

# Macroarray analysis of gene expression in a marine pseudotemperate bacteriophage

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**ABSTRACT:**  $\phi$ HSIC is a pseudotemperate phage that infects *Listonella pelagia*, isolated from Mamala Bay, Oahu, Hawaii.  $\phi$ HSIC has a circularly permuted genome 37, of length 966 nt, containing 47 putative open reading frames (ORFs). The pseudolysogenic interaction results in sigmoidal growth curves yielding simultaneous production of high host and phage abundances. The purpose of the present study was to determine patterns of phage gene expression in response to conditions that favor lytic or lysogenic interactions. To evaluate the gene expression patterns of this phage, macroarrays were generated by dotting PCR amplicons of each ORF onto a filter membrane. Viral gene expression over the course of an infection cycle was examined, as was gene expression in the HSIC-1a pseudolysogen grown at normal (39 ppt) and low (11 ppt) salinity. Viral gene expression was significantly higher in the 39 ppt treatment in 2 experiments for nearly every ORF. Free phage and intracellular phage concentrations were significantly lower in the 11 ppt treatment. The results of these experiments indicate that changes in environmental conditions, such as lowered salinity, favor a lysogenic-like relationship in the HSIC-1a pseudolysogen by modulating phage gene expression. These experiments have provided a window into the expression of  $\phi$ HSIC phage genes in response to environmental changes, and may be a model for how phages and their hosts respond to changing conditions in the marine environment.

**KEY WORDS:** Pseudolysogeny · Gene expression analysis · Salinity stress

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## INTRODUCTION

Genomic sequencing can provide valuable information on genome organization, putative gene content, evolutionary relationships between organisms, and metabolic pathways. However, information on gene regulation and expression cannot be gleaned from sequence information alone. Techniques such as Northern blotting and real-time PCR have been used to detect and measure levels of phage gene expression, but these techniques are limited to the study of a few genes at any one time (Sumby & Waldor 2003, Ventura & Brüssow 2004, Frye et al. 2005). As such, array technology has been developed to study whole genome expression in an organism.

Analysis of viral gene expression using array technology has led to the mapping of the entire infection

process over the course of a lytic infection (Duplessis et al. 2005, Lua et al. 2005, Semenova et al. 2005). Unfortunately, many genes of marine bacteriophages have no identifiable homologs in genetic databases. As a result, it is difficult to draw conclusions about their function. However, data from the expression analysis of a viral genome can lead to preliminary classification of unknown genes by comparing the initial time of expression and changes in expression levels from unknown genes to the known genes around it (Lua et al. 2005). Finally, viral genome expression analysis may lead to the use of anti-sense RNA to develop anti-phage treatments that can prevent phage infection, thereby reducing the negative effect that phages can have on bacteria (Duplessis et al. 2005).

$\phi$ HSIC, a temperate phage with pseudotemperate characteristics, was one of the first marine phages to be

sequenced (Jiang et al. 1998, Williamson et al. 2001, Paul et al. 2005).  $\phi$ HSIC infects the bacterium *Listonella pelagia*, and the infected strain is known as HSIC-1a pseudolysogen. Growth of the pseudolysogen is characterized by sigmoidal growth curves with high production of host cells and phage particles ( $>10^{10}$  ml<sup>-1</sup>) as has been observed for other pseudolysogens (Moebus 1997a,b,c). Probing of southern transfers of host genomic DNA indicated chromosomal integration of the prophage, yet phages were not inducible by Mitomycin C (Williamson et al. 2001). Infected cells were also homoimmune, a hallmark of lysogeny. Genomic sequencing of the phage did not yield any recognizable lysogeny genes. In fact, only 8 out of 47 genes could be assigned a putative phage function (large and small terminase subunits,  $\beta$ -subunit of DNA polymerase, capsid protein, tail tape-measure protein, lysin, helicase, and resolvosome helicase) (Paul et al. 2005). In an effort to putatively identify the gene function, we initiated expression analysis of  $\phi$ HSIC by macroarray studies.

Salinity is thought to influence viral lysis in other phage–host systems (Husson-Kao et al. 2000, Lunde et al. 2005, Gnezda-Meijer et al. 2006, Williamson & Paul 2006). While most studies have focused on dairy phages, 2 have extensively examined environmental phages, resulting in conflicting results in regards to the effect of salinity on lysogeny (Husson-Kao et al. 2000, Gnezda-Meijer et al. 2006). In lysogenic strains of *Streptococcus thermophilus*, sodium chloride concentrations beyond the cell's normal range resulted in almost immediate cell lysis. Bacteriophage fragments were observed by electron microscopy, indicating that the bacteriophages produced were not stable (Husson-Kao et al. 2000). On the other hand, increased concentrations of sodium chloride led to an increase in the phage latent period during induction experiments with *Vibrio* spp. (Gnezda-Meijer et al. 2006).

Induction studies with CTX $\phi$ , a phage that infects *Vibrio cholerae*, showed that when the salinity was at or below 0.1% (w/v), the majority of transducing particles were inactivated. CTX $\phi$  was most stable at salinities above 0.5%. Additionally, the frequency of spontaneous induction of  $\phi$ LC3, a *Lactococcus lactis* phage, significantly decreased as the osmolarity of the solution was increased from 0 to 1.0%. This indicates that the stability of the prophage was increasing as salinity increased (Faruque et al. 2000, Lunde et al. 2005).

Previous salinity experiments with the  $\phi$ HSIC/*Listonella pelagia* marine phage–host system have shown that growth and phage production were stimulated at high salinities and depressed at low salinities (Williamson & Paul 2006). These data provided a method to experimentally manipulate the lysogenic response in the HSIC-1a pseudolysogen and further

explore gene expression studies that might help identify the genes involved in the lysogeny of this phage–host system.

Macroarrays have been successfully used to study a number of different organisms including the bacteriophage Xp10 (Semenova et al. 2005), *Sinorhizobium meliloti* (Bergès et al. 2003, Becker et al. 2004) and *Lactococcus lactis* (Xie et al. 2004) as well as microbial gene diversity in environmental samples (Jenkins et al. 2004, Steward et al. 2004). While not as sensitive as microarrays, macroarrays are less expensive to produce and require less instrumentation to analyze while providing a quantitative measurement of gene expression (Bergès et al. 2003, Steil et al. 2003, Becker et al. 2004, Xie et al. 2004, Zheng et al. 2004, Semenova et al. 2005). To better understand the molecular lysogeny switch in a marine phage and to understand lysogeny in the marine environment, macroarrays were used in the present study to analyze the infection cycle of  $\phi$ HSIC during a synchronous infection, and to study  $\phi$ HSIC gene expression in normal and low salinity conditions.

## MATERIALS AND METHODS

**Cultivation and growth of *Listonella pelagia* and the HSIC-1a pseudolysogen.**  $\phi$ HSIC was isolated from Mamala Bay, Oahu, Hawaii, and infects *Listonella pelagia*, a marine heterotrophic bacterium (Jiang et al. 1998). The uninfected host strain is known as *L. pelagia* and the infected strain is referred to as the HSIC-1a pseudolysogen. The HSIC-1a pseudolysogen is a second generation lysogen, as the first generation, HSIC-1e, was unstable and the virus segregated from the host (Williamson et al. 2001). All experiments were conducted at 28°C in a bench-top incubator with shaking at 150 rpm and all plates were incubated at 28°C. *L. pelagia* and the HSIC-1a pseudolysogen were both grown in ASWJP+PY (3.1% w/v); the salinity of ASWJP is 33 ppt, and the addition of peptone and yeast increase the salinity of the media to 39 ppt. To lower the salinity of the media to 11 ppt (0.39% w/v), the amounts of NaCl and MgSO<sub>4</sub> × 7 H<sub>2</sub>O used to make ASWJP+PY were reduced.

**PCR of open reading frames.** Forward and reverse primers were designed for all 47  $\phi$ HSIC open reading frames (ORFs) using Primer 3 software ([http://frodo.wi.mit.edu/cgi-bin/primer3/primer3\\_www.cgi](http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi)). In addition, 16S rDNA primers were used to amplify host DNA to generate a positive control for RNA extraction efficiency. Primer sequences are listed in Appendix 1 (available online as Supplementary Material at: [www.int-res.com/articles/suppl/a049p001\\_app.pdf](http://www.int-res.com/articles/suppl/a049p001_app.pdf)). PCR reactions for all the ORFs except 7, 20, 30, and 42

had a total volume of 50  $\mu\text{l}$ : 2  $\mu\text{l}$  of HSIC template DNA at a concentration of 4.25 ng  $\mu\text{l}^{-1}$ , 1  $\mu\text{l}$  (100  $\mu\text{M}$ ) each of both the forward and reverse primer, 25  $\mu\text{l}$  of Promega PCR Master Mix 2X (Promega), and 21  $\mu\text{l}$  of sterile deionized water. The PCR Master Mix comprised 3 mM  $\text{MgCl}_2$ , 400  $\mu\text{M}$  each of dATP, dCTP, dGTP, and dTTP, and 1.25 U *Taq* DNA polymerase in a Promega proprietary reaction buffer.

ORFs 7, 20, 30, and 42 did not yield sufficient amplicons for blotting and were cloned using Topo TA kit (Invitrogen). DNA was isolated from the cloned cells using the Promega Wizard Plus Miniprep DNA Purification. PCR was done to amplify the clones, the DNA was diluted to 5 ng  $\mu\text{l}^{-1}$  and 1  $\mu\text{l}$  of the dilution was mixed with 1  $\mu\text{l}$  (100  $\mu\text{M}$ ) each of the M13 forward and reverse primers (Invitrogen), 25  $\mu\text{l}$  of Promega PCR Master Mix 2X, and 22  $\mu\text{l}$  of sterile deionized water.

PCR reactions were heated at 95°C for 2 min to ensure template denaturation followed by 40 cycles of denaturation at 94°C for 45 s, annealing at the appropriate temperature (either 51 or 53°C as determined by trial and error) for 45 s, and extension at 72°C for 2 min. There was a final extension at 72°C for 10 min. Products were confirmed by gel electrophoresis on 1% agarose gels stained with EtBr and purified using a Zymo Clean & Concentrator™-25 kit (Zymo Research). Purified PCR products were quantified using the Hoechst 3328 method (Paul & Meyers 1982).

**Macroarray production.** PCR products quantified by the Hoechst 3328 method (Paul & Meyers 1982) were diluted in RNase-free water. To denature the DNA, 10 mM EDTA and 0.4 M NaOH were added to the DNA and the mixture was then boiled for 10 min. DNA was neutralized after boiling by the addition of an equal volume of cold 2 M ammonium acetate.

Macroarrays were produced by dotting 100 ng of each ORF in duplicate on charged nylon membrane (0.45  $\mu\text{m}$ , Osmonics) using a Bio-Rad 96-well dot blotter. Of the 16S rDNA positive control, 1 ng was spotted in every corner. A schematic of the array illustrating ORF location can be found in Appendix 2 (available online as Supplementary Material at: [www.int-res.com/articles/suppl/a049p001\\_app.pdf](http://www.int-res.com/articles/suppl/a049p001_app.pdf)). A low vacuum was then used to pull the samples through the dot-blotter. Once all the liquid was through, 500  $\mu\text{l}$  of 0.4 M NaOH was applied to neutralize the DNA. The filter was removed from the blotter and crosslinked using a UV crosslinker (FB-UVXL-1000, Fisher Scientific).

**Control blots.** To detect cross-hybridization by host DNA, ORFs 7, 8, 9, 12, 18, 31, 33, 35, 37, 41, 44, and the *Listonella pelagia* 16S rDNA were dotted in triplicate on a 0.45  $\mu\text{m}$  charged nylon membrane. Five ml of overnight culture of uninfected *L. pelagia* was transferred into 50 ml ASWJP+PY, and cultures were grown until the optical density of the culture was between

0.400 and 0.600, mid-log phase. The culture was split into two 25 ml samples to avoid overloading RNeasy columns with cellular material, and RNA was extracted. cDNA probes were made with 5  $\mu\text{g}$  of RNA and hybridized to the blots overnight.

ORFs 17 and 42 were dotted 20 times each on a membrane along with 4 negative control spots and 4 *Listonella pelagia* 16S rDNA spots to test the precision of the method. Twenty ml of an overnight culture of HSIC-1a was transferred into 200 ml of ASWJP+PY.  $\phi$ HSIC lysate was added to the culture when the optical density was between 0.4 and 0.6 at a multiplicity of infection (MOI) of 1. At 60 min post-infection (p.i.), RNA was extracted from the culture. Five  $\mu\text{g}$  of RNA was used to make the cDNA probe, and probes were hybridized to the blots overnight.

**Synchronous infection experiments.** Twenty-five ml of an overnight culture of uninfected *Listonella pelagia* was transferred into 250 ml of ASWJP+PY in a 500 ml flask. When the absorbance at 600 nm ( $A_{600}$ ) was between 0.4 and 0.6, the flask was placed on ice for 10 min to slow cell growth. After 10 min,  $\phi$ HSIC phage lysate with a known titer of  $10^{10}$  was added for an MOI of 1. The culture was kept on ice for 30 min to allow adsorption while inhibiting the infection process. The culture was transferred into a Beckman centrifuge bottle and spun at  $3020 \times g$  for 10 min at 4°C to remove unattached phages. The supernatant was drained off and the pellet resuspended in an equal volume of ASWJP+PY.

The resuspended pellet was then transferred to a sterile flask, and 50 ml of culture were immediately removed for the 0 min p.i. sample. The flask was placed back in the shaker at 28°C. Fifty ml samples were taken every 20 min for 1 h, and RNA was extracted from all the samples.

**Gene expression analysis at varying salinities.** Two overnight cultures of HSIC-1a cells were spun in a Beckman centrifuge at  $3020 \times g$  and the pellets washed twice with sterile ASWJP to remove unattached phages. After the second wash, the pellets were resuspended in either 39 ppt ASWJP+PY or 11 ppt ASWJP+PY. The resuspended pellets were then diluted even further in the appropriate salinity media so that the initial optical density of both cultures was 0.05 at 600 nm.

Optical density measurements were taken once an hour for 10 h and again at 24 h. Phage production was measured every 2 h from  $T_0$  to  $T_{10}$  and again at  $T_{24}$ . At  $T_4$ ,  $T_6$ , and  $T_8$ , samples were removed for RNA extraction. Previous experiments indicated that RNA extraction was most efficient at these time-points. There was also more cellular matter in the 39 ppt sample than in the 11 ppt sample, leading to column overloading and loss of sample during RNA extraction. To accommo-

date for that, 2 samples of 2 ml each were withdrawn from the 39 ppt, and 2 samples of 5 ml each were withdrawn from the 11 ppt sample.

**RNA extraction.** For all RNA extractions, the Qiagen RNeasy mini-kit protocol was followed with minor changes. Briefly, after the sample was spun for 10 min at  $10\,000 \times g$  at  $4^{\circ}\text{C}$ , the supernatant was poured off and the pellet air-dried for 5 min. The cell pellet was then resuspended in 750  $\mu\text{l}$  Buffer RLT that had been mixed with 10  $\mu\text{g} \mu\text{l}^{-1}$  of  $\beta$ -mercaptoethanol (Sigma Chemicals). For the synchronous infection experiments, samples were mixed with muffled glass beads and bead-beaten for 1 min, put on ice for 1 min, bead-beaten for 1 min, and then subjected to a final ice chill. In the salinity experiments, an additional bead-beating/ice step was added to ensure that the cell walls were being completely lysed.

After bead-beating was complete, the sample was spun down to pellet out the glass beads and transferred to a fresh 1.5 ml tube. A total of 350  $\mu\text{l}$  of 100% ethanol was added before the sample was split and applied to 2 RNeasy columns (Qiagen). At that point, RNA was extracted following the Qiagen RNeasy Mini protocol for Isolation of Total RNA from Bacteria. The RNA was concentrated and further purified using the Qiagen RNeasy Mini Protocol for RNA Cleanup and Optional On-Column DNase Digestion with the RNase-free DNase set. Quantification of RNA was done with Ribogreen (Molecular Probes) according to the manufacturer's protocol.

**cDNA probe production, hybridization and chemiluminescent detection.** cDNA probes were produced using the SuperArray TrueLabeling RT Kit (SuperArray Bioscience) with one modification. In place of the gene-specific primer mix provided by SuperArray, random primers (Promega, catalog #C1181) were mixed with the viral RNA sample to make the annealing mixture. Biotin-16-dUTP (Roche Applied Bioscience) was used for labeling. The recommended amount of RNA for a probe was between 2.5 and 5  $\mu\text{g}$ . For both the synchronous infection experiment and the salinity experiment, 2.5  $\mu\text{g}$  of RNA was used to make the probes.

The SuperArray protocol for pre-hybridization, hybridization, and washing of the arrays was followed with some modifications: the volume of the reagents was doubled, and the amount of hybridization solution was tripled. This was done to account for the size difference between the HSIC macroarrays and the typical arrays used by SuperArray. All blots were put in hybridization tubes, and hybridization and washing were carried out at  $60^{\circ}\text{C}$  in a rotisserie style hybridization oven (Fisher Scientific). After the probe was removed from the hybridization tubes, the blots were washed in a series of increasing stringency washes

( $2 \times \text{SSC}$ , 1% SDS and  $0.1 \times \text{SSC}$ , 0.5% SDS) at  $60^{\circ}\text{C}$  as recommended by SuperArray. The protocol for chemiluminescent detection was developed from the SuperArray protocol, and was done using their Chemiluminescent Detection Kit (SuperArray Biosciences) using doubled reagent volumes. Maximum emission of CDP-Star typically occurs 2 to 4 h after application. As such, the blots were wrapped in plastic wrap (Saran<sup>TM</sup>), placed in the dark for 2 h, and then placed on X-Ray film (Fisher Scientific).

**Array analysis.** Blot images were processed using the AlphaImager<sup>TM</sup> System (Alpha Innotech). X-Ray films were photographed using the AlphaImager camera system and converted to TIFF files. Spot density analysis was done using the AlphaEase FC<sup>©</sup> system. Blots were split into quadrants and the *Listonella pelagica* 16S rDNA and negative control specific to the quadrant were used for normalization in all experiments. For the synchronous infection experiments, the negative control served as background and was manually subtracted from the spot density value. The subtracted value was then divided by the average 16S rDNA spot density to give the normalized value that was used in all further data analyses. For the salinity experiments, either the negative control or a blank spot on the blot served as the background value which was automatically subtracted from the sample spot density using the AlphaEase FC<sup>©</sup> software, and spot density was then divided by the subtracted 16S rDNA spot density to give a normalized expression value. In this way, for all experiments, expression of each gene was compared to the 16S ribosomal DNA which was set as the maximum expression value.

**Identification of promoter and termination sites.** In the  $\phi$ HSIC genome, ORFs located near one another had similar patterns of expression. This prompted us to manually search the genome for  $-10$  and  $-35$  promoter sites using FruitFly software ([www.fruitfly.org/seq\\_tools/promoter.html](http://www.fruitfly.org/seq_tools/promoter.html)). TransTerm software ([www.genomics.jhu.edu/TransTerm/transterm.html](http://www.genomics.jhu.edu/TransTerm/transterm.html)) was used to examine the genome for putative termination sites.

**Real-time PCR.** A Taqman probe was designed for  $\phi$ HSIC ORF 11, and the Operon Oligo Analysis and Plotting tool kit ([www.operon.com/oligos/toolkit.php](http://www.operon.com/oligos/toolkit.php)) was used to check that the primers and the probe were not complementary or self-complementary. Forward and reverse primer sequences for ORF 11 are listed in Appendix 1. The Taqman probe sequence was 5'-FAM-CCCCATCCCAACGCGCAAAGAG-TAMRA-3'.

Duplicate 1 ml samples were taken from the 11 and 39 ppt treatments at  $T_4$ ,  $T_6$ , and  $T_8$  during the salinity experiment. Samples were centrifuged for 10 min at  $10\,000 \times g$ , the supernatant aspirated off, and the pellet air-dried for 5 min. The dried pellets were then stored at  $-80^{\circ}\text{C}$  until the DNA could be extracted. To extract

the DNA, the Promega Genomic DNA extraction kit (Promega) protocol for Gram-negative bacteria was followed, and DNA was quantified using the Hoechst 3328 method (Paul & Meyers 1982).

A standard curve was made with diluted  $\phi$ HSIC DNA that ranged from  $10^5$  to  $10^1$  copies per reaction. DNA from all 3 time-points was diluted to 1:100 and 1:10000 and duplicate reactions were done. Each reaction mixture was 500 nM of both the forward and reverse primers, 100 nM of the probe, 25  $\mu$ l of the Taqman master mix (Roche Applied Biosystems), 5  $\mu$ l of the template at the appropriate dilution, and 19.45  $\mu$ l of sterile water for a total reaction volume of 50  $\mu$ l. The PCR reactions were heated at 95°C for 10 min to ensure template denaturation and then underwent 40 cycles of denaturation at 95°C for 45 s, annealing at 53°C for 1 min, and extension at 72°C for 1 min.

**Effects of salinity on growth of uninfected *Listonella pelagia*.** As there were concerns that low salinity might affect host physiology, leading to the observed differences in cell growth between the treatments, growth curves were done using uninfected *L. pelagia* cultures at 2 different salinities. Five ml of an overnight culture of uninfected *L. pelagia* was added to either 50 ml of 39 ppt ASWJP+PY or 50 ml of 11 ppt ASWJP+PY. Flasks were put in a bench-top shaker set at 28°C and shaken for 24 h. Optical density measurements ( $A_{600}$ ) were taken every 1 h from  $T_0$  to  $T_8$  and again at  $T_{24}$ .

**Statistical analysis.** To classify genes based on time of expression during the synchronous infection experiment, the highest normalized expression value was divided by the lowest normalized expression value for the ORF in question. The  $\log_2$  of the ratio was then found to give the  $M$  value (Becker et al. 2004). If the  $M$  value was  $\geq 0.80$ , then there was a greater than 2-fold increase in gene expression between the 2 time-points. Genes with the highest  $M$  value at 0 min p.i. were classified as early; genes that had the highest  $M$  values at either 20 or 40 min p.i. were classified as middle genes; and genes with the highest  $M$  values at 60 min p.i. were labeled late genes.

The number of plaque-forming units (PFUs) from the salinity experiment was first log-transformed before the statistical significance of the differences between the 2 treatments was calculated using paired  $t$ -tests in Minitab v. 13. Phage production and cell growth were plotted in SigmaPlot v. 8.0. Average normalized

gene expression values for the salinity experiment were plotted in SigmaPlot v. 8.0 and negative values were set to zero. The difference between the salinity treatments was calculated in Excel by dividing the average normalized expression value for the 39 ppt treatment by the average 11 ppt treatment expression value and the  $\log_2$  of the ratio was then determined. If the  $\log_2$  was  $\geq 0.80$  or  $\leq -0.80$ , there was a significant difference in expression between the 2 treatments (Dudoit et al. 2002, Becker et al. 2004). All gene expression values that were equal to zero in the salinity experiments were set to 0.1 so that the  $\log_2$  of the ratio could be calculated.

## RESULTS AND DISCUSSION

### Gene expression studies: controls

To demonstrate that host cDNA was not hybridizing to the blots and thereby causing a false positive, RNA was extracted from uninfected *Listonella pelagia* and a cDNA probe was made. There was faint hybridization to 3 of the 11 ORFs dotted (ORFs 33, 35, and 37) as shown in Fig. 1A. As hybridization was slight for these ORFS and undetectable for the other 8, we concluded that cross-hybridization by host DNA was not significant.

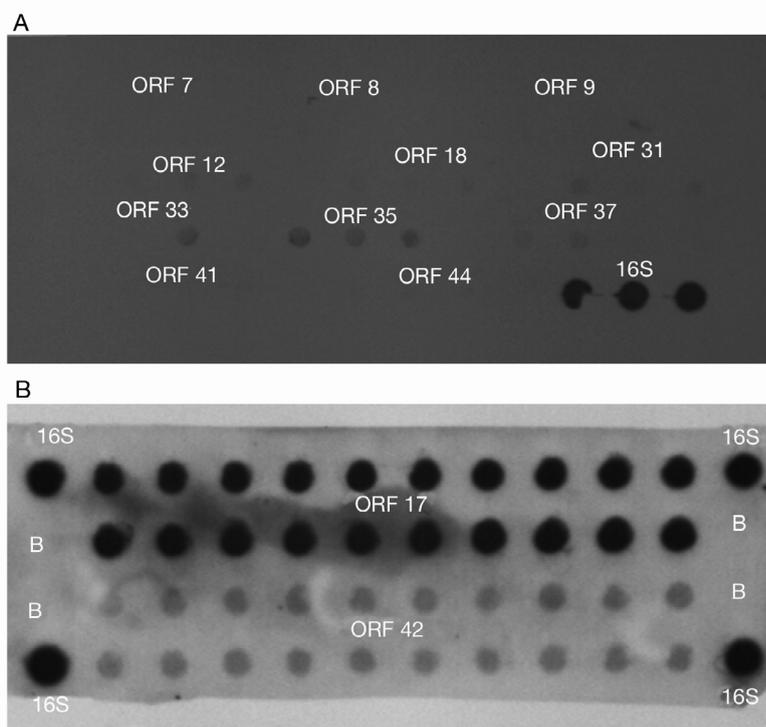


Fig. 1. Control blots. Blots used to test (A) specificity and (B) precision of the methods. ORF: open reading frame

To test the precision of our method, ORFs 17 and 42 were dotted 20 times each on a nylon membrane (Fig. 1B). During a preliminary synchronous infection, ORF 17 was highly expressed while ORF 42 was expressed at much lower value, which was the reason these 2 ORFs were chosen for this experiment. The coefficient of variation for ORF 17 was 1.5 and 2.5% for ORF 42. From these results, we concluded that this method provided a precise measurement of the varying levels of gene expression in hybridization experiments.

### Phage gene expression during an infection cycle

After it was shown that hybridization was repeatable and changes in gene expression levels could be analyzed, experiments were done to study gene expression over the infection cycle. By slightly altering the method used by Kobiler et al. (2005), a synchronous infection experiment was designed. This ensured that secondary infections were not occurring in some cells while other cells were undergoing a primary infection (Kobiler et al. 2005).

To classify genes based on time of expression, the ratio of the highest expression to the lowest expression was found for each of the 47 ORFs. For example, the lowest level of expression for ORF 1 was seen at 60 min p.i., while the highest expression value was seen at 0 min p.i. Therefore, the expression value from 0 min p.i. was divided by the 60 min p.i. value for ORF 1. The  $\log_2$  of the ratio was then calculated to give the  $M$  value. If the  $M$  value was  $\leq -0.80$  or  $\geq 0.80$ , then there was a significant change in expression between the 2 time-points (Dudoit et al. 2002, Becker et al. 2004).

Plots of the expression values for each ORF according to time of expression are shown in Fig. 2. Early genes include ORF 2 (the putative DNA polymerase), ORF 42 (the putative helicase), and several phage-associated proteins (ORFs 46 and 47) (Fig. 2A). As shown in Fig. 2B, 8 ORFs were classified as middle genes. The function of most of the ORFs is still unknown; however, ORF 31 is the putative tail tape-measure protein. Six ORFs were classified as late genes using this method (Fig. 2C). The large terminase subunit (ORF 8) and the major capsid protein (ORF 18) are both late genes.

Twelve ORFs (7, 12, 15, 16, 17, 23, 27, 29, 30, 33, 37, and 46) did not have  $M$  values above the cut-off value; therefore, expression levels for these ORFs did not significantly change during the experiment (Fig. 2D). As such, these ORFs were designated as continuously expressed. Half of these ORFs (7, 12, 17, 33, 37, and 46) are structural phage genes located near putative promoter sites, and it is possible that they are continuously expressed for continuous production of phage.

Eleven putative promoters including a putative host promoter, and 12 rho-independent terminators were found scattered throughout the genome as determined by bioinformatics analysis. In Fig. 3, the genome map of  $\phi$ HSIC has been arranged with each ORF patterned according to peak expression, and includes the location of the putative promoters and termination sites. Early genes are clustered at the beginning and the end of the genome, while the middle, late, and continuously expressed genes are randomly distributed in the middle of the genome. The location of several of the promoters agrees with the temporal classification of the genes. For example, there is a promoter for ORFs 1 through 6 located after ORF 47, and there is a termination site found between ORFs 6 and 7. ORFs 7 and 8, the small and large terminase subunits, respectively, have a separate promoter and termination site compared to the ORFs surrounding them.

Additionally, a host promoter site with a  $-35$  start site and a  $-10$  TATA box was located between ORFs 46 and 47. ORF 46 is a conserved hypothetical bacteriophage protein with strong hits to phages isolated from *Streptococcus thermophilus* and *Bacillus clarkii*, while ORF 47 has several hits to different phage-associated primases (Paul et al. 2005). Primases are responsible for producing the RNA primer that is necessary for the initiation of DNA replication.

While no lysogeny module has been identified in the  $\phi$ HSIC genome, it is hypothesized that the lysogeny genes are located somewhere in the early gene cluster found from ORFs 38 through 47 and 1 through 6 on the circularly permuted genome. ORF 47 shared some characteristics with a tyrosine integrase (Paul et al. 2005). The putative identification of some of the genes in this region as well as the presence of the host promoter supports this hypothesis. The lysogeny module of the temperate phage Sfi21 is located between the host lysis and replication modules on that phage's genome (Ventura et al. 2002). ORF 37 is a putative lysozyme gene while a putative primase gene has been located at ORF 47 (Paul et al. 2005). It is possible that the lysogeny module is located within this region as 6 out of 9 early genes in this region have no known function.

The putative small and large terminase subunits (ORFs 7 and 8) had relatively constant levels of expression throughout the course of the infection, although ORF 8 is technically a late gene with an  $M$  value of 1.11 at 60 min p.i. In phages that use the sequence-independent headful packaging mechanism such as  $\phi$ HSIC, the small terminase subunit is responsible for site-specific assembly and DNA recognition, while the large terminase subunit prepares the DNA for packaging into the prohead and

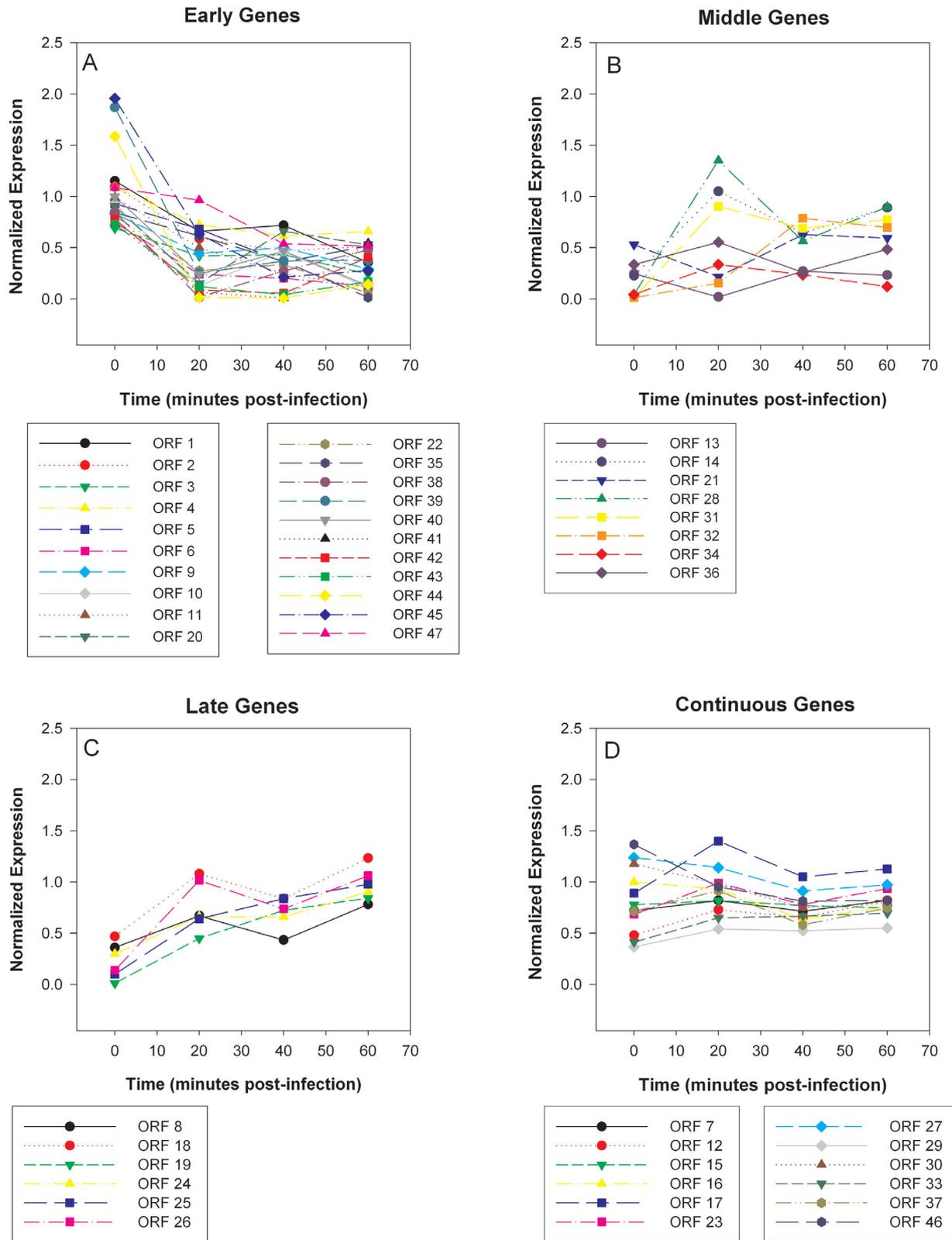


Fig. 2. Expression values for all  $\phi$ HSIC open reading frames (ORFs). ORFs are grouped by time of peak expression into (A) early, (B) middle, and (C) late genes during the synchronous infection. All genes that had relatively continuous levels of gene expression throughout the infection cycle are shown in (D)

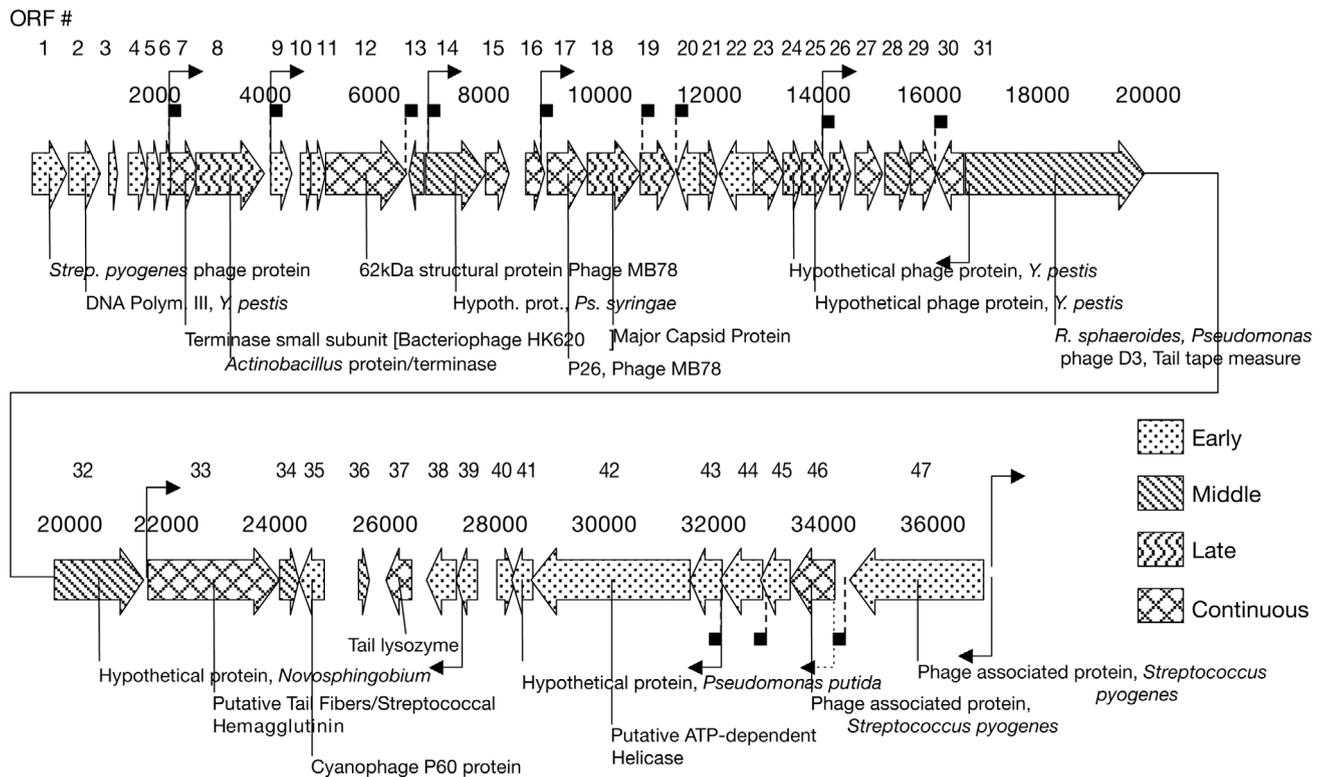


Fig. 3.  $\phi$ HSIC gene expression map. Solid black arrows are putative viral promoters, the dotted black arrow is the putative host promoter, and the black boxes with dashed lines are the putative termination sites. Early: ORFs that have been classified as early genes; Middle: middle genes; Late: late genes; Continuous: ORFs that do not have significant change in expression with time. *Strep. pyogenes*: *Streptococcus pyogenes*; *Y. pestis*: *Yersinia pestis*; *Ps. syringae*: *Pseudomonas syringae*; *R. sphaeroides*: *Rhodobacter sphaeroides*

has ATPase activities (Isidro et al. 2004). The 2 subunits also work together to form a complex that moves the DNA into the empty capsid through the portal.

Late genes that were significantly up-regulated from their initial expression value included the major capsid protein (ORF 18) and the large terminase subunit (ORF 8). Capsid proteins have been identified as late genes in the vibriophage KVP40, and both terminase subunits are known late genes in the lytic cycle (Parreira et al. 1996, Miller et al. 2003, Casjens et al. 2005). There are 6 late genes in the  $\phi$ HSIC genome. These genes have been putatively identified as the large terminase subunit, the major capsid protein, a structural protein, possible tail fibers, and several unknown phage proteins.

Pseudolysogenic cultures are known for the simultaneous production of high numbers of host cells and phage particles (Moebus 1997a,b,c). It follows then that gene transcription in pseudotemperate phages would occur continuously, albeit at varying levels throughout the infection cycle. Mapping the infection cycle of HSIC-1a with microarrays has given molecular evidence for pseudolysogeny.

### Effects of salinity on the growth of uninfected *Listonella pelagia*

To determine if salinity was affecting viral gene expression, it was first necessary to test that the observed changes in the growth of the pseudolysogenic cells were not a result of the changes in salinity affecting *Listonella pelagia* cell growth. Virtually identical growth curves were obtained for the uninfected *L. pelagia* cells grown at salinities of 11 or 39 ppt (Appendix 3, available at: [www.int-res.com/articles/suppl/a049p001\\_app.pdf](http://www.int-res.com/articles/suppl/a049p001_app.pdf)). Therefore, we hypothesize that lowered salinity does not noticeably affect host cell growth, and differences in viral gene expression are most likely the result of the effects of salinity changes on viral gene expression.

### Expression analysis of $\phi$ HSIC grown at different salinities

Previous experiments with HSIC-1a had shown that changes in media salinity led to significant differences in both cell growth and phage production. It was

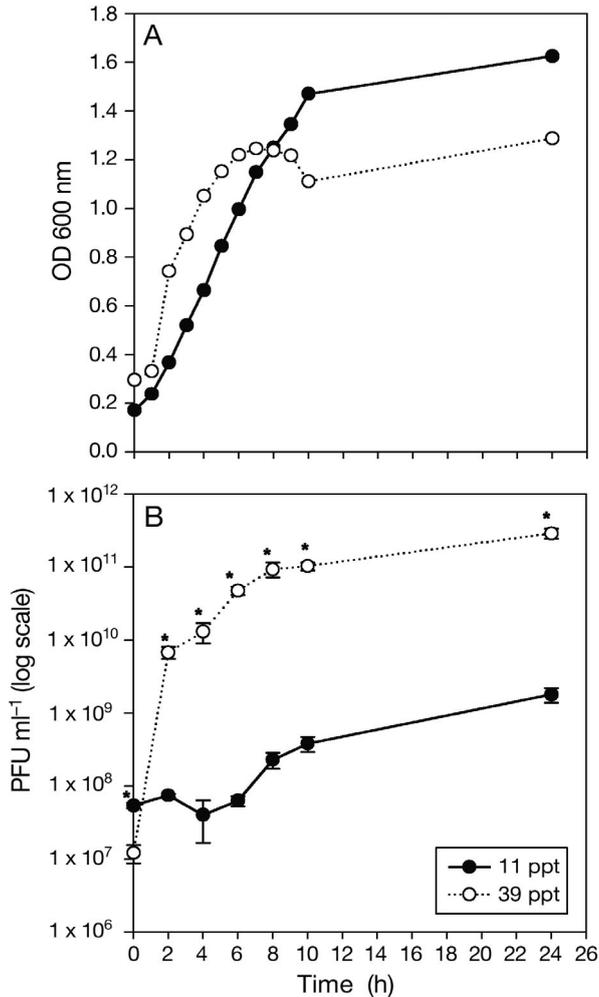


Fig. 4. (A) Cell growth and (B) phage production by HSIC-1a when grown in ASWJP+PY with a salinity of either 11 or 39 ppt. Error bars  $\pm$ SD represent duplicate measurements. \*: statistically significant difference. OD: optical density; PFU: plaque-forming units

hypothesized that these changes in salinity were changing the interactions of an as yet unidentified repressor molecule (Williamson & Paul 2006). As ionic strength decreases, interactions between charged molecules become more stabilized and bind tighter to one another (Bell & Koudelka 1993). It has been hypothesized that, in the  $\phi$ HSIC system, the repressor molecule has a greater affinity for the operator sites and, as such, HSIC-1a cells enter the lysogenic state when the salinity of ASWJP+PY is lowered. To understand what occurs at the lysogenic switch on the molecular level during growth at differing salinities, gene expression analysis of HSIC-1a cells growing in both 11 and 39 ppt ASWJP+PY was done. Phage production and growth were also measured.

Cell growth of the HSIC-1a strain was dramatically different between the 11 and 39 ppt treatments in the

first 8 h, as shown in Fig. 4A. Between  $T_1$  and  $T_2$  (1 and 2 h post-resuspension in the appropriate salinity media, respectively), there was a sharp increase in cell growth in the 39 ppt treatment compared to the 11 ppt treatment. The growth rate of the 39 ppt culture was  $1.2 \text{ h}^{-1}$ , while that of the 11 ppt treatment was  $0.63 \text{ h}^{-1}$  at these 2 time-points. After  $T_2$ , cell growth steadily and rapidly increased in the 39 ppt treatment until  $T_8$ , at which point it decreased until the  $T_{10}$  reading. By  $T_{24}$ , the optical density of the 39 ppt culture had increased again to 1.3. Conversely, cell growth in the 11 ppt treatment was much less than that of the 39 ppt culture until  $T_8$  when the optical density of the 11 ppt culture equaled that of the 39 ppt culture. Growth in the 11 ppt culture was greater for the rest of the experiment. The sigmoidal growth curve characteristic of pseudolysogenic cultures was not observed in the low salinity treatment.

Fig. 4B shows the changes in free phage concentrations in the 11 and 39 ppt treatments over the course of 24 h. Free phage concentration was significantly higher in the 11 ppt treatment at the initial sampling point,  $5.43 \times 10^7 \text{ PFU ml}^{-1}$  compared to  $1.22 \times 10^7 \text{ PFU ml}^{-1}$  in the 39 ppt culture. However, by  $T_2$ , the number of PFU  $\text{ml}^{-1}$  in the 39 ppt treatment increased to  $6.80 \times 10^9$ , while there were  $7.45 \times 10^7 \text{ PFU ml}^{-1}$  in the 11 ppt treatment. This trend continued for the rest of the experiment, and the differences in phage production were significant at  $T_2$ ,  $T_4$ ,  $T_6$ ,  $T_8$ ,  $T_{10}$ , and  $T_{24}$  ( $p < 0.05$ ).

After the  $T_{24}$  measurement, the cultures were transferred into fresh ASWJP+PY of the appropriate salinity and continued for another 30 h. At  $T_{54}$ , the  $A_{600}$  measurement for the 39 ppt treatment was still high (1.10). The cell density of the 11 ppt culture was high as well, even though it had dropped from an  $A_{600}$  of 1.6 at  $T_{24}$  to 1.3 at  $T_{54}$ . By  $T_{54}$ , the 11 ppt culture had a free phage concentration of  $1.70 \times 10^{10} \text{ PFU ml}^{-1}$ , and the 39 ppt treatment had a concentration of  $1.30 \times 10^{11} \text{ PFU ml}^{-1}$ , which was significantly greater ( $p < 0.05$ ).

Normalized gene expression values for the salinity experiment were calculated in a way similar to the synchronous infection experiment. However, in the salinity experiment, the AlphaEase<sup>®</sup> software was used to automatically subtract the background from the spot density value before normalization to the *Listonella pelagia* 16S rDNA, whereas in the synchronous infection experiment, the background was manually subtracted from the spot density value before normalization. Several ORFs had negative expression values. Negative values do not mean that there was negative gene expression; rather they indicate that the spot density of the ORF was lower than that of the negative control. To account for this, all negative values were set to zero for graphs.

To determine if there was a significant difference in gene expression between the 2 treatments and to isolate any patterns that may have occurred, the ratio of expression in the 39 ppt treatment to that in the 11 ppt treatment was calculated at both time-points. The  $\log_2$  of the ratio was then found. In the case where a gene had zero or negative expression, values were set to 0.1 so that the ratio could be found and the  $\log_2$  value could be determined to give the  $M$  value. If the  $M$  value was  $\leq -0.80$  or  $\geq 0.80$ , then the levels of hybridization were considered to be significantly different between the 11 and 39 ppt treatment as well as between the 2 time-points (Dudoit et al. 2002, Becker et al. 2004). Negative  $M$  values signify that expression in the 11 ppt treatment was significantly greater than that of the 39 ppt treatment.  $M$  values for each ORF at the 2 different time-points are listed in Table 1.

The average normalized expression values of each ORF for both the 11 and 39 ppt treatments at  $T_4$  are plotted in Fig. 5A. Overall, higher gene expression values were seen in the 39 ppt treatment than in the 11 ppt treatment for many of the phage genes at this time-point. Expression values for the 11 ppt treatment are greater than the 39 ppt treatment values at ORFs 1 through 7, 11, 12, 15, and 16. ORFs 10, 23, and 47 have almost equal expression values to their counterparts in the 39 ppt treatment at  $T_4$ . However, only ORFs 2 through 6 and 16 have significantly higher levels of gene expression in the 11 ppt treatment than in the 39 ppt treatment.

The average normalized gene expression values for the 11 and 39 ppt treatments at  $T_6$  are plotted in Fig. 5B. At  $T_6$ , ORFs 5 and 16 were the only ones that had higher gene expression in the 11 ppt treatment than in the 39 ppt treatment, but neither was significantly greater. Additionally, many of the genes that were not expressed at  $T_4$  in the 11 ppt treatment, such as the latter half of the genome from ORF 32 onwards, were expressed by  $T_6$ . In fact, except for ORFs 21, 29, and 43, all were expressed at  $T_6$  in the 11 ppt treat-

Table 1.  $M$  values ( $\log_2$  of ratio highest/lowest normalized expression value) for each open reading frame (ORF). Significant  $M$  values are shown in bold.  $T_4$  and  $T_6$  are time points 4 and 6 h, respectively. Hypo.: hypothetical; Pol.: polymerase

ORF	Predicted function	$M$ value	
		$T_4$	$T_6$
1	Hypo. protein	-0.66	<b>1.51</b>
2	DNA Pol. III, $\beta$ -subunit	<b>-1.33</b>	<b>1.48</b>
3	Unknown	<b>-1.04</b>	<b>1.41</b>
4	Unknown	<b>-2.61</b>	<b>2.55</b>
5	Unknown	<b>-0.98<sup>a</sup></b>	-0.08
6	Unknown	<b>-2.00</b>	<b>1.35</b>
7	Terminase, small subunit	<b>-0.87</b>	<b>1.14</b>
8	Terminase, large subunit	<b>1.24</b>	<b>1.05</b>
9	Unknown	<b>2.32</b>	<b>4.58</b>
10	Unknown	0.11	0.18
11	Unknown	0.60 <sup>a,b</sup>	<b>0.92</b>
12	60 kDa structural phage protein	-0.54	0.74
13	Unknown	0.74	<b>1.00</b>
14	Head morphogenesis protein	<b>1.75</b>	<b>1.93</b>
15	Unknown	-0.30	<b>1.71</b>
16	Unknown	<b>-1.13</b>	-0.20
17	Hypo. phage protein	0.23	<b>1.55</b>
18	Capsid protein	0.37	<b>1.23</b>
19	Unknown	<b>2.78</b>	<b>1.89</b>
20	Unknown	<b>6.65</b>	<b>5.97</b>
21	Unknown	<b>4.73</b>	<b>3.86<sup>a</sup></b>
22	Unknown	<b>2.86</b>	<b>2.75</b>
23	Hypo. phage protein	0.07	0.67
24	Hypo. phage protein	<b>1.33</b>	<b>1.07</b>
25	Hypo. phage protein	<b>2.43</b>	<b>2.59</b>
26	Unknown	<b>2.46</b>	<b>2.53</b>
27	Unknown	0.66	<b>1.97</b>
28	Unknown	0.38	<b>1.09</b>
29	Unknown	<b>0.93</b>	<b>3.59</b>
30	Unknown	<b>1.46</b>	<b>2.14</b>
31	Tail tape-measure protein	<b>1.98</b>	<b>3.86</b>
32	Hypo. protein	<b>1.35</b>	<b>1.92</b>
33	Tail fibers	<b>1.10</b>	<b>1.60</b>
34	Unknown	<b>0.81</b>	<b>1.65</b>
35	Cyanophage P60 protein	<b>2.62</b>	<b>3.16</b>
36	Unknown	<b>2.20</b>	<b>2.58</b>
37	Baseplate hub subunit/Tail lysozyme	<b>2.72</b>	<b>4.76</b>
38	Unknown	<b>2.30</b>	<b>3.32</b>
39	Unknown	<b>4.14</b>	<b>1.59</b>
40	Unknown	<b>0.93</b>	<b>1.21</b>
41	Hypo. phage protein	<b>2.58</b>	<b>2.42</b>
42	Helicase	<b>2.82<sup>a</sup></b>	<b>2.46</b>
43	Unknown	<b>2.53<sup>a</sup></b>	<b>3.82<sup>a</sup></b>
44	Unknown	<b>2.33<sup>a</sup></b>	<b>1.42</b>
45	Unknown	<b>2.74</b>	<b>1.69</b>
46	Hypo. phage protein	<b>0.98</b>	<b>3.14</b>
47	Primase/integrase	0.17	<b>3.62</b>

<sup>a</sup>Initial expression in either the 11 or 33 ppt treatment was either zero or negative and so 0.1 was substituted  
<sup>b</sup>False positive

ment. In the 39 ppt treatment, all genes were expressed as well.

In the 39 ppt treatment at both  $T_4$  and  $T_6$ , 27 ORFs were significantly greater (Table 1). The majority of these were early genes, but there were also middle,

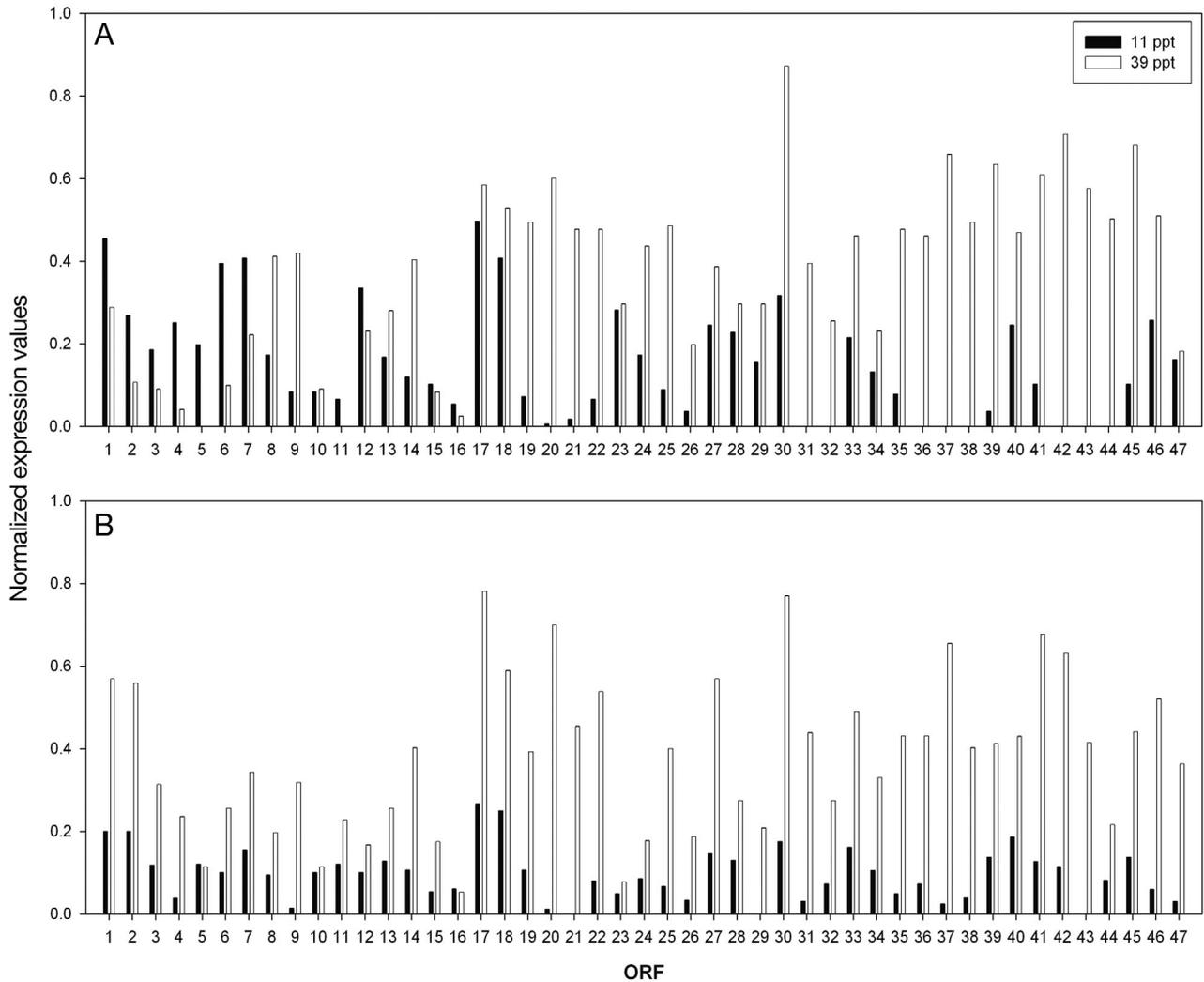


Fig. 5. Average normalized gene expression values for 11 and 39 ppt salinity treatments at (A)  $T_4$  (4 h) and (B)  $T_6$  (6 h)

late, and uniformly expressed genes. Some of the genes that were consistently greater in the 39 ppt treatment include the large terminase subunit (ORF 8), the head morphogenesis protein (ORF 14), the helicase protein (ORF 42), the tail lysozyme (ORF 37), and several unknown proteins. This trend can be seen in Fig. 5. Gene expression was never significantly greater at both time-points measured in the 11 ppt treatment during the experiment.

ORFs that decreased in expression with time in the 39 ppt treatment include ORFs 8, 9, 12, 23, 24, 25, 30, 38, 42, 43, 44, and 45 (Fig. 5). For the most part, gene expression in the 39 ppt treatment either increased or stayed constant during the salinity experiment. Gene expression in the 39 ppt treatment was significantly greater than that in the 11 ppt treatment for many of the genes, especially the early genes suspected to be involved in the integration of the  $\phi$ HSIC genome and

initiation of DNA replication as well as the large terminase subunit. Other genes that had high levels of expression in the 39 ppt treatment included ORFs 9, 14, 17, 30, 39.

#### Free phage concentration versus intracellular phage concentration

The HSIC-1a lysogen is unstable, and  $\phi$ HSIC has previously segregated from *Listonella pelagia* by multiple platings (Williamson et al. 2001). Due to this instability, it was hypothesized that segregation was occurring in the low salinity treatments, which would explain the low number of PFUs and low gene expression values. Quantitative real-time PCR was done using host cell DNA extracted from samples taken at  $T_4$ ,  $T_6$ , and  $T_8$  to determine the cellular viral genome

concentrations. ORF 11 was chosen for this procedure because, even though it has a cryptic function, it satisfied all the guidelines for the target sequence in order to successfully design a real-time PCR probe.

Using the results of the real-time PCR and bacterial counts, we were able to approximate the number of intracellular phage genes per host cell. Table 2 lists the viral genomes per cell for the 11 and 39 ppt treatments. The number of viral genomes increased from 1.94 at  $T_4$  to 3.15 at  $T_6$  in the 39 ppt treatment, and from 1.19 to 1.52 in the 11 ppt treatment during the same time period. This should not imply that there were fractional viral genomes, but reflect the average state of cellular phage genome content. The number of viral genomes decreased in both treatments from  $T_6$  to  $T_8$  by about 1-fold, which is expected in the 39 ppt treatment as that is when the optical density of the culture begins to decrease. However, the decrease in viral genomes at the 11 ppt treatment was unexpected, as this is the time-point where the optical density of the 11 ppt culture surpasses that of the 39 ppt culture (Fig. 4A).

The number of viral genomes per cell in the 39 ppt treatment increased from 2 to 3 from  $T_4$  to  $T_6$  before dropping back down to 2 at  $T_8$ . In the salinity experiment, gene expression in the first half of the genome decreased overall from  $T_4$  to  $T_6$  but, in the second half of the genome, gene expression increased overall (Fig. 5). However, in the same experiment during the same time period, the number of viral genomes in cells essentially remained the same, ranging from 1.2 to 1.5. At  $T_8$ , the number of viral genomes in the cell decreased to 0.5 in the 11 ppt treatment.

Limited host density, suggested to be a possible factor in influencing the lysis/lysogeny decision, did not appear to be a factor in these experiments (Stewart & Levin 1984). The differences between host cell abundance in the 11 and 39 ppt treatments were not significant (data not shown). It is not surprising that cellular levels of viral DNA were higher in cells actively producing  $\phi$ HSIC, i.e. those growing in the 39 ppt medium. It is also noteworthy that the low salinity cells did not segregate (lose viral DNA copies).

It has been previously hypothesized that HSIC-1a cells enter the lysogenic state when the salinity of the growth medium is decreased due to increased effi-

ciency of the repressor molecule (Williamson & Paul 2006). At  $T_4$ , 8 ORFs had higher expression in the 11 ppt treatment than in the 39 ppt treatment. Seven of those were significantly greater, and of those 7 there is a putative function for only one of them (ORF 2). However, this relationship is reversed at  $T_6$  and there was no significant gene expression in the 11 ppt culture at this time-point. The putative transcriptional repressor may have been located in ORFs 1 to 7 and 16, since they apparently were initially activated in the low salinity treatment. Additional work employing phage mutations in these ORFs would be needed to help ascribe a role to these genes in the general repression of phage genes caused by low salinity.

## CONCLUSIONS

In the present study, it is shown that HSIC-1a cells grown at low salinity produce nearly 2 orders of magnitude less phages, and gene expression is significantly less at most ORFs compared to the 39 ppt treatment. Even as cell growth in the low salinity treatment caught up to cell growth in the normal salinity treatment, intracellular and free phage concentrations remained significantly lower. Further experiments showed that lowered salinity did not appear to affect uninfected *Listonella pelagia* cell growth. Therefore, the presence of the phage affected the physiology of the host, resulting in lower initial growth at low salinities. Finally, synchronous infection experiments led to the classification of  $\phi$ HSIC ORFs into time classes corroborated by the identification of putative promoters and termination sites for several ORFs in the  $\phi$ HSIC genome.

Pseudotemperature phage production may fluctuate along with gene transcription throughout a growth experiment but new phage production is always occurring. There was a clear increase in phage gene expression in most of the ORFs in the 39 ppt treatment. The implication is that, at low salinity, there was a repression of nearly all phage ORFs, which was most likely a stress response to low salinity possibly by host control. This is consistent with the idea that lytic phages are found under favorable conditions, 39 ppt in this case, while low salinity leads to the lysogenic response and gene expression is shut down. Alternatively, some of the phage ORFs (1 to 7, 16) may have participated in the low salinity repression process.

Data gathered from various salinity experiments indicate that decreased salinity increases the latent period of the phage as well. It may be that a repressor protein has a greater affinity and/or binding to phage DNA at low salinity that impedes lytic phage production, as was previously suggested for the  $\phi$ HSIC system

Table 2. Number of viral genomes per cell in the 11 and 39 ppt salinity treatments at  $T_4$ ,  $T_6$ , and  $T_8$  (4, 6 and 8 h, respectively)

Time	11 ppt	39 ppt
$T_4$	1.19	1.94
$T_6$	1.52	3.52
$T_8$	0.5	2.3

(Williamson & Paul 2006). Conversely, it is also possible that the host RNA polymerase is unable to bind to the viral promoters to begin transcription, or that transcription machinery is not as efficient at lowered salinities.

These experiments have shown that unfavorable environmental conditions, such as low salinity, may be responsible for the transition of HSIC-1a cultures from the lytic life-cycle to the lysogenic life-cycle. Lower levels of viral gene expression in the 11 ppt treatment support this conclusion, as does the decrease in free and intracellular phage concentrations even as cell growth is increasing. The exact mechanism of this transition is still unknown. It is possible that the decreases in salinity are leading to an increase in repressor molecule-protein interactions within the cell, which would inhibit viral gene transcription. It is also possible that, in 11 ppt media, there are changes in ionic strength of the host cell cytoplasm, leading to changes in viral gene expression. In either case, these observations are consistent with a shift toward lysogeny under unfavorable conditions.

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