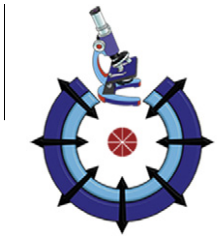
Journal of Genetic Engineering and Biotechnology (2012) **10**, 177–184



Academy of Scientific Research & Technology and
National Research Center, Egypt

Journal of Genetic Engineering and Biotechnology

www.elsevier.com/locate/jgeb

ARTICLE

Cloning and expression of a new bacteriophage (*SHPh*) DNA ligase isolated from sewage

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Received 22 February 2012; revised 21 May 2012; accepted 27 May 2012
Available online 28 June 2012

KEYWORDS

DNA ligase;
T4 bacteriophage;
DH5α bacteria;
E. coli

Abstract During the last 50 years, major advances in molecular biology and biotechnology have been attributed to the discovery of enzymes that allow molecular cloning of important genes. One of these enzymes that has been widely acknowledged for its role in the development of biotechnology is the T4 DNA ligase. This enzyme joins the break in the DNA backbone structure by creating a phosphodiester bond between 5′ PO_4 and 3′ OH ends, in an ATP dependent multi-step reaction, thus allowing the ligation of related and foreign DNA sequences. Due to its role in modern DNA recombinant technology, there is a high demand on DNA ligase to allow the ligation of target DNA inserts into a chosen vector as part of DNA cloning technology. To closely look at ligase sequence diversity, a bacteriophage that infects *DH5α* (commercial lab strain of *Escherichia coli*) was isolated from sewage system in Hebron, Palestine. The DNA ligase gene of this phage was cloned and its sequence was compared to the NCBI database. The new bacteriophage ligase, named (South Hebron Phage, *SHPh*) DNA ligase, shows homology to T even bacteriophage DNA ligases posted in the NCBI database with 35 nucleotide differences, an indication of existed diversity among T even DNA ligation enzymes that can be used as markers in phage classification.

1. Introduction

Bacteriophages are considered as the most abundant organisms on earth that exist almost in all environments particularly where bacteria flourish, such as in wastewater and sewage [12]. One of these bacteriophages that is extensively studied is the T4 bacteriophage responsible for producing one of the most important enzymes used in molecular cloning named the T4 DNA ligase [11,19].

Bacteriophage T4 is a double stranded DNA virus of about 169 Kb encoding approximately 300 genes that produce the virus proteins [4,8]. T4 DNA ligase is the most important enzyme encoded by bacteriophage genome and is used for virus metabolism, genome replication, recombination and repair. This enzyme mediates the phosphodiester bond formation between 5′ PO4 and opposing 3′ OH groups in a DNA molecule. It is encoded by gene 30 (*gp30*), with full length of 487 amino

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Peer review under responsibility of National Research Center, Egypt

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http://dx.doi.org/10.1016/j.jgeb.2012.05.005

acids and molecular weight of 55.3 kDa [2,4,18,29]. Although many nucleic acid ligases have been characterized, the T4 DNA ligase is the most commonly used enzyme in molecular cloning.

DNA ligase is a universal enzyme that is present in almost all living organisms; it is a housekeeping enzyme that is required for survival functions and cellular processes related to breaks correction in the DNA backbone structure. It has essential roles in DNA replication, repair and recombination [24]. Bacteriophage DNA ligases utilize ATP as a co-factor similar to Archaea and Eukarya DNA ligases [27]. Ligation reaction is energy dependent and consists of three successive steps involving two covalent reaction intermediates. In the first step; ligase is activated by the covalent attachment between α-phosphate of AMP molecule and the enzyme creating a ligase-AMP intermediate and releasing inorganic pyrophosphate (PP_i). In the second step; the AMP group is transferred from ligase to the 5′ end phosphate group of the DNA molecule creating an AMP-DNA intermediate. In the third step; the hydroxyl group on the 3′ end of the break in the substrate attacks the phosphate on the 5′ end of the opposing nucleic acid strand creating continuous backbone structure of the DNA molecule and releasing a free AMP [6,11,21,24,27].

Enterobacteriaceae are gram negative rods that include the *Escherichia coli* and *Shigella*. There are several DNA ligases posted on the GenBank belong mostly to Enterobacteria phages, such as the T4 bacteriophage [30]. There are several protein markers used to differentiate between T4 phage superfamily, including the capsid proteins gp20 and gp23 [8]. These markers however are not specific enough in differentiating between T4 and T-like bacteriophages [8]. DNA ligases are highly conserved among these strains with identity score over 98%, but still can show variations that can be used as tools in distant bacteriophage classification. In this study, a DNA ligase was cloned from a bacteriophage that was able to host on a commercially designed strain of *E. coli* and its sequence was compared with related DNA ligases. The sequence supports the idea that ligases can be used as markers to classify bacteriophages.

2. Materials and methods

2.1. Phage (SHPh) isolation

Samples of wastewater (0.5 ml) were collected from open undeveloped sewage in an area in south Hebron, Palestine and used to infect 5 ml overnight culture of *DH5α*, a commercial strain of *E. coli*, to isolate a strain-specific phage. The mixture was allowed to incubate overnight at 37 °C with continuous shaking at 200 rpm. In the morning of the next day, 1.0 ml of the viral culture was transferred to sterile microfuge tube, and one drop of chloroform was added and mixed by inversion before centrifugation at 12,000 rpm for 2 min. The supernatant was transferred to a new microfuge tube before it was centrifuged again. The supernatant was filtrated by 5 cc syringe (A0.22micron) filter disc, the filtrate (enriched phage prep) was stored at 4 °C and used later to lyze bacterial cells grown on agar plates a night before performing plaque assay. To determine whether the new isolated phage is a T even virus, 2 capsid protein markers *gp20*, *gp23* were checked using specific primers designed to amplify the full lengths of these two genes. Primer information is shown in Table 1 and the PCR conditions were as follows: initial denaturation at 95 °C for 2 min, 25 cycles of 95 °C for 30 s, 54 °C for 30 s, 72 °C for 2 min, followed by a final extension at 72 °C for 10 min.

2.2. Isolation and amplification of SHPh DNA ligase

Genomic DNA was isolated from viral extract using commercially available EZ-DNA isolation kit (Genomic DNA isolation kit/ Biological Industries). To amplify the DNA ligase gene, PerlPrimer software was used to design primers based on the T4 DNA ligase sequence accession number X00039 as described in Table 1. The gene was amplified by PCR using high fidelity proofreading thermostable *Taq* DNA polymerase. PCR amplification was carried out with a thermal cycler under the following conditions: initial denaturation at 95 °C for 2 min, 25 cycles of 95 °C for 30 s, 54 °C for 30 s, 72 °C for 2 min, followed by a final extension at 72 °C for 10 min. The gene was then purified from agarose gel to be used in the cloning step.

2.3. Competent cell preparation

Competent cells were prepared according to protocol as described by Sambrook et al. 2001 [25] with modifications as follows: One hundred ml of LB (1L of LB contained 10 g trypton, 10 g NaCl, 5 g yeast extract) were infected with 5 ml overnight culture of *DH5α* and incubated at 37 °C for about 3 h until cell density reached 0.6 OD at 600 nm. Fifty milliliter of the culture was transferred to conical tube and centrifuged at 2000 rpm for 10 min. The supernatant decanted and the pellet re-suspended with 10 ml of ice cold 0.1 M $CaCl_2$ before centrifugation at 2000 rpm for 10 min. The supernatant decanted and the pellet re-suspended in 1.0 ml of ice cold 0.1 M $CaCl_2$. Competent cells were aliquoted in 50 µl volumes and stored in 1.5 ml Eppendorf tubes at −80 °C for subsequent use.

Table 1 The information of the PCR primers and restriction enzyme recognition sites (underlined) used in this study.

Primer name	Accession No.	5′–3′ sequence	Aim of use
T4 F cl	X00039.1	TAAAGTTAGAACCACGTACCAC	Initial DNA ligase amplification and cloning into
T4 R cl	X00039.1	GCTTTCATAGACCAGTTACCT	pGEM®-T Easy cloning vector.
T4 F ex	X00039.1	GGGGGATCCTAAAGTTAGAACCACGTACCAC	Cloning in the expression vector pPROEXHTb.
T4 R ex	X00039.1	GGGCTCGAGGCTTTCATAGACCAGTTACCT	Recognition sites for *BamH1* (F), and *Xho1* (R).
Gp23 F	X01774.1	CCGGACTGAATTTCGTCACT	Amplification of the marker protein *gp23*
Gp23 R	X01774.1	AACCCGAAGGTTCCCTAAAA	Full length primers
Gp20 F	X16055.1	TCCCATTTGGAGAATACAATGA	Amplification of the marker protein *gp20*
Gp20 R	X16055.1	ACCTTCCATTAAAAATCCTCT	Full length primers

2.4. Cloning of DNA ligase gene into pGEM®-T easy cloning vector

The gene was purified from agarose gel using AccuPrep® Gel Purification Kit (Bioneer, K-3035) in preparation for cloning into the pGEM®-T Easy (Promega/A1360) cloning vector. The ligation reaction incubated overnight at 4 °C using standard cloning procedures [25] and transformed into *DH5α* competent cells by heat shock method [25] as follows: The transformed cell culture was plated onto labeled agar plates containing ampicillin, Isopropyl β-D-1 Thiogalactopyranoside (IPTG) and X-Gal. The plates were sealed and incubated overnight at 37 °C. White positive colonies were selected and the presence of the insert gene was verified by PCR.

2.5. Subsequent cloning of DNA ligase gene into expression vector

In order to transfer the DNA ligase gene named here *SHPh* into the expression vector pPROEX HTb that contains His-tag to facilitate protein purification; 2 sets of primers were designed with *BamHI* - recognition site added at the 5′ end and *XhoI* - recognition site added at the 3′ end (Table 1). Both the purified DNA ligase gene and pPROEX HTb expression vector were double digested with *BamHI* (Biolabs/ R0136S) and *XhoI* (Biolabs/ R0146S) restriction enzymes. The digested DNA ligase gene was ligated with the digested pPROEX HTb expression vector via ligation reaction containing 2X ligation buffer, 61 ng pPROEX HTb vector, 46 ng of insert and three Weiss units of commercial T4 DNA ligase. Ultra pure water added to a final reaction volume of 10 µl. The ligation reaction was allowed to incubate overnight at 4 °C before it was used for transformation into *DH5α* competent cells. In the following day, pPROEX HTb expression vector containing a DNA ligase gene was transformed into *DH5α* bacterial cells as described above except that the transformed cell culture was plated onto labeled agar plates containing only ampicillin as a selectable marker. Colonies were PCR-tested for the presence of the ligase gene and glycerol stock prepared from positive clones. To confirm the orientation of the coding sequence in the expression vector, *SHPh* was sequenced (three clones) in the Heredity Lab at Bethlehem University sequencing facility.

2.6. His-tag protein purification

Five hundred ml LB containing 500 µl of ampicillin (50 mg/ml) were infected with 5 µl ligase expression clones stored at -80 ° C and allowed to incubate at 37 °C until cell density reached 0.6 at OD 600 nm. The culture was induced by the addition of IPTG to a final concentration of 1 mM before overnight incubation at 37 °C. Following induction, the cell culture centrifuged at 3000 rpm, 4 °C for 10 min, and the supernatant was discharged while the cell pellet resuspended with 50 ml Phosphate Buffer Saline (PBS) before it was centrifuged one more time at 3000 rpm, 4 °C for 10 min. The supernatant discharged and the cell pellet stored at −80 °C until the following day. The pellet of each tube resuspended in 1 ml lysis buffer containing PMSF, 5 mg lysozyme, and 10 mM imidazole. After 10 min incubation at room temperature, the mixture was vortexed before centrifugation for 10 min, at 13,000 rpm 4 °C to separate supernatant from pellet which was kept at −20 °C to be checked on SDS–PAGE. Ligase purification was carried out using affinity chromatography using 50% Ni–NTA Superflow columns according to Qiagen protocols. One milliliter of Ni Nickel resin equilibrated with washing buffer (20 mM of imidazole). Following resin settlement, the buffer was removed and the resin mixed with supernatant (i.e., total protein extract) that was separated from the previous step. The mixture incubated under continuous shaking at room temperature for 60 min to allow nickel resin binding with the His-tag of the recombinant ligase. The mixture poured into the column and the flow-through collected for leakage checkup on SDS–PAGE. The

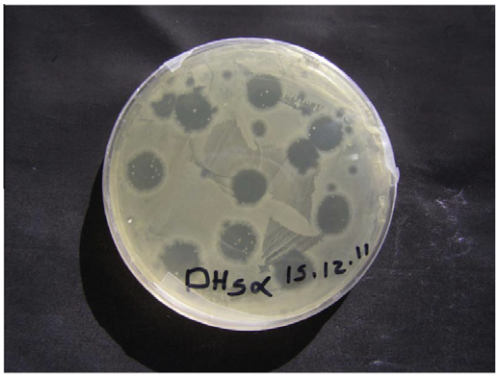

Figure 1 Plaque assay. Effect of the isolated bacteriophage on *DH5α*. The bacteriophage was able to lyse *DH5α E. coli* strain and generate clear zones representing the areas where the phage particles were pipetted.

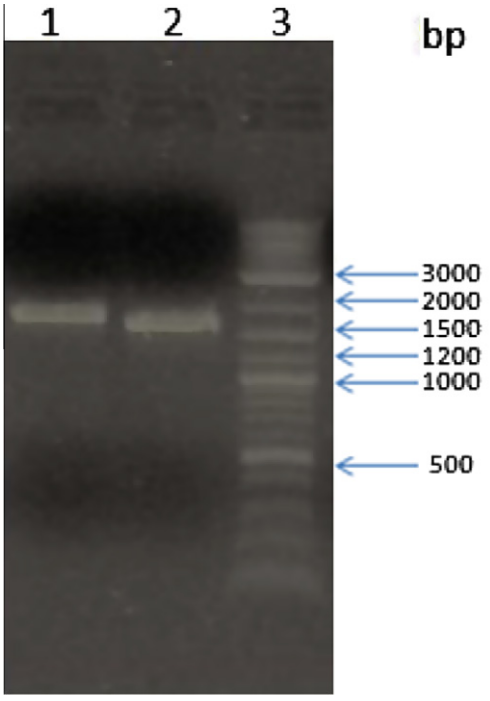

Figure 2 Ethidium bromide-stained agarose gel of amplified PCR amplicons representing T even bacteriophage surface protein markers. Lane 1 represents a 1720 bp amplicon of the *gp23*, whereas Lane 2 represents the *gp20* of approximately 1601. Lane 3 contains the DNA ladder.

beads washed three times with 1 ml washing buffer and the three washing samples were kept for SDS–PAGE checkup. Recombinant protein was then eluted four times with 500 µl elution buffer (250 mM imidazole). All purification steps took place on ice and samples were stored at −20 °C until they were loaded on SDS–PAGE.

3. Results and disussion

3.1. A strain specific bacteriophage was isolated

A *DH5α* strain-specific bacteriophage was isolated from enriched bacteriophage culture using a standard plaque assay [9]. The bacterial culture was infected by phage particles isolated from wastewater collected from sewage system south Hebron city in Palestine. Multiple culturing and subsequent infections resulted in the isolation of a bacteriophage that could invade the commercially available *E. coli*. After multiple incubations and subculturing using new media, it is assumed that if a limited number of the phage is capable of infecting the *DH5α* strain, then after multiple cultures the number of these phages will multiply. The *DH5α* is an engineered commercial strain that shows some resistance to a good number of bacteriophages. The fact that the enriched phage particles were able to grow on this strain means that a *DH5α* strain-specific bacteriophage has been isolated and the phage is apparently capable of recognizing surface receptor proteins specific for *DH5α* strain. When purified phage particles were pippeted on agar plates containing *DH5α* and allowed to incubate at 37 °C, clear areas started to appear after approximately 6 h, an indication of the ability of this virus to host on the *DH5α* strain. The plaques or clear zones appeared as a result of cell lysis by bacteriophage (Fig. 1) [14].

The phage (South Hebron Phage, *SHPh*) was isolated from wastewater, a medium that is considered a very rich source for bacteriophages because it is a natural environment of bacteria, particularly *E. coli*, and so the presence of bacteria is an

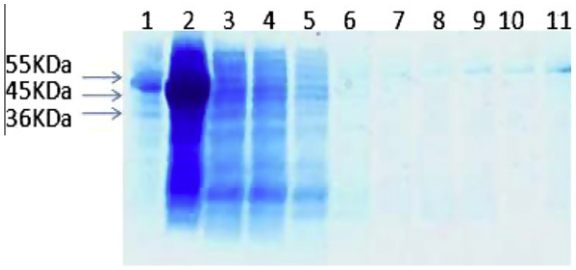

Figure 4 SDS–PAGE gel photo of the purirfied DNA ligase protein. Visualization of the purified DNA ligase protein as appeared on 10% resolving SDS–PAGE gel. Lane 1 contains high molecular weight protein ladder. Lane 2 shows the total protein extract from cell lysate. Lane 3 from flowthrough. Lanes 4–6 from washing 1, washing 2, and washing 3, respectively. Lanes 7–11 show the different elutions containing the DNA ligase. The protein size is about 55.3 kDa and comes out in the elutions.

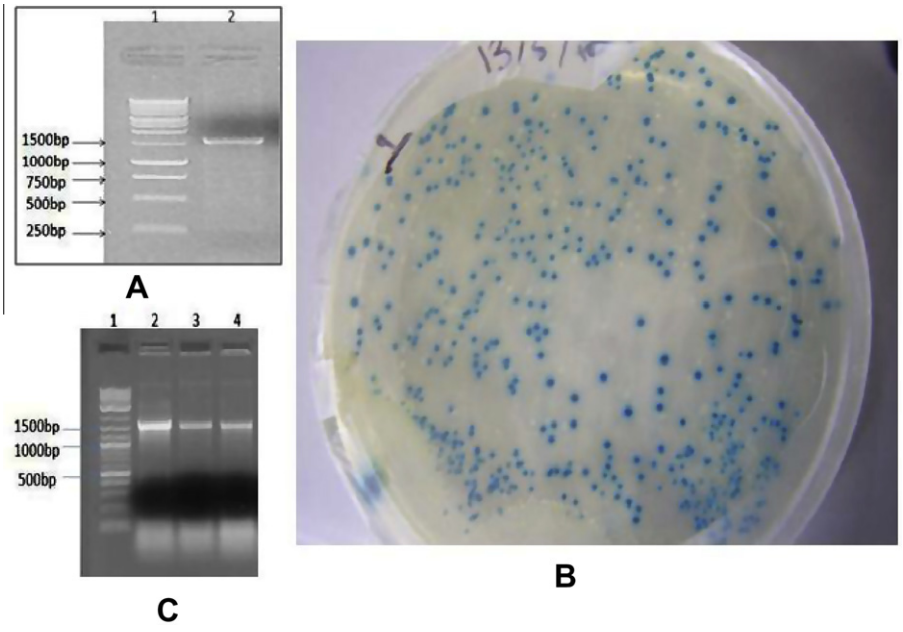

Figure 3 Cloning of DNA ligase gene. (A) The ligase gene was PCR amplified and loaded on 1% agarose gel, stained with EtBr and visualized using gel documentation system (right lane). The left lane is showing DNA ladder. The amplified band expected size is 1561 bp and matches 1500 bp on the ladder. (B) Blue/white selection of positive cloning. The ligase gene was purified from agarose gel and cloned into the pGEM®-T Easy cloning vector as described in materials and methods. The transformed cell culture was plated onto agar plates containing ampicillin, IPTG and X-Gal. The plates were sealed and incubated overnight at 37 °C. White colonies represent positive cloning and were selected for subsequent verification. (C) PCR verification of positive cloning in pGEM®-T Easy cloning vector. The ligase gene was PCR amplified and loaded on 1% agarose gel, stained with EtBr and visualized using gel documentation system (right lanes). Lane 1 contains the DNA ladder. Lane 2 shows the amplified gene from bacterial colony. Lane 3 amplified gene from liquid culture. Lane 4 from purified pGEM®-T Easy plasmid. Specific band matches the corresponding size of the bacteriophage DNA ligase.

indicator of bacteriophage abundance because the phages are abundant where bacteria exist [7,9,12,13].

To check whether the isolated bacteriophage belongs to the T even phages, two bacteriophage markers of the major capsid proteins *gp20* and *gp23* were amplified by specific PCR primers and produced positive results as shown in (Fig. 2), an indication of T even bacteriophage. These markers, however, can only be specifically used to identify members of the T4 phage superfamily [8]. Genomic comparison has provided evidence for the existence of a large and diverse superfamily of T4-like phages in the environment, with over 150 phages infecting over 30 gram-negative bacteria possessing related morphology [8].

A number of phages that can host on *E. coli* have been isolated and intensely studied, but few if any of the well-characterized ones infect the *DH5α* strain. The *SHPh* ability to host on this commercially available strain supports the new trend to use bacteriophages in therapies and modern

```
>lcl|57029 SHPh
Length=1464
 Score = 2510 bits (1359),  Expect = 0.0
 Identities = 1429/1464 (98%), Gaps = 0/1464 (0%)
 Strand=Plus/Plus

SHPh   1     ATGATTCTTAAAATTCTGAACGAAATAGCATCTATTGGTTCAACTAAACAGAAGCAAGCA  60
             |||||||||||||||||||||||||||||||||||||||||||||||||| ||||||||||||
Gp30   1     ATGATTCTTAAAATTCTGAACGAAATAGCATCTATTGGTTCAACTAAGCAGAAGCAAGCA  60

SHPh   61    ATTCTTGAAAAGAATAAAGATAATGAATTGCTTAAACGAGTATATCGTCTGACTTATTCT  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Gp30   61    ATTCTTGAAAAGAATAAAGATAATGAATTGCTTAAACGAGTATATCGTCTGACTTATTCT  120

SHPh   121   CGTGGGTTACAGTATTATATCAAGAAATGGCCTAAACCTGGTATTGCTACCCAGAGTTTT  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Gp30   121   CGTGGGTTACAGTATTATATCAAGAAATGGCCTAAACCTGGTATTGCTACCCAGAGTTTT  180

SHPh   181   GGAATGTTGACTCTTACCGATATGCTTGACTTCATTGAATTCACATTAGCTACTCGGAAA  240
             |||||| |||||| |||||||||||||||||||||||||||||| ||||||||||||||||
Gp30   181   GGAATGCTGACTATTACCGATATGCTTGACTTCATTGAATTCACGTTAGCTACTCGGAAA  240

SHPh   241   TTGACTGGAAATGCAGCAATTGAGGAATTAACTGGATATATCACCGATGGTAAAAAAGAT  300
             |||||||||||||| |||||||||||||||||||||||||||| || || ||||||||||||
Gp30   241   TTGACTGGAAATGCGGCAATTGAGGAATTAACTGGATATATTACTGACGGTAAAAAAGAT  300

SHPh   301   GATGTTGAAGTTTTGCGTCGAGTGATGATGCGAGACCTTGAATGTGGTGCTTCAGTATCT  360
             |||||||||||||||||||| |||||||||||||||||||||||| ||||||||||||||||
Gp30   301   GATGTTGAAGTTTTGCGTCGGGTGATGATGCGAGACCTTGAATGCGGTGCTTCAGTATCT  360

SHPh   361   ATTGCAAACAAAGTTTGGCCAGGTTTAATTCCTGAACAACCTCAAATGCTCGCAAGTTCT  420
             |||||||||||||||||||||||||||||||||||||||||||||||||||| |||||||||
Gp30   361   ATTGCAAACAAAGTTTGGCCAGGTTTAATTCCTGAACAACCTCAAATGCTTGCAAGTTCT  420

SHPh   421   TATGATGAAAAAGGCATTAATAAGAATATCAAATTTCCAGCCTTTGCTCAGTTAAAAGCT  480
             |||||||||||||||||||||||||||||||||||||||||||||||||| ||||||||||||
Gp30   421   TATGATGAAAAAGGCATTAATAAGAATATCAAATTTCCAGCCTTTGCCCAGTTAAAAGCT  480

SHPh   481   GATGGAGCTCGGTGTTTTGCTGAAGTTAGAGGTGATGAATTAGATGATGTTCGTCTTTTA  540
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Gp30   481   GATGGAGCTCGGTGTTTTGCTGAAGTTAGAGGTGATGAATTAGATGATGTTCGTCTTTTA  540

SHPh   541   TCACGAGCTGGTAATGAATATCTAGGATTAGATCTTCTTAAGGAAGAGTTAATTAAAATG  600
             |||||||||||||||||||||||||||||||||||||||||||||||||||| ||||||||
Gp30   541   TCACGAGCTGGTAATGAATATCTAGGATTAGATCTTCTTAAGGAAGAGTTAATCAAAATG  600

SHPh   601   ACCGCTGAAGCCCGCCAGATTCATCCAGAAGGTGTGTTGATTGATGGCGAATTGGTATAC  660
             || || ||||| |||||||||||||||||||||||| ||||||||||||||||||||||||
Gp30   601   ACTGCAGAAGCTCGCCAGATTCATCCAGAAGGTGTGTTAATTGATGGCGAATTGGTATAC  660

SHPh   661   CATGAGCAAGTTAAAAAGGAGCCAGAAGGCCTAGATTTTCTTTTTGATGCTTATCCTGAA  720
             |||||||||||| |||||||||||||||||||||||||||||||||||||||||||||||||
Gp30   661   CATGAGCAAGTTGAAAAGGAGCCAGAAGGCCTAGATTTTCTTTTTGATGCTTATCCTGAA  720
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Figure 5 The NCBI-Blast analysis of SHPh DNA ligase gene with gp30 T4 DNA ligase (Accession number X00039). Full-length mRNA is shown (1464 bp).

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SHPh   721   AACAGTAAAGCTAAAGAATTCGCCGAAGTAGCTGAATCACGTACTGCTTCTAATGGAATC  780
             |  |||||||||||||||||||||||||||||||||||||||||||||| ||||||||| |||
Gp30   721   ATTAGTAAAGCTAAAGAATTCGCCGAAGTAGCTGAATCACGTACTGCATCTAATGGCATC  780

SHPh   781   GCCAATAAATCTTTAAAGGGAACCATTTCTGAAAAAGAAGCACAATGCATGAAGTTTCAG  840
             |||||||||||||||||||||||||||||||||||||||||| ||||||||||||||||||
Gp30   781   GCCAATAAATCTTTAAAGGGAACCATTTCTGAAAAAGAAGCTCAATGCATGAAGTTTCAG  840

SHPh   841   GTCTGGGATTATGTCCCGTTGGTAGAAATATACAGTCTTCCTGCATTTCGTTTGAAATAT  900
             |||||||||||||||||||||||||||||||| |||||||||||||||||||||||||||
Gp30   841   GTCTGGGATTATGTCCCGTTGGTAGAAATATACGGTCTTCCTGCATTTCGTTTGAAATAT  900

SHPh   901   GATGTACGTTTTTCTAAACTAGAACAAATGACATCTGGATATGATAAAGTAATTTTAATT  960
             |||||||||||||||||||||||||||||||| || ||||||||||||||||||||||||
Gp30   901   GATGTACGTTTTTCTAAACTAGAACAAATGACATCAGGTTATGATAAAGTAATTTTAATT  960

SHPh   961   GAAAACCAGGTAGTAAATAACCTAGATGAAGCTAAGGTAATTTATAAAAAGTATATTGAC  1020
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Gp30   961   GAAAACCAGGTAGTAAATAACCTAGATGAAGCTAAGGTAATTTATAAAAAGTATATTGAT  1020

SHPh   1021  CAAGGTCTTGAAGGTATTATTCTCAAAAATATCGATGGATTATGGGAAAATGCTCGTTCA  1080
             |||||||||||||||||||||||||||||||| ||||||||||||||||||||||||||||
Gp30   1021  CAAGGTCTTGAAGGTATTATTCTCAAAAATACCGATGGATTATGGGAAAATGCTCGTTCA  1080

SHPh   1081  AAAAATCTTTATAAATTTAAAGAAGTAATTGATGTTGATTTAAAAATTGTAGGAATTTAT  1140
             |||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||
Gp30   1081  AAAAATCTCTATAAATTTAAAGAAGTAATTGATGTTGATTTAAAAATTGTAGGAATTTAT  1140

SHPh   1141  CCTCACCGTAAAGACCCTACTAAAGCGGGTGGATTTATTCTTGAGTCAGAGTGTGGAAAA  1200
             |||||||||||||||||||||||||| ||||||||||||||||||||||||||||||||||
Gp30   1141  CCTCACCGTAAAGACCCTACTAAAGCAGGTGGATTTATTCTTGAGTCAGAGTGTGGAAAA  1200

SHPh   1201  ATTAAGGTAAATGCTGGTTCAGGCTTAAAAGATAAAGCCGGTGTAAAATCGCATGAACTT  1260
             |||||||||||||||||||||||||||||||| |||||||| |||||||| |||||||||
Gp30   1201  ATTAAGGTAAATGCTGGTTCAGGCTTAAAAGACAAAGCCGGCGTAAAATCACATGAACTT  1260

SHPh   1261  GACCGTACTCGCATTATGGAAAACCAAAATTATTATATTGGAAAAATTCTAGAGTGCGAA  1320
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Gp30   1261  GACCGTACTCGCATTATGGAAAACCAAAATTATTATATTGGAAAAATTCTAGAGTGCGAA  1320

SHPh   1321  TGCAACGGTTGGTTAAAATCTGATGGCCGCACTGATTACGTTAAATTATTTCTTCCGATT  1380
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Gp30   1321  TGCAACGGTTGGTTAAAATCTGATGGCCGCACTGATTACGTTAAATTATTTCTTCCGATT  1380

SHPh   1381  GCGATTCGTTTACGTGAAGATAAAACTAAAGCTAATACATTCGAAGATGTATTTGGTGAT  1440
             |||||||||||||||||||||||||||||||||||||||||| ||||||||||||||||
Gp30   1381  GCGATTCGTTTACGTGAAGATAAAACTAAAGCTAATACATTCGAGGATGTATTTGGTGAT  1440

SHPh   1441  TTTCATGAGGTAACTGGTCTATGA  1464
             |||| ||||||||||||||||||
Gp30   1441  TTTCGTGAGGTAACTGGTCTATGA  1464
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Figure 5 *(continued)*

environmental technologies as bacteriophages can be efficient factors in destroying pathogenic cells [15,16,22,28]. This is particularly important due to the emerging concern about antibiotic resistance as researchers have become interested in the use of bacteriophages in replacement of antibiotics to detect and combat pathogenic bacteria [1,3,15,17,20,23,26].

3.2. Cloning of SHPh DNA ligase

The *SHPh* DNA ligase gene of about 1.5 Kb was amplified using specific forward and reverse primers (T4 F a and T4 R a respectively), which were designed using PerlPrimer v1.1.16 software based on the T even upstream and downstream coding sequence of posted DNA ligase sequences available on the GenBank. The highly specific band was amplified from genomic DNA prepared from virus particles isolated from lysed bacteriophage-bacterial culture. The PCR amplified DNA loaded on 1.5% agarose gel and visualized by UV light. The amplicon size was 1561 bp (Fig. 3A). The amplified fragment matched the known sizes of the DNA ligase genes available on the GenBank. The insert was then cloned into pGEM®-T-Easy cloning vector and positive clones were verified by

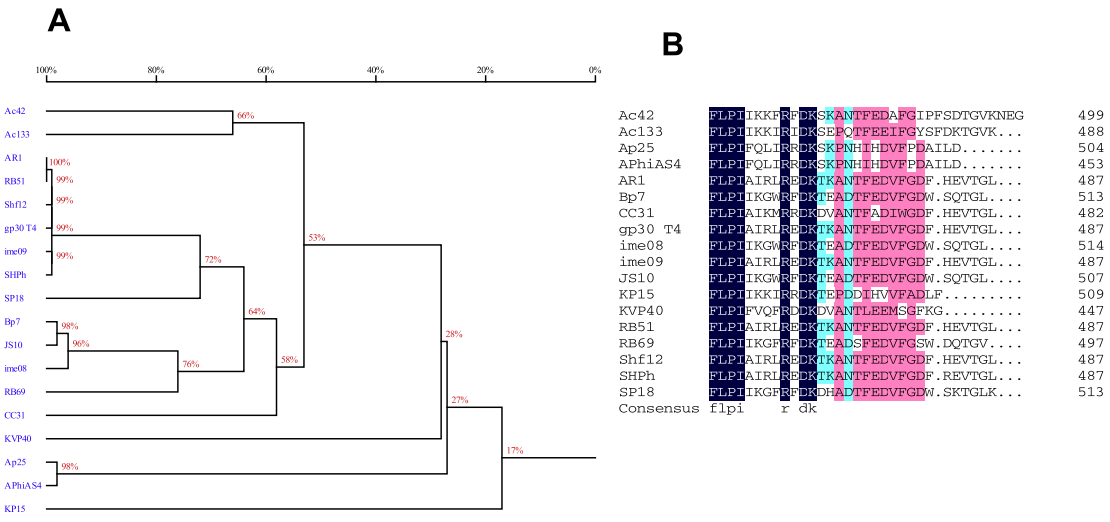

Figure 6 Amino acid sequence information of *SHPh*. (A) The homology tree of several bacteriophage DNA ligases with the amino acid sequence of SHPh ligase used as the outgroup sequence. The alignment of whole sequence was used to construct the neighbor-joining tree using DNAMAN version 4.15 software. Numbers refer to homology degrees among the analyzed phages. (B) Sequence blast result of DNA ligase showing c-terminal amino acid differences between SHPh sequence and the bacteriophage DNA ligases analyzed above.

three methods. First, by the antibiotic ampicillin as only the transformed cells containing the cloning vector would survive, second, by screening blue-white colonies on a selection media containing X-gal that would be broken by β-galactosidase enzyme encoded by *lacZ gene*. If cloning has failed, then the *lacZ gene* is not disrupted and as a result the β-galactosidase enzyme is functional to break X-gal and produce negative blue-colored colonies, while white colonies are indicative of positive cloning due to the disruption of *lacZ gene* (Fig. 3B). The third method depended on PCR amplification of the gene to confirm that the white colonies grown on agar plates containing ampicillin/IPTG/X-Gal were positive clones for the *SHPh* DNA ligase gene (Fig. 3C). The pGEM®-T-Easy is a high copy number cloning vector, which has 3′-T overhangs at its insertion site (Promega, technical manual) that facilitates ligation and cloning of a PCR amplified insert that has complementary 3′-A overhang which is provided to both sides by *Taq* DNA polymerase [30].

To check whether a secreted gene was cloned, we subsequently recloned the *SHPh* DNA ligase gene into the pPROEX HTb expression vector and verified the cloning by PCR and sequencing. The expression vector is a medium size of about 4779 bp with ampicillin resistant gene and N-terminus six tandem histidine (6xHis) residues to facilitate purification on Nickel ion (Ni–NTA)-containing columns. The expression in pPROEX HTb produced the desired recombinant protein with N-terminal 6 His-tag, which bound the nickel ions in the column and later displaced by a high concentration of imidazole, a step that allowed the protein's purification [10]. The *DH5α* clones containing the expression vector with *SHPh* DNA ligase gene were IPTG induced for enzyme expression and the protein purified and samples loaded on SDS–PAGE gel electrophoresis. Protein bands matched the expected size of the DNA ligase of about 55 kDa, indicating that the gene could be expressed to produce the enzyme (Fig. 4).

The SHPh DNA ligase coding sequence was used to carry a nucleotide BLAST (Basic Local Alignment Search Tool) at the NCBI (National Center for Biotechnology Information) website (http://www.ncbi.nlm.nih.gov/). It revealed that SHPh DNA ligase has high similarity with gp30 T4 ligases. The obtained results scored 2510 bits (1359), with 0.0 E. value, the identity percentage was 98% (1429/1464), without any gap (Fig. 5). Most variations, however, are at the 3rd letter in the codon. The results also revealed that the *E. coli* RB51 phage (Accession number FJ839693.1) and *gp30* T4 ligase (Accession number X00039.1) were the closest to our gene with some 35 variations at nucleotide level from the highest score (data not shown).

To have a closer look at the variation at the protein level, the *SHPh* ligase sequence was translated using expasy translation tool (http://www.expasy.ch/tools/dna.html), and the resulted amino acid sequence blasted against protein database at NCBI. A homology comparison with the amino acid sequences of several ligases was constructed (Fig. 6A) using DNAMAN version 4.15 (Lynnon BioSoft). Several T4 and *Shigella* phages appear to be closed relatives to SHPh phage. To identify specific regions that can be used as fingerprints to identify closed relatives, we analyzed the sequences from several closed and distant relatives and found an area at the c-terminal end of the ligase that can be used to design specific primers (Fig. 6B). This area possesses four amino acids that their nucleotides differences can be used for designing specific primers (Fig. 6B). This indicates that despite the fact that most ligases are highly conserved, they can still show some sequence differences possibly to avoid host cell restriction enzymes. This may emphasize the speculation that bacteriophages play a crucial role in the evolution of their bacterial hosts and the emergence of new pathogens [5]. It also suggests that the DNA ligases can be used as markers to differentiate between Enterobacteria and several other phages (Fig. 6).

4. Authors' contribution

A. AL-M carried out the experimental work and provided the first draft of the manuscript. F. AL-R designed and supervised

the work. Both authors have read and approved the final manuscript.

Acknowledgments

The authors would like to thank Dr. Yaqoub Ashhab for advice and Dr. Rami Arafeh for carefully reading the manuscript, Mrs. Amal Abu Rayan for carrying out the sequencing, and Mrs. Asma Tamimi for technical support. The project was support by an IIRG Grant from Palestine Polytechnic University.

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