Genome sequences characterizing five mutations in RNA polymerase and major capsid of phages \( \phi A318 \) and \( \phi As51 \) of \textit{Vibrio alginolyticus} with different burst efficiencies

Liu \textit{et al.}
Genome sequences characterizing five mutations in RNA polymerase and major capsid of phages ϕA318 and ϕAs51 of Vibrio alginolyticus with different burst efficiencies

Wangta Liu2, Ying-Rong Lin1, Ming-Wei Lu3, Ping-Jyun Sung4, Wei-Hsien Wang1,4 and Chan-Shing Lin1*

Abstract

Background: The burst size of a phage is important prior to phage therapy and probiotic usage. The efficiency for a phage to burst its host bacterium can result from molecular domino effects of the phage gene expressions which dominate to control host machinery after infection. We found two Podoviridae phages, ϕA318 and ϕAs51, burst a common host V. alginolyticus with different efficiencies of 72 and 10 PFU/bacterium, respectively. Presumably, the genome sequences can be compared to explain their differences in burst sizes.

Results: Among genes in 42.5 kb genomes with a GC content of 43.5%, 16 out of 47 open-reading frames (ORFs) were annotated to known functions, including RNA polymerase (RNAP) and phage structure proteins. 11 strong phage promoters and three terminators were found. The consensus sequence for the new vibriophage promoters is AATAAAGTGCCCTATA, where the AGTG bases of −8 through −12 are important for the vibriophage specificity, especially a consensus T at −9 position eliminating RNAP of K1E, T7 and SP6 phages to transcribe the genes. ϕA318 and ϕAs51 RNAP shared their own specific promoters. In comparing ϕAs51 with ϕA318 genomes, only two nucleotides were deleted in the RNAP gene and three mutating nucleotides were found in the major capsid genes.

Conclusion: Subtle analyses on the residue alterations uncovered the effects of five nucleotide mutations on the functions of the RNAP and capsid proteins, which account for the host-bursting efficiency. The deletion of two nucleotides in RNAP gene truncates the primary translation due to early stop codon, while a second translational peptide starting from GTG just at deletion point can remediate the polymerase activity. Out of three nucleotide mutations in major capsid gene, H53N mutation weakens the subunit assembly between capsomeres for the phage head; E313K reduces the fold binding between β-sheet and Spine Helix inside the peptide.

Keywords: RNA polymerase truncation, Spine Helix, Mutation in F-loop

Background

Vibrio strains are reported to be fatal pathogens not only to humans but also to aquaculture fish and shellfish. Several virulence factors that they produce—including hemolysin, caseinase, gelatinase, lipase, and phospholipase—are detrimental to animal health [1-4]. For instance, an extracellular protease with endopeptidolytic and exopeptidolytic activities from a pathogenic V. pelagius strain is responsible for the lethal effect and vibriosis in turbot.

The different virulence factors are produced at different stages of infection. Hemolysin-producing bacteria include V. parahaemolyticus, V. alginolyticus, V. cholerae, V. hollisae, Aeromonas veronii, and V. anguillarum (Listonella anguillarum).

Vibrio alginolyticus, a Gram-negative halophilic bacterium that frequently occurs in the normal microbiota of marine environments, is a pathogen of epizootic outbreaks, which causes serious mortality of fish and shellfish and also can transmit to humans. The disease caused by V. alginolyticus often has lethal consequences for fish larvae throughout the world, and it has become one of the
major limiting factors in aquaculture in developing countries. For example, it was a causal agent of vibriosis outbreaks in grouper sea bream *Sparus aurata* [2]. *V. alginolyticus* causes several symptoms in sea bream: septicemia, hemorrhaging, dark skin, and ulcers on the skin surface. Some fish accumulate fluid in the peritoneal cavity or have hemorrhagic livers. In several cases of high-mortality outbreaks, *Vibrio alginolyticus* and *Vibrio splendidus* biovar II were the primary organisms found in moribund clam larvae (*Ruditapes decussatus*), and these two species processed extracellular products collaboratively at high concentrations to kill the hemocytes after four hours of incubation. Neurotoxic effects caused by the supernates from diseased strains of *Vibrio alginolyticus* and *Vibrio anguillarum* caused several symptoms in trout, including convulsions, wriggling, contortive swimming and respiratory arrest [2].

Chemotherapeutic agents are effective to control pathogenic bacteria in aquaculture. Nevertheless, emergence of antibiotic-resistant bacteria has become a critical issue [3]. As a result of management practices in production cycles, the use of probiotics to prevent pathogenic bacterium growth in aquaculture is a practice to reduce both issues on antibiotic resistance and drug residues in food. One alternative is to use bacteriophages to control bacterial growth, which is similar to phage therapy in medical care. This idea has been proposed as a cure for coral disease [5]. A variety of bacteriophages that can quickly lyse the pathogens have high potential for future applications in aquaculture.

The burst size of a phage represents an overall outcome that is so important for phage therapy and probiotic usage. The molecular interactions between phage and its host under certain environmental conditions are complicated to resolve. Sequences of bacteriophage genomes provide a magnificent tool to investigate interactions with hosts. In addition, virophages can be used as a new typing scheme for pathogen identification and as candidate agents for phage therapy and probiotics. For example, the roles of lytic bacteriophages in cholera epidemics were investigated, such as filamentous CTX φ, myovirus ICP1, and podovirus ICP3 of *Vibrio cholerae*, causing infant and adult deaths for centuries [6]. Though quite a large number of *Vibrio* phages have been described, only a few of complete genome sequences are known. Genome sequences of lytic virophages are new keys for molecular typing, pathogenesis, and even assessment of therapeutic efficacy. Complete genomes of some phages of *V. cholerae*, *V. para-haemolyticus*, and *V. vulnificus* were documented, yet no genome for *V. algino-lyticus* podovirus has been reported. In this study, we characterized the genome sequences of two newly-isolated phages that can lyse *V. alginolyticus* ATCC 17749 with different efficiencies.

### Results

#### Host range, burst size, and thermal stability

The plaques of φA318 that formed in 4 hours at 25°C were very clear at their center and at the margin of the edge; sizes of the plaques were about 3 mm in diameter, which increased to 5 mm overnight (Figure 1A). The phage concentration in each plaque infected and fully lysed the amounts of host bacterium up to a 50x volume ratio. In comparison, the plaque size of φAs51 only reached 1 mm overnight (Figure 1A), and the amplification should not be greater than 3-5x dilution ratio. The EM morphology of phages φA318 and φAs51 was indistinguishable from each other. The icosahedral head was almost isometric, with a size of 50–55 nm, and the shells on the head capsids sketched out at least three visible rings, although the tail fiber was barely distinguishable from the capsid shell in the micrograph (Figure 1B). The tail was 12 nm long, elongating from 25 nm wide in the head-neck connector to 5 nm in the nozzle. They are highly similar to the bacteriophages of the family *Podoviridae* [7].

The susceptibility of other *Vibrio* strains to phage φA318 and φAs51 was also investigated by the agar overlay method. Among six other Vibrios, *V. damsela* and *V. harveyi* were found to be susceptible to both phages φA318 and φAs51, while the other four species could not be infected even at an MOI of more than 100. For measurement of the burst size, a bacterial culture in the early exponential growth phase was split into glass tubes with equal numbers of *V. alginolyticus* cells (MOI = 1.0), and then plaque titers were calculated within 4 hours of incubation. According to the one-step method [8], the burst size of phage φA318 was 72 PFU per infected cell, while that of φAs51 was 10 PFU/cell (Figure 1C).

A thermal stability test was carried out to analyze the heat resistance of phages at pH 7.5-8.0. The phages were incubated at 25 - 80°C for one hour. As Figure 1D shows, the relative phage titers were measured at different temperatures. After incubation at 50°C for one hour, phage φA318 retained almost 100% infectivity, while φAs51 decreased to 80%. When the temperatures rose higher than 70°C, nearly all phages were inactivated after 15 minutes of incubation.

#### The φA318 genome

φA318 genome was sequenced by a combination of shotgun sequencing (Figure 2 and Table 1). Open reading frames (ORFs) were determined using a combination of RAST visual inspection and translated tBLASTx searches [9]. The genome consists of a single double-stranded DNA molecule of 42544 bp with a GC content of 43.5%. We have annotated 47 open reading frames (The genome was submitted to GenBank with access no. KF322026 for phage φA318; [GenBank:KF322026]; Additional file 1: annotation
A. Plaque size

B. TEM micrograph

C. Burst size

D. Thermal stability

Figure 1 (See legend on next page.)
Figure 1 Different characteristics between phages \( \phi A318 \) and \( \phi As51 \). (A) The plaque sizes within 16 h after phage infection at 25°C. (B) Transmission electron micrographs of phage particles. Virions were negatively stained with uranyl acetate. The bars represent a length of 100 nm. (C) One-step growth curves of phages in \( V. \) alginolyticus at 25°C for \( \phi A318 \) and 37°C for \( \phi As51 \). The burst sizes were calculated by the amplifying ratio of Pt/P0 (phage titer at the plateau phase is divided by the inoculating phage titer). (D) Thermal stability of phages at various temperatures one hour. Samples were taken to determine the titer of the surviving particles and to calculate the percentage of infectious phage. In the stationary phase, standard errors (SE) for each phage were consistent at 16-20% (SE/mean), while the standard errors (SE) were approximate 16-72% (SE/mean) during the exponential phase.

Like most of members of the closely-related T7 family, \( \phi A318 \) is flanked by terminal repeats, suggesting similar replication strategies: phiL-122, 124, and 126 were promoter-like and repeats. Promoter sequences for most gene transcription among T7 supergroup phages, which are driven by phage-encoded RNAPs, are recognized very specifically. \( \phi A318 \) encodes an RNAP with highly conserved motifs to that of T7. Employing the UGENE program for searching at repeats with the conditions of >13 bases and >95% homology, we predicted 16 promoter consensus sequences from 126 repeat regions: 11 putatively strong promoters and six weak ones. Most of those have T7-like homologues (Figure 3). The consensus sequence from −7 to +1 is identical to that of T7, except that A at −6 was replaced by C. However, bases of −8 through −12, which are important for promoter specificity, differ from T7, T3, K11, and SP6 [10,11]. The consensus at −9 for T7 and SP6 is C, any change leading to inactive [10]. Since \( \phi A318 \) and K1E have T and G, respectively, at this position, it is unlikely that the T7 or K1E RNA polymerase will initiate transcription from \( \phi A318 \) promoters. \( \phi A318 \) RNAP has different promoter specificity.

\( \phi A318 \) appears to have phage promoters throughout the region of the T7 analogues. We proposed that the promoters of phi122, phi124, and phi125 in \( \phi A318 \) are equivalent to \( \phi OR \) involved in packaging and possibly replication [10]. Unlike phages gh-1 and K1E, one phage-specific promoter (phi-A8 at position 1741) was found immediately upstream of the protein kinase and RNA polymerase genes in \( \phi A318 \), suggesting that the autoregulation controls these two genes together. The genome contains a strong σ70 promoter (at 229–257 nt) responsible for early gene transcription, including phage RNA polymerase. The end of transcription of the early genes in \( \phi A318 \) is marked by GCCCCTGATCttaagagTCAGGGC TTTT CCTT, a rho-independent terminator (TE, near position 5957) immediately following the RNA polymerase gene, the equivalent position at 7128 to 7161 in K1F of such terminator. PEG 4 in \( \phi A318 \) is similar to gene 0.7 in T7, which could encode a kinase to phosphorylate host RNA and efficiently inactivate the host gene expression [12]. Two terminators, GCCCCTGCTCacttGTTAGGGGC TTATTTTT at position 24971 and GAGGGACT ctaag AGTCCCTCCTTCTTT at position 38217, were found. Terminator at 24871 was a potential analogue to the T7
A: Genome alignment to ϕA318

B: Genome alignment to phage SP6

C: Genome alignment by aggressive Mauve

Figure 2 (See legend on next page.)
T3 terminator, located inside the gene encoding major capsid; terminator at 38217 was just the end of tail fibers.

Genome comparisons between ϕA318 and ϕAs51
Analyses of nucleotide restriction enzyme polymorphisms and protein profiles showed no difference between ϕA318 and ϕAs51 (data not shown); the amplification rates were, however, significantly distinct for the two phages. It was worthwhile to sequence the whole genomes to investigate the context. ϕAs51 genome of 42542 bp (GenBank access no. KF800937; [GenBank:KF800937]; Additional file 2: annotation for ϕAs51 genome) was almost the same as ϕA318, except that five single nucleotides were mutated sporadically in the genome. Triple checks by PCRs and re-sequencings showed that two deletions of Adenosine were in RNAP gene while single-nucleotide mutations in three sites also occurred in the major capsid protein.

In comparison with gene sequences of other T7-like RNA polymerases, ϕA318 and ϕAs51 RNAP genes shared five consensus regions, corresponding to residues 421–425, 537–538, 563–575, 627–641, and 811–813 residues in the T7 RNA polymerase. As shown in our previous publication [7], the motifs were highly conserved among the T7-like phages. In phage ϕA318, the sequences of the motifs were DFRGR (motif T7-421), DG (motif T7-537), PSEKPQDIYGAVS (motif T7-563), RSMTKKPVMTLPYGS (motif T7-627), and HDS (motif T7-811), respectively. In the region of ϕAs51 RNAP gene, two nucleotides were deleted at 1545 nt, resulting in that the RNA polymerase molecules were translated into two pieces: one N-term fragment of the 515 residues plus three additional residues and the second fragment for C-term with just one additional Met as a start codon (Figure 4A). The analysis of nucleotide sequence revealed that the ribosome binding site AGAAGAAT upstream of a GTG was

| Table 1 | The genes in genomes of phages ϕA318 and ϕAs51 were annotated to known functions |
|---------|---------------------------------------------------------------------|
| Class   | T7 | K1E | ϕA318 ORF | Start | End  | Function                  | Aliases                                             |
|---------|----|-----|-----------|-------|------|----------------------------|-----------------------------------------------------|
| I       | gene 0.3? | 3   | 1023      | 1916  | gp6 protein | Inhibits EcoB and EcoK host restriction |
| I       | gene 0.7 | 4   | 1990      | 2886  | protein kinase |
| I       | gene 1  | 5   | 3173      | 5957  | RNA polymerase*     | ϕAs51: 3173.4727 and 4615.5955 |
| II      | gene 4A | 9   | 6997      | 9093  | DNA primase          |                                               |
| II      | gene 5  | 12  | 10263     | 12806 | gp13            | Putative DNA polymerase                        |
| II      | gene 6  | 19  | 16306     | 16599 | hypothetical protein | Era10.24 Phage exonuclease 40.13 |
| I       | gene 1.3 | 24  | 19109     | 20107 | gp24            | SP6gp25 Adenylation_DNA_ligase_like; K1-5 gp24 |
| III     | gene 8  | 29  | 21233     | 22861 | head portal protein |                                             |
| III     | gene 9  | 30  | 22861     | 23769 | scaffolding protein |                                             |
| III     | gene 10B| 31  | 23817     | 24968 | major capsid protein*|                                             |
| III     | gene 11 | 32  | 25042     | 25689 | tail protein        |                                               |
| III     | gene 12 | 33  | 25692     | 28313 | tail protein        |                                               |
| III     | gene 13 | 36  | 31035     | 34724 | internal virion protein | K1E gp34 internal protein |
| III     | gene 17 | 37  | 34759     | 35694 | gp37            | K1E endosialidase adaptor protein; SP6gp38 tail fiber/adaptor |
| III     | gene 18 | 39  | 35889     | 36233 | hypothetical protein | Era10.45 Phage terminase, small subunit 33.01 |
| III     | gene 19 | 40  | 36233     | 38107 | large terminase subunit |                                             |
|         |       |     | 40747     | 41136 | similar to cell wall hydrolases | involved in spore germination |

*Five nucleotide mutations were found in ϕAs51, which can be referred to text.
suitable for the second translation of ϕAs51 RNAP gene to a product of the exactly same sequence as the C-term of ϕAs318 RNAP. With use of the threading method of PHYRE² to predict the 3-dimentional structures for these two fragments of ϕAs51 RNAP, the result revealed that the truncation join was located in the junction of palm and fingers. As Figure 4B shows, the tail of ϕAs51 RNAP N-term fragment formed a clip (arrows) to hold the ϕAs51 RNAP C-term fragment at the position on palm.

Three mutations of single nucleotide were all in the first codon of the residue, and therefore, totally altered the residues. The mutation at 23973th nucleotide resulted in mutating Histidine at 53th residue to be Asparagine (H53N); 24171th nucleotide mutation changed Isoleucine at 119th residue to Valine (I119V); and 245789th nucleotide mutation changed Asparagine at 515th residue to Lysine (E313K). The mutation at 23973th nucleotide resulted in inactivates the promoter function [10]. Since ϕAs318 and K1E have T and G, respectively, at this position, it is unlikely that the T7 or K1E RNA polymerase will initiate transcription from ϕAs318 promoters. ϕAs318 RNAP has different promoter specificity. This exclusivity of promoter specificity seems to be the trend among the T7-like phages, and it seems that there must be some selective pressure that resulted in this feature.

Electron microscopy revealed that phage ϕAs318 and ϕAs51 particles were morphologically similar to type C in Bradley's classification of Podoviridae phage (Figure 1) [13]. The podovirus family is characterized by phages with a nearly isometric icosahedron head and a short non-contractile tail [13,14]. Furthermore, the lengths of the icosahedral capsid and tail of phage ϕAs318 were morphologically indistinguishable from those typically observed for ϕAs51.

In phages ϕAs318 and ϕAs51, the sequences of the motifs were DFRGR (motif T7-421), DG (motif T7-537), PSEKPOQIVAYGS (motif T7-563), RSMTKVKPMVLTPY GS (motif T7-627), and HDS (motif T7-811), respectively. Strikingly, two nucleotides were deleted in the region of ϕAs51 RNAP gene, which resulted in the RNA polymerase molecules being translated into two pieces separated at the 515th residues (1545 nt); the N-term fragment increased three additional residues in its C-terminus while the second fragment started from a suitable upstream ribosome binding site AGAGAAT and added just one codon GTG for Met to form exactly the same product as the C-term of ϕAs318 RNAP (Figure 4A). We suggested that the two fragments could form a 2-subunit-like

Discussion

This is the first Podoviridae genomes of Vibrio alginolyticus phages that are documented in GenBank. 16 out of 47 ORFs were annotated to known functions, while other ORFs were similar to enterobacteria phages K1E and SP6. 11 strong phage promoters and three terminators were predicted, which are located slightly differently from the closest partners, though genomic organizations were highly homologous [10,12]. Most of promoters have T7-like homologues, except that A at −6 was replaced by C and specificity region on −8 to −12 (Figure 3). In ϕAs318 and ϕAs51 phage promoters, the consensus sequence from −7 to +1 is CCCTATAG. However, bases of −8 through −12, which are important for promoter specificity, differ from T7, T3, K11, and SP6. The consensus at −9 for T7 and SP6 is C; any change of this nucleotide inactivates the promoter function [10]. Since ϕAs318 and K1E have T and G, respectively, at this position, it is unlikely that the T7 or K1E RNA polymerase will initiate transcription from ϕAs318 promoters. ϕAs318 RNAP has different promoter specificity. This exclusivity of promoter specificity seems to be the trend among the T7-like phages, and it seems that there must be some selective pressure that resulted in this feature.

Electron microscopy revealed that phage ϕAs318 and ϕAs51 RNAP gene to a product of the exactly same sequence as the C-term of ϕAs318 RNAP. With use of the threading method of PHYRE² to predict the 3-dimentional structures for these two fragments of ϕAs51 RNAP, the result revealed that the truncation join was located in the junction of palm and fingers. As Figure 4B shows, the tail of ϕAs51 RNAP N-term fragment formed a clip (arrows) to hold the ϕAs51 RNAP C-term fragment at the position on palm.

Three mutations of single nucleotide were all in the first codon of the residue, and therefore, totally altered the residues. The mutation at 23973th nucleotide resulted in mutating Histidine at 53th residue to be Asparagine (H53N); 24171th nucleotide mutation changed Isoleucine at 119th residue to Valine (I119V); and 245789th nucleotide mutation changed Asparagine at 515th residue to Lysine (E313K). The structures of ϕAs318 and ϕAs51 major capsid proteins were predicted from models of HK97 and T7 capsids by PHYRE² with >93% confidence. In comparing with the ϕAs318 capsid, the mutation effects of Asn-53 on ϕAs51 major capsid protein broke the presumed cation bridge between His-53 of two capsomere subunits in ϕAs318 capsid (Figure 5A and C). Glu-313 residue of β1 in P-domain formed two hydrogen bonds with the O = C of

![Image](http://www.biomedcentral.com/1471-2164/15/505)
enzyme to function for the RNA polymerization but weaken RNAP and lose 88% bursting ability.

Virus maturation corresponds to a transition from an initial non-infectious to an infectious and robust virion. PHYRE2 matched the major capsid protein of ϕA318 and ϕAs51 to phage T7 capsid structure PDB:3IZG [15,16]. The 3-dimensional prediction clearly revealed that the capsids of the two new phages were characterized by A-domain, Spine Helix, P-domain, E- and F-loops. Three β-strands are located in P-domain, where βJ is in the center of the three. The matches of residues, however, were not by one-on-one basis. The 53th residue of ϕA318 and ϕAs51 capsid was ruled onto 156th residue in F-loop of PDB:3IZG while 115-135th residues of ϕA318 and ϕAs51 were corresponding to 170-190th residues of Spine Helix in PDB:3IZG. The prediction clearly showed Spine Helix and βJ is located in P-domain. The pair of His53 is in the F-loops of two capsomere subunits or between the subunit G and hexamers. A fusion of the scaffolding protein to be δ-domain, residues 2–103, in HK97 capsid was not found in the new phages, suggesting that the dynamic of forming matured capsid of ϕA318 and ϕAs51 may differ from HK97. Based upon our predicted capsid that was well aligned with the structure of residues 104–385 in the HK97 coat subunits, which form a mixture of hexameric and pentameric capsomers upon expression, they may still share some degree of expansion intermediates with the HK97 capsid [17]. For example, 415 coat subunits (60 hexamers and 11 pentamers) assemble with a dodecameric portal and ~60 copies of the viral protease to

Figure 4 Location of nucleotide deletion in ϕs51 and proposed products of subunits. (A) Two nucleotides were deleted found after 1545 nt in ϕAs RNAP. The proposed new ribosomal binding site was underlined. (B) Two fragments of peptides were predicted by PHYRE2 for 3D structures. Green represents the N-term of full RNAP, while the cyan is for C-term. The predicted structures were superimposed together onto T7 RNAP skeleton.

Liu et al. BMC Genomics 2014, 15:505
http://www.biomedcentral.com/1471-2164/15/505
form proheads; or the prohead particles exhibit spine helix bent and P-domain β-sheet twist. Veesler et al. [17] used an HK97 subunit mutation that prevents formation of non-covalent interactions in virus-like particles and stops maturation at the expansion intermediate of the particles without E-loop “chainmail” interactions. Our results of φAs51 also showed E313K in βJ may be involved in the spine helix bending and P-domain β-sheet twisting, while H53N mutation may prevent non-covalent interactions of E-loop chainmail. When Histidine at 53th is present in the F-loop, adjacent to E-loop, divalent cations like Ni\(^{2+}\) and Ca\(^{2+}\) will build a bridge for such interactions.

Capsid instability in φAs51 with residues Asn-53 (53 N) and Lys-313 (313 K) can account for the thermal stability data in Figure 1. We proposed that the deletion effects of RNAP contributed higher percentages of losing burst ability for φAs51 phage than the point mutations occurring in the major capsid protein. Our findings suggest that the genome sequencing provides a security check prior to application of phages in therapy; thereafter, the mutation rate for each hotspot can be validated for managing the medication plan. The complicate mechanisms still lie ahead for us to solve. Once phage decreases gene activities, the host gains a niche to suppress the phage propagation; as a seesaw does [11]. Further investigations will discover the details.

**Conclusions**

Five nucleotide mutations sporadically spread in the φAs51 genome of about 42500 nucleotides. Those small differences may be the causes to distinguish the lower propagation ability and thermal tolerance of φAs51 from the superior φA318 in plaque size. Subtle analyses on the residue alterations uncovered the effects of five nucleotide mutations on the functions of the RNAP and capsid proteins, which account for the host-bursting efficiency. The deletion of two nucleotides in RNAP gene truncates the primary translation due to early stop codon, while a second translational peptide starting from GTG just at deletion point can remediate the polymerase activity. Out of three mutations in major capsid gene, H53N mutation weakens the subunit assembly between capsomers for the phage head; E313K reduces the fold binding between β-sheet and Spine Helix inside the peptide. Genome sequencing has been done for phage therapy and probiotics to detect a tiny mutation but great effects
which can provide early prediction of treatment efficacy or potential side effects.

Methods
Bacterial strains and growth conditions
Vibrio strains were bought from the Bioresource Collection and Research Center, Taiwan, including V. alginolyticus ATCC 17749, V. carchariae ATCC 35084, V. damsela ATCC 35356, V. harveyi ATCC 14126, V. parahaemolyticus ATCC 17802, V. pelagius ATCC 25916, and V. vulnificus BCRC15431. The Vibrio strains were maintained in Bacto Brain Heart Infusion medium (BHI; Becton-Dickinson Co, USA) supplemented with 3% NaCl (Panreac Quimica, France). For long-term preservation, bacteria were frozen at −80°C in BHI supplemented with 1% NaCl and 25% glycerol (Nihon Shiyaku, Japan). The strains were streaked onto modified seawater yeast extract (rich MSWYE) agar plates consisting of 23.4 g NaCl, 6.98 g MgSO$_4$·7 H$_2$O, and 0.75 g KCl in 1000 ml distilled water [18]. The pH was adjusted to 7.6 with 1 N NaOH, followed by the addition of 5.0 g of proteose peptone (HIMEDIA Lab, India), 3.0 g of yeast extract (HIMEDIA Lab, India), and 20.0 g of agar per liter.

Isolation and titer of bacteriophages
Water samples were collected from aquaculture waterways in southern Taiwan between 2008 and 2010. The water was centrifuged at 10,000 × g for 30 minutes, and the supernatants were filtered through a 0.45 μm microfilter. After centrifugation and microfiltration, 20% MSWYE medium [7,8] and 1% overnight-grown Vibrio alginolyticus were added to the filtrate and incubated at 25°C for 4–24 hours to enrich the phage growth. The bacterial debris was removed by two centrifugations at 10,000 × g for 30 minutes. After enriching the phages in the field samples with the target bacterium Vibrio alginolyticus for 4–16 hours, phase-containing supernatants were incubated with the indicator strain for 5 minutes, suspended in low-percentage agar (top agar), and plated onto solid agar plates, so the target strain would form a bacterial lawn with the formation of plaques. The ten largest plaques from each plate were picked and re-amplified three times in bacterium-containing broth using a 5× volume ratio. The plaques were picked into 200 μl MSWYE broth for further titer counting or production amplification. To prepare bacterial cells for determination of phage concentrations, the host Vibrio alginolyticus was freshly inoculated as a 1% volume of seed from overnight culture into 10 ml of rich MSWYE broth, and it grew to an OD$_{600}$ of 0.3-0.4 in about 2 hours. After a series of dilutions, 10 μl of diluted phage were added to 200 μl of bacterial cells, incubated for 5 minutes, mixed with 3 ml of top agar (rich MSWYE with 0.5% agar), and then poured onto the solid surface of a 2% agar plate. The plaques were counted in 3–5 hours; the titer per ml was calculated as 100 × (dilution factor) × (plaque count).

Electron microscopy
Preparation of phage particles for electron microscopy has been described elsewhere [19]. In brief, bacteriophage particles were applied to parafilm to produce a spherical drop. Carbon-coated nitrocellulose films were fabricated on copper grids, which were placed face down on the sample drop for 1 min to absorb the particles. After being briefly washed twice in 10 μl of 10 mM Tris buffer (pH 8) (Amresco, USA), the samples were then rinsed twice with freshly prepared 2% uranyl acetate (UA; Sigma-Aldrich, USA) in Tris–HCl (pH 8.0) and stained for 60 seconds. Following each step of absorption, washing, and UA staining, the grids were blotted with filter paper until almost dry. The finished grids were dried in vacuo overnight. Images of phage particles were taken at a magnification of 40,000× and a defocus of 3 μm, using a 200-kV electron microscope (JEOL JEM-2010, equipped with a Gatan-832 CCD camera).

DNA Preparation for bacteriophage
For propagation of phage, 10 ml of phage stock was added to 100 ml of V. alginolyticus (3 × 10$^8$ CFU ml$^{-1}$) cultured in MSWYE and incubated in a shaker at 25°C for 3–5 hours until the lysate was clear. The remaining cells and debris were removed by two centrifugation cycles at 10,000 × g for 30 minutes. The supernatant, with a titer of 2 × 10$^{10}$ PFU ml$^{-1}$, was stored at 4°C as a phage stock. To concentrate phages using a standard PEG protocol [7,20], solid NaCl and polyethylene glycol 8000 (Fluka, Germany) were added, and precipitation was performed overnight at 4°C. After centrifugation, the phage particles were resuspended in 2 ml of SM buffer and treated with DNase I and RNase A (Sigma, USA) to remove contaminating nucleic acids from the host. The polyethylene glycol was extracted by adding an equal volume of chloroform (TEDIA, USA) until the interface was clear. The aqueous phase containing the phage was treated with proteinase K (Invitrogen, USA) and sodium dodecyl sulfate (SDS; MD Bio, Inc., USA) at 56°C for 1 h. Phenol extraction was carried out three times at room temperature, and the aqueous phase was further extracted with a 1:1 mixture of equilibrated phenol (Sigma, USA) and chloroform. DNA precipitated by a 2x volume of cold ethanol was redissolved in deionized water.

Adsorption and phage burst size
As described previously [7,8] for the adsorption of phage on bacteria, 1 ml of broth containing host cells and phages was taken at the 5th and 10th minutes and centrifuged to remove bacteria and bound phage, and free phage in the supernatants was then measured. Similarly,
the burst size was measured as described previously [7]. In brief, *V. alginolyticus* cells were grown in 20 ml of medium to mid-exponential phase (OD<sub>600</sub> = 0.3-0.5) and mixed with 0.1 ml phage solution (MOI = 1). Samples of 1 ml of the mixture were taken at intervals and immediately subjected to centrifugation at 14,000 x g for 3 minutes to remove bacteria. The phage titers in the solutions were determined by the agar overlay technique. Using the growth curve for MOI = 1 according to the one-step criteria, the burst size (Bs) of phage was calculated as Bs = Pt/P0, where Pt is the phage titer at that plateau phase and P0 is the initial infective titer.

**Genome sequencing and annotation**

Similar to the shotgun sequencing described elsewhere, approximately 5 μg of the bacteriophage genomic DNA was randomly sheared by nebulization, and DNA sequencing was performed at Mission Biotech according to the manufacturer’s protocol for the Genome Sequencer GS Junior System (Roche Diagnostic). Low quality sequences of the reads generated by the GS Junior sequencer were trimmed off. *De novo* assembly of the shotgun reads was performed with the GS Assembler software. Sequence assembly and analyses were performed essentially as previously described. Protein-encoding genes (PEG) were predicted using The RAST Server ( Rapid Annotations using Subsystems Technology; http://rast.nmpdr.org/) [21] and analyzed with the SEED-Viewer (http://www.theseed.org/wiki/Main_Page) [22]. Protein-coding genes were also checked using the *ab initio* gene-finding program Glimmer v3.02 [23]. rRNA and tRNA genes of the draft assembly were identified using RNAmmer [24] and tRNAscan-SE [25]. Automatic functional annotation results obtained by the RAST were further compared with the proteins in the GenBank database using PSI-BLAST algorithm [31].

**Multiple sequence alignment**

To determine the classification status of the newly isolated ϕAs318 and ϕAs51 phages, sequence data for the genes encoding the DNA polymerase, DNA ligase, RNA polymerase, single-strand DNA binding protein, and capsid proteins of enterobacteria phages (T7, SP6, K1E, and K1F) and *Vibrio cholerae* phages (N4, VP4, and ICP3) were employed to find highly homologous regions. Complete genome sequences of four enterobacteria phages and three *Vibrio Podoviridae* phages were acquired from National Center for Biotechnology Information, NIH, USA: T7 (39,937 bp) [GenBank:NC_001604], SP6 (43,769 bp) [GenBank:NC_004831], K1E (45,251 bp) [GenBank:NC_007637], K1F (39,704 bp) [GenBank:NC_007456], ICP3 (39,162 bp) [GenBank:NC_015159], N4 (38,497 bp) [GenBank:NC_013651], and VP4 (39,503 bp) [GenBank:NC_007149]. The T7 RNA polymerase sequence is also the same as PDB:1QLN (RCSB-PDB ID), and SP6 RNA polymerase is from NC_004831 [GenBank:NC_004831]. The threading predictions by PHYRE ver2 [15] were iterated to predict the tertiary structures of ϕAs318 and ϕAs51 RNA polymerase.

Sequences of individual genes which were retrieved from the genome sets were then aligned using ClustalW with default options [28]. The complete RNA polymerase sequences were analyzed by the neighbor-joining method using the NEIGHBOR program in Phylogeny Inference Package (PHYLIP) [29]. Distances were calculated using the DNADIST program of PHYLIP and displayed in TreeView. ClustalW, PHYLIP, and TreeView were bundled in the BioEdit program version 7.0.5 [30]. The multiple sequence alignment for the phage genomes was constructed in the Mauve using MUSCLE3.6 algorithm [31].

**Additional files**

Additional file 1: ϕAs318.gbk. It is in GenBank format. Annotation for ϕAs318 genome.

Additional file 2: ϕAs51.gbk. It is in GenBank format. Annotation for ϕAs51 genome.

**Competing interests**

The authors declare that they have no competing interests.

**Authors’ contributions**

CSL conducted the study and wrote the manuscript. CSL and YRL did the experiments. CSL, WL, MML, PJS and WHW analyzed the sequences and functions. All authors read and approved the final manuscript.

**Acknowledgements**

This research fund is partially supported by grants from the National Science Council, Taiwan (NSC99-2313-B-110-002-MY3 and NSC102-2313-B-110-002-MY3), and the Ministry of Education, Taiwan (NSYSU95-99C031701; the second term of Top University Program: NSYSU00-02C030205 and NCHU 100-S05-09) under the ATU plan. We thank Professors Hsiuh-Wen Chang, MH Tai, Chih-Chuang Liaw, Chen-Tung Arthur Chen and Houng-Yung Chen, as the grant organizers of the ATU plan. Also we appreciate Kenneth B. Lin and Dr. Simon White for comments and editing.

**Author details**

1. Department of Marine Biotechnology and Resources, Asia-Pacific Ocean Research Center, National Sun Yat-sen University, Kaohsiung 80424, Taiwan.
2. Department of Biotechnology, Kaohsiung Medical University, Kaohsiung, Taiwan.
3. Department of Aquaculture, National Taiwan Ocean University, Keelung, Taiwan.
4. National Museum of Marine Biology and Aquarium, Pingtung 944, Taiwan.

Received: 18 November 2013 Accepted: 30 May 2014
Published: 21 June 2014
