A Hypothetical Model for VHML Phage Conversion of *Vibrio harveyi*

JANE OAKEY[1,2], BRADFORD CULLEN[1] AND LEIGH OWENS[1]

[1]*James Cook University, Townsville, Queensland 4811*

[2]*Oonoonba Veterinary Laboratory, Queensland Department of Primary Industries, Animal and Plant Health Service, Townsville, Queensland 4811*

ABSTRACT

The bacteriophage VHML (*Vibrio harveyi* myoviruslike) originated from a toxin producing strain of *Vibrio harveyi*. It has been demonstrated previously that the presence of the VHML prophage can induce virulence to *V. harveyi* in the laboratory. Here, a hypothetical model for phage virulence conversion of *V. harveyi* is presented. Through the nucleotide sequence determination of the entire VHML genome (43,193 bp), we have identified putative phage structural genes consistent with the physical characteristics of the virions as observed by TEM. We have also identified putative genes consistent with integration of the genome, supporting the theory that VHML integrates the host *V. harveyi* genome as a prophage. In addition, we have identified a potential toxin gene on the VHML genome. This gene includes DNA sequence similar to the reported active site of the ADP-ribosylating group of toxins. These ADP-RT's include toxins from other bacteria reported previously to be a result of bacteriophage conversion. This presentation will illustrate how the phage genes could cause infection of *Vibrio harveyi* host cells, integration of the phage genome into the hosts' chromosome and subsequent production of the putative toxin, thereby conferring virulence to *Vibrio harveyi*.

INTRODUCTION

The *Vibrio harveyi* myovirus-like bacteriophage (VHML), was first found in *Vibrio harveyi* ACMM 642 (Oakey and Owens, 2000). This bacterial isolate was isolated in northern Queensland from *Penaeus monodon* larvae with vibriosis (Muir, 1987), and subsequently was shown to produce at least one extracellular toxic product, or exotoxin (Harris and Owens, 1999). VHML is morphologically similar to the myovirus family of bacteriophages by virtue of a rigid tail (approx. 150 nm) covered by a contractile sheath. TEM studies also showed an icosahedral capsid (approx. 40 nm diameter), and the presence of tail fibres at the base of the tail sheath (Oakey and Owens, 2000). The host range of VHML has been determined to be narrow, consisting of 4 out of 17 strains of *V. harveyi* tested, one strain of *V. alginolyticus* (out of 17 non-*V. harveyi* vibrionaceae tested) and one commercial strain of *E. coli* (JM109) out of 5 enterobacteriaceae tested (Oakey and Owens, 2000).

Oakey, J., B. Cullen and L. Owens. 2005. A hypothetical model for VHML phage conversion of *Vibrio harveyi. In* P. Walker, R. Lester and M.G. Bondad-Reantaso (eds). Diseases in Asian Aquaculture V, pp. 457-464. Fish Health Section, Asian Fisheries Society, Manila.

Subsequent analyses showed that infection of naïve strains of *V. harveyi* by VHML in the laboratory caused a number of changes to the host cells - increased haemolysis; the production of additional extracellular products that cross reacted with monoclonal antibodies raised to the *V. harveyi* ACMM 742 exotoxin; and apparent virulence in previously avirulent strains, as indicated in larval penaeid bioassays (Munro *et al*., 2003).

However, it was unknown whether genes within the bacteriophage genome encoded the products associated directly with these changes (haemolysins and/or toxins), or whether the changes were a result of the bacteriophage somehow altering the regulation of transcription/ translation of host genes. Therefore we have determined the entire sequence of the bacteriophage genome in order to identify putative proteins encoded and, hence, to try and ascertain the model of virulence conversion of *V. harveyi* by VHML (Oakey *et al*., 2002). This paper presents a hypothetical model, based on the nucleotide sequence data, for infection of host cells. An open reading frame (ORF) thought to be associated with the increase in virulence of host cells to penaeids will also be discussed.

NUCLEOTIDE SEQUENCE OF THE VHML GENOME

Sequence analysis of the VHML genome, as described in detail by Oakey *et al*. (2002), showed that the genome length was 43, 193 bp, consisting of 57 ORF's (Genbank Accession AY133112). Of these, 37 could be identified through comparison with amino acid sequences in the Genbank database, using a combination of sequence similarity; the E value, or significance, of the sequence matches; and the presence of conserved domains within the predicted amino acid sequences. From the 37, 28 could be assigned a putative function based upon the translated sequence. Some of these will be further discussed in this paper.

STRUCTURAL GENES

Genes for the 'head', or capsid, proteins were located together on the VHML genome and were designated ORF21 to ORF24. ORF's 21 and 22 had highly significant matches to terminase subunits (DNA packaging/capsid assembly) of enterobacteriaceae phages, ORF23 was similar to the head/tail connector protein (or portal protein) of a *Wolbachia* sp. phage, and ORF24 was similar to the capsid structural protein gene of the same phage. Figure 1 depicts these ORF's in relation to the structure of the phage as seen by TEM.

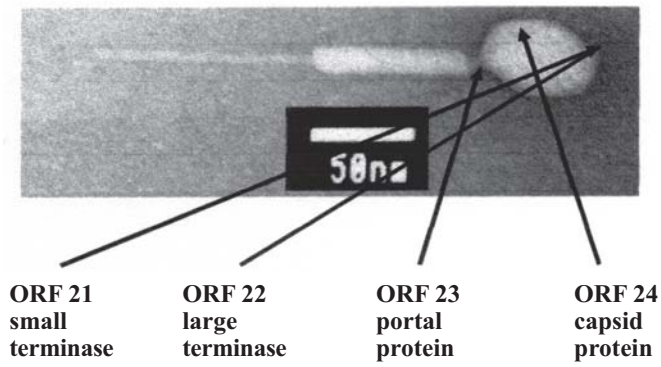

Figure 1. Relationship between identified ORFs and the structure of the bacteriophage capsid.

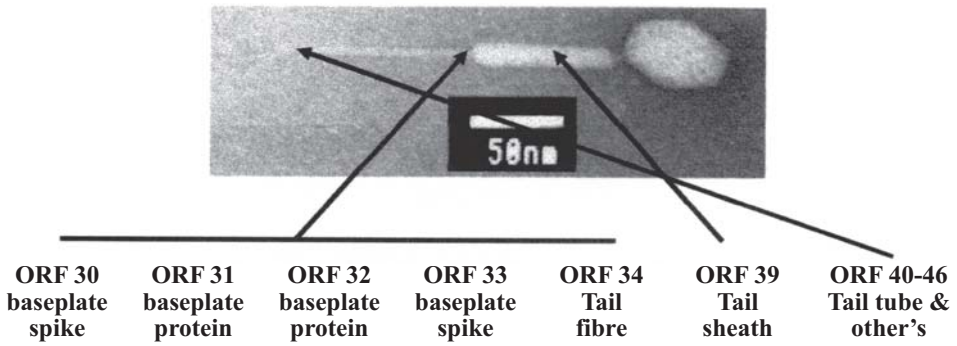

Figure 2. Relationship between identified ORFs and the structure of the bacteriophage tail.

Genes encoding the tail proteins were located in two blocks of the genome, one consisting of the genes for the baseplate at the base of the tail sheath and the genes for the tail fibres (ORF's 30 to 34), and the other consisting of the genes for the tail sheath, tail tube, tail length determinator and other tail associated proteins (ORF's 39 to 46). Within the former block, the baseplate assembly proteins ORF31 and 32 show general similarity to analogous proteins from other phages throughout their entire gene length. However, the baseplate spike and tail fibre genes (ORFs 30, 32 and 33) display similarity with comparable phage genes only in the N-terminal half of the sequence, the the C-terminal sequence having no significant similarity to any other sequence in the Genbank database. The second gene block contains ORFs with highly significant translated matches to homologous proteins from the enterobacteria phage P2. Figure 2 depicts these ORF's in relation to the structure of the phage as seen by TEM.

GENES ASSOCIATED WITH LYSOGENY

Unlike structural genes, the genes associated with lysogeny are not located in blocks but dispersed through the genome. ORF sequence searches have shown that ORF10 shows high similarity to the amino acid sequence of a recombinase from *V. cholerae*. Similarly, ORF37 showed a significant match with a transposase. The presence of both of these genes suggests that VHML may integrate into the host cell's chromosome. In addition, we noted that the phage genome termini consisted of inverted terminal repeat sequences of 33 bp. This suggests that integration may occur in a similar manner to transposition and, unlike some other P2-like myoviruses, the genome cannot circularise in the event of the host chromosome lacking a specific attachment site. Bacteriophage such as P2 and phi-ctx have 'sticky' ends or single (-) stranded termini consisting of direct repeats enabling these termini to self-anneal and generate a circular form similar to a plasmid within the host cell.

A lysogenic phage requires a repressor gene to 'switch off' those genes associated with structure and replication in order to remain within the host over many replication cycles. In addition, an anti-repressor protein is required to 'switch on' these genes to induce the lytic cycle of such phages. Within VHML, we have identified the ORF6 gene product as a putative phage repressor, with a significant match to a repressor reported for a myovirus of *E. coli;* and ORF48 as an anti-repressor gene, having similarity to the amino acid sequence of the rha antirepressor of phi-80.

HYPOTHETICAL MODEL FOR INFECTION OF HOST CELLS BY VHML

Using the putative genes described above, a hypothetical model can be proposed for the infection of host cells by the VHML phage. This model is summarised in Fig 3 where infection is split into five stages:

(1) receptor recognition by the tail fibres;

(2) receptor recognition by the baseplate spikes;

(3) sheath contraction and tail insertion through the host cell wall;

(4) mobilisation of phage genome into the host cell; and

(5) integration of the genome into the host chromosome using recombinase and/or transposase.

It is thought that integration occurs at site-specific locations recognised by the recombinase enzyme to be similar to the inverted terminal repeats of the phage genome. This enzyme could bind the two inverted repeat regions thus bringing them together with the proposed attachment site (see Fig. 3) prior to integration into the chromosome by transposase or a combination of the two enzymes. This would imply that recombination occurs in a similar manner to transposition. Transposition as a means of integration has been observed for a number of other bacteriophages, such as phage Mu (Zou *et al*., 1991).

It can be seen that, in order for infection to occur using this model, a host cell must display the receptor proteins for the tail fibres and for the baseplate spikes, and must possess a specific attachment sequence within its chromosome. These three limiting factors may explain the narrow host range of VHML. The specificity of the receptor recognition may also explain the presence of previously unidentified sequence within the genes for the tail fibres and baseplate spikes.

Once integrated into the host chromosome, it is thought that the repressor gene (putatively identified as ORF6) will repress all structural and replication-associated genes within the phage genome, as documented for other lysogenic phages (Dottin *et al*., 1975). The phage then exists as a prophage and will be replicated with the host chromosome for many generations of bacterial growth, where all daughter cells possess this prophage. However, when the anti-repressor protein (putatively identified as ORF48) is transcribed and translated, this protein prevents the action of the repressor, and replication of the phage genome and structural components occurs. Previous texts have indicated that host stress may trigger the anti-repressor activation or directly inactivate the repressor protein (Shearwin *et al*., 1998). Indeed, we have previously noted that VHML could be induced to replication through the addition of sublethal concentrations of the mutagen mitomycin C to a bacterial culture containing the prophage (Oakey and Owens, 2000).

GENES ASSOCIATED WITH VIRULENCE OF HOST CELLS

No genes were identified within the phage genome that showed similarity with any previously reported bacterial toxin. However, ORF17 was putatively identified as a DNA adenine methyltransferase (*Dam*) gene, having high amino acid sequence similarity to a Dam of *E. coli* with a highly significant match, and the presence of the conserved protein domain of this group of enzymes. Dam's have recently attracted interest as powerful transcriptional

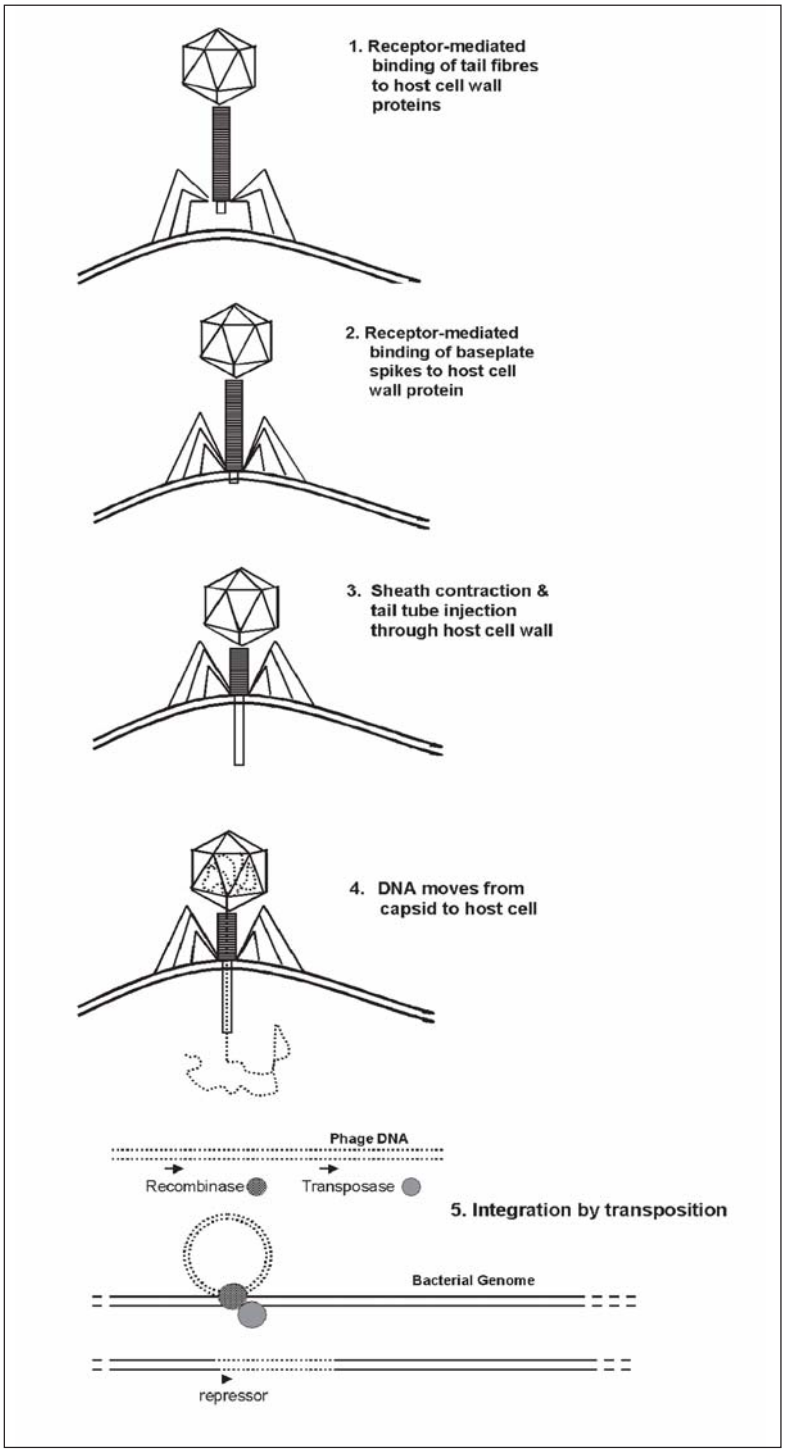

Figure 3. Hypothetical model for the infection of host cells with VHML.

regulators, having the ability to turn genes on and off. Low *et al*. (2001) reported that this most likely occurred by alteration of the affinity of other regulatory proteins for DNA. In addition, Dam's have been reported as essential components of virulence in a number of bacteria. For example, the virulence of some strains of *E. coli* causing urinary tract infections, *V. cholerae, Salmonella typhimurium*, and *Yersinia pseudotuberculosis* have all been shown to be dependent upon Dam regulation of virulence genes. The absence of this enzyme rendered these strains to be avirulent (Braaten *et al*., 1994; Heithoff *et al*., 1999; Portello *et al*., 1999; Julio *et al*., 2001). It may be hypothesized, therefore, that Dams may play a similar role in *V. harveyi*, where a previously inactive gene becomes 'switched on' through the presence of the VHML prophage and its encoded Dam, resulting in the production of a toxin. The presence of a powerful transcriptional regulator also may be responsible for the up-regulation of haemolysin, or the 'switching on' of an additional haemolysin, as has been noted phenotypically in infected strains of *V. harveyi* (Munro *et al*., 2003, in press).

Another point of interest regarding ORF17 is that this is longer than many other reported adenine methyltransferase genes. Upon translation, the 'extra' sequence includes amino acid sequence similar to that reported by Barth *et al*. (1998) as the conserved site of ADP-ribosylating toxins (ADPRT). Specifically, the amino acid arrangement matches that of the group 2 ADPRT's which act upon the G protein and includes cholera toxin; and that of the group 4 ADPRT's which act upon actin and tend to have a neurotoxic effect such as that of the C2 toxin of *Clostridium botulinum*. These active sites all consist of an essential S-T-S motif with an upstream arginine (R) residue and two downstream glutamic acid (E) residues. Group 2 toxins have an additional conserved histidine (H) residue between the S-T-S and the R. The conserved domains reported by Barth *et al*. (1998) compared with the translated sequence of the corresponding region of VHML ORF17 are shown in Fig 4.

Further research is required to determine if adenine methyltransferase is the catalyst required to stimulate virulence in *V. harveyi* strains, or whether this gene also encodes for a toxic element, or whether virulence is a combination of these factors.

Figure 4. Comparison of partial amino acid sequence of VHML ORF 17 with conserved active sites of ADP-Ribosylating toxins (ADPRT's) reported by Barth *et al*. (1998). * = sequential amino acid as encoded from the ORF; x = number of spanning amino acids between residues involved in the active sites.

CONCLUSION

In conclusion, it is likely that the host range of VHML depends upon the presence of at least two host cell wall proteins and upon a specific nucleotide sequence in the host cell's chromosome. Subsequent virulence by the host cell may be a result of transcriptional up-regulation by a VHML-encoded DNA methyltransferase and/or the presence of a phage-encoded potential toxin related to the ADP-ribosylating group of bacterial toxins.

This raises a few questions with which we must provide answers if we are to overcome, or even understand, vibriosis disease in farmed penaeids. Firstly, are all virulent strains of *V. harveyi* attributable to bacteriophages? Certainly, bacteriophages have been linked to a virulent strain in Thailand (Ruangpan *et al*., 1999), and a second virulent strain in Australia has been found to possess a prophage which can be induced to replicate into viable virions, although studies to link this phage to virulence have not yet been conducted (J. Oakey, unpublished). However, it has been determined that these three phages are not the same. Indeed, they appear to belong to three different taxonomic families of bacteriophage. A more widespread study into a number of virulent strains would be required to ascertain the frequency of the phage conversion phenomenon.

Another point of interest may be that of phage therapy for vibriosis. If bacteriophages are present in virulent strains of *V. harveyi*, can lytic phages suggested for use as therapeutics infect and lyse these bacteria? The phenomenon of exclusion of closely related phages by an infected bacterial cell is well documented (Lu and Henning, 1994; Myung and Calendar, 1995; Nesper *et al*., 1999). Prophages may encode proteins which prevent entry of phage nucleic acids into the host cell or encode for an exclusionary exonuclease which destroys incoming linear nucleic acids such as those of other phages. The specificity of these enzymes may vary between phages. In our laboratory, we have noted such a 'battle of the phages' in Vibrio cultures, where some phages exclude infection of another, while other phages apparently have some mechanism to remove a prophage from a host and so infect the host themselves (Payne, 2002). The use of lytic phages for therapeutic purposes must, therefore, be validated against phage-containing strains.

The models presented in this paper are purely hypothetical and should be taken as such. It must be noted that site-directed mutagenesis has not been conducted to sequentially identify the functions of these genes experimentally. However, using the VHML nucleotide sequence, the vast number of sequences in the Genbank database and a thorough review of the literature concerning the mode of action of the putatively identified proteins and bacteriophages in general, we believe that our hypotheses are highly plausible.

REFERENCES

Barth, H., Preiss, J.C., Hofman, F. and Aktories, K. 1998. Characterisation of the catalytic site of the ADP-ribosyltransferase *Clostridium botulinum* C2 toxin by site directed mutagenesis. Journal of Biological Chemistry 273, 29506-29511.

Braaten, B.A., Nou, X., Kaltenbach, L.S. and Low, D.A. 1994. Methylation patterns in pap regulatory DNA control pyelonephritis-associated pili phase variation in *E. coli*. Cell 76, 577-588.

Dottin, R.P., Cutler, L.S. and Pearson, M.L. 1975. Repression and autogenous stimulation in vitro by bacteriophage lambda repressor. Proceedings of the National academy of Science USA 72, 804-808.

Harris, L.J. and Owens, L. 1999. Production of exotoxins by two luminous *Vibrio harveyi* strains known to be primary pathogens of *Penaeus monodon* larvae. Diseases of Aquatic Organisms 38, 11-22.

Heithoff, D.M., Sinsheimer, R.L., Low, D.A. and Mahan, M.J. 1999. An essential role for DNA adenine methylation in bacterial virulence. Science 284, 967-970.

Julio, S.M., Heithoff, D.M., Provenzano, D., Klose, K.E., Sinsheimer, R.L., Low, D.A. and Mahan, M.J. 2001. DNA adenine methylase is essential for viability and plays a role in the pathogenesis of *Yersinia pseudotuberculosis* and *Vibrio cholerae*. Infection and Immunity 69, 7610-7615.

Low, D.A., Weyand, N.J. and Mahan, M.J. 2001. Roles of DNA adenine methylayion in regulating bacterial gene expression and virulence. Infection and Immunity 69, 7197-7204.

Lu, M.J. and Henning, U. 1994. Superinfection exclusion by T-even-type coliphages. Trends in Microbiology 2, 215-216.

Muir, P. 1987. Factors affecting the survival of *Penaeid prawns*. PhD Thesis. James Cook University, Townsville, Australia.

Munro, J., Oakey, J., Bromage, E. and Owens, L. 2003. Experimental bacteriophage-mediated virulence in strains of *Vibrio harveyi*. Diseases of Aquatic Organisms 54, 187-194.

Myung, H. and Calendar, R. 1995. The old exonuclease of bacteriophage P2. Journal of Bacteriology 177, 497-501.

Nesper, J., Blass, J., Fountoulakis, M. and Reidl, J. 1999. Characterization of the major control region of Vibrio cholerae bacteriophage K139: immunity, exclusion, and integration. Journal of Bacteriology 181, 2902-2913.

Oakey, H.J. and Owens, L. 2000. A new bacteriophage, VHML, isolated from a toxin-producing strain of *Vibrio harveyi* in tropical Australia. Journal of Applied Microbiology 89, 701-709.

Oakey, H.J., Cullen, B.R. and Owens, L. 2002. The complete nucleotide sequence of the *Vibrio harveyi* bacteriophage VHML. Journal of Applied Microbiology 93, 1089-1099.

Payne, M. 2002. Cross infection of bacteriophage between *Vibrio harveyi, V. cholerae* and *V. mimicus*. BSc Honours Thesis. James Cook University, Townsville, Australia.

Portello, F.G.-D., Pucciarelli, M.G. and Casadesus, J. 1999. DNA adenine methylase mutants of *Salmonella typhimurium* shows defects in protein secretion, cell invasion and M cell cytotoxicity. Proceedings of the National Academy of Science USA 96 11578-11583.

Ruangpan, L., Danayadol, Y., Direkbusarakom, S., Siurairatana, S. and Flegal, T. 1999. Lethal toxicity of *Vibrio harveyi* to cultivated *Penaeus monodon* induced by a bacteriophage. Diseases of Aquatic Organisms 35, 195-201.

Shearwin, K.E., Brumby, A.M. and Egan, J.B. 1998. The Tum protein of coliphage 186 in an antirepressor. Journal of Biological Chemistry 273, 5708-5715.

Zou, A.H., Leung, P.C. and Harshey, R.M. 1991. Transposase contacts with mu DNA ends. Journal of Biological Chemistry 266, 20476-20482.