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Isolation and Partial Characterization of Bacteriophage from Oyster

Young Hee KIM
Dept. of Microbiology, Dongeui University, Pusan 614-714, Korea
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The bacteriophage from the fresh oyster, *Crassostrea Virginica* which is specific to the marine bacterium was isolated and characterized. Among the four different vibrio species and the five different serotypes of *Vibrio parahaemolyticus* host strains tested, only two strains of *Vibrio parahaemolyticus* possessing K17, K52 antigens were highly sensitive to the phage. The size of the isolated plaque was 0.4 mm and the electron microscopic head size of the isolated phage was about 67 nm long and 83 nm wide. PFU/ml was 1.25×10^{11}. The phage was sensitive to chloroform but resistant to acetone or methanol. The assay of the isolated phage nucleic acid was deoxyribonucleic acid. The restriction enzyme pattern showed 14 fragments from *Hind* III and 4 fragments from *Eco R* I. Two different antigenic groups showed similar restriction enzyme patterns.
Key words : Bacteriophage, *Crassostrea Virginica, Vibrio parahaemolyticus,* plaque, PFU: plaque-forming units, deoxyribonucleic acid, *Eco R* I, *Hind* III.

1. INTRODUCTION

The rapid change of the marine environment creates lots of stress to the bioorganisms and their adaptability to nature or existence called much attention. Specifically marine microbes and their genetic material exchange in the aquatic condition has been studied and today the presence of viruses is an indicator of estuarine environmental pollution as well as bacterial pollution control.

The occurance and distribution of bacteriophages (Bancroft, 1970; Koga *et al.*, 1981; Nordeen, 1983; Ju *et al.*, 1987; Hahn *et al.*, 1991) in marine environments have been investigated by previous workers (Chen *et al.*, 1966; Baross *et al.*, 1978; Liston *et al.*, 1978; Groubert *et al.*, 1994; Buchrieser *et al.*, 1995). However, relatively little is known about the relationship between ecological changes and the genetic nature of these bacteriophages (Valaentine *et al.*, 1966; Yamamoto *et al.*, 1970; Maniatis, 1982; Hatsumi, 1984; Muntada, 1995; Young *et al.*, 1995). And also there have not been many extensive studies of bacteriophages from seafood and sediment although bacteriophages active aganist marine bacteria have been isolated but only for phage-typing purposes.

Detection of bacteriophages from shell fish like the oyster, *Crassostrea Virginica*, in its natural habitat (Motes, 1994; Sun *et al.*, 1995) which has a world wide distribution (Nakanishi, 1966; Sklarow, 1973) and increasing consumption were shown to invariably harbor high titers of phages whose hosts were halophilic bacteria like Vibrio species (Kaysner, 1990).

The quantitative incidence of phages from shell fish originating from marine sources was shown to increase with increasing temperatures of sea water, uncooked or undercooked oysters consumption habit and also with the applications of phage hosts.

Because of ensuing oyster consumption there is a strong demand for detailed and continuous investigations of bacteriophages from estuarine environments, their morphological changes, distribution and transfer of genetic material in determining phenotypic characteristics which may affect patho-

genicity for bacterial pollution control and other related studies. Thus this paper describes the isolation of bacteriophages specific to vibrio species found in oysters from 1994 to 1995 and deals with morphological traits, some physical properties and genomic nature of phages in part to analyze the genetic properties of recent isolates.

2. Materials and Methods

2.1 Organisms and culture conditions

The host strains used in this study were *Vibrio vulnificus, Vibrio mimicus, Vibrio fluvialis, Vibrio furnissi* and 5 different serotypes of *Vibrio parahaemolyticus* K17, K25, K30, K52 and K57. All were Kanagawa phenomenon positive and obtained from Japan National Institute of Health. Oyster samples were taken over a 7-day period from 1994 to 1995. Nutrient broth with 2% maltose and 3% NaCl was used as the liquid medium for the host strain. For solid medium nutrient agar with 3% NaCl and 0.7 % agar for top agar was used for phage isolation.

2.2 Phage isolation

The material for phage isolation was commercially purchased fresh oysters. They were taken to the laboratory immediately in an ice box and used within 24 hrs. They were chopped and minced in a mortar. Prepared oysters were seeded into a culture of host strains and incubated for phage growth for 48 hrs at 37℃. Phage supernatant was obtained by centrifugation at 12,000×g for 10 min and then filtering the supernatant through a 0.22μm membrane filter. The filtrate was mixed with an equal volume of soft agar (Bacto beef extract 3 g, peptone 5 g, 3% NaCl, 0.7% agar, pH 7.2) at 50℃ and overlaid on agar (peptone 5 g, yeast extract 3 g, soluble starch 5 g, 0.1M MgSO4 10 ml, 0.1M $CaCl_2$ 10 ml, H2O 1000 ml, agar 15 g, pH 7.2) by using the double agar overlaying technique. The plates were scored for plaque formation after an overnight incubation. Well seperated single plaque was stabbed with a sterile needle or a toothpick and suspended in SM phage buffer (NaCl 5.8 g, $MgSO_4$ 2 g, 1M Tris-HCl (pH 7.5) 50 ml, 2% gelatin solution 5 ml, H2O 1000 ml). Ten-fold dilutions were plated by the double agar overlay method to obtain well-isolated plaques, and the process was repeated three times more to obtain unique phage isolates. The number of plaques varied from a few to confluent lysis. Harvested phage was kept with a few drops of chloroform in a refrigerator for later assay.

2.3 Phage assay

Adams method was employed for phage assay

2.4 Purification of phage particles

Purification of phage stocks was performed by the addition of 10% polyethylene glycol to precipitate phage particles on ice incubation for 1 hr. Phage pellets were harvested by centrifugation, added SM buffer and chloroform, and then vortexed. 40% of CsCl centrifugation was followed and phage pellets were recovered. They were dialyzed overnight aganist two changes of SM buffer at 4%. Purified phage was concentrated and stored at 4℃ or at -20℃.

2.5 Electron microscopy

A more or less pure concentrated suspension of phage particles was prepared for examination under an electron microscope. Specimens for microscope were prepared by suspending the phage pellet in ammonium acetate and applying a drop to a carbon-coated grid for 1 min to 2 min, followed by negative staining with 1% aqueous uranyl acetate for 1 min and examined by an electron microscope (JEOL 1200 EX-2).

2.6 Phage nucleic acid extraction and electrophoresis

Phage nucleic acid was isolated by phenol extraction and ethanol precipitation. Isolated nucleic acid was treated overnight with deoxyribonuclease and ribonuclease. Phage nucleic acid was cleaved with restriction enzymes according to the specifications of the suppliers. And the samples were run on an electrophoresis on 1% agarose gels in TAE buffer (40 mM Tris, 20 mM sodium acetate, 2 mM EDTA, pH 8.0). The gels were stained in ethidium bromide (0.5 μg/ml) final and the bands were detected under a UV illuminator.

3. Results and Discussion

The frequency of marine environmental pollution threats is increasing and raw sea food consumption is still much dependable in many geographic regions of the world including the Korean penninsula (Hatsumi, 1984; Ju *et al* 1987). Viruses and marine microorganisms associated with shell fish are affected by salinity, changes in the levels and kinds of nutritional characteristics, temperatures and many other ecological factors.

The high frequency of phage isolation from shell fish prompted a quantitative study of the actual numbers of specific Vibrio phage from molluscan shellfish on a seasonal basis. The incidence of increasing phage isolation with increasing temperatures of sea water during the- summer (data not shown) was noticeable.

Phage isolates have been characterized with respect to host range, unfortunately not all of the suspected samples were susceptable to the host. Among the four Vibrio species and five K-serotype pilot strains of *Vibrio parahaemolyticus* which were employed for phage isolation, only two strains of *Vibrio parahaemolyticus* possessing K17 and K52 antigens were sensitive to the phage. The average size of the plaque was 0.4 mm which was slightly smaller than that of other author (Ju et al 1987). The morphology of the plaque is shown in Fig. 1. The titer obtained by the soft-agar-overlay technique was in the range of 10^{10} -10^{11} PFU/ml. The isolated phage was unaffected by storage over chloroform for long periods of storage. A stock was routinely filter sterilized and it had no effect on phage titers and it was consistent with the result of Hatsumi.

The electron micrographs of the isolated phage are shown in Fig. 2 and Fig. 3. Vibrio phages were classified into 4 groups of morphology and 3 tail types: a tail with a contractile sheath, a six-sided head and a tail, a six-sided head with a tail but the tail is shorter than the head diameter or maximal dimension. The isolated phage had an elongated six-sided head but it was not clear whether it

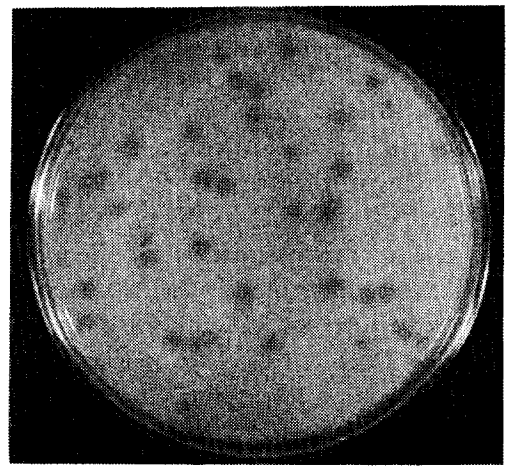

Fig. 1. Plaque morphology of 10^{-9} dilution of phage possessing K52 antigen of *Vibrio parahaemolyticus* from oysters.

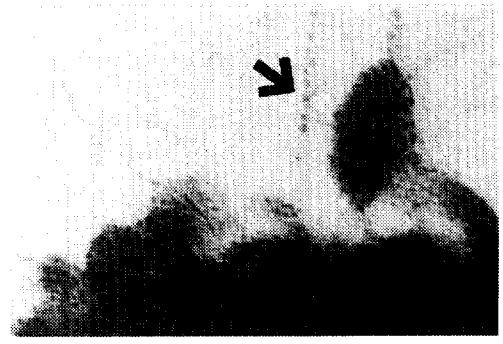

Fig. 2. Electron microscopy of *Vibrio parahaemolyticus* K52 phage from oysters.

Fig. 3. Electron micrograph of a negatively stained preparartion of *Vibrio parahaemolyticus* K17 phage from oysters. Bar represents 100 nm.

had a terminal appendage or tail as seen either in Fig. 2 or Fig. 3. The phage head measured 67 nm in length 83 nm in width (Fig. 3).

Nucleic acid of the isolated phage was analyzed after overnight treatment with deoxyribonuclease (DNase) and ribonuclease (RNase), followed by the addition of trichloroacetic acid (TAE) plus 1N KOH, also overnight, it was found that the phage-contained deoxyribonucleic acid because of its resistance to RNase and its ability to make discrete bands when it was subjected to restriction endonuclease digestion. Restriction endonucleases *BamH* I, *Eco R* I, *Hind* III, *Pst* I were applied according to the instruction of the manufacturers to observe the patterns of the isolated phage genomes. As seen in Fig. 4, isolated DNA was serially diluted and separated according to size by agarose gel electrophoresis. When 17 μl of DNA was electrophoresed in TAE buffer, digested to completion with *Eco R* I restriction endonuclease gave 4 bands in lane E and H, and 14 bands in lane D and I with *Hind* III, no cuts from in lane B and K with *Bam* HI, in lanes C and J with *Pst* I digestion respectively. As seen in Fig. 4, the restriction fragment of two antigenic groups showed similar patterns with *Eco R* I and *Hind* III digestion suggesting identity between two serovars.

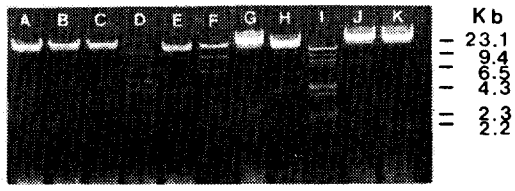

Fig. 4. Restriction patterns of isolated phage DNA of *Vibrio parahaemolyticus* K52 (A-E), K17 (G-K) from oysters. Lane A, undigested DNA of K52: Lanes B and C, digests of *Bam*H I and Pst 1: Lane D, *Hind* III: Lane E, *EcoR* 1: Lane F, lambda DNA *Hind* III marker: Lane G, undigested DNA of K17: Lane H, *EcoR* 1: Lane I, *Hind* III: Lane J, *Pst* I: Lane K, *Bam*H I digests.

Further studies with genomes should be conducted to determine whether the oyster phage carries any virulence factor by hybridization with host strain toxin probe which may or may not cause infection of humans.

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생굴로부터 bacteriophage의 분리 및 부분특성

김영희
동의대학교 미생물학과
(1996년 3월 29일 접수)

시판중인 생굴을 대상으로 Vibrio 속을 숙주로하는 bacteriophage의 분리를 시도하였다. 4 종의 Vibrio 속과 5 혈청형의 *Vibrio parahaemolyticus*를 실험대상으로 한 결과 *Vibrio parahaemolyticus* 2 혈청형에서만 bacteriophage가 분리되었다.

분리된 phage 의 plaque 크기는 0.4 mm였으며 전자현미경적 형태는 미부가 뚜렷하지 않은 육각형의 두부가 관찰되었고 크기는 67 nm×83 nm 였으며, PFU/ml은 1.25×10^{11}이었다. 분리된 phage는 chloroform에 감수성을 나타내었다.

분리된 phage의 genomic 특성을 규명하기 위하여 핵산을 분리한 결과 DNA로 판명되어졌으며 두 혈청형 모두 제한효소 처리한 결과 *Eco R* I 으로 4부위의 절단양상과 *Hind* III로 부터 14부위 절단양상이 관찰되었다.