Differential efficiency of induction of vibriophage (φALP (VC)-3) encoding virulent genes in naturally occurring non O1 *Vibrio Cholerae*

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Introduction

Vibrios contain nearly all known bacterial genetic elements, including integrons, episomes, plasmids, transposons, and bacteriophages, making them an attractive model for the study of genome plasticity (Fabiano *et al*., 2006). Vibriophages are those viruses infecting the family *Vibrionaceae*. Viruses are believed to contribute significantly to the marine microbial loop and nutrient cycling in the oceans, and may also serve as agents of gene transfer in the marine environment. (Fuhrman, 1999). The production of temperate phages is dependent on the number of lysogenic bacteria and the presence of an inducing agent, whereas virulent phage production depends on the encounter rate between phages and host cells (Markus *et al*., 1996). Release of mature phage requires removal of the repressor, a process called induction. The SOS response due to bacterial DNA damage can lead to significantly higher levels of prophage induction. One of the most effective and widely used inducing agents is mitomycin C. It was demonstrated that marine bacterial isolates were inducible by mitomycin .Jiang and Paul (1994) found that more than 40% of marine bacterial isolates screened contained inducible phages. Some of these phages are known to be capable of changing various host characteristics (Siddiqui and Bhattacharyya 1982). Pedulla *et al*. (2003) venture that "bacteriophages perhaps represent the biggest unexplored reservoir of sequence information in the biosphere".

Bacteriophage encoding virulence factors can convert their bacterial host, in a process known as phage lysogenic conversion, from a non pathogenic strain to a virulent strain or a strain with increased virulence. (Carlos *et al*., 2003) The cholera toxin gene, a key virulent gene of *vibrio cholerae*, was observed to be laterally acquired through a filamentous phage (ctx Φ) genome (Waldor and Mekalanos, 1996).

Characterization of these lysogens may provide insight into the emergence of newer pathogenic clones of *V. cholerae*. Present study is on the vibriophages isolated from the backwaters of southwest India. In this work we aimed to compare efficiency of prophage induction and further lytic development, after treatment of bacterial cultures with various agents potentially affecting prophage maintenance. Vibriophage φALP(VC)-3 and its host was analyzed by detecting the virulent gene cassettes by PCR. Molecular characteristics should be useful baseline information to trace the emergence of pathogenic variants by horizontal gene transfer.

Materials and Methods:

Bacterial strain and its identification

Vibrio strains were isolated on TCBS from the water samples collected from the backwaters of Alleppy and Cochin. The identity of the strains was confirmed by the 16S rRNA gene analysis to species level. They were used as host for phage induction and isolation.

Prophage induction agents

Six induction agents were tested for analyzing their differential efficiency as induction agents- 1 mg/ml mitomycin C (Sigma Chemical Co., St. Louis, Mo) (an inhibitor of DNA synthesis and a well-known prophage inductor), 0.2 mg/ml nalidixic acid (HiMedia, Mumbhai, India) (an antibiotic from the group of quinolones), 200 mM NaCl (a salt concentration which was demonstrate to induce prophage) and 3 mM H_2O_2 (agents that induce oxidative stress, like hydrogen peroxide), Ultraviolet radiation (an inhibitor of DNA synthesis and a well-known prophage inductor), and temperature (60^0 C).The filtrates were tested for the presence of bacteriophages by diluting and plating onto lawns of vibrios by the soft agar overlay method with modification (Adams, 1959). Phages from representative plaques were used for the production of high-titer stocks

Physiochemical characterisation

Bacteriophage coat proteins were analyzed by SDS- PAGE under denaturing conditions (Laemmli as adapted by Sambrook *et al*, 1989.). Electron microscopy was done at IIHR, Bangalore using Transmission Electron Microscope (TEM) (Model Joel 1011)

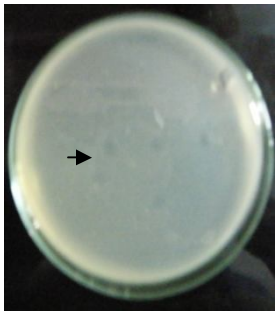
Molecular characterisation

Phage DNA was extracted by phenol:chloroform method (Sambrook *et al*; 1989). *V.Cholerae* genes located in the *V. cholerae* ancestral chromosome, the VPI-I, and the chromosome II, i.e. ctxA, ctxB,zot, ace, toxR, tcpA and hlyA were determined by PCR. Genes of the VPI-II, nanH (encoding neuraminidase) and ninT (encoding bacteriophage-like integrase), were determined by multiplex PCR (Mitra *et al.,* 2001). Host DNA was isolated by boiling method. The presence of these genes were checked in the phage genome of φALP (VC)-3

Results:

Isolation and identification of vibriophage

The isolate which was Gram negative rods/cocci, positive for cytochrome oxidase test and with ability to ferment glucose with or without gas in the MOF test was segregated as Vibrios and 15 of them were identified as *Vibrio cholerae* by 16s rRNA gene partial sequence analysis. Induction of the *Vibrio cholerae* strains with Mitomycin C (0.2µg/ml) produced translucent plaques with bull's eye morphology (fig 1A). TEM image (fig-1B) indicated the morphology of head and tailed phage and was classified in the group Caudovirales and family siphoviridae



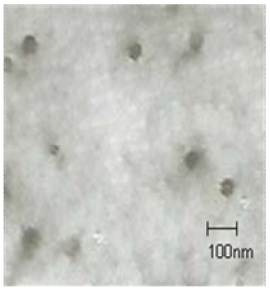


Fig. 1(A)**.**Plaques of φALP (VC)-3 and (B) Transmission electron micrograph of φALP (VC)-3

Protein profiling

Protein profiling by denaturing SDS-PAGE and silver staining indicated that the phage has more than one kind of capsid protein (Fig 2).

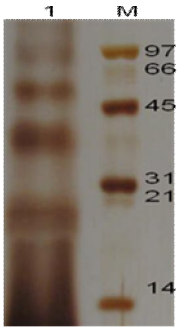


Fig 2.SDS-PAGE (Lane 1-marker, Lane 2- φALP (VC)-03)

Differential prophages induction by various inducers

Efficiencies of induction of prophages and their further development vary considerably in response to different induction agents. Mitomycin was found to produce maximum induction in the lysogen under study.Nalidixic acid which was not reported earlier was also found to be a potent inducing agent. Temperature, 200mM NaCl and 3 mM H_2O_2 were not found to be as effective as the other three. Moreover, efficiency of progeny phage production might be modulated by other factors like temperature or bacterial growth rate. (Fig-3)

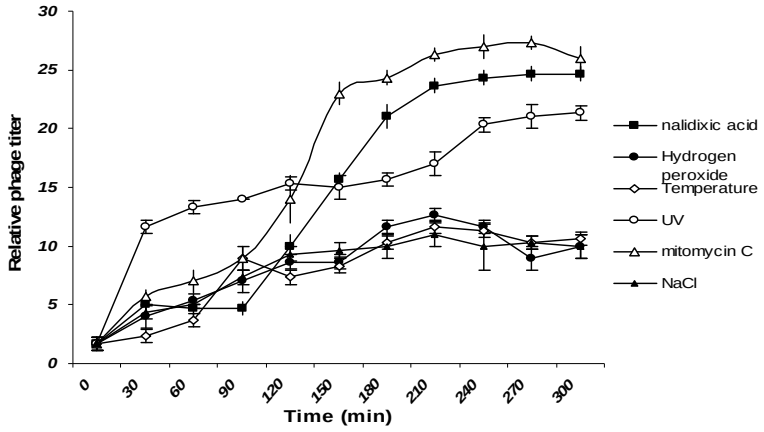


Fig.3 Efficiency of prophage induction and further development of φALP (VC)-03 with Mitomycin C (open triangles), nalidixic acid (closed squares), UV (open circles), Hydrogen peroxide (closed rhombus), NaCl (closed triangle), temperature (open rhombus)

Molecular Characterisation:

The *Vibrio cholerae* strain ALP (VC)-3 revealed amplicons of the genes zot, ace, toxR, nan H, ninT and hlyA by PCR (Fig: 5).Vibriophage φALP (VC)-3 also gave amplicons for the genes hyl A and Zot.(Fig 6). Further sequencing and analysis has revealed significant sequence similarity between the host and the phage genes which can be related to the chances of horizontal gene transfer

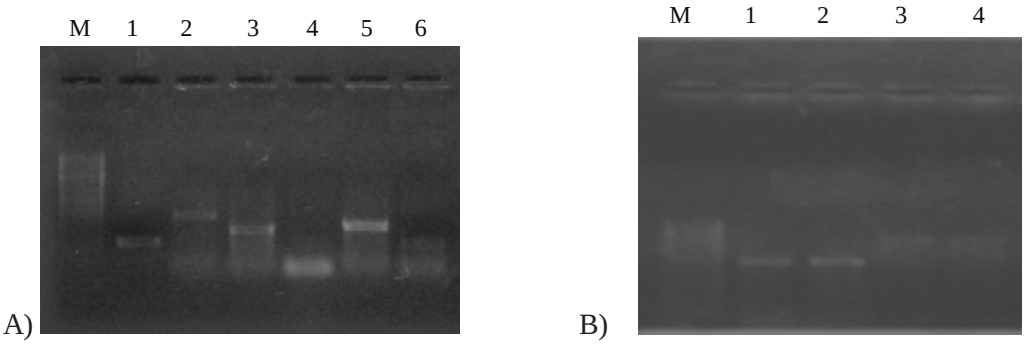


Fig 4. PCR amplicons of host ALP (VC)-3 genes (**A**) Lane M, DNA marker(100bp DNA ladder):Lanes 1-6,amplicons of *tox R, nin T, zot .ace, nan H, hyl A* respectively. (**B**)PCR amplicons of vibriophage φALP (VC)-3 and host ALP (VC)-3 M, DNA marker (100bp DNA ladder): Lanes 1-4; amplicons of *hyl A* (phage),*hyl A* (host),*zot* (phage),*zot* (host)

References:

Adams, M. H. (1959). *Interscience Publishers*, London, United Kingdom.

Carlos Canchaya, Ghislain Fournous, Sandra Chibani-Chennoufi,Marie-Lise Dillmann and Harald Bru¨ ssow(2003) .Phage as agents of lateral gene transfer. *Current Opinion in Microbiology*, 6:417–424

Fabiano, L. T., K. E. Klose., and AVIB group (2006) *Vibrio* 2005: The first international conference on biology of *vibrios. J. Bact.* 188, 4592-4596.

Fuhrman, J. A. (1999). Marine viruses and their biogeochemical and ecological effects. *Nature* 399:541–548.

Jiang, S. C., and J. H. Paul. (1994). Seasonal and diel abundance of viruses and occurrence of lysogeny/bacteriocinogeny in the marine environment. *Mar. Ecol. Prog. Ser.* 104:163–172

Laemmli, U. K. (1970). *Natur*e 227:680–685.

Markus G Weinbauer and curtis A. Suttle.,(1996),.Potential significance of lysogeny to bacteriophage production and Bacterial mortality in coastal waters of the Gulf of Mexico. *Appl. Environ. Microbiol*. p. 4374–4380 vol. 62, No. 12

Mitra RK, Nandy RK, Ramamurthy T, et al. (2001) Molecular characterizationof rough variants of Vibrio cholerae isolated from hospitalizedpatients with diarrhea. *J. Med Microbiol*; 50:268-76

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Pedulla, M.L., Ford, M.E., Houtz, J.M., Karthikeyan, T., Wadsworth, C., Lewis, J.A., Jacobs-Sera, D., Falbo, J., Gross, J., Pannunzio, N.R., *et al.* (2003). Origins of highly mosaic mycobacteriophage genomes. *Cell 113*, 171–182.

Sambrook,J., Fritish, E., and Maniatis, I (2000). "*Molecular Cloning, a Laboratory manual*."(C.S.H. Laboratory, Ed.), 3rd edition.

Siddiqui, K.A.I. and Bhattacharyya, F.K. (1982) Role of temperate phage in determining Iytic phage sensitivity and serotype of *Vibrio cholerae. Infect. Immun.* 37, 847-851.

Sunny C. Jiang, John H. Paul. (1996) Occurrence of lysogenic bacteria in marine microbial communities as determined by prophage induction *Mar Ecol Prog Ser* 142: 27-38,

Takeya, K., Zinnaka, Y., Shimodori, S., Nakayama, Y.,Arnako, K. and Iida, K. (1965) Lysogeny in El tor Vibrio. *Proc. Cholera Res. Symp.* lst, pp. 24-28.

Waldor, M. K., and J.J. Mekalanos (1996). Lysogenic conversion by a filamentous phage encoding cholera toxin.

Science 272, 1910-1914