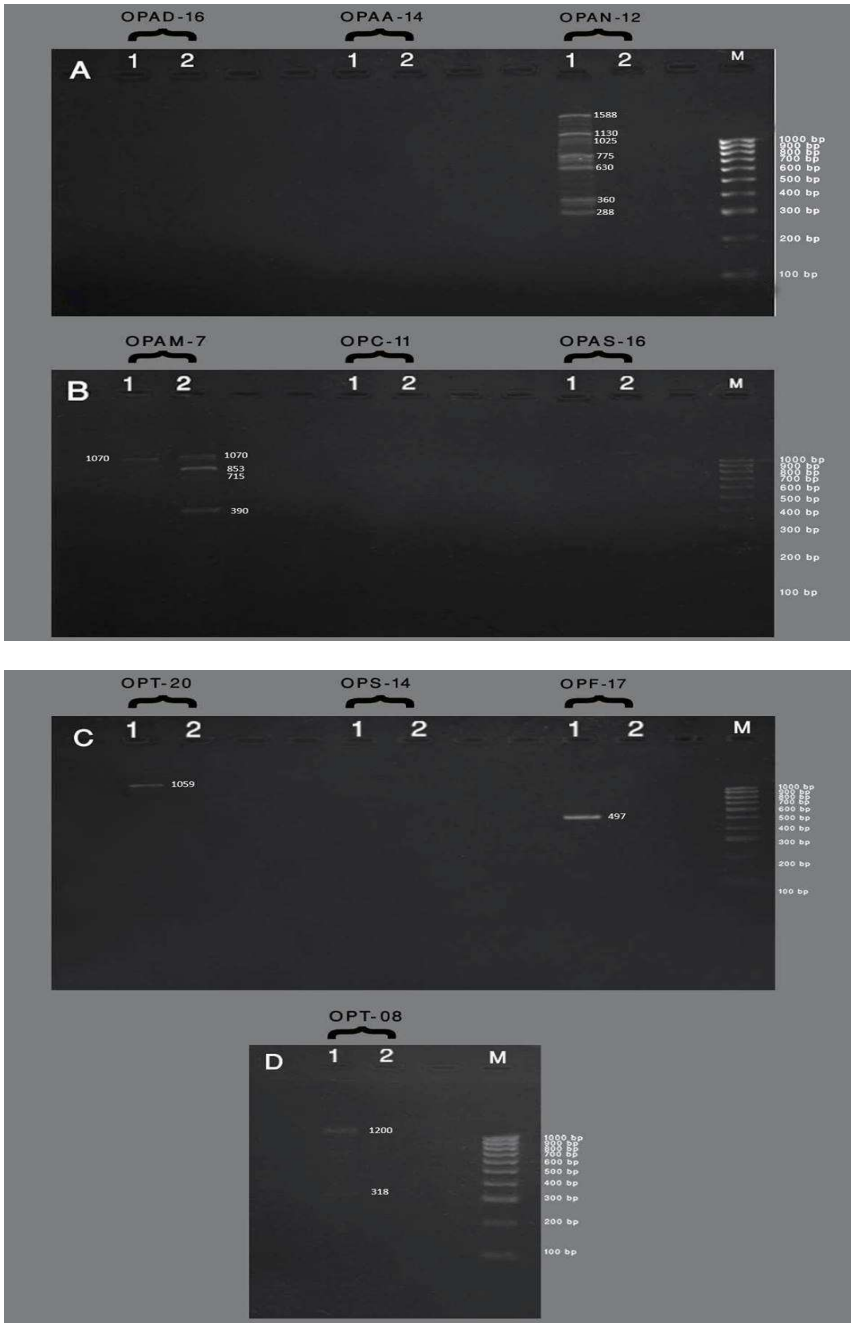

Figure (6): RAPD patterns with primers (A) OPAD – 16, OPAA-14 & OPAN-12, (B) OPAM – 07, OPAC-11 & OPAS- 16, (C) OPT – 20, OPS-14 & OPF-17 and (D) OPT – 08. Lane M: DNA ladder; lane 1: phage SmI and lane 2: phage EdI.

For OPAN – 12 primer, 7 DNA unique bands were appeared only with induced phage from *Serratia marcescens ss marcescens* (SmI). These sizes ranged from 288-1588 bp. According to OPAM – 07primer, five DNA unique bands showed to the induced phages from *Serratia marcescens ss marcescens* (SmI) appeared one band and four bands to *Entrobacter cloacae ss dissolvens* (EdI). The ranged in size bands were found between 390 and 1070 bp. Regarding the DNA primer OPT – 20 primer and OPF – 17primer, showed to one unique band was recorded to induced phage of *Serratia marcescens ss marcescens* (SmI) for each primer used. The size of these bands were at 1059 bp & 497 bp respectively.

Moreover, primers OPT – 08 generated to 2 unique bands with *Serratia marcescens ss marcescens* (SmI) induced phage. The size of the bands generated ranged between 318 and 1200 bp. Table (5) showed that 16 unique DNA bands and Polymorphism percentage were obtained by different primers.

Table (5): The unique polymorphic amplified DNA bands and Polymorphism percentage for each primers.

Primers	No. of Bands	Band size (bp)	SmI induced phage	EdI induced phage	Polymorphism %
OPAD – 16	0	0	0	0	0%
OPAA – 14	0	0	0	0	0%
OPAN – 12	7	288-1588	1	0	25%
OPAM – 07	5	390-1070	1	1	50%
OPC – 11	0	0	0	0	0%
OPAS – 16	0	0	0	0	0%
OPT – 20	1	1059	1	0	25%
OPS – 14	0	0	0	0	0%
OPF – 17	1	497	1	0	25%
OPT – 08	2	318-1200	1	0	25%

Genetic distances relationship among the two induced phages were studied based on their similarity indices data of RAPD- PCR analysis (table 6). Genetic distance value between induced phages SmI and EdI was 0.85 and phage EdI and SmI was 1.0 .

Table (6): Similarity indices among two induced phages isolates based on RAPD analysis.

Induced phages	SmI	EdI
SmI	0	1
EdI	0.8571	0

DISCUSSION

In the present study, two bacterial strains were isolated from potato tuber showing soft rot symptoms. These two bacterial strains were identified as *Serratia marcescens ss marcescens* and *Entrobacter cloacae ss dissolvens.* These results are in agreement with those obtained by **[12,13,14].**

Both of the isolated bacteria were found to be lysogenic bacteria. Two temperate phages designated SmI and EdI were induced from *Serratia marcescens ss marcescens* and *Entrobacter cloacae ss dissolvens,* respectively, using U.V irradiation. Similar results were obtained by **[8]**, who used U.V irradiated to induce temperate phage from *Serratia marcescens*. Moreover, **[22],** used norfloxacin (1 mg/ ml^{-1}) for induction of temperate phages from *Enterobacteriaceae* sp.

The induced SmI and EdI phages formed clear circular plaques of 0.2 and 0.3 mm in diameter and their thermal inactivation points were found to be 66°C and 54°C, respectively. Moreover, different characteristics of the two induced phages were studied (*i.e.* longevity *in vitro*, dilution end point, tolerant to pH and osmotic shock) were studied. In addition, tolerance of the two phages (SmI and EdI) to different chemical agents (formaldehyde., chloroform., ethanol., SDS and ascorbic acid) was also studied. The results were similar to those obtained by **[23,17].**

The host range of each of the two induced phages was studied. Each of the two phages has a narrow host range. The results were agreement with those obtained by **[24,25].**

The particles of the induced phages (SmI and EdI) were found to be of a head and tail type. Both phages have hexagonal icosahedral heads and contractile tails. These two phages seem to be members

of family *Myoviridae*. Also, **[26]** examined temperate phage from *Erwinia carotovora*, the particles have an icosahedral head and a long non-contractile tail with a base plate.

Electron micrographs of *Serratia marcescens ss marcescens* infected with the induced phage (SmI) showed marked signs in cell structure compared with healthy ones. These marked signs such as changes in the form of the cell wall, coagulation of cytoplasmic membrane and virus particles were appeared inside the cell in the late phase. These results were similar to those obtained by **[27,28].**

The SmI and EdI induced phages isolates contained ds-DNA as a viral genome. This result is in agreement with those of **[11,9].** RAPD-PCR based methods dependent on laboratory condition such as template DNA concentration, PCR and electrophoresis settings among others **[29,30].** In this study, RAPD-PCR analysis of SmI and EdI induced phages DNA, showed that among the 16 bands, all bands are unique polymorphic, the size of amplified fragments ranged from 288 to 1588bp. Similar results were obtained by **[11], when used** the RAPD-PCR technique to study the genetic diversity between 26 different bacteriophages infecting different bacterial strains.

CONCLUSION

According to the obtained results it can be concluded that:

- *Serratia marcescens ss marcescens* and *Entrobacter cloacae ss dissolvens* were found to contain prophages. The temperate phages were induced by U.V irradiation from *Serratia marcescens ss marcescens* and *Entrobacter cloacae ss dissolvens* and designated SmI and EdI, respectively.
- The induced phages were found to be of head and tail types and belong to family *Myoviridae*. Ultrathin sections of infected *Serratia marcescens ss marcescens* cells with induced phage (SmI) showed marked signs in cytoplasm and cell membrane, *i.e.* changes in the form of the cell wall and coagulation of cytoplasmic membrane and virus particles were appeared inside the cell in the late phase.
- The RAPD-PCR indicated that, sixteen bands were unique polymorphism with all primer used.

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