Volume : 3 | Issue : 6 | June 2014 • ISSN No 2277 - 8179

Research Paper

Induction of Temperate Vibriophage Φ Knm4 From the Environmental Non O1 Vibrio Cholerae by Various Biotic and Abiotic Agents.

Biology

KEYWORDS : Vibrio cholerae, Vibriophage, Phage induction, Mitomycin

Linda L	Microbial Genetics Laboratory Department of Biotechnology Cochin University of Science and Technology Thrikkakara Post Cochin-682022
Jeena A	Microbial Genetics Laboratory Department of Biotechnology Cochin University of Science and Technology Thrikkakara Post Cochin-682022
Linda L	Microbial Genetics Laboratory Department of Biotechnology Cochin University of Science and Technology Thrikkakara Post Cochin-682022
Sarita G. B*	Microbial Genetics Laboratory Department of Biotechnology Cochin University of Science and Technology Thrikkakara Post Cochin-682022

ABSTRACT

Bacteriophages are viruses infecting bacterial hosts. Phages are metabolically inert in their extra-cellular form, reproducing only after infecting suitable host. While providing a valuable resource to the development of modern biotechnology, their ability to mobilize and transfer toxin genes in the environment is viewed with concern. Environmental samples were collected from mangrove ecosystems of Kannamally track off Cochin. Vibrios isolated from the water samples on TCBS plates and characterized using biochemical and molecular tools, were used as host for phage isolation by the enrichment technique. Prophage was induced by means of different biotic and abiotic agents viz. mitomycin C, nalidixic acid, NaCl, H2O2, ultraviolet radiation and temperature (60°C). The results presented in this report clearly indicate that different prophages may be induced with different efficiency and their development may be modulated by environmental factors.

Introduction

Vibriophages are viruses infecting the family Vibrionaceae. Phages 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 (Weinbauer and Suttle, 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 (Ogg et al., 1978). One of the most effective and widely used inducing agents is mitomycin C. More than 40% of marine bacterial isolates contained inducible phages. Some of these phages are capable of changing various host characteristics (Canchaya et al., 2003). Present study compares efficiency of prophage induction and further lytic development of vibriophage Φ KNM4 from an environmental non O1 V. cholerae by various induction agents.

Materials and Methods:

Isolation and Identification of vibrios

Vibrio cholerae strain KNM4 was isolated from water samples collected from coastal mangrove ecosystems of Kannamally (09°52′43.3″N 76°15′50.6″E), off Cochin in Kerala, India. The serially diluted samples were plated onto Thiosulphate Citrate Bile salt Sucrose (TCBS) agar (HiMedia, Mumbai, India) plates by the spread plate method and incubated at 37°C for 24 hours. Isolated single colonies were picked, purified on nutrient agar (NA) plates and stored as 20% glycerol stock.

The isolate was characterized upto genus level as outlined by Buchanan and Gibbons (1974). Genomic DNA was isolated as described by Esteban et al. (1993). 16S rRNA gene (1.5kb) was amplified by PCR using the primer pair: 16SF5'AGTTTGATCCTGGCTCA3' and 16SR 5' ACGGCTACCTTGTTACGACTT 3' (Shivaji et al., 2000). The products were sequenced by Sanger's dideoxy method using ABI 3730 Excel (SciGenom Labs Pvt Ltd, Cochin, Kerala). Homology was analysed using BLAST software (http://www.ncbi.nlm.nih.gov/blast) and the identity of the sequences was established (Altschul et al., 1990).

Isolation of temperate phages

V. cholerae strain KMN4 was grown in Luria broth (LB) (HiMedia, Mumbai) at 37°C to get O.D600 =0.2. Mitomycin C (Sigma Chemical Co, USA) was added at 1µg/mL and incubated overnight at 37°C. The culture supernatant was sterilized by filtration through 0.22 µm membrane (Millipore, USA). The filtrate was used as putative phage lysate (Faruque et al., 1998).

The filtrates were tested for phages by soft agar overlay method (Adams, 1959). Tetrazolium staining was done to visualize the phages more clearly (Pattee, 1966).

Purification and partial characterization of phage

The phage was purified and concentrated using polyethylene glycol (PEG) 8000 as described by Boulanger (2009). Optimal MOI was determined according to Lu et al. (2003).

The phage DNA was extracted as described by Sambrook et al. (2000). The DNA was visualized by 1% agarose gel electrophoresis, stained by ethidium bromide (Sigma Chemical Co., USA). Bacteriophage coat proteins were analyzed by SDS- PAGE under denaturing conditions (Laemmli, 1970)

Prophage induction studies

Vibrio cholerae strain KNM4 at mid log phase was treated with following agents: 1 mg/mL mitomycin C (Sigma Chemical Co, USA), 0.2 mg/mL nalidixic acid (HiMedia, India), 200 mM NaCl and 3 mM H_2O_2, Ultraviolet radiation and temperature (60°C).

Analogous experiments but without addition of induction agents were performed. Relative phage titer is the ratio of phage titres in induced and non induced cultures.

Results:

Isolation and Identification of Vibrios

Vibrio- like isolates which were Gram negative, oxidase positive, fermentative, with or without gas production on MOF media, and which showed yellow/green coloured colonies on TCBS (Thiosulfate Citrate Bile salt Sucrose) agar were segregated as Vibrio sp.

Strain KNM4 was identified as non O1Vibrio cholerae by 16S rRNA gene partial sequence analysis. The sequence was submitted in Genbank and the accession number obtained as KJ734982.

Isolation of temperate phages

Phage induction of the environmental Vibrio cholerae strain KNM4 with Mitomycin C produced translucent plaques with bull's eye morphology. The vibriophage hence forth is referred as ΦKNM4.

Volume : 3 | Issue : 6 | June 2014 • ISSN No 2277 - 8179

Purification and partial characterization of phage

The lysogenic phage ΦKNM4was purified by repeated plating. The optimal MOI of ΦKNM4 was four phages per bacterium. Protein profiling by denaturing SDS-PAGE and silver staining indicated that the phage ΦKNM4 had more than one kind of capsid protein (Fig 1). The results indicated that coat protein possessed 4 major proteins and many minor proteins of very low molecular weight. The four major bands were ~97 KDa, ~55KDa, ~40KDa and ~21 KDa. Phage DNA was isolated successfully and was found to be more than 21.2 kb (Fig 2).

Fig 1 and Fig 2 about here

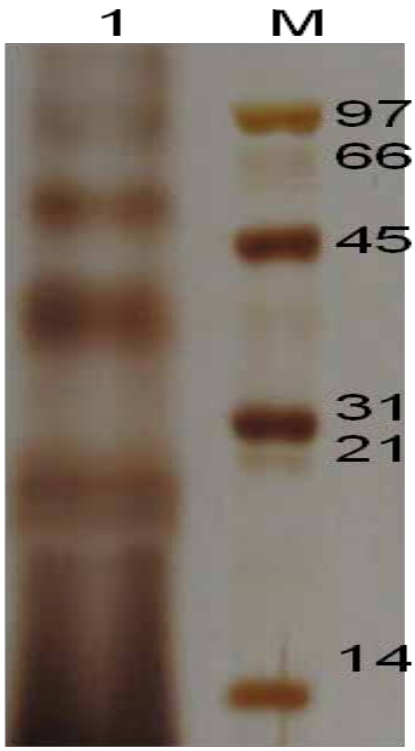


Fig 1.SDS-PAGE :Lane M-marker, Lane 1 -ΦKNM4

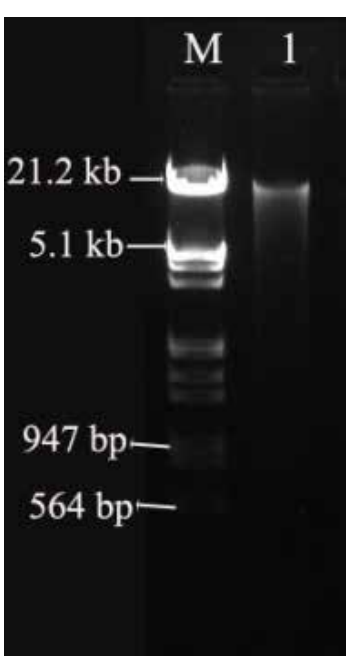


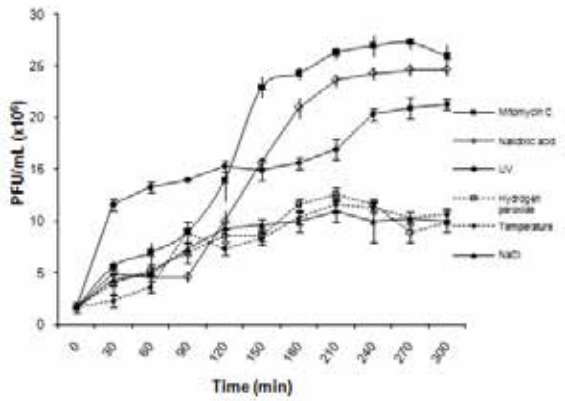
Fig 2: Agarose gel (1%) electrophoresis of phage DNA. Lane M-Lamba DNA/EcoR 1/Hind III Double Digest, Lane 1 - Phage DNA

Prophage induction studies

Efficiencies of induction of the prophages and their further development varied considerably in response to different inducing agents (Fig 3). Mitomycin was observed to be most efficient in induction of the lysogen under study. Nalidixic acid which was not reported earlier as an inducing agent, also proved to be a potent inducer. UV was also shown to induce phages significantly. Temperature (60°C), 200 mM NaCl and 3 mM H_2O_2 were not found to be effective in inducing the prophages. The induction of ΦKNM4 was highest in presence of antibiotics and lowest in 200 mM NaCl.

Fig 3 about here

Fig.3: Differential efficiency of induction of ΦKNM4 by various induction agents



Discussion:

Bacteriophages are natural viral pathogens of bacteria and coexist with their hosts in the same ecological niches (Fuhrman, 1999). V. cholerae is known to be primarily a human pathogen which can persist in the aquatic environment in unexplained ecological associations (Canchaya et al., 2003). The induction, isolation and characterization of the temperate phages in environmental Vibrio cholerae was part of the study on vibriophages to understand the causes of seasonal outbreaks of cholera along the west coast of Kerala, India. The bull's eye morphology of the plaques, not as clear as that of the typical lytic plaques is a unique character of the lysogenic plaques (Faruque et al., 1998).

Concentration and purification of viral particles are prerequisites for structural and functional characterization of phages. Concentration of phages was by PEG - NaCl precipitation as the efficiency of this method is independent of phage concentration (Boulanger 2009). The method allows a 100-fold phage concentration, even after low speed centrifugation with negligible loss of infectivity. Multiplicity of infection is the ratio of the phage particles to the infected bacteria. MOI giving maximum yield per infection is considered as the optimal MOI (Adams and Wassermann, 1956).

The induction studies with various inducing agents were a test of the biotic and abiotic factors which can cause changes in the bacterial genome. Any agent that can provoke the bacterial SOS response is a prophage induction agent (Kimmitt et al., 2000). The potential of nalidixic acid, a quinolone antibiotic as an induction agent is a novel report from our study. Since the antibiotics can challenge microbial populations they are considered as environmental pollutants. The use of mitomycin as induction agent is well studied in various prophages (Mandal and Chatterjee, 1987). Prophage induction is possible in natural ecosystem due to abiotic factors. Release of mature phage requires removal of the repressor (Takeya et al., 1965). Phage induction in the absence of external factors is called spontaneous induction which implies expression of the prophage genes. This can be a major driving force in bacterial population dynamics (Bossi et al., 2003).

Conclusions

Lysogenic phages encoding virulence factors can convert their bacterial host from a non pathogenic strain to a virulent strain or a strain with increased virulence. The presence of inducible lysogenic phages in environmental isolates of V. cholera is viewed with concern for their ability to mobilize and transfer toxin genes in the environment.

Acknowledgments

This study was supported by Department of Biotechnology, Cochin University of Science and Technology, Cochin, Kerala, India. The first author gratefully acknowledges the Council of Scientific and Industrial Research, Govt. of India for research fellowship.

REFERENCE

1) Adams MH (1959): Bacteriophages. N. Y. Wiley- Interscience. | 2) Adams, M. H., & Wassermann, F. E. (1956). Frequency distribution of phage release in the one-step growth experiment. Virology, 2(1), 96-108. | 3) Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990). Basic local alignment search tool. Journal of molecular biology, 215(3), 403-410. | 4) Bossi, L., Fuentes, J. A., Mora, G., & Figueroa-Bossi, N. (2003). Prophage contribution to bacterial population dynamics. Journal of bacteriology, 185(21), 6467-6471. | 5) Boulanger, P. (2009). Purification of bacteriophages and SDS-PAGE analysis of phage structural proteins from ghost particles. In Bacteriophages (pp. 227-238). Humana Press. | 6) Buchanan, R. E., & Gibbons, N. E. (1974). Bergey's Manual of Determinative Bacteriology 8ed. | 7) Canchaya, C., Fournous, G., Chibani-Chennoufi, S., Dillmann, M. L., & Brüssow, H. (2003). Phage as agents of lateral gene transfer. Current opinion in microbiology, 6(4), 417-424. | 8) Esteban, E., Snipes, K., Hird, D., Kasten, R., & Kinde, H. (1993). Use of ribotyping for characterization of Salmonella serotypes. Journal of clinical microbiology, 31(2), 233-237. | 9) Faruque, S. M., Alim, A. A., Albert, M. J., Islam, K. N., & Mekalanos, J. J. (1998). Induction of the lysogenic phage encoding cholera toxin in naturally occurring strains of toxigenic Vibrio cholerae O1 and O139. Infection and immunity, 66(8), 3752-3757. | 10) Fuhrman, J. A. (1999). Marine viruses and their biogeochemical and ecological effects. Nature, 399(6736), 541-548. | 11) Kimmitt, P. T., Harwood, C. R., & Barer, M. R. (2000). Toxin gene expression by shiga toxin-producing Escherichia coli: the role of antibiotics and the bacterial SOS response. Emerging infectious diseases, 6(5), 458. | 12) Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227(5259), 680-685. | 13) Lu, Z., Breidt Jr, F., Fleming, H. P., Altermann, E., & Klaenhammer, T. R. (2003). Isolation and characterization of a Lactobacillus plantarum bacteriophage, ΦJL-1, from a cucumber fermentation. International journal of food microbiology, 84(2), 225-235. | 14) Mandal, D., & Chatterjee, S. N. (1987). Mitomycin-induced prophage induction in Vibrio cholerae cells. Indian journal of biochemistry & biophysics, 24(6), 305-307. | 15) Ogg, J. E., Shrestha, M. B., & Poudayl, L. (1978). Phage-induced changes in Vibrio cholerae: serotype and biotype conversions. Infection and immunity, 19(1), 231-238. | 16) Pattee, P. A. (1966). Use of tetrazolium for improved resolution of bacteriophage plaques. Journal of bacteriology, 92(3), 787 | 17) Sambrook, J., Fritsch, E. F., & Maniatis, T. (2000). Molecular cloning (Vol. 2, pp. 14-9). New York: Cold spring harbor laboratory press. | 18) Shivaji, S., Vijaya Bhanu, N., & Aggarwal, R. K. (2000). Identification of Yersinia pestis as the causative organism of plague in India as determined by 16S rDNA sequencing and RAPD-based genomic fingerprinting. FEMS microbiology letters, 189(2), 247-252. | 19) Weinbauer, M. G., & Suttle, C. A. (1996). Potential significance of lysogeny to bacteriophage production and bacterial mortality in coastal waters of the Gulf of Mexico. Applied and environmental microbiology, 62(12), 4374-4380. |