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Identifying Exclusion Genes of Bacteriophage HK239

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Senior Thesis for University Honors Program

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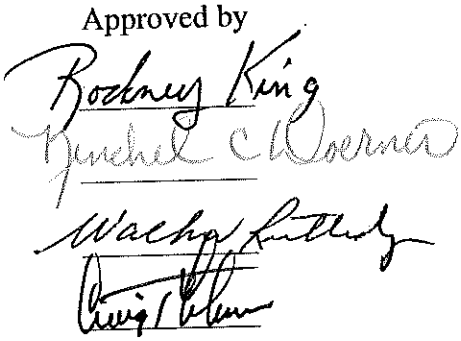
Approved by



2006

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Abstract

Temperate bacteriophage can adopt either a lytic or a lysogenic lifestyle. In the lytic lifestyle, the bacteriophage infects a cell, uses the host's cellular machinery to replicate, and lyses the cell to release the phage progeny. In the lysogenic lifestyle the bacteriophage genome is integrated into the bacterial genome to generate a prophage. Most of the genes of the prophage are repressed. However, those genes that are expressed have a wide variety of functions including exclusion, the prevention of phage infection. Lysogens of bacteriophage HK239 have the ability to exclude a wide variety of phage, including λ, T4rII, P1*vir*, P2, and HK022. The goal of this research was to understand how HK239 prevents the growth of this diverse group of phages, by identifying the gene or genes that are responsible for the exclusion function. To this end, a library of HK239 DNA fragments was constructed and transformed into *Escherichia coli*. After the transformants were screened for phage resistance, one clone was found to exclude HK022 and a λφ80 hybrid. The recombinant plasmid was isolated, and the sequence of the cloned DNA revealed the presence of a φ80 *cor* homolog. In φ80, the *cor* gene is responsible for preventing infection by rendering the phage binding site inaccessible.

Introduction

Temperate bacteriophage can adopt a lytic or lysogenic lifestyle. In both lifestyles, the bacteriophage injects its genome into a bacterial cell. During the lytic cycle, bacteriophage use the host's cellular machinery to replicate its genome and produce the phage-encoded proteins. At the end of the cycle, the cell lyses and releases new phage to repeat the cycle of infection. In the lysogenic lifestyle, the bacteriophage integrates its genome into the host chromosome, creating a prophage. Most of the prophage genes are not expressed; however, those that are expressed encode a variety of functions, including virulence and exclusion mechanisms (Cann, 2001). Exclusion mechanisms (see Table 1) vary among phage and range from exonucleases, which degrade injected DNA, to the death of the single infected cell, effectively preventing the spread of the virus to the rest of the colony.

Bacteriophage Lambda has one of the more extensively studied superinfection exclusion systems, the Rex system. Located within Lambda's immunity region, the Rex system excludes Lambda, T4, and T4rII (Engelberg-Kulka, 1998). Lambda exclusion by Rex differs from immunity in that the phage repressor is not involved. The system is comprised of two genes: *rexA* and *rexB*. Upon infection and replication of T4, *RexA* activates *RexB*. Activated *RexB* forms an ion channel in the plasma membrane and

allows monovalent cations to flow into the cell. The loss of membrane potential disrupts ATP production and kills the cell. This prevents the phage from infecting other lysogens in the colony (Snyder, 1995). Bacteriophage P2 has an exclusion mechanism that differs greatly from the Rex system. In P2, the *old* gene encodes an exonuclease, an enzyme which degrades DNA. The *old* exonuclease degrades double-stranded DNA, single-stranded DNA, and RNA, but will not circular DNA. This system effectively excludes Lambda by degrading its DNA when upon infecting a P2 lysogen (Myung, 1995).

Another exclusion mechanism is found in some strains of *E. coli*. These strains have retained a cryptic prophage-like element called e14. A gene on this prophage encodes a protease called Lit that is responsible for cleaving EF-Tu, an elongation factor essential for protein synthesis. The proteolytic activity of Lit is induced when a short peptide called "gol" (growth on Lit) is synthesized. The gol peptide is a T4 coat protein, and it has been suggested that there are high levels of this protein late in the T4 growth cycle. The protease activity induced by gol is responsible for killing the host before T4 can complete its growth cycle. As the mechanism comes into play late in the growth cycle, the EF-Tu cleavage does not prevent all infections from spreading (Gottesman, 1998).

Other, less understood mechanisms of exclusion can be found in phages K139 and P22. Bacteriophage K139 is known to infect *Vibrio cholera*, the bacterium that is responsible for cholera outbreaks. The phage contains a gene, called *glo*, that encodes for a 13.6 kDa periplasmic protein. The gene is expressed during the phage's lysogenic phase and is responsible for early inhibition of infection by competing phage (Nesper, 1999). Bacteriophage P22 infects *Salmonella* and has evolved mechanisms to exclude

other *Salmonella* bacteriophage. P22 has two exclusion systems, encoded by the SieA and SieB (superinfection exclusion) proteins. Both of these systems exclude bacteriophage L, MG178, and MG40 but work independently of one another. The gene product of *sieB* is believed to cause abortive infection, and the gene product of *sieA* is thought to be a cytoplasmic membrane protein, which excludes the phage by preventing its entry into the cell (Hofer, 1995).

A mechanism of exclusion similar to P22's *SieA* system is used by bacteriophage φ80. Lysogens of φ80 exclude N15, T1, T5, and other φ80 phage. The φ80 *cor* gene product interferes with FhuA. FhuA is a membrane protein which contains a β barrel motif that allows for transport of ferrichrome and antibiotic related compounds. Bacteriophage enter the lysogen by binding to FhuA. The *cor* gene product interacts with FhuA to block infection and also stops the transport function of the protein. The *cor* gene product is hypothesized to be a transmembrane protein (Uc-Mass, 2004). Our study indicated that this may be an exclusion mechanism used by HK239.

Bacteriophage HK239, the focus of this study, is unique in that, unlike most phage, it excludes a wide variety of phage, including P2, T4rII, P1*vir*, Lambda, and HK022 (Dhillon, T. S. and Dhillon, Elvera K. S., 1973). The bacteriophage was isolated from cow dung in Hong Kong in the early 1970s. The phage forms clear plaques on most *E. coli* strains with the exception of K-12 strain HfrC and C strain C1a, on which it forms turbid plaques (Dhillon, Elvera K. D. and Dhillon, T. S., 1973). In their characterization of HK239, Dhillon and Dhillon successfully created a mutation in the phage genome, called *exc*, that allowed the growth of lambda, HK022, P1*vir*, P2, and T4rII (Dhillon, T. S. and Dhillon, Elvera K. S., 1973). No additional work was done on

HK239. As a result, many samples of the phage, including the *exc* mutant, have been lost. In this project we began characterizing HK239v the only known viable sample of the phage. HK239v is a virulent mutant that can only adopt a lytic lifestyle. Since this phage is unable to form lysogens, we could not attempt to re-isolate the *exc* mutant. Therefore, to identify the gene or genes responsible for exclusion, fragments of the HK239 genome were cloned and screened for exclusion activity.

Methods

Growth of Bacteriophage

Serial dilutions of HK239 (10^{-3} – 10^{-9}) were made in TMG (10 mM Tris, 10 mM $MgSO_4$, and 0.01% gelatin). One hundred µL of each dilution were added to an overnight culture of 50µL of *E. coli* strain LE392. Three mL of top agar (10 g tryptone, 5 g yeast extract, 5 g NaCl, and 8 g agar in 1L) were added to the mixtures and poured onto plates. After an overnight incubation at 37°C, plaques were picked and placed in 1.0 mL TMG. The suspended plaques were incubated overnight at 4°C. The plate lysate method was used to generate high titer stocks of phage for genomic DNA isolation. An agar plug containing a phage plaque was added to 100 µL of an overnight LE392 culture. The cells were incubated for twenty minutes at 37°C. Following incubation, 2.5 mL of molten top agarose (1% w/v Bacto®-tryptone, 0.8% w/v NaCl, and 0.6% w/v agarose) were added to the infected cells, mixed, and then poured onto a prewarmed plate. This was done for each plate. Once the top agarose had solidified, the plates were incubated overnight at 37°C. After allowing time for plaque formation, 2 to 3 mL of SM buffer (0.01% gelatin, 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 8 mM $MgSO_4$) were added to each plate. The top agarose was scraped off the plate, transferred to a high speed 30 mL centrifuge tube, incubated at room temperature for 30 minutes, and centrifuged for 10

minutes at 8,000-10,000 x g and 4°C. The supernatant was transferred to a fresh tube, and chloroform was added to 0.3% (v/v). The phage lysate was stored at 4°C.

Isolation of HK239 DNA

The HK239 DNA was extracted using the Wizard® Lambda Preps DNA Purification System. This kit facilitates phage precipitation and phage coat removal. As the kit directed, the lysate was treated with nuclease mixture (40 µL per 10 mL lysate) and incubated at 37°C for 15 minutes. Then 4 mL of phage precipitant solution were added, and the lysate was incubated on ice for 30 minutes. Following incubation the lysate was centrifuged at 10,000 x g for 10 minutes. The supernatant was removed and the pellet resuspended in 500 µL phage buffer. Once the sample was transferred to a microfuge tube, the sample was centrifuged at 10,000 x g for 10 seconds. The supernatant was transferred to a new tube, and 1 mL of resin was added. The solution was then transferred to a syringe barrel and applied to a minicolumn. The column was washed with 2 mL of 80% isopropanol. Then the resin in the column was dried by centrifugation (10,000 x g for 2 minutes). Following centrifugation, 100 µL of water preheated to 80°C were added to the column and was centrifuged for 20 seconds at 10,000 x g. The eluted DNA was later examined on a 1% agarose gel.

Construction of Phage DNA Library

Single restriction enzyme digests with EcoRI, HindIII, and BamHI were performed on the purified HK239 DNA and the cloning vector, pUC18 (extracted from RK726 using a QIAprep® Miniprep kit). Digests consisted of 10 µL DNA, 2 µL of appropriate 10x buffer, 1 µL BSA, 1µL enzyme, and 6 µL H_2O. The digests were

incubated at 37°C for one hour. The digested HK239 DNA and pUC18 DNA, a λ HindIII marker, and undigested controls were electrophoresed on a 1% agarose gel and stained with ethidium bromide.

The restriction digests were typically phenol chloroform extracted twice followed by two extractions with an equal volume of chloroform. The aqueous fraction was collected and concentrated using a centricon concentrator (Centricon 100). The plasmid DNA suspended in H_2O was dephosphorylated as follows: 7 μL of plasmid, 3 μL of 10X Alkaline phosphatase buffer, 19 μL H_2O, and 1 μL CIP enzyme. The reaction was incubated at 37°C for 30 minutes. The dephosphorylated DNA was phenol chloroform extracted and centricon purified as outlined above.

The HK239 fragments from the EcoRI digest and the dephosphorylated plasmid were ligated together as follows: 10 μL dephosphorylated plasmid, 1 μL digested phage DNA, 2 μL buffer, 6 μL H_2O, and 1 μL T4 DNA ligase. The reaction was incubated at 14°C overnight. The ligation was heat inactivated the next day by a 20-minute incubation in a 65°C water bath. Afterwards, the reaction was centricon purified and further concentrated in a speed vac to a volume of 95 μL.

Transformations

Chemically competent cells were made using a calcium chloride protocol (*Molecular Cloning*, 3rd ed.). *E. coli* strain XL-1 Blue (see Table 2) was grown in 100 mL LB at 37°C to an O.D. of approximately 0.35. The culture was transferred to 50 mL tubes, incubated on ice for 10 minutes, then centrifuged at 2700 x g 4°C for 10 minutes. The supernatant was decanted, and the tubes were inverted for one minute at room

temperature to drain the remaining media. Each pellet was resuspended in 30 mL of an ice cold $MgCl_2$-$CaCl_2$ solution (80 mM $MgCl_2$ and 20 mM $CaCl_2$). The centrifugation was repeated, and the pellet was resuspended in 2 mL ice cold $CaCl_2$. The cells were aliquoted into microcentrifuge tubes (200 µL per tube) and stored at -80°C.

Two hundred µL of *E. coli* XL-1 Blue cells were mixed with 15 µL of ligation reaction and incubated on ice for 30 minutes. The mixture was transferred to a 42°C water bath for 90 seconds and immediately placed on ice afterwards. After cooling, 2 mL of SOC (2% w/v Bacto® tryptone, 0.5% w/v Bacto® yeast extract, 0.01 M NaCl, 2.5 mM KCl, 0.02 M $MgSO_4 \bullet 7H_20$, and 0.02 M glucose; pH 7.0) were added to the cells and incubated at 37°C without shaking for one hour. Aliquots of the transformation mix were plated onto LB plates containing ampicillin (amp, 100 µg/mL), X-gal (40 µg/mL) and IPTG (0.1 mM).

Identifying clones with HK239 inserts

Transformants with inserts were identified using blue-white screening. Blue-white screening utilizes vectors containing the *lacZ* gene and *E. coli* strains that lack *lacZ*. *LacZ* encodes the β galactosidase protein, which will hydrolyze X-gal. X-gal, when broken down, produces a blue precipitate and causes the cells to turn blue. Vectors used for blue-white screening contain a multiple cloning site in the lacZ gene. When a fragment is inserted into this site, *lacZ* is disrupted and no functional β galactosidase is produced. As a result, colonies with insertions are white, while colonies without insertions are blue (Geocites, 2005). White transformants were streaked onto LB amp/X-

gal plates for purification. Overnight cultures of purified colonies were grown in 5 mL of TB supplemented with ampicillin.

Screening for phage exclusion

To test for phage resistance, a cross-streak test was performed. A loopful of phage W248 (a λ φ80 hybrid) was streaked down the center of an LB plate and allowed to soak into the agar. Cultures of the transformants and the control XL-1 cells were then streaked perpendicular to the phage streak, allowing them to pick up phage. The infected cells either did not grow or grew poorly beyond the phage streak. Normal growth would indicate that the clones were resistant to phage infection. The plates were incubated overnight at 37°C, and the transformants were checked the following day for sensitivity.

One transformant that tested positive for W248 resistance was characterized further. Dilutions of phages P2, λhφ80, λimmHK022, HK022, W10 (λ), W14 (λvir), W30 (λb2Δ), HK0283 (clΔ), HK022 (WT), and HK0228 were spotted onto a lawn of the transformant. The plate was incubated overnight at 37° and checked for plaques the following day.

Sequencing of cloned HK239 DNA.

Recombinant Plasmid was extracted from the positive transformant using a Qiaprep® Spin Miniprep Kit (Qiagen), and the DNA was examined on a 1% agarose gel stained with ethidium bromide. Sequencing reactions for the recombinant plasmids consisted of the following: 1 μL of plasmid was added to 1 μL M13 primer (forward or reverse), 4 μL H_2O, and 4 μL Big Dye 3.1 (Applied Biosystems). The reactions were

amplified in 25 cycles under the following conditions: 96°C for 30 seconds, 50°C for 15 seconds, and 60°C for 4 minutes. The completed reactions were purified by alcohol precipitation. Forty microliters of room temperature 75% isopropanol was added to each reaction and incubated for fifteen minutes at room temperature. The samples were then centrifuged at max speed, at room temperature, for twenty minutes. The supernatant was removed, and the pellet was washed with 150 μL of 75% isopropanol. The samples were centrifuged at max speed at room temperature for ten minutes. The alcohol wash was repeated once. To remove any remaining isopropanol, the samples were dried in a speed vac for fifteen minutes. The pellets were suspended in 20 μL TSR (template suppression reagent) and denatured at 95°C for five minutes and then cooled at 4°C for two minutes. The samples were then loaded on an ABI 310. After the samples were analyzed by the ABI Prism® Genetic Analyzer Software, they were edited using Vector NTI. BLAST searches (NCBI) were performed to find homologous sequences.

Testing for P2 Exclusion

P2 does not plate well on *E. coli* strain XL1 Blue, so it was necessary to transform the recombinant plasmid AW22 into a suitable plating strain, C1a (RK876, see Table 2). An overnight culture of C1a was grown from a colony. Electrocompetent cells were made as outlined. The cultures were chilled on ice for 15 minutes before being centrifuged for ten minutes at 1900 x g. The supernatant was discarded, and the cells were resuspended in 5 mL ice cold water. The centrifugation was repeated once. The cell pellet was once more resuspended in water and centrifuged. After the supernatant was discarded, the pellet was resuspended in 1.5 mL 10% glycerol, transferred to a

microfuge tube, and centrifuged at 6000 rpm for ten minutes. The supernatant was discarded, and the cells were resuspended in 40 μL 10% glycerol. For the electroporation 2 μL of recombinant plasmid were added to 40 μL electrocompetent C1a cells. The cells were transferred to a cuvette with 1 mm gap width for higher transformation efficiency and electroporated at 25 μF, 2.5 kV, and 200 ohms. One mL of SOB (20 g tryptone, 5 g yeast extract and 0.5 g NaCl with H_2O to 1L, pH 7.0) was added immediately, and the cells were transferred to a 15 mL tube. After one hour of incubation at 37°C, 5 μL and 10 μL aliquots were plated onto LB amp X-gal plates and incubated overnight at 37°C.

Clones were purified, and single colonies were selected for overnight cultures. A lawn of one of the transformants was generated, and dilutions of P2 were spotted onto the lawn. The plate was incubated overnight at 37 °C and checked for plaques the following day.

Results

Construction of HK239 Library

HK239 genomic DNA and pUC18 plasmid were singly digested with restriction enzymes EcoRI, HindIII, and BamHI. Plasmid pUC18 was linearized by each enzyme as expected because pUC18 has a single site for each of these restriction enzymes. EcoRI generated at least ten fragments of HK239 DNA, whereas HindIII generated seven fragments, and BamHI generated two fragments (see Figure 1). Because EcoRI generated the most fragments, this digest was used to construct the library. The fragments from the EcoRI digest were ligated into pUC18 and transformed into XL-1 Blue cells.

Transformation

Forty white colonies and three blue colonies were obtained. The white colonies were streaked for purification on LB amp, X-gal plates. The remainder of the ligation mixture was plated, and 114 additional white colonies were obtained. These clones were also purified. Overnight cultures of each purified transformant were grown in 5 mL TB supplemented with amp (100 μg/mL).

Screening of Transformants

To identify clones with exclusion phenotypes, a cross-streak test was performed. In the cross-streak test (see Figure 2), transformants from the overnight cultures were streaked perpendicularly across a primary streak of phage W248. As the transformants were streaked across the phage streak, the cells were infected by the phage. If the transformants were sensitive to the phage, they would eventually be lysed. One transformant, AW22, displayed uniform growth across the plate. This indicated that AW22 was resistant to W248. Further analysis was performed on this transformant to confirm its exclusion properties.

To determine the range of exclusion, dilutions of various phage were spotted onto a lawn of AW22. XL-1 Blue cells served as the control. The test revealed that λ could form plaques on AW22 but HK022 could not. This indicated that AW22 was resistant to HK022 but sensitive to λ (see figure 3). P2 does not plate well on XL-1 Blue; therefore, pAW22 was transformed into *E. coli* strain C1a. Dilutions of P2 were spotted on a lawn of C1a that had the recombinant plasmid. Plaque formation was observed, demonstrating that pAW22 does not confer resistance to P2 (data not shown).

The resistance to HK022 and φ80 is mediated by a φ80 cor homolog

The recombinant plasmid from clone AW22 was isolated and sequenced. A BLAST search of the sequencing data revealed the presence of a φ80 *cor* homolog. In φ80, the *cor* gene product prevents superinfection of φ80, N15, T1, T5, and other phage. There was a strong homology between the two sequences (see figure 4), suggesting that this may be responsible for some of the exclusion properties of HK239. The few

discrepancies, however, have been confirmed by sequencing. Three amino acid substitutions appear to have been made: R45A, E71K, and Y91N. In φ80, S92 is followed by a stop codon. In HK239, S92 is followed by a valine, a serine, and then a stop codon.

Discussion

Bacteriophage HK239 is unusual in that its lysogens can exclude a variety phage. When Dhillon and Dhillon (1973) isolated a mutant defective for exclusion of λ, HK022, P1*vir*, and P2, they hypothesized that a single gene was responsible for the wide range of exclusion. However, our results suggest otherwise. Clone AW22 was found to exclude W248 and HK022, but not Lambda or P2. This suggests that other genes are responsible for the exclusion of these unrelated phage.

The sequencing data revealed a homolog of the φ80 *cor* gene in the AW22 recombinant plasmid. The *cor* gene is responsible for preventing superinfection of φ80 lysogens (Matsumoto, 1985). The interaction of Cor with FhuA prevents the entry of phage into the host (Uc-Mass, 2004). W248 is a λφ80 hybrid in which the immunity region of φ80 has been replaced with that of Lambda. W248 may have been excluded because it is mostly φ80. Although it is probably responsible for the exclusion phenotype, the *cor* homolog in AW22 has not yet been inactivated to show that it is the gene responsible for the exclusion phenotype.

Clone AW22 did not exclude many of the phage that lysogens of HK239 have been reported to exclude. Therefore, it was necessary to try to isolate clones that exclude these phage. To identify the genes responsible for Lambda, P2, T4rII, and P1*vir*

exclusion, a second HK239 DNA library was created using HindIII fragments of HK239 DNA. The resulting transformants were tested for λ exclusion in the same manner that the EcoRI transformants were tested. Two candidates were identified, but the recombinant plasmids contained active lysis genes. As a result, the transformants exhibited poor growth, making the exclusion phenotype doubtful. We are currently sequencing the genome of HK239. This will require about the same amount of time as searching for the gene by cloning and screening, and it will provide us with a wealth of information. The sequence will allow us identify candidate genes in silico. The genomic sequence will also us to determine how HK239 is related to other members of the phage community.

Exclusion is of interest in HK239 because it can exclude a wide variety of phage, which makes HK239 unusual. We have identified a phi80 *cor* homolog that appears to be responsible for excluding some phage, but we have yet to identify other exclusion genes. These unidentified genes may be homologous to known exclusion genes in other phage or they may encode a novel exclusion mechanism. Exclusion is important in understanding bacteriophage interactions, and it can influence bacterial and bacteriophage evolution. Like any biological entity, phage must adapt to their environment. Phage that can form a lysogen and prevent other phage from entering and lysing the host bacterium will have a selective advantage.

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Yahoo. What is Blue/White colony selection (or screening) and how does it work? http://www.geocities.com/jsonnentag/iguana/bluwht.htm

Appendix

Phage/Prophage	Gene	Phage Excluded	Reference
λ	*rexA, rexB*	λ, T4, T4rII	Engelberg-Kulka, 1998
P2	*old*	λ	Myung, 1995
e14	*Lit*	T4	Gottesman, 1998
P22	*sieA*	L, MG178, MG40	Hofer, 1995
P22	*sieB*	L, MG178, MG40	Hofer 1995
φ80	*cor*	N15, T1, T5, φ80	Uc-Mass, 2004

Table 1: A summary of phage exclusion mechanisms.

Strain	Genotype
XL-1 Blue	F' ::Tn*10 proA*$^+$*B*$^+$ *lacI*^{q} *Δ(lacZ)M15/recA1 endA1 gyrA96* (Nal^r) *thi hsdR17* ($r_k^-m_k^+$) *supE44 relA1 lac* (Bullock, 1987)
C1a (RK876)	*E. coli* C wild type; F$^-$ restriction defective (Maniatis, 1982 and Woodcock, 1989)
LE392	F$^-$ e14$^-$(McrA$^-$) *hsdR514*($r_k^-m_k^+$) *glnV44 supF58 lacY1 or Δ(lacIZY)6 glaK2 galT22 metB1 trpR55* (Sambrook, 1989)

Table 2: A summary of strain genotypes.

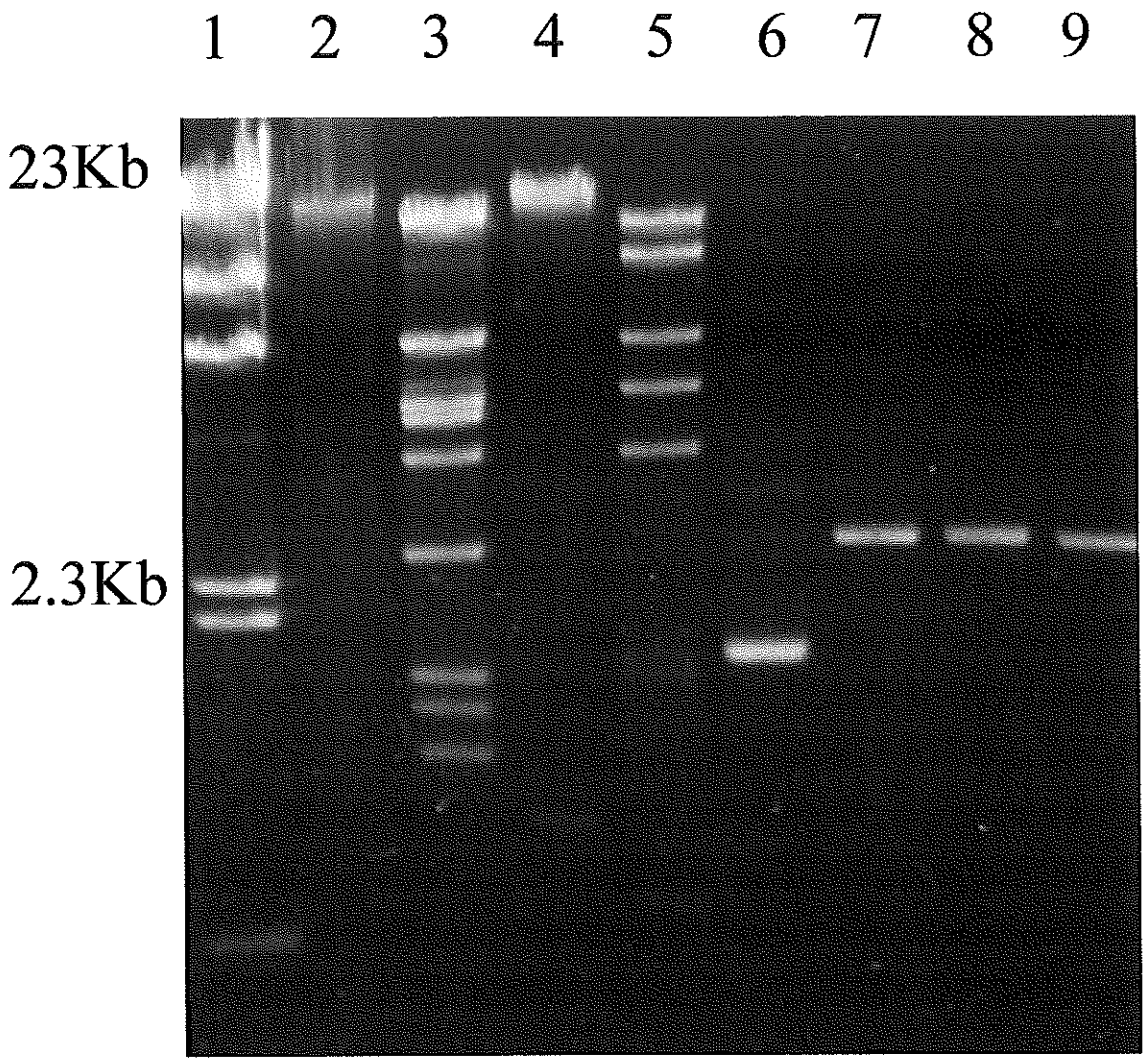

Figure 1: Restriction digests of HK239 and pUC18 on 1% agarose gel. Lane 1 contains a λ HindIII marker. Lane 2 contains uncut HK239 DNA. In lanes 3-5 are single restriction digests of HK239 using EcoRI, BamHI, and HindIII in that order. Lane 6 contains uncut pUC18, and lanes 7-9 contain pUC18 cut with EcoRI, BamHI, and HindIII respectively.

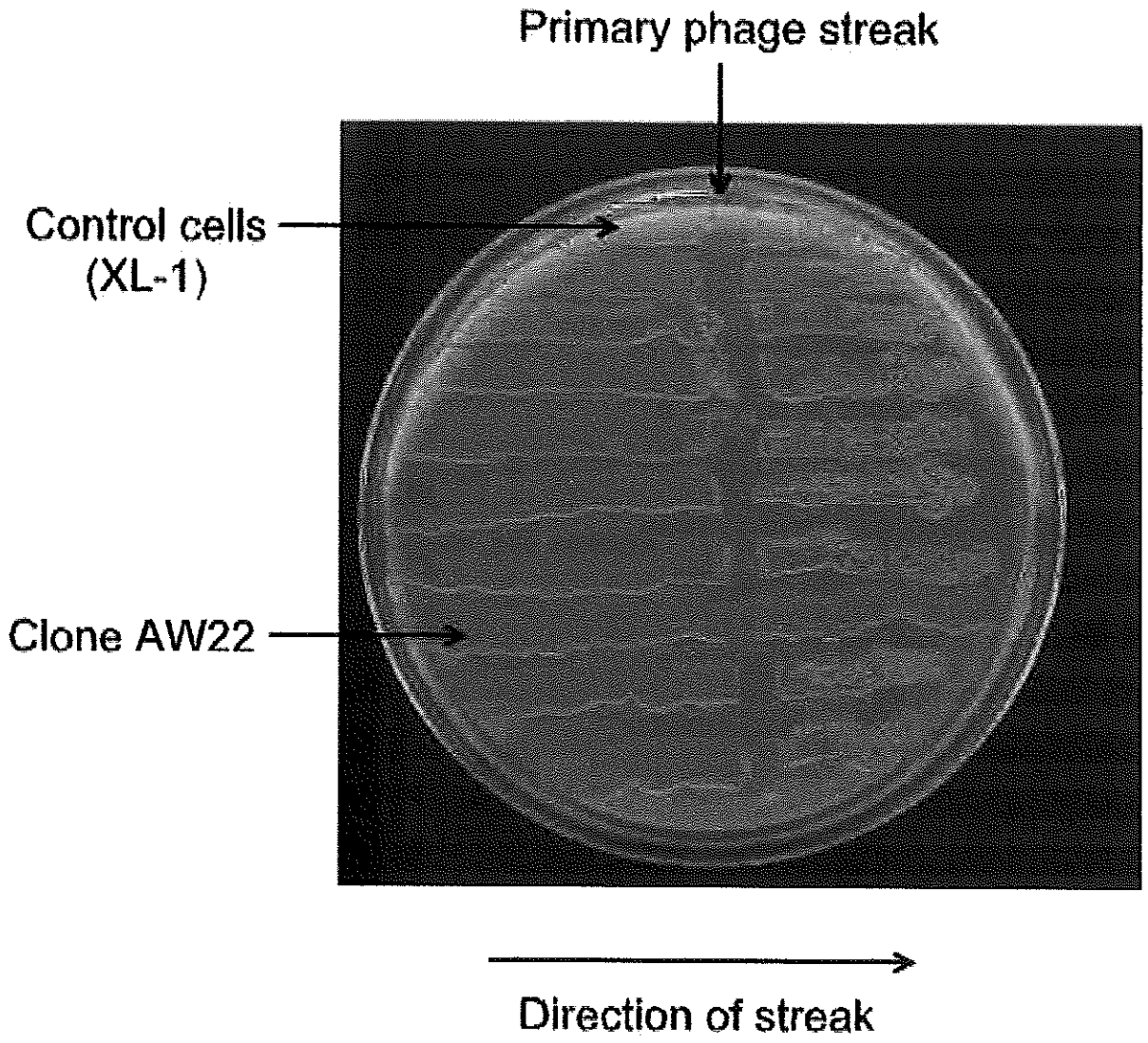

Figure 2: Cross-streak test for exclusion. The primary phage streak is W248. Control cells show interrupted growth when crossing the phage streak. All clones tested on this plate resemble control cells except for clone AW22, which had uninterrupted growth across the plate.

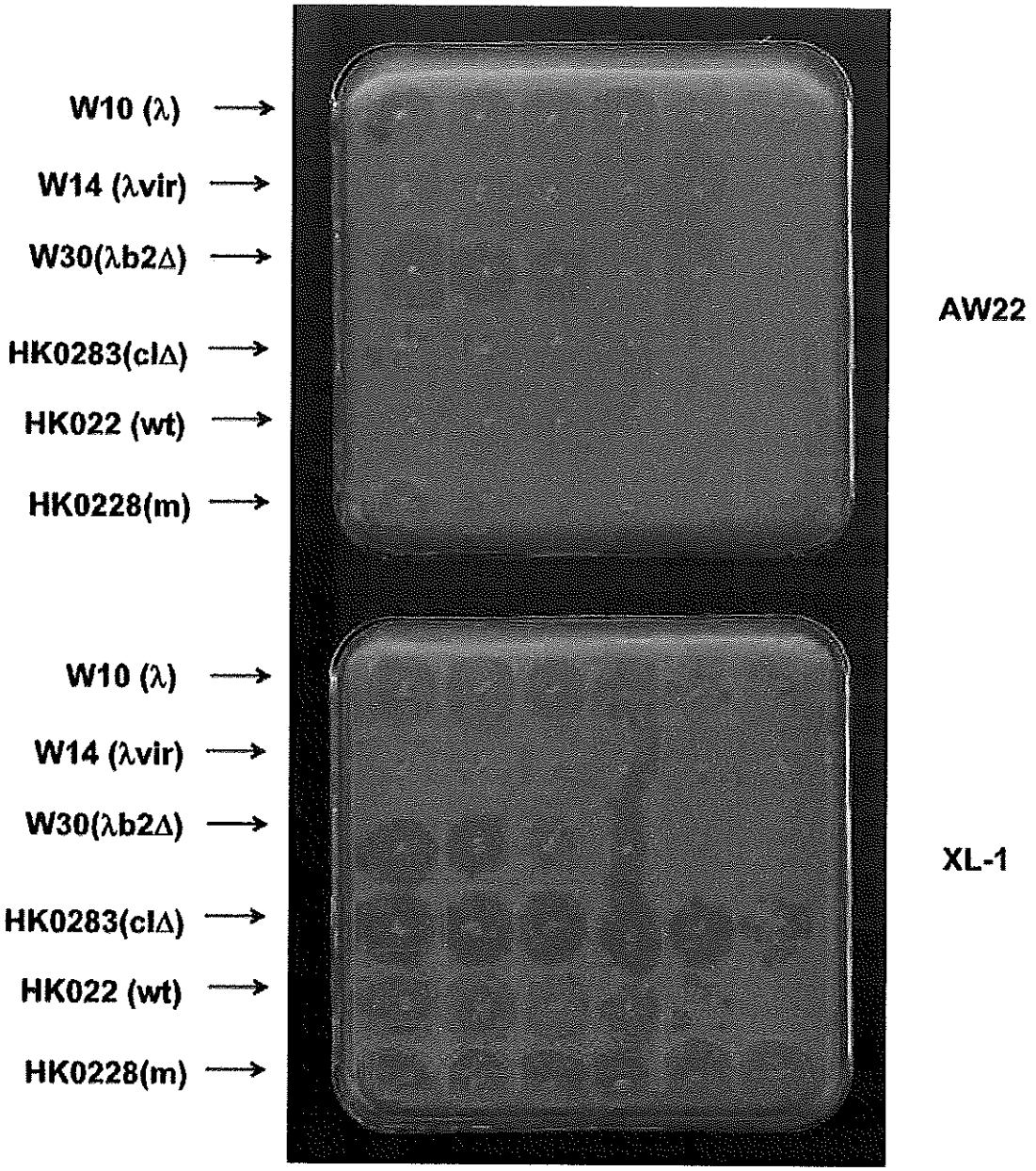

Figure 3: Plaque formation of the indicated phage on lawns of AW22 and XL-1 (control). Bacteriophage λ was able to form plaques on both AW22 and the control. HK022 did not form plaques on AW22 whereas it did on the control.

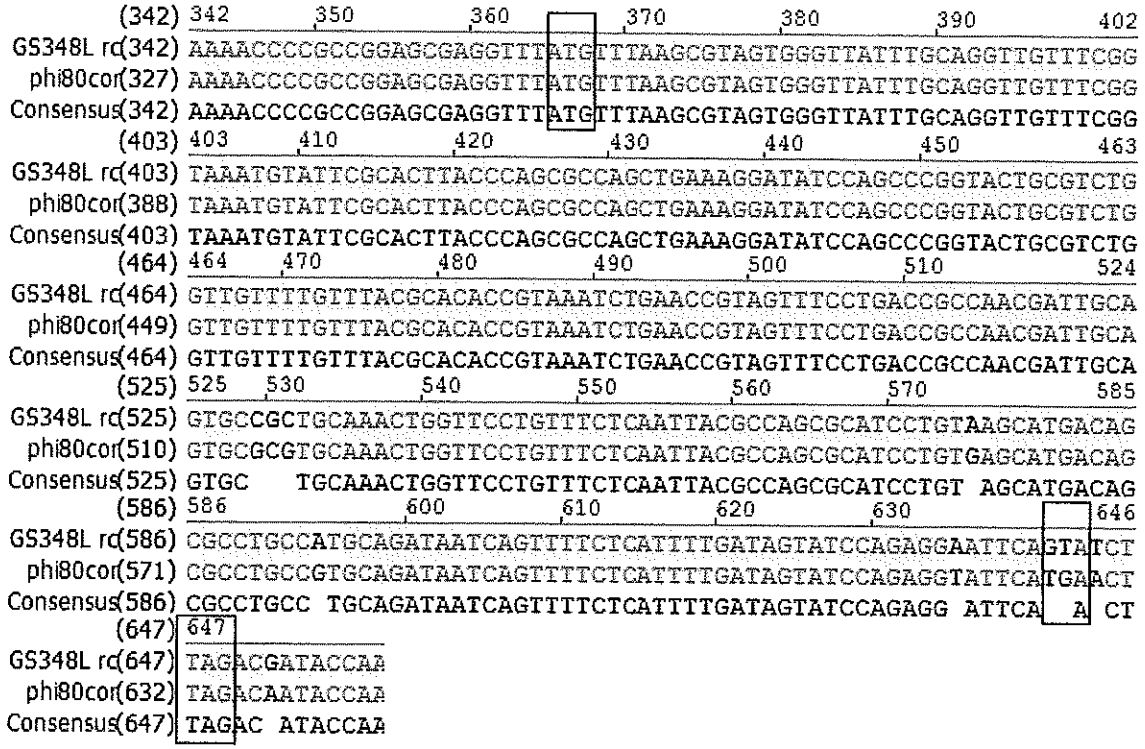

Figure 4: Alignment of φ80 *cor* gene and the HK239 homolog (this data is from clone GS348). Yellow shading shows areas of homology. The first black box (between 360 and 370) indicates the start codon, and the second black box (between 630 and 646) shows the stop codon for φ80 *cor*. The red box indicates the stop codon for the HK239 homolog.