Luminous *Vibrio harveyi* Associated with Tea Brown Gill Syndrome in Black Tiger Shrimp

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ABSTRACT: Luminous *Vibrio harveyi* (VH1039) isolated from tea brown gill syndrome (TBGS) black tiger shrimp, *Penaeus monodon*, was examined as a lysogenic bacteriophage host. Temperate phages found were morphologically identified as siphoviruses of various sizes (i.e., heads of 20-100 nm diameter and tails of 20-200 nm in length). The colonies of VH1039 were highly variable upon extended cultivation. This variability might be caused by transposon like activity of these phages. Lysogenic VH1039 caused no disease symptoms in the shrimp. This study also showed that luminescence was influenced by temperature and pH conditions but that it was not critical to shrimp pathogenicity. The relationship of the phage to the TBGS shrimp is being studied.

KEY WORDS: *Vibrio harveyi*, luminescence, tea brown gill syndrome, TBGS, bacteriophage, *Penaeus monodon*

INTRODUCTION

Vibrio harveyi (VH) is one of many luminous bacteria like *V. fisheri*, *Photobacterium leiognathi* and *P. phosphoreum*. It is found abundantly in marine and estuarial habitats (Hastings *et al*. 1981, Jiravanichpaisal *et al*. 1994). Some studies have reported that VH is a devastating pathogen of penaeid larvae, especially those of the black tiger shrimp (Baticados *et al*. 1990, Lavillla-Patogo *et al*. 1992). However, previous studies have also reported that it is a normal constituent of the non-pathogenic flora of marine animals (O'Brien & Sizemore 1979, Ruby & Morin JG 1979). All these luminous bacteria, whether normal flora or pathogens, can exhibit luminescence in marine animals. A recent study found that luminous VH isolate and a bacteriophage could be found in black tiger shrimp exhibiting tea brawn gill syndrome (TBGS). A bioassay study showed that intramuscular injections of this VH strain into juvenile shrimp causes no symptoms, but that the combination of bacteriophage and VH caused shrimp death (Ruangpan *et al*. 1998). *Vibrio* species, including VH, are major hosts of marine bacteriophages (Proctor 1997). Most bacteriophages are temperate phages and they have been reported to cause diversity in bacterial hosts by acting as transposon-like elements. It is well known that some temperate phages in bacterial hosts play major roles in causing diseases in humans (Cheetham & Katz 1995, Reid & Mekalanos 1995). In marine shrimp, however, there is still no confirmed evidence that temperate phages lead to disease.

In the present paper we report highly variable characteristics of *Vibrio harveyi* VH1039 isolated from TBGS black tiger shrimp. This bacterial strain is lysogenic and luminous. We report the optimal physical conditions for its growth and luminescent expression. The temperate phage was isolated and morphologically identified. Moreover, there was evidence to show that a transposon (Tn3) might also reside in this strain.

MATERIALS AND METHODS

Cultivation and identification of VH1039

TBGS in black tiger shrimp was first reported in 1998 (Ruangpan *et al*. 1998). A luminescent bacterial strain was isolated from moribund TBGS shrimp and it was later identified as VH1039 by biochemical tests. The bacterium was cultured on TCBS agar, on tryptic soy agar (TSA) and in tryptic soy broth (TSB). It was incubated at various temperatures (from room temperature to 37 °C), in media of various percentages of NaCl (between 0.5-8%), and various alkalinity (pH 5-12). Under these physical conditions, the strain grew well and colony morphology and luminescence production were investigated. The bacterium was also subjected to confirmatory biochemical tests and antibiotic sensitivity tests were carried out using BBL Sensi-Discs (BBL).

Isolation and examination of temperate phages

To investigate the possibility that VH1039 was infected with temperate phages, it was cultured in TSA with 3% NaCl

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for 18-20 hours at temperatures between 30°C and 33°C. Bacterial colonies were then fixed in a solution of 0.5% glutaraldehyde in 4% paraformaldehyde, stained with 2% phosphotungstic acid and examined by transmission electron microscopy.

To stimulate the production of higher quantities of phage particles, VH1039 was cultured in TSB with 3% NaCl at 30-33°C for 18-20 hours to reach OD 0.6-0.7 at 590 nm. The medium was then divided into portions containing different concentrations of mitomycin-c (from 1-250 ug/ml) and allowed to incubate at 37°C for another 18-20 hours. These suspension were also examined by transmission electron microscopy. To concentrate and purify phage particles, bacterial cells were precipitated by adding NaCl to a final concentration of 1 M followed by immersion in an ice bath for 1 hour before centrifugation at 12,000 g for 20 minutes. After this, bacterial debris was eliminated from the supernatant by repeated centrifugation under the same conditions. Bacteriophage suspended in the supernatant was then precipitated by adding polyethylene glycol 8000 at a concentration of 10% followd by immersion in an ice bath for 1 hour before pelleting by centrifugation at 12,000 g for 20 minutes. The pellet was resuspended in phage buffer (20 mM Tris-HCl, pH 7.5 contianing 10 mM $MgSO_4$). The solution was finally filtered through a 0.22 m filter membrane before examination by the transmission electron microscopy.

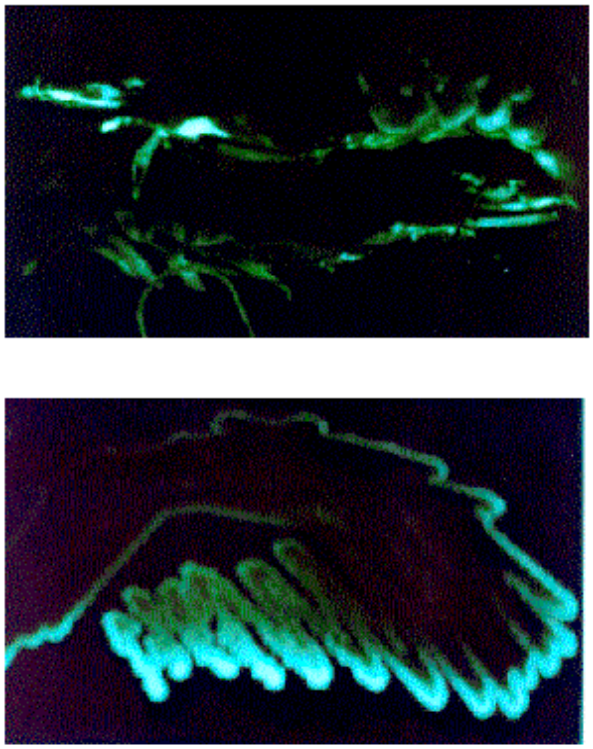

Figure 1. Luminescence of VH infected *P. monodon* (top) and VH on TSA with 3% NaCl (bottom).

RESULTS

Optimal condition for culture of VH1039

VH1039 grew well from room temperature (28-30°C) to 33°C, but the best temperature for growth was 33°C. It tolerated but did not grow at 35°C or above. At higher temperatures like 37°C, it exhibited strong luminescence, but little or none at temperatures from room temperature to 33°C. However, luminescense weakened after incubation for 4-5 days, regardless of the incubation temperature. The luminescense could be reactivated in approximately 6 hours after switching the cultures from a lower to a higher temperature (e.g., 30°C to 37°C). If the temperature was lowered once again, the luminescence faded.

Salinity also influenced the growth of VH1039. It grew well in 2-3% NaCl but also persisted in percentages of NaCl from 1% up to 6.5%. It grew well between pH 7-9, but not at pH lower than 5, and slowly at any pH above 9. Also, at pH 7-9 it exhibited stronger luminescence than at lower pH. The luminescent shrimp and luminescent VH are shown in Fig. 1.

Antimicrobial sensitivity and biochemical tests

VH1039 was tested with antimicrobial agents to examine for the possibile presence of transposons. Among the antimicrobial agents tested were neomycin, kanamycin, streptomycin, trimethoprim, tetracyclin and ampicillin. It was resistant to only ampicillin, a characteristic of Tn3. In biochemical tests, it expressed an unusual character for *Vibrio* species by producing H_2S in 24-48 hours in triple sugar iron (TSI) medium.

Variation in colony morphology

When VH1039 was sub-cultured in 3% NaCl TSA and TCBS, it presented variable colonial morphology. The appearance of the colonies was diverse in size, shape and color (Fig. 2). In spite of these diverse forms, tests identified each colony as biochemically identical. This variation appeared only in solid culture media incubated for periods from 3 to 5 days or longer. The variation was not evident upon daily subculture.

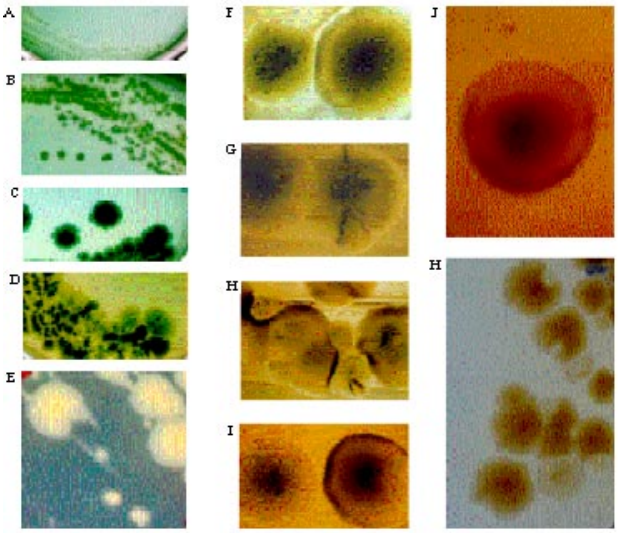

Figure2. Various colony types of VH 1039; (A)clear and (B) clouded colonies; (C-D) colonies with black pigments on TCBS agar, some small and rough (C), others big and smooth (D); (E) margins of big and small colonies on the same plate; (F-J) other shapes and colors of colonies; (K) colony partly digested.

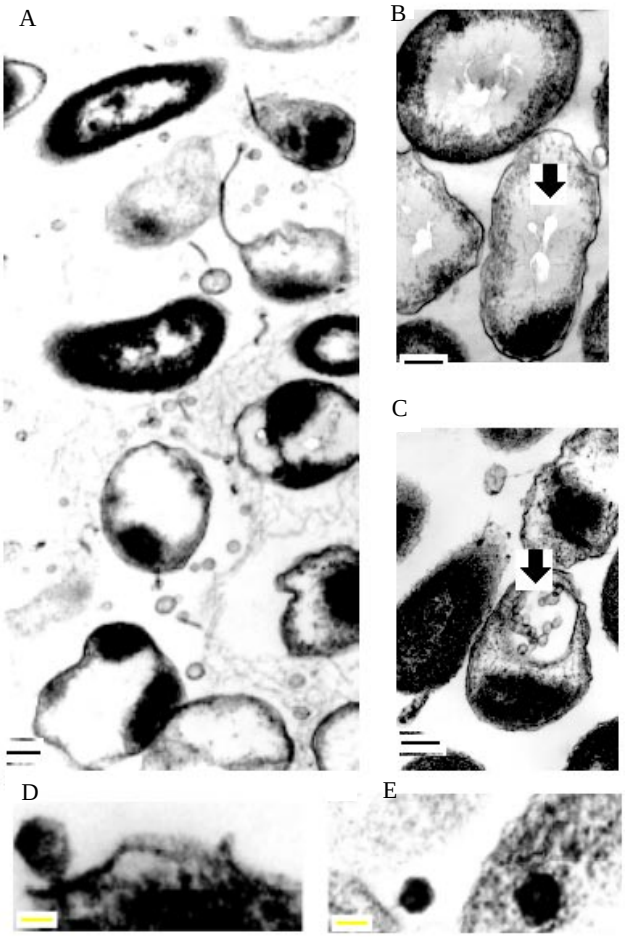

Figure 3. TEM of various sizes of bacteriophages with VH 1039. (A) Phage particleoutside VH 1039; (B) phage particle inside VH 1039 (arrow); (C) unidentified particle inside VH 1039 (arrow); and (D-E) phage adhered to VH 1039 (bars 100 nm).

Detection of temperate phages in VH1039

Studies using the electron microscopic showed that VH1039 could produce a siphovirus or λ-like phage particles with icosahedral heads and a filamentous tails. The sizes of the phages produced varied, with head diameters of 20 – 100 nm and a tail lengths of 20-200 nm. The sizes of the head and the tails were not related (Figure 3 and 4). Induction of phages (using mitomycin-C) and extraction (i.e., using the NaCl/PEG technique) resulted in higher density yields. However, the use of mitomycin-C (optimal at 5 µg/ml) generally resulted in the formation of separate heads and tails (Figure 4).

DISCUSSION

Bacterial luminescense is reportedly autoinduced, with each genus or species of luminous bacteria producing a different autoinducer. However, the major autoinducer of VH has been reported to be a long chain aliphatic aldehyde. Autoinducers are accumulated during bacterial growth and their synthesis triggers lux gene expression. The electron transport proceeds by reaction of luciferase enzyme which catalizes the reaction amongst reduced flavin mononucleotide ($FMNH_2$), oxygen and a long chain aliphatic aldehyde to produce flavin mononucleotide (FMN) and an aliphatic carboxylic acid which emits the light (Fisher *et al*. 1995). Our results showed that temperature may also influence expression of luminescence. The temperature may either stimulate luciferase activity and/or the production and/or function of the autoinducer. Logically, since VH1039 does not grow at higher temperatures like 37°C, it would seem unlikely that autoinducer production would be involved and it is more likely that temperature affects enzyme or autoinducer activity. We also found that alkalinity affected luminescence expression. Higher than optimal-pH media gave strong lumi-

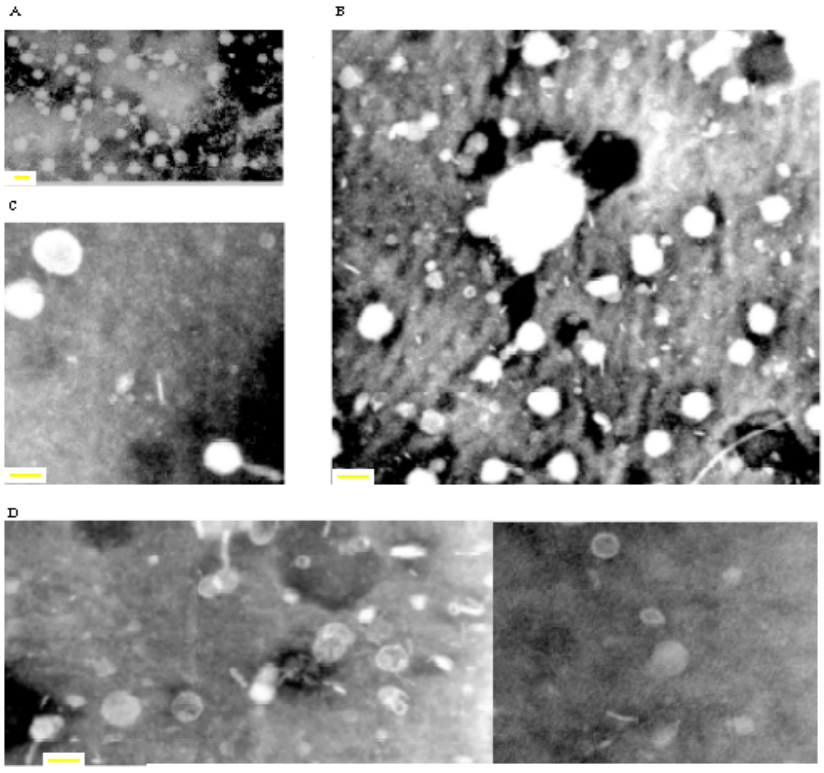

Figure 4. Temperate phage induced with mitomycin-C and isolated by NaCl/PEG. (A-B) mostly phage heads; (C) complete phage particle; (D) phage tails are seperated from the heads (bars 100 nm).

nescence. This combination (temperature and alkalinity) might find some application in manipulation of VH strains for higher production of poly-3-hydroxybutyrate (PHB), a raw material of interest in the plastic industry due to its properties of thermoplasticity, water resistance and biodegradability. It was previously reported that the production of PHB is related to luminous expression and that it is controlled by the lux autoinducer (Sun et al. 1994).

The variable morphology of VH1039 colonies is of some interest. This variability might involve the fact that it is a lysogenic host of a temperate phage(s). Indeed, the variability of other bacteria has also been reported to be due to transposon-like behavior of bacteriophages (Reidl & Makalanos 1995, Belas *et al*. 1984). This may be the best explanation for variable morphology of bacterial colonies since the biochemical tests did not change for each variety.

To study the possibility of transposon element integration, we checked for antibiotic drug sensitivity and found that VH1039 was resistant to ampicillin. This might mean that it contains a Tn3 element. We have recently isolated another luminescent *Vibrio* strain (VHN1) which acts similar to VH1039 in that it causes shrimp mortality in the laboratory only when combined with a bacteriophage partner. This new strain can resist ampicillin, neomycin, kanamycin, and streptomycin. Thus, in addition to Tn3, this *Vibrio* strain may also contain Tn5 (known to carry resistance to neomycin, kanamycin and streptomycin) and Tn 10 (known to carry resistance to tetracycline). This strain must be carefully handled due to its antibiotic resistance and its potential for pathogenicity. To confirm the presence of Tn3 in VH1039, we are currently using PCR and sounthern blot hybridization tests.

According to this work and the previous work of Ruangpan et al. (1998), VH1039 cannot induce TBGS on its own, even though it contains a temperate phage(s). The phage partner that cooperates with VH1039 in causing TBGS has still not been isolated and characterized. Although TBGS shrimp exhibit tea brown pigment at their gills, the only obvious histopathological changes evident are devastation of the hepatopancreas which contains both bacteria and phage. It is possible that the tea brawn pigment in the gills is a coincident symptom, and not the main cause of shrimp death. For an explanation of the tea brown color in TBGS shrimp, the unusual characteristic of H_2S production by VH1039 may be considered. It is possible that H_2S produced by the bacterium in the shrimp could react with Fe^{++} in the shrimp pond water to produce FeS which would precipitate in shrimp gills to produce a tea brown color.

The phage partner of VHN1 is a lytic phage that was isolated from shrimp pond water in screening tests for lytic phages of *Vibrio*. The combination of VHN1 with this phage can cause shrimp death in several hours after intra muscular injection while neither the phage nor the bacterium alone cause mortality. Nor is there any mortality when this phage is combined with VH1039. The situation appears to be complex, with the probable interaction of several bacterial strains and specific bacteriophage partners. They can apparently combine under appropriate circumstances to cause shrimp mortality.

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