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ORIGINAL ARTICLE

Virulence Changes to Harveyi Clade Bacteria Infected with Bacteriophage from *Vibrio owensii*

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Abstract *Vibrio owensii* is one of the most virulent vibrios known being able to kill crustacean larvae at 10^2 CFU ml^{-1}. This study describes virulence changes to naïve strains of *Vibrio harveyi* and *Vibrio campbellii* when infected with the bacteriophage VOB from a closely related species *V. owensii* 47666-1. The bacteriophage from *V. owensii* was induced into lytic phase by using mitomycin C at 100 ng ml^{-1}. One strain of *V. harveyi* and two strains of *V. campbellii* from 29 tested containing no prophage were susceptible to lysogenic conversion with VOB. Virulence changes induced in Harveyi clade bacteria included the up-regulation of protein secretion, statistically significant increased haemolysin and chitinase production and increased mortality to nauplii of *Penaeus monodon*. No change in siderophore production was observed. Bacteriophage VOB is likely to be responsible for some of the virulence factors expressed by *V. owensii*. As this bacteriophage is able to infect strains of *V. harveyi* and *V. campbellii* this phage may contribute to increased virulence of other vibrios in aquaculture and in the natural environment.

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Introduction

Members of the *Vibrio harveyi* clade are major pathogens of many aquatic organisms including vertebrates and invertebrates. The Harveyi clade consists of eleven species: *V. harveyi*, *V. campbellii*, *V. rotiferianus*, *V. alginolyticus*, *V. parahaemolyticus*, *V. mytili*, *V. natriegens*, the newly described *V. azureus* [29], *V. sagamiensis* [30], *V. owensii* [2], *V. communis* [4] which is a junior synonym of *V. owensii* [3, 8] and most recently, *V. jasicida* [31]. Many isolates of this clade particularly *V. harveyi*, *V. campbellii* and *V. owensii*, are bioluminescent and contribute to a very important penaeid hatchery disease called luminescent vibriosis. Numerous virulence factors have been implicated in severe outbreaks of luminous vibriosis. The possession of toxic extracellular proteins such as proteases, phospholipases, haemolysins [10] and cysteine proteases [11] reportedly increase the virulence of this marine bacterium. In addition, virulence-related products such as siderophores [17], bacteriocins [12] resistance plasmids [6] and chitinases [25] have also been implicated in the virulence of Harveyi clade members.

It was suggested that virulent isolates within *V. harveyi* are rare and that virulence is likely to be due to a mobile genetic element such as plasmids or bacteriophages [20]. Virulence factors encoded on bacteriophages may confer a wide range of traits to their hosts including toxin production and immunity to superinfection [18]. The involvement of bacteriophage in the virulence of *V. harveyi* has been documented previously by several authors [14, 16, 21]. One strain of *V. campbellii* (ACMM 642) originally reported as *V. harveyi* and one of the new species of *V. owensii* (47666-1)

(previously *V. harveyi* 47666-1) have been shown to cause devastating disease in prawns in northern Australia [7]. A bacteriophage VHML from *V. campbellii* 642 was confirmed to confer virulence to naïve strains of *V. harveyi* [14]. While it was reported that *V. owensii* strain 47666-1 was more virulent than strain ACMM 642, causing 100 % mortality to *Penaeus monodon* larvae at 10^2 CFU ml^{-1} [13], there is no information yet to confirm whether a bacteriophage in this species confers virulence. This strain has been shown to be infected with *Vibrio harveyi* Podovirus-like (VHPL) bacteriophage, classified in the family Podoviridae [15], which should now be called *Vibrio owensii* Podovirus like (VOPL) as this is more accurate and the previous name is not established in the literature. Accordingly, this bacteriophage is characterised by an icosahedral head ($\sim$70 nm diameter), short stumpy tail, and has a linear double stranded DNA genome. Also a filamentous bacteriophage about 2–3 µm has been seen in TEM of strain 47666-1. The methodology herein could not separate the two possible phages so the term Vibrio owensii bacteriophage (VOB) is used throughout this paper.

As our library of *V. owensii* strains only consists of three strains and *V. owensii* is a member of the Harveyi clade, it was decided to attempt to infect *V. harveyi* and *V. campbellii* with phage from *V. owensii*. This paper describes phenotypic changes to naïve strains of the Harveyi clade when infected with VOB.

Materials and Methods

Strain Identification

Once bacterial strains were found to be susceptible to VOB, the host bacteria were typed by multilocus sequence analysis (MLSA) using two loci, *mreB* and *topA* shown to work perfectly for the Harveyi clade [3].

Bacterial Isolates and Growth Conditions

All isolates used in this study were from the Microbiology and Immunology culture collection at James Cook University, Australia. Frozen isolates were cultured in PYSS (bacteriological Peptone, Yeast, and Sea Salt) agar and incubated overnight at 28 °C. Several isolated colonies from an agar plate were suspended in PYSS broth. The suspension was incubated at 28 °C in an orbital incubator shaken at 110 rpm until the appropriate cell density was achieved. The isolate was then prepared for storage by the addition of 10 % glycerol (w/v), stored at −70 °C for long-term storage and at –20 °C for working stocks.

Bacteriophage Extraction and Concentration

A loop of *V. owensii* 47666-1 from the −20 °C working culture was inoculated into PYSS broth [16]. The culture was incubated overnight at 28 °C to give a mid to late exponential phase culture corresponding to an optical density of 0.95–1.00 at 600 nm. This corresponded to a cell density of approximately 2×10^9 colony forming units (CFU) ml^{-1}. The culture was induced into the lytic cycle by the addition of 100 ng ml^{-1} mitomycin C (Sigma-Aldrich, Castle Hill, NSW, Australia). Induced cultures were returned to incubation for 15–18 h on an orbital shaker at 28 °C at 110 rpm. Cells were centrifuged at $5{,}000 \times g$ for 10 min followed by passage of supernatant through 0.45 µm disposable Millipore filter membranes (Millipore, Billerica, MA, USA) and this cell-free supernatant (CFS) was transferred to polycarbonated centrifuge tubes (Beckman, Palo Alto, CA USA). The CFS was spun through ultracentrifuging at $200{,}000 \times g$ for 4 h. The pelleted phage was resuspended in 0.01 original volume sterile SM buffer (5.8 g sodium chloride, 2 g magnesium sulphate, 50 ml 1 mol l^{-1} Tris pH 7.5, 5 ml of 2 % gelatin and 945 ml distilled water).

Infection and Detection of Phage from *V. owensii* 47666-1

Those bacterial strains which were shown to have no prophage by Oakey and Owens [16] were challenged with the bacteriophage from *V. owensii* 47666-1 using methods described therein. Bacteria were cultured in 100 ml aliquots of PYSS and incubated as stated previously until $O.D._{600}$ was approximately 0.2. The cultures were aseptically divided into four equal aliquots. A volume of 500 µl of concentrated VOB phage from *V. owensii* 47666-1 was added to two of the aliquots and 500 µl SM buffer added to the other two. All tubes were returned to incubation for 2 h to allow potential infection to occur. All infected strains were stored as previously described. All tubes with added extract and one of the 'blank' tubes were induced with 100 ng ml^{-1} mitomycin C and further incubated. Optical density readings were taken periodically throughout the experiment to a maximum of 30 h post-induction. A decrease in the $O.D._{600}$ which was not evident in either the control or the induced control was taken as a presumptive infection with VOB.

Preparation and Concentration of Cell-Free Supernatant

The procedure for the preparation of CFS extract was modified from Harris and Owens [7] and Munro et al. [14]. Sterile PYSS was inoculated with a stab from a strain of Harveyi clade bacteria from an overnight culture. This culture was grown to a density corresponding to an absorbance of 0.95–1.05 at 600 nm. Disposable cuvettes (2 ml) (Sarstedt, Adelaide, Australia) were used to measure absorbance in an Ultrospec III spectrophotometer (Amrad

Pharmacia Biotech, Boronia, Australia). Each culture of bacteria with and without the phage VOB was lysed using mitomycin C at a dose of 100 ng ml^{-1}. They were returned to incubation for 5 h prior to pelleting by centrifugation at 12,200×*g* for 20 min. The supernatant was harvested and filtered through a 0.45 μm followed by 0.22 μm membrane filter (Millipore, North Ryde, NSW, Australia). To ascertain that the bacteria had been removed from the lysates, a loopful of supernatant was streaked onto PYSS agar plate and was observed for bacterial growth. The lysates that showed no growth on plates were further processed with the concentration method.

A 50–300 times concentration of the supernatant was achieved by membrane filtration. Two membranes and filter devices were used:

(1) For the preparation of small volumes of cell-free supernatant extract (CFSE), the supernatant was spun through a Centriprep-30 concentrator filter (Amicon, Beverly, MA, USA) at 1,500×*g* using a swinging bucket rotor in a Clements 2000 bench top centrifuge (Clements, Sydney, Australia). In this method, a 15-ml supernatant was concentrated to approximately 500 μl in 90 min.
(2) For the preparation of larger volumes, approximately three litres of supernatant was concentrated by filtration through a 68 mm YM 50 (50 kDa) (Amicon, Beverly, MA USA) in an Amicon (Millipore, Sydney, Australia) model 8200 ultrafiltration stir-cell at 4 °C, under pressure of nitrogen gas applied at 100 psi. In this method, a 3,000-ml supernatant was concentrated to approximately 15 ml in 2 days.

Analysis of CFSE Protein Profiles by SDS-PAGE

Each CFSE was analysed by discontinuous sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) under denaturing conditions. Equal amount of each CFSE was mixed with PBS (phosphate buffered saline) to achieve a uniform protein concentration. The mixed protein samples were then diluted with equal volume of SDS reducing buffer, mixed, boiled at 100 °C for 5 min and used immediately in SDS-PAGE. One-dimensional poly-acrylamide gels of 7.5 cm length, using 0.75 mm spacers, with a 12 % separating gel and 4 % stacking gel were used and run by the method of [20] and [14]. A Bio-Rad Mini PROTEAN 3 Electrophoresis Cells (Bio Rad Laboratories, Regents Park, NSW, Australia) was used to electrophorese the gels with 1× SDS running buffer (pH 8.3). Samples were prepared by mixing equal volumes of concentrated CFSE with reducing buffer. Twenty-four microliters of each sample was loaded into the wells. A broad range SDS-PAGE molecular weight marker standard (Bio-Rad, Sydney, Australia) was included in each run to indicate protein size of samples. Electrophoresis was performed at 125 V until the tracking dye left the bottom of the gel. Once finished, gels were removed from glass plates and were stained using Coomassie blue stain.

Haemolysis Production for Strains Infected with VOB from *V. owensii* 47666-1

Dual layered agar plates were used to demonstrate haemolysin production in the four naïve strains infected with phage from *V. owensii* 47666-1. Strains were plated onto PYSS agar plates and incubated overnight. Individual colonies were transferred onto dual layered agar plates using sterile toothpicks and incubated overnight. Both the colony diameter and the area of haemolysis were measured in mm.

Screening of *V. harveyi* Strains for Siderophore Production

Siderophore production was measured by chrome azurol sulphate (CAS) agar method [22]. The MM9 salts solution of the original recipe was excluded and sterile $MgCl_2$ was added to the CAS solution post-autoclave. Single colonies from overnight cultures of Harveyi clade strains on PYSS agar were touched with the tip of a sterile wooden toothpick. The tip of the toothpick was then lightly pressed onto the surface of a CAS agar plate. The plates were incubated at 28 °C for two days before results were ascertained. The ratio between halo diameter and colony diameter was considered as relative quantification of the amount of siderophore produced.

Chitin Degradation Assay for Strains Infected with VOB from *V. owensii* 47666-1

Chitin overlay agar was used to demonstrate chitin degradation in the four Harveyi clade strains infected with phage from *V. owensii* 47666-1. Individual strains were plated onto PYSS agar plates and incubated overnight. Colonies were transferred from PYSS agar plates onto chitin overlay agar plates using sterile toothpicks and were incubated overnight. Both the colony diameter and the area of clearing were measured in mm.

Challenge of *P. monodon* Larvae

The modified method used by Muir [13], Harris and Owens [7] and Munro et al. [14] was performed in bath challenge experiments with *P. monodon* nauplii. The seawater where the nauplii came from in the prawn hatchery was used instead of autoclaved seawater. Nauplii were challenged

with 10^6–10^7 CFU ml^{-1} of bacteria per flask of 15 larvae in 150 ml seawater. These flasks were replicated five times. An equivalent volume of PYSS broth was used as a broth control. The larvae were then assessed for survival after 48 h from initial challenge.

Statistical Analysis

All data were checked for Normality via a Q–Q plot. Haemolysin and chitin assays were not Normal, therefore a $\log_{10}$ transformation was conducted. Once the data was Normalised, analysis between groups was performed by One-way ANOVA and comparisons of individual means were performed using LSD multiple comparison using the computer program SPSS^{TM} version 11.

Results

Phage Infection to Naïve Strains of Harveyi Clade by Optical Density (OD)

The induction with mitomycin C in *V. owensii* strain 47666-1 (Fig. 1) caused a decrease in OD values as noted from about 2 h after induction and onwards, indicating lysis. The experiments indicated that out of 29 naïve strains [16] challenged with bacteriophage from *V. owensii* 47666-1, only 3 strains (*V. campbellii* 643, *V. campbellii* 30 and *V. harveyi* 12) could be infected using optical density as an indicator (*V. campbellii* 643 in Fig. 2 and *V. harveyi* 20 in Fig. 3 as a negative). Naïve strains *V. campbellii* 643, 30 and *V. harveyi* 12 were used in the virulence assay as strains able to be infected with the phage from *V. owensii* 47666-1. Naïve strain *V. harveyi* 20 (Fig. 3) was used as a representative of negative strains as it did not show a decline in OD value. The cultures receiving mitomycin C but no bacteriophage did not show cell death confirming that they harboured no native prophage.

SDS-PAGE Analysis of Cell-Free Supernatants

Generally, the presence of bacteriophage caused an up-regulation of certain extracellular proteins of bacterial cultures (Figs. 4, 5). These results showed that strains *V. campbellii* 643, 30 and *V. harveyi* 12 infected with bacteriophage VOB from *V. owensii* 47666-1 showed an up regulation of protein with each having acquired a specific protein band of 55 kDa. Polyclonal and monoclonal antibody staining of this 55 kDa protein band showed that this was the most consistent change related to the presence of the bacteriophage (data not shown).

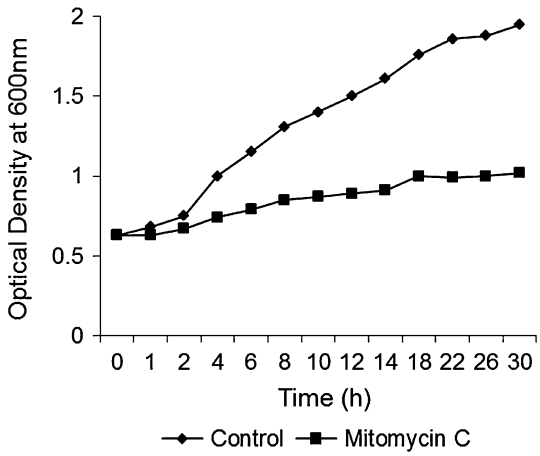

Fig. 1 Optical density changes of *V. owensii* 47666-1 over time after induction with mitomycin C

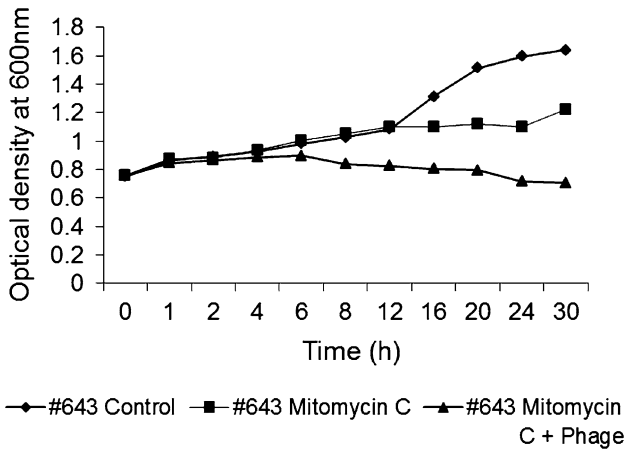

Fig. 2 Optical density changes of *V. campbellii* 643 infected and uninfected with phage from *V. owensii* 47666-1 over time after induction with mitomycin C

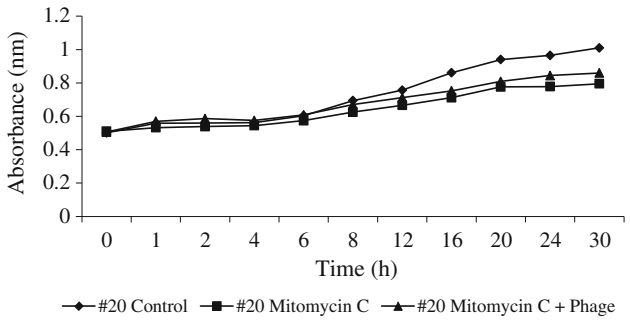

Fig. 3 Optical density changes of *V. harveyi* 20 infected and uninfected with phage from *V. owensii* 47666-1 over time after mitomycin C induction

Based from these SDS-PAGE gels it was evident that the presence of phage from *V. owensii* 47666-1 caused an increase in band intensity and some additional protein

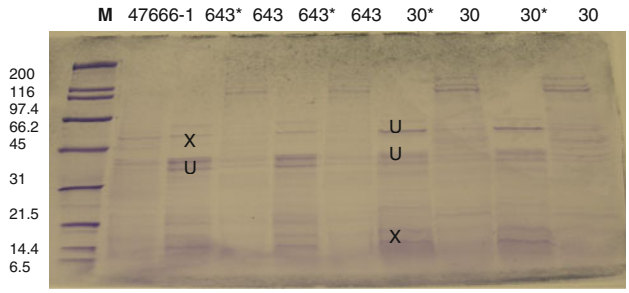

Fig. 4 Protein profile of *V. campbellii* 643 and 30 (SDS-PAGE 12 %, Coomassie blue-stained) showing cell free supernatant extract (CFSE). *Note* (*) bacteriophage infected strain, (*X*) extra protein band, (*U*) up-regulation of protein band, (*M*) broad-range marker

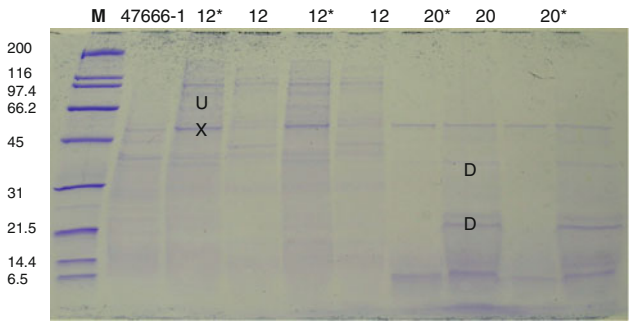

Fig. 5 Protein profile of *V. harveyi* strain 12 and 20 (SDS-PAGE 12 %, Coomassie blue-stained) showing cell free supernatant extract (CFSE). *Note* (*) bacteriophage infected strain, (*X*) extra protein band, (*U*) up-regulation of protein band (*D*) down-regulation of protein band, (M) broad-range marker

bands in *V. campbellii* 643, 30 and *V. harveyi* 12 that was not apparent in the same strain without the bacteriophage. There was also some down-regulation of some bands particularly in strain 30 around 45 kDa and strain 20 at ~32 and ~22 kDa (Figs. 4, 5).

Haemolysin Production

The presence of bacteriophage enhanced the haemolytic activity of bacterial cultures.

As a group, the haemolysin halo and colony diameter of naïve Harveyi clade strains infected with phage from *V. owensii* 47666-1 differed significantly ($F = 28.84$, $DF = 8, 72$, $P < 0.001$ and $F = 20.542$, $DF = 8, 72$, $P < 0.001$ respectively) from strains without the phage (Table 1). In *V. campbellii* 643 and *V. harveyi* 12, a significant increase in terms of halo diameter and colony diameter ($P < 0.001$) was observed (Fig. 6). However, this was not evident in *V. campbellii* 30 which showed no significant difference in both halo and colony diameter from the same individual strain without the phage. The negative control (*V. harveyi* strain 20) did not elicit a

Table 1 The *P* values of the comparisons of parent uninfected strains with the same strains exposed to bacteriophage VOB for various phenotypic changes

Parent Strain	Bacteriophage infected strain				
	Haemolysin		Chitinase		Mortality
	Halo	Colony	Halo	Colony	
12	**0.000**	**0.000**	**0.005**	0.354	**0.000**
20	0.661	0.954	**0.005**	0.135	**0.044**
30	0.311	0.461	**0.004**	0.070	**0.000**
643	**0.003**	**0.002**	**0.029**	0.460	**0.000**

Bolded values are significantly different at the 0.05 level

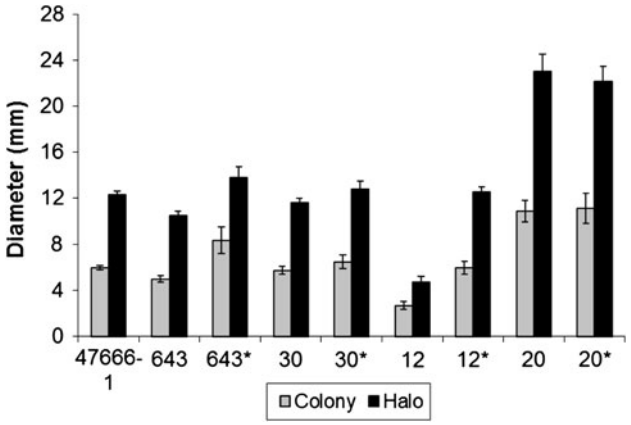

Fig. 6 Mean colony diameter and haemolysin diameter of strains from the Harveyi clade with and without the bacteriophage. *Note* (*) bacteriophage-infected strain

significant difference ($P > 0.05$) in either measurements. Using Pearson correlation, there was an association ($P < 0.05$) between an increase in halo diameter and an increase in mortality rate of prawn nauplii.

Siderophore Production

Most of the Harveyi clade infected and uninfected with phage VOB were negative for siderophore production having a mean of less than 1.3 except *V. harveyi* 20 that gave the strongest reaction in CAS agar plates of 1.4 (Table 2) but both with and without the phage.

Chitin Degradation Assay

The ability of the bacteria to secrete chitinase in chitin-enriched medium was tested with phage infected and naïve Harveyi clade strains (Fig. 7). It can be seen that as a group, the log of the zone of chitin clearance (mm) from Harveyi clade strains with the phage was significantly greater ($F = 5.160$, $DF = 8, 63$, $P < 0.001$) than the strains without the phage (Table 1). The clear zone of the

Table 2 Siderophore production, measured by halo ratio (HR) values, for strains from the Harveyi clade with or without the bacteriophage VOB calculated after 48 h

Strain of bacteria	HR values
V. owensii # 47666-1	1.2
V. campbellii #643	1.1
V. campbellii #643 + phage	1.1
V. campbellii #30	1.1
V. campbellii #30 + phage	1.1
V. harveyi #12	1.1
V. harveyi #12 + phage	1.1
V. harveyi #20	1.4
V. harveyi #20 + phage	1.4

Less than 1.3 HR is considered negative

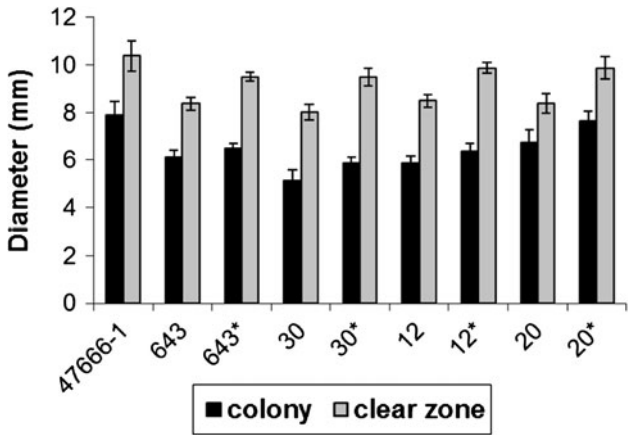

Fig. 7 Mean colony diameter and clear zone diameter on a plate for detecting chitinase from strains of the Harveyi clade with and without the bacteriophage. *Note* (*) bacteriophage-infected strain

naïve *V. campbellii* 643, *V. campbellii* 30, *V. harveyi* 12 and *V. harveyi* 20 infected with the phage significantly increased ($P < 0.05$) compared to the same strains without the phage (Table 1).

The colony sizes of all strains tested did not show significant differences between phage-infected and uninfected strains (Table 1).

Bath Challenge of Larvae of *P. monodon*

As a group, there was a significant increase in mortality rate among strains infected with bacteriophage from *V. owensii* 47666-1 ($F = 82.824$, $DF = 9, 40$, $P < 0.001$) compared to strains of the Harveyi clade without the bacteriophage (Table 1, Fig. 8). *Vibrio owensii* 47666-1 caused significantly higher ($P < 0.001$) mortality in the larval prawns than any other bacterial strains under study (Fig. 8).

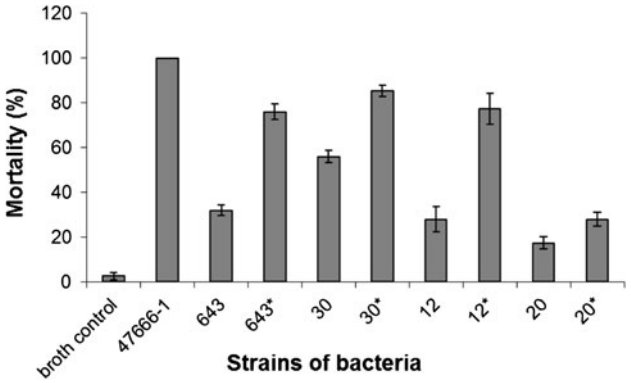

Fig. 8 Mean mortality and standard error from five replicate flasks of nauplii of *Penaeus monodon*, 48 h after being bath challenged with the strains of the Harveyi clade. *Note* (*) bacteria with bacteriophage

Discussion

The decrease in optical density (OD) values after induction with mitomycin C was taken as a presumptive indication of infection by bacteriophage. Three out of 29 naïve bacterial strains could be infected with phage extracted from *V. owensii* 47666-1 and mimic the results of Oakey and Owens [16] when they infected bacteriophage VHML from *V. campbellii* 642 into 36 naïve bacterial strains. Oakey and Owens found that VHML has a narrow range and only infected four of the 36 strains tested. However, some other species of *Vibrio*, notably *V. cholerae* (ACM #2773/ATCC #14035) outside the Harveyi clade [19] and *V. alginolyticus* [16] could be infected by VHML. Of note was that the strains of the Harveyi clade successfully infected were mostly different between the two bacteriophages. VHML infected *V. harveyi* 12, *V. harveyi* 20 *V. campbellii* 45 and *V. campbellii* 645 whilst VOB infected *V. harveyi* 12, *V. campbellii* 30 and *V. campbellii* 643. This suggests that the two phages may have different receptor sites as modes of infection. However, changes in OD readings are only presumptive of an infection, hence further tests need to be undertaken to confirm bacteriophage infection to naïve strains under study.

A method employing SDS–polyacrylamide gel electrophoresis was applied to detect the presence of extracellular proteins and possible up regulation of proteins from VOB. It was confirmed that the presence of bacteriophage in naïve strains caused these strains to produce additional extracellular proteins compared to the same individual strains receiving no phage. These results are similar to the findings of Munro et al. [14] with bacteriophage VHML from the virulent bacterium, *V. campbellii* ACMM 642. The infection of VHML caused up regulation of proteins which was not evident in the same individual strains uninfected with the phage.

Haemolytic activity has been considered an important virulence property [9]. Zhang and Austin [32] showed that a pathogenic isolate, *V. harveyi* VIB 645, produced exotoxin with a high titre of haemolytic activity against Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*) erythrocytes. In our study, infection of some naïve strains from the Harveyi clade with VOB resulted in a significant increase ($P < 0.001$) in both halo of haemolysis and colony diameter as compared to the same uninfected strains. With Pearson's correlation, there was an association ($P < 0.05$) between an increase in halo diameter and an increase in mortality rate of prawn nauplii. Bacteriophage-infected *V. campbellii* 30 did not show any difference in either halo or colony size compared to the uninfected parent strain. However, although it was suggested by Liu et al. [9] that haemolysin might contribute to pathogenicity, not all pathogenic vibrios possess this character. Sudheesh and Xu [24] found pathogenic *V. parahaemolyticus* to be a poor producer of haemolysin but highly virulent to *P. monodon* with an LD_{50} value of 1×10^5 CFU/prawn. Toranzo et al. [27] also demonstrated that haemolysins were able to be produced by both pathogenic and non-pathogenic marine vibrios from striped bass thus, it is not yet possible to determine conclusively what role the haemolysins play in the pathogenesis of vibriosis.

The ability to sequester iron from the host fluids has also been suggested as one of the virulence factors associated with vibriosis [1]. This can be achieved by means of iron-sequestering systems such as siderophore production. Production of siderophores in avirulent strains of *V. harveyi* infected with bacteriophage from a virulent strain, *V. owensii* 47666-1, was investigated using the universal assay of Schwyn and Neilands [22]. The pathogenic strain, *V. owensii* 47666-1, showed no significant siderophore production in this study which coincides with the results obtained by Harris [6]. An avirulent strain, *V. harveyi* 20, showed much larger halo ratio (HR) value and hence higher levels of siderophore production. However, there was no siderophore enhancement observed as it produced the same HR value when challenged with the phage. There are possible reasons why siderophore production in strains of the Harveyi clade under study did not elicit significant differences. One might be because most of the avirulent strains used in this study were slow growers in the agar except *V. harveyi* 20 in which colonies spread quickly throughout the plate in just a few hours of incubation. Schwyn and Neilands [22] stated that the halo size was dependent on growth conditions where slowly growing cultures produced smaller zones than faster growing ones. Another possible reason is the host group from which the bacterium where isolated. Owens et al. [17] demonstrated that invertebrate isolates produced the lowest level of siderophore activity as compared to vertebrate isolates. In this study, all of the isolates were from invertebrate host, hence the mechanisms of iron uptake were less pronounced.

The use of chitin as nutrient source is quite widespread among microbes [5]. It has been reported that *V. harveyi* produces chitinase for the degradation of chitin to biologically useful soluble oligosaccharides [26]. Further, chitinases probably aid the invasion of the pathogen as well as provide a source of nutrients in the form of amino acids [5]. Significant up-regulation of chitinase production in bacteriophage-infected strains from the Harveyi clade was shown in this study. However, the colony diameter between infected and uninfected strains did not differ significantly.

The virulence of *V. harveyi* to *P. monodon* larvae has been studied by several authors [7, 20, 23]. Doses of 10^2 of *V. owensii* 47666-1 have shown to cause 100 % mortality after 48 h of incubation [7]. In this study, we used approximately 10^6 CFU ml^{-1} doses of bacterial culture of *V. owensii* 47666-1 and 100 % of the infected larvae died. All of the naïve strains from the Harveyi clade infected with VOB caused a significantly greater mortality rate than the same ones unifected. These results were similar to those of Munro et al. [14] where naïve strains of *V. harveyi* were infected with bacteriophage VHML from *V. campbellii* 642 and mortality rate was higher than with uninfected strains. It can therefore likewise be suggested that the presence of bacteriophage VOB causes or at least enhances the pathogenicity of *V. owensii* 47666-1 to prawn larvae.

Based on the mitomycin C results, *V. harveyi* strain 20 was chosen as a negative strain unable to be infected with the phage. However, results from SDS-PAGE, chitinase assay and prawn bioassay suggest otherwise. This strain might have been lysogenised by VOB upon infection but failed to lyse with the dose of mitomycin C used. Perhaps this is a mutation in, and/or a failure of, the gene *recA* which is the mechanism under which mitomycin C induction is believed to work.

In the course of experiments, different doses of mitomycin C were used according to the methods of different authors: doses of 30 ng ml^{-1} [16] and 50 ng ml^{-1} [14]. However, these methods failed to show a decrease in OD values after the 30-hours of observation. The authors herein used a 100 ng ml^{-1} dose and this led to a considerable fall in OD in *V. harveyi* 12, *V. campbellii* 30 and 643 but not in *V. harveyi* 20; hence the authors presumed *V. harveyi* 20 to be uninfected with the phage. Different types of bacteriophage may respond variably to inducing agent such that some phages can be induced at a lower dose e.g. 30 ng ml^{-1} [16] and some at a higher dose e.g. 1 μg ml^{-1} [28]. The result of SDS-PAGE analysis showed that infection with phage might have changed the biochemical activity of this strain resulting in the down-regulation of proteins. Experiments with chitin degradation and the bioassay have demonstrated virulence with high chitinase excretion on chitin overlay agar and high mortality to prawn nauplii when challenged with the phage. Further work is required to understand induction failure or confirmation of VOB infection of *V. harveyi* 20.

In conclusion, the presence of bacteriophage from *V. owensii* 47666-1 enhanced the virulence using several assays with four naïve strains from the Harveyi clade as a model. There was significant up-regulation of proteins based on SDS-PAGE results, up-regulation of haemolysins, chitinases and greater mortality to *P. monodon* larvae. It is therefore suggested that the presence of VOB may either partly or fully confer virulence to *V. owensii* 47666-1. Despite VOB being a presumptive podovirus or a filamentous phage, the results herein are surprisingly similar to effects seen when the myovirus VHML was introduced into the Harveyi clade members [14]. Therefore there is an imperative need to sequence VOB to see how similar it is to VHML.

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