Evidence for Phage-Induced Virulence in the Shrimp Pathogen *Vibrio harveyi*

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ABSTRACT

Vibrio species comprise the most frequently encountered bacterial pathogens of cultivated shrimp, and *V. harveyi* is amongst the most virulent. Most *V. harveyi* strains are luminescent on agar media and also in infected shrimp that are suffering from luminescent disease or luminous bacteriosis. However, not all isolates of *V. harveyi* are highly virulent. Some can be injected at high dose (10^5-10^7 cells per g shrimp body weight) without causing shrimp mortality, while other isolates are lethal at 10^3 per g shrimp body weight or less. In addition, virulence is often lost upon continuous subculture. Simple differentiation of virulent and avirulent isolates has not been successful, although virulence factors including various enzymes (e.g., proteases and lipases), siderophores and proteinaceous toxins have been identified. Because of this and the genetic diversity of *V. harveyi*, it has been suggested that virulence is acquired via mobile genetic elements. Indeed, recent work has suggested that 2 quite different bacteriophages, one from the family *Myoviridae* and the other from the family *Siphoviridae*, can change the phenotype of *V. harveyi* isolates from non-virulent to virulent. The host range for both bacteriophages is relatively narrow. A similar phenomenon occurs in *V. cholerae*, where conversion to virulence is mediated by a filamentous phage (*Inovirus*) from the family *Inoviridae*. Altogether, the current information suggests that there may be diverse groups of phages and complementary *Vibrio* hosts that could mediate virulence in *V. harveyi* and make the process quite complex. It also suggests that virulence of other *Vibrio* pathogens of shrimp may be influenced by bacteriophages. Thus, the use of bacteriophages for biological control of pathogenic *Vibrio* species in aquaculture should include environmental impact studies on the potential for transfer of virulence or antibiotic resistance genes.

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INTRODUCTION

The bacterium *Vibrio harveyi* is a straight to curved motile rod that is a gram negative and oxidase positive. It is a facultative anaerobe that is usually luminescent at some phase of growth in appropriate culture media and also in diseased shrimp, leading to the name luminescent disease for both larval (Lavilla-Pitogo *et al*., 1990) and juvenile (Jiravanichpaisal *et al*., 1994) shrimp. From protein comparisons and DNA fingerprinting, Pizzutto and Hirst (1995) proposed that virulent isolates within *V. harveyi* were rare and did not appear to comprise a genetically uniform group. Virulence appears to be associated with factors such as production of extracellular enzymes and toxins (Harris and Owens 1999; Lee *et al*., 1999; Shinoda, 1999) and might have resulted from transfer of mobile genetic elements, as suggested by Pizzutto and Hirst (1995). In this brief review, we examine the role that bacteriophages might play in the horizontal transfer of virulence factors amongst *V. harveyi* strains that are pathogenic to shrimp and how this might impact on disease control strategies for shrimp farms.

BACTERIAL VIRULENCE ACQUIRED FROM BACTERIOPHAGES

Freeman (Freeman, 1951) was the first to report a bacteriophage encoding the diphtheria toxin gene of *Corynebacterium diphtheria*. Since then, several virulence genes encoded by bacteriophages have been identified (Boyd *et al*., 2001) and some of these are summarized in Table 1. Morphologically and genetically different bacteriophages may provide virulence factors for a single host species and in some cases a single bacteriophage may be capable of infecting more than one species of bacteria (Boyd *et al*., 2000; Faruque *et al*., 1999).

Table 1. Some genes for virulence factors carried by bacteriophages (Boyd *et al*., 2001).

Host bacteria	Bacteriophages	Gene	Virulence factor
Escherichia coli	933, H-19B	*Stx*	Shiga toxins
	ΦFC3208	*Hly2*	Enterohemolysin
	λ	*Lom*	Serum resistance
	λ	*Bor*	Host cell envelope protein
Shigella flexneri	Sf6	*Oac*	O-antigen acetylase
	sfll, sfV, sfX	*Gtrll*	Glucosyl transferase
Salmonella enterica	SopΦ	*SopE*	Type III effector
	Gifsy-2	*sodC-1*	Superoxide dismutase
	Gisfy-2	*NanH*	Neuraminidase
	Gisfy-1	*GipA*	Insertion element
	ε^{34}	*Rfb*	Glucosylation
Vibrio cholerae	CTXΦ	*CtxAB*	Cholera toxin
	K139	*Glo*	G-like protein
	VPΦ	*Tcp*	Toxin co-regulated pilus (TCP) pilin
Pseudomonas aeruginosa	ΦCTX	*Ctx*	Cytotoxin
Clostridium botulinum	Phage C1	*C1*	Neurotoxin
Staphylococcus aureus	NA	*see, sel*	Enterotoxin
	Φ13	*entA, sak*	Enterotoxin A, staphylokinase
	TSST-1	*Tst*	Toxic shock syndrome toxin-1
Streptococcus pyogenes	T12	*SpeA*	Erythrogenic toxin
Corynebacterium diptheriae	β-phage	*Tox*	Diphtheria toxin

In most cases where virulence factors are directly acquired, the host bacteria are converted from non-virulent to virulent strains by a process called **lysogenic conversion**. This involves infection by a **temperate** or **lysogenic bacteriophage** (as opposed to a lytic bacteriophage) that is able to exist as a stable plasmid or integrate into the host bacterial chromosome (a process called **lysogeny**) where it resides as a **prophage**. The result is a stable bacterial strain that may express genes from the acquired prophage. The prophage may be induced into a lytic cycle by physical (e.g., UV light) or chemical factors (e.g., mitomycin C). Practically speaking, exposure of a bacterial suspension to a temperate bacteriophage will result in lysis of many and conversion of some of the host cells. An important feature of the converted bacteria (called **lysogenic strains**) is that they are refractile to lysis when re-exposed to a free bacteriophage that is already resident as a prophage.

Lysogenic conversion by bacteriophages can result in the horizontal transfer of elements called pathogenicity islands that may contain more than 40 kb of DNA and change bacterial virulence by quantum leaps (Miao and Miller, 1999). Bacteriophages can survive harsh conditions that may eliminate bacterial populations and they can spread DNA to a whole population, bypassing the need for growth of clones or genetic exchange by intercellular contact. From Table 1 it can be seen that many of the virulence factors are toxins, but other factors include enzymes, cell attachment molecules (pili) and type III contact-dependent protein secretion effectors. The latter can act like a bacterial syringe to allow simultaneous injection of several proteins that modify host cell physiology (Miao and Miller, 1999).

In addition to the direct encoding of virulence factors, it has recently been found that bacteriophage-bacteriophage interactions may also be involved in the development of bacterial virulence (Boyd *et al*., 2001). This may take the form of one bacteriophage helping another in mobilization of DNA, provision of infection receptors or potentiation of encoded virulence genes (Boyd *et al*., 2001). Some of these such as the TCP pilin required for CTXΦ to infect *V. cholerae* are shown in Table 1.

LYSOGENIC STRAINS OF *V. HARVEYI*

The first indication that *V. harveyi* non-virulent for shrimp might be converted to virulence by a temperate bacteriophage came with the report of shrimp mortality from "tea brown gill syndrome" in Thailand (Ruangpan et al. 1999). Moribund shrimp had hepatopancreatic infections of *V. harveyi* but the purified isolate (*VH* 1039) was not pathogenic. Subsequent transmission electron microscopy of infected shrimp tissue revealed the presence of a bacteriophage together with *VH* 1039 (Fig. 1). Based on morphology (60 nm diameter icosahedral head and 100 nm unornamented tail) the putative phage partner of *VH* 1039 was tentatively placed in the family *Siphoviridae* of the order *Caudovirales*. However, attempts to amplify the bacteriophage in culture failed and this made it impossible to study *V. harveyi* 1039 and its phage partner in detail.

In 2000, Oakey and Owens (2000) described the successful use of mitomycin C to induce lytic cycles of natural prophages in 13 (46%) of 28 *V. harveyi* isolates tested. One of these was a stable, toxin-producing strain (*VH* 642) previously isolated from diseased shrimp larvae (Harris and Owens, 1999). The bacteriophage from *VH* 642 had an icosahedral head of 40-50 nm diameter, a sheathed rigid tail of 150-200 nm length (Fig. 2) and contained double stranded DNA of approximately 40 kbp. These features placed it in the family

Myoviridae of the order *Caudovirales*. It was called "*V. harveyi* myovirus-like" or VHML and it was able to infect several different isolates of *V. harveyi* (4 out of 15 tested) but also one isolate of *V. alginolyticus* (ACMM 102) and *Escherichia coli* JM 109. Subsequent analysis of VHML showed that the total genome consisted of 43,193 bp (GenBank AY133112) encoding 57 putative open reading frames (Oakey *et al*., 2002). Of these, 34 had BLASTp similarity to genes of temperate phages or bacteria known to contain them. Some of these genes were related to baseplate and tail genes of P2-like bacteriophages, also in the family *Myoviridae* but 20 of the putative genes had no similarity anything in the database.

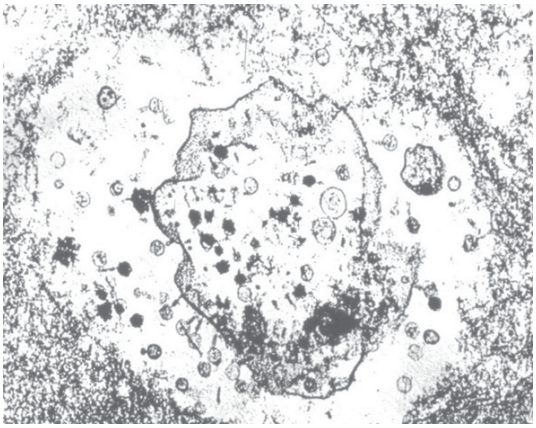

Figure 1. Transmission electron micrograph of tissue from a Thai shrimp specimen showing gross signs of "tea brown gill syndrome". Many phage particles can be seen both free and attached to the wall of a lysed bacterial cell. Some parts of phage particles can also be seen inside the bacterial cell.

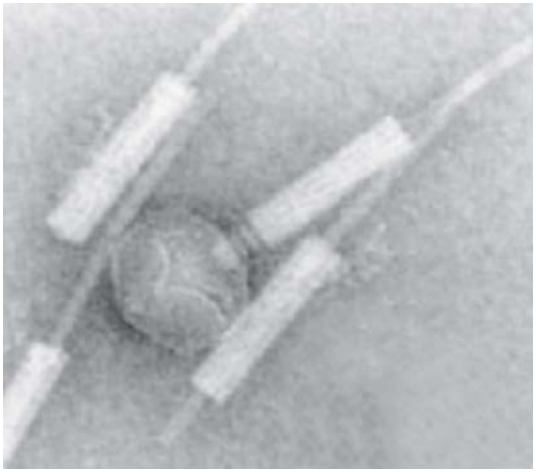

Figure 2. Myophage VHML from Australia.

In Thailand, a second siphophage has been recently found (unpublished). Morphologically, it resembles that described above for *VH* 1039 (Fig. 3), but it was isolated from shrimp culture pond water together with its partner *V. harveyi* (*VH* 1114). This phage has been named VHS1 (*V. harveyi* Siphophage 1). It forms large clear plaques on lawns of *VH* 1114 and very much smaller plaques on two other strains of *V. harveyi* but not with other species of bacteria tested including *E. coli, V. cholerae* and *V. parahemolyticus*. It contains double stranded DNA of approximately 44 kbp as measured by pulse field gel electrophoresis, and 40% of this has been sequenced and deposited at GenBank (Accession numbers: AF480606), AF480607, AF480608, AF480609, AF480610, AF480611 and AF465603). One of the clones (AF465603) contains a sequence with significant homology to a Type A DNA polymerase from *Vibrio cholerae* (NC_002505), SP01 phage of *Bacillus subtilis* (Okubo *et al*. 1964; Stewart *et al*. 1998) and the Siphophage T5 (Leavitt& Ito 1989).

Lysis occurs only with *VH* 1114 cells in the exponential phase of growth and cells subcultured from the middle or edges of plaques give rise to colonies that are refractory to lysis by VHS1. This indicates the presence of a prophage that can be confirmed by the presence of VHS1 DNA in Southern blots of chromosomal DNA extracts (Fig. 4). In addition, treatment of these cells with mitomycin C induces lysis (a large reduction in optical density) while similar treatment of unconverted cells causes none. Membrane filtered supernatant solution from converted *VH* 1114 treated with mitomycin C causes plaque formation with unconverted VH 1114. A PCR method has been devised for detection of VHS1 DNA using a pair of primers PH102-8F, 5'-TCG CGC ATG TTA CGA GC-3', and PH102-3R, 5'-TAC CGG GCT GAA TCG C-3' designed from a VHS1 phage fragment (GenBank accession number AF465603). The amplification protocol consists of 35 cycles with denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute and extension at 72°C for 2 minutes. The primary denaturation and final extension are 94°C for 10 minutes and 72°C for 7 minutes, respectively. The specific amplified fragment is 1038 bp.

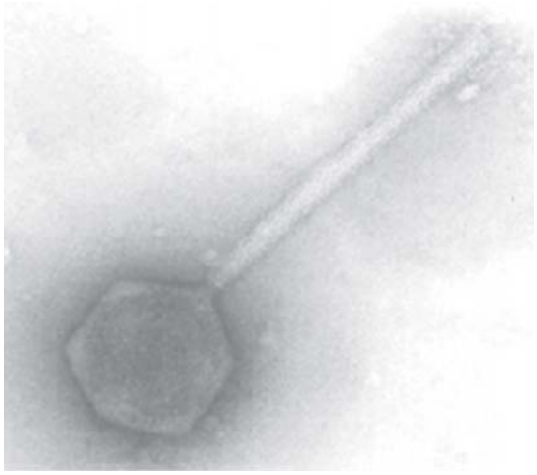

Figure 3. Siphophage VHS1 from Thailand.

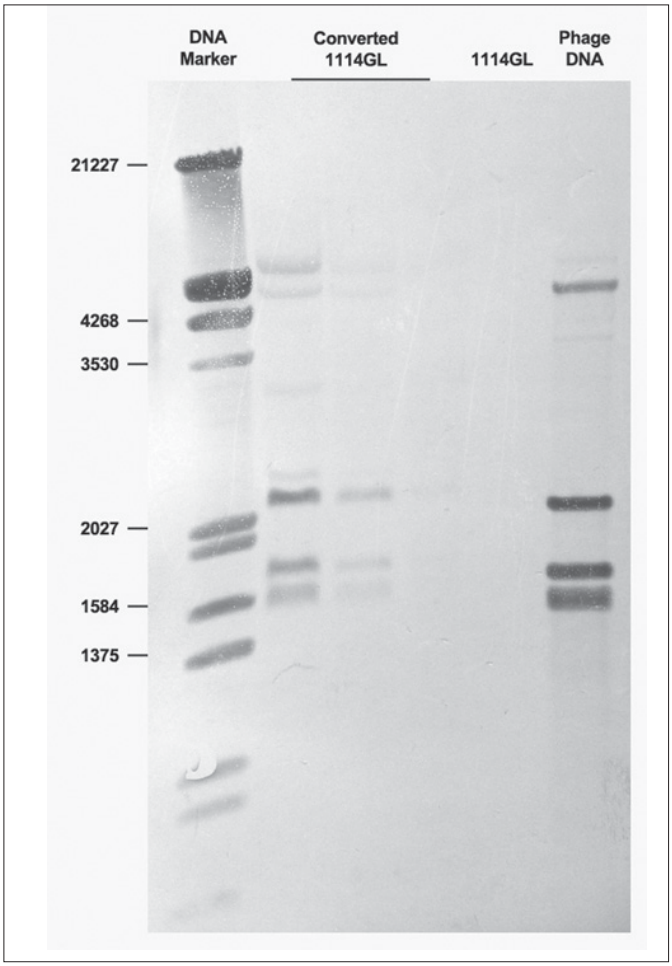

Figure 4. Southern blot of lysogenic and normal VH 1114 DNA cut with restriction enzymes and compared to VHS1 DNA cut with the same restriction enzyme. The membrane was probed with DIG labeled DNA for the molecular weight marker (not cross-reacting with the bacterial or phage DNA) and a cocktail of VHS1 DIG labeled probes. Three dilutions of converted VH 1114 were used.

The order *Caudovirales* contains 3 families (*Myoviridae, Siphoviridae* and *Podoviridae*) of tailed viruses that infect Bacteria and Archaea (Van Regenmortel *et al*., 2000). The family *Siphoviridae* has approximately 300-400 species and the family *Myoviridae* approximately 200-400 species assigned or not (Van Regenmortel *et al*., 2000). The siphophage VHS1 and the myophage (Oakey and Owens, 2000) described above are the only two phages that have been reported so far from *V. harveyi*. The *Myoviridae* are reported to have a wide range of genome sizes from approximately 30-160 kbp while those of the *Siphoviridae* cover a narrower range from approximately 30-50 kbp (Van Regenmortel *et al*., 2000).

LYSOGENY AND VIRULENCE IN *V. HARVEYI*

The report on "tea brown gill syndrome" from Thailand (Ruangpan *et al*., 1999) showed that pure cultures of *VH 1039* isolated from moribund shrimp were not pathogenic to test shrimp in the laboratory even at 10^7 colony forming units (cfu) per shrimp, unless they were mixed with cell-free extracts of diseased shrimp prior to injection. Since tissues of the diseased shrimp showed the presence of a putative siphophage and since the tissue extracts were not lethal when injected alone, it was proposed that *VH* 1039 was converted to a virulent form by the phage. However, efforts to confirm this hypothesis were unsuccessful due to the inability to isolate stable lysogenic strains of *VH* 1039 or to amplify the phage in culture.

Munro et al. (2003) were able to use VHML from *VH* 642 to lysogenize 4 natural isolates of *V. harveyi* that had previously been shown to be prophage-free by treatment with mitomycin C. The treated isolates all showed increased virulence for nauplii of *P. monodon* and this correlated with the production of new proteins in the cell free culture extracts. Although an overall increase in haemolysin activity was seen for the converted isolates, it was unclear whether this was directly related to the increase in virulence. On the other hand, monoclonal antibodies (MAb) raised against semi-purified toxins of *VH* 642 did react with some of the protein bands from the cell free extracts of the converted bacteria, suggesting that they had acquired the ability to produce the toxins from the incorporated prophage either directly or by induction of previously silent genes. Similarly, *V. harveyi* 1114 lysogenized by VHS1 is approximately 100 times more virulent for *P. monodon* that the parent strain (TW Flegel, unpublished).

So far, no potential genes for toxins or enzymes associated with virulence have been identified in the genome of VHS1. However, Oakey *et al*. (2002) did find a putative gene for an ADP-ribolylating toxin (APDRT). Some of the toxins in this group act on actin filaments to produce a neurotoxic effect and this might explain the weakness and intermittent swimming motion of penaeid larvae exposed to *VH 642* containing VHML (Harris and Owens, 1999). They also found an ORF containing a putative DNA adenine methyltransferase protein that may be involved in altering the expression of host bacterial genes. Indeed, a search of the VHML genome for translated sequences matching amino acid sequences of T1 and T2 toxins of *VH 642* as reported by Harris and Owens (1999) gave negative results and it is possible that these genes are bacterial genes whose expression is altered by VHML. Further work is needed to identify potential toxin genes in these phages or genes that might be responsible for up-regulation of host bacterial genes for toxins or other virulence factors.

COMPARISON OF *V. HARVEYI* BACTERIORPHAGE GENOMES

Comparison of the available DNA sequences of VHS1 (GenBank accession numbers above) with the total genome of VHML (Oakey *et al*., 2002) revealed no significant homology. This included the sequences for the primers used in VHS1 detection. This might have been expected given the fact that the two viruses are from different families. The fragments of VHS1 had no significant sequence homology to other known sequences in GenBank, except for a putative DNA polymerase gene (in GenBank AF465603DNA) with significant homology to type A DNA polymerase from *Vibrio cholerae* (NC_002505), from SP01 phage of *Bacillus subtilis* (Okubo *et al*., 1964; Stewart *et al*., 1998) and from siphophage T5 (Leavitt and Ito, 1989). By contrast, VHML had no recognizable DNA polymerase. Obviously, VHML and VHS1 are very different.

IMPLICATIONS FOR AQUACULTURE

The fact that two apparently unrelated bacteriophages can have similar properties of lysogeny and induction of increased virulence in *V. harveyi* suggests that we may just be scratching the surface of a rather larger phenomenon. It is probable that there are even more phage types for *V. harveyi* waiting to be discovered and that other *Vibrio* species pathogenic to shrimp also have lysogenic phages. Indeed, using mitomycin C for induction of cell lysis by prophages, Oakey and Owens (2000) found that 30 (48%) of 63 *Vibrio* isolates tested were positive for the presence of prophages. This included 11 (70%) of 16 unidentified *Vibrio* isolates tested and a number of other single isolates of *Vibrio* species including *V. vulnificus*, another known shrimp pathogen. Although the single tested isolate of another important shrimp pathogen *V. parahaemolyticus* was negative for prophages, it is likely that screening of more isolates would have revealed their presence.

Clearly there is an inherent risk for shrimp farmers in this phenomenon. In addition to the danger of virulence conversion, we also have to consider that bacteriophages can act as transducing shuttles for tag-along DNA from their bacterial hosts. When a single phage has more than one bacterial host species, there is potential for intermixing amongst the bacterial host genomes. From the human health point of view, a particular concern in this regard is the spread of antibiotic resistance genes. It also means that molecular taxonomists may face a difficult task in defining the limits of bacterial "species". These difficulties may be of little immediate concern to shrimp farmers when compared to the possibility of rapid horizontal transfer of virulence factors throughout a pond bacterial population upon accidental introduction of a lysogenic phage. At this time, it is difficult to assess the extent of the practical risk to aquaculture from such a phenomenon, but it would be prudent to make some assessments. One part of this task would be to examine the possible routes by which bacteriophages might enter the rearing system. For example, VHS1 is relatively resistant to heat and although its ability to lyse cells is lost after boiling for 10 minutes, it still retains its ability to lysogenize. This being the case, one wonders whether the conditions used to prepare crustacean headmeal and other natural shrimp feed components are sufficient to fully inactivate bacteriophages that might be present.

The use of lytic bacteriophages is among the recent innovations proposed for control of bacterial pathogens in shrimp ponds. This has the potential for very specific control without the negative environmental impact that results from the widespread use of antibiotics and disinfectants. As part of the safety studies for development of such technology, it would be important to examine the possibility for genetic exchange amongst the lytic and lysogenic or temperate phages of the target bacteria.

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