ORIGINAL ARTICLE

COLIPHAGE HSA AS A MODEL FOR ANTIVIRAL STUDIES/SPECTRUM BY SOME INDIGENOUS BACTERIOCIN LIKE INHIBITORY SUBSTANCES (BLIS)

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ABSTRACT
Coliphage HSA was isolated from a raw sewage sample (collected from a local sewage treatment plant). The phage was analyzed by spot and tube lysis followed by plaque assay. Phage titre (plaque forming units i.e. PFU) was found to be 4.2 x 10^3 PFU/mL. Further purification of the phage was achieved by acid-precipitation method. Genomic identification of the coliphage HSA (done by fluorescent staining using acridine orange) revealed it to be a dsDNA bacterial virus. Staphylococcin188, Enterocins AAR-71, AAR-74, and Erwiniocin NA4 were screened for their antiphage activity by plaque assay. Accordingly, all the bacteriocin preparations possess demonstrable antiphage activity witnessed as a reduction in PFU after treatment. In the case of Staphylococcin 188, the number dropped up to 40 PFU/mL, Enterocin AAR-71 and Erwiniocin NA4 treatment reduced it to a zero PFU level, while Enterocin AAR-74 could reduce PFU to 50 (after addition of a constant volume, 500uL, of each of the crude bacteriocin preparations). Transmission Electron Microscopy studies revealed the phage to have an icosahedral head with a long tail and tail fibers.

Keywords: Coliphage, bacteriocins, plaques, acid precipitation, antiphage activity.

INTRODUCTION

The phage (bacterial virus), like the virion particle, consists of a nucleic acid surrounded by a protein capsid. Most coliphages have tails, the tips of which have the ability to bind to specific molecules on the surface of their target bacteria (Sarkar *et al.*, 2004). Each kind of bacterium has its own phages, which can be isolated which can be isolated from the habitat (sewage, pus, soil, marine water and hot springs etc.) of a particular bacterial host (Kutter, 1997).

Conventional methods of lytic phage enumeration measure phage directly; by its ability to lyse the infected cells in agar and thus giving rise to plaques, (zones of lysis/clearing in confluent lawns of specific host cells) or, indirectly by assessing culture activity that is inhibited in the presence of phage (Rasool, 2002).

Bacteriocins (also referred as bacteriocin-like inhibitory substances-BLIS) are protein bacterial products with high therapeutic bioactivity (Riley, 2002). Staphylococcins (bacteriocins by staphylococci) are higher molecular mass molecules (dissociable into smaller units) with a broad based bioactivity (Franz *et al*., 1999). Similar to their structural heterogeneities, staphylococcins show wide functional diversity and are active not only against other staphylococci but also against many distant microorganisms (De-Oliveira *et al*., 1998).

Enterocins are gaining interest because of their inhibitory activity against many food related pathogens including *S. aureus* and *L. monocyotgenes*. *E. faecium* AL40 produced enterocin which inhibited the growth of all enterococcal indicator strains isolated from municipal sewage. This ability of enterocin indicates its possible use in biotechnological environmental processes (Laukova *et al.,* 1993). *Erwinia carotovora NA4* (an isolate from diseased potatoes) has been found to produce a proteinaceous antibacterial substance, Erwiniocin NA4. It has a broad spectrum activity against many gram-positive and gram-negative bacteria including phytopathogens (Jabeen *et al.*, 2004). One of the most important benefits of bacteriocins would include the possible use of effective anti-phage activity against human viral infections. By following the research plan it will be easier to infer their grand antiviral activity. This may provide a platform to use these agents as contra viral alternatives (Wachsman *et al*., 1999).

MATERIALS AND METHODS

Bacterial strain
Culture of *E. coli* ATCC 0241 was procured from Culture Collection Center of Department of Microbiology, University of Karachi. MacConkey's agar and Brain Heart Infusion (BHI) agar were used for the maintenance and preservation of the bacterial culture. Cells were grown in Luria basal broth (LBB) for the phage studies. Culture of *S. aureus* AB188, *Enterococcus faecalis* AAR-71 and AAR-74 were maintained on BHI agar and the phytopathogen *Erwina carotovora* NA4 was maintained on Nutrient agar.

Isolation of coliphage from sewage was performed by the

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method of Benson (1994).
Acid precipitation technique was applied to purify the phages (Bachrach, 1971). Bacterial debris was removed by centrifugation at 3000rpm for 30 minutes. Phages were precipitated from the clear supernatant by drop wise addition of 1N HCl until a final pH of 3.9 was reached, then placed in refrigerator for 24 hrs. Clear upper fluid was removed and the rest was centrifuged at 6000rpm for 60 minutes. The sedimented phages were suspended in borate buffer (pH. 9).

Plaque assay, tube and spot lysis of the isolated phage were performed as per (Douglas, 1975).
Fluorescent staining technique was performed to determine the nucleic acid type of the phage as per Bradley (1967). Droplets of pure phage suspension were dried on glass slides, fixed in Carnoy's fluid and stained in 1% Acridine Orange in McIlvaine's buffer at pH 3.8. After soaking in 0.15N Na2HPO4 for 15 minutes, the slides were examined under 2570Aº UV source. A green fluorescence indicated dsDNA or dsRNA, while a red colour indicated ssDNA or ssRNA. Then the slide was treated with Molybdic acid solution for a few seconds to see if it is RNA or DNA.

Transmission electron microscopy of Coliphage HSA
Method of Daw and Falkiner (1996) was followed for this purpose. Five micro liters of the sample (acid precipitated coliphage HSA) was applied to a freshly carbon coated (300 mesh sized) copper grid and the excess fluid was blotted off after 5 minutes. The grid was washed four times in sterile distilled water. For negative staining 1% uranyl acetate was used and after 4 minutes the excess fluid was blotted away. The grid was then air dried and observed under JOEL JEM-100SX (Japan) Transmission Electron Microscope after an acceleration of 80kV. Areas of interest were imaged at 40,000 magnifications. The micrographs were exposed for 1 second and developed in full strength Agfa developers for 5 minutes.

Anti-phage activity of staphylococcin188, Enterocins AAR-71, AAR-74 and Erwiniocin NA4
Antiphage activity (based on PFU) of Staphylococcin188 (2mg/mL), Enterocins AAR-71, AAR-74 (4mg/mL), and Erwiniocin NA4 (3mg/mL) was performed using different concentrations of the bacteriocins (100-1000µL) with the 100µL of the phage lysate (Waschsman *et al.*, 1999).

RESULTS

Coliphage HSA was isolated from the local sewage sample. Manifestation of phage activity was determined by tube lysis method i.e. clearance (cell lysis) after 6 hrs incubation compared to the control. In case of spot lysis, zone of clearance appeared on the lawn of the culture. Further purification of the phage lysate was carried out by acid precipitation followed by centrifugation (for sedimentation at 6000rpm (figure 1). Transmission Electron Microscopy of coliphage HSA revealed that the phage contained an icosahedral head with a long tail and tail fibers. The size of the phage was 125 nm in length (figure 3). Fluorescent staining technique revealed the phage to be a dsDNA bacterial virus (table 1). Antiphage activity of Staphylococcin188, Enterocins AAR-71 & AAR-74, and Erwiniocin NA4, was observed by plaque assay. In case of Staphylococcin 188, PFU/mL dropped from 5040 (control) to 40 PFU/mL (figure 4A). For Enterocin AAR-71 PFU/ml was reduced from 620 to zero PFU/mL while by Enterocin AAR-74 PFU/ml was reduced from 4.2 x 10^3 to 50 PFU/mL (figures 4B and 4C). Erwiniocin NA4 also reduced the PFU/ml from 4.2 x 10^3 to zero PFU/mL (figures 2 and 4D).

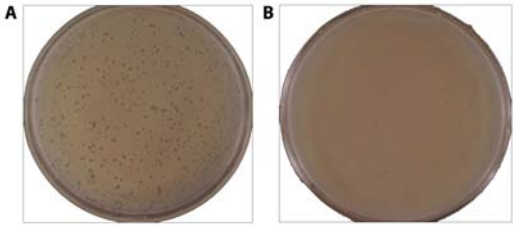

Fig. 1: Phages were present in sediment (A) but absent in supernatant (B) by Acid Precipitation Technique.

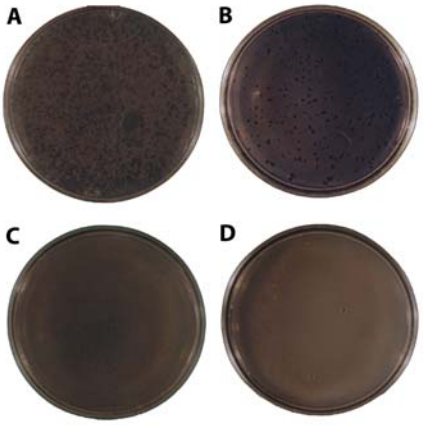

Fig. 2: Antiphage activity of Erwiniocin NA4: A) Lysate 100µL, B) Lysate 100µL + Erwiniocin NA4 100µL, C) Lysate 100µL + Erwiniocin NA4 500µL and D) Lysate 100µL + Erwiniocin NA4 1000 µL.

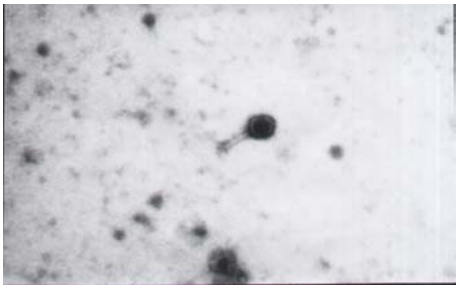

Fig. 3: Transmission electron micrograph of tailed coliphage HSA. The size of the phage was 125 nanometers in length (magnification: 40,000X).

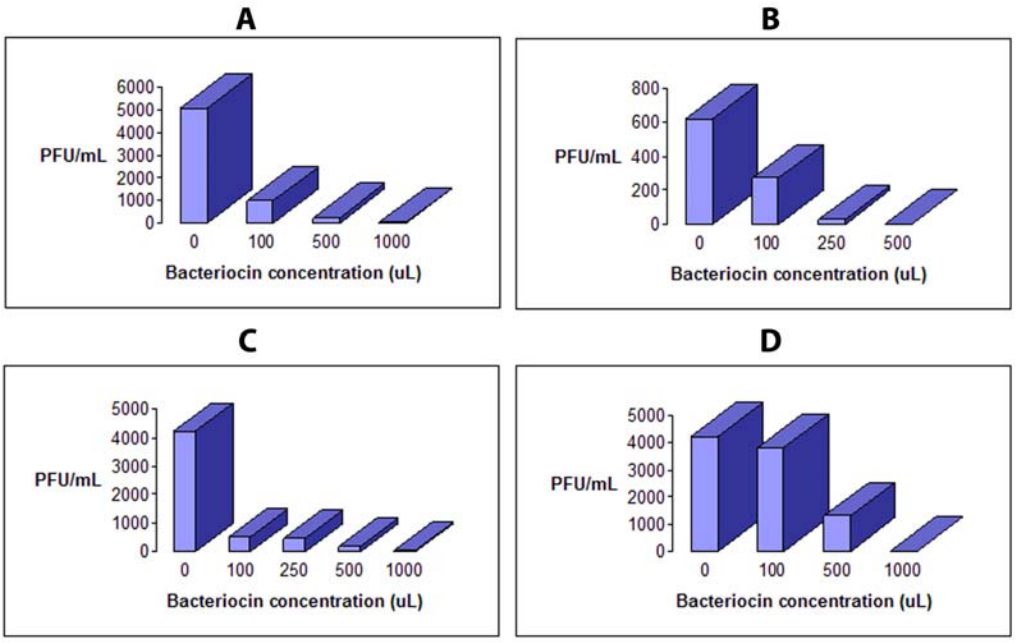

Fig. 4: A) Antiphage activity of Staphylococcin188: B) Antiphage activity of Enterocin AAR-71, C) Antiphage activity of Enterocin AAR-74 and D) Antiphage activity of Erwiniocin NA4.

Table 1: Fluorescent Acridine Orange-UV staining of Coliphage HAS

Nucleic Acid	Treatment	
dsDNA	Na2HPO4	Molybdic acid
	Green	Green

DISCUSSION

Bacterial viruses present an attractive model for elucidating the antiphage activity of bacteriocins as they are relatively easier to handle and do not require cell line culture for their growth. The present study was undertaken in order to assess the antiphage activity potential of indigenous bacteriocin preparations. The present study was undertaken. The observations will ultimately help to argue about the antiviral (with reference to human-animal specific viruses) potential of these protein antimicrobials. For this purpose, coliphage HSA (against *E. coli)* was isolated from raw sewage (with the titre of 4.2 x 10^3 PFU/mL). By Acid (HCl) precipitation and electron microscopic techniques have shown that the isolated phage belongs to the T-even series as T-odd phages are sensitive to the acid (Bachrach, 1971).

To observe the exact *in vitro* picture of the antiphage activity, PFU/mL was determined in the presence (and absence) of different concentrations of the bacteriocins. Results indicated that the lower survival rate of the phage (based on PFU/mL) at increasing concentration of bacteriocin (as shown in figures 4A, 4B, 4C and 4D) is because of the antiphage activity of bacteriocins. Antiphage activity has been observed to be present in a number of proteins like Aklavin, an antibiotic like substance extracted from Streptomycetes (Strelitz *et al.*, 1955), and in the Lipopolysaccharide (LPS) of the bacterial membrane (Zerov, 1976).

Bacteriocins (particularly those of low molecular weight) enter the phage head, thus getting direct access to the nucleic acid and exerting their deleterious effect. On the other hand, higher molecular weight bacteriocins compete for the receptors on the host cell, thus causing the drop in phage titre. In case where the phage DNA infected the host, some interference by the bacteriocin could be expected leading to the suppression of host protein synthesis that is carried out by the phage, and plaques are not produced (Wachsman *et al.*, 2003). There is no effect of the bacteriocins on the *E. coli* cells as they are resistant to the effects of the bacteriocins and thus it cannot be said that the reduction in the PFU/ml was due to killing of the phage host cells by the bacteriocins.

The results of the present study are in agreement with Wachsman *et al.* (1999; 2003) who have shown antiviral activity of Enterocin CRL35 against (HSV) type 1 and 2 and it was noticed that inhibition of HSV spreading by Enterocin CRL35 is due to the prevention of mainly late glycoprotein synthesis.

ACKNOWLEGEMENTS

This study was funded by Karachi University research grant to Dr. Sheikh Ajaz Rasool, Department of Microbiology, University of Karachi.

REFERENCES

Bachrach U and Friedman A (1971). Practical Procedures for the Purification of Bacterial Viruses. Applied Microbiol., **22**(4): 706-715.

Benson HJ (1994). Microbiological Applications, Wmc Brown Publishers, Oxford. 6th ed., pp.120-139.

Bradley DE (1967). Bacteriological Reviews. A publication of the American Society for Microbiology., **31**(4): 230-314.

Daw M. A and Falkiner FR. (1996). Bacteriocins: Nature, function and structure. *Micron.,* **27**: 467-479.

De-Oliveira S S, Abrantes J, Cardoso M, Sordelli D and Bastos MC (1998). Staphylococcal strains involved in bovine mastitis are inhibited by *Staphylococcal aureus* antimicrobial peptides. *Lett. Appl. Microbiol.,* **27**: 287-291.

Douglas J. (1975.) Bacteriophages. Chapman and Hall publishers, 4th ed., London. pp.20-46.

Franz C M A P, Holzapfel W H and Stiles ME (1999). Enterococci at the cross-roads of food safety. *Inter. J. Food Microbiol.,* **47**: 1-24.

Jabeen N, Rasool SA, Ahmad S, Ajaz M and Saeed S (2004). Isolation, identification and bacteriocin production by indigenous diseased plant and soil associated bacteria. *Pak. J. Biol. Sci.,* **7**(11): 1893-1897.

Kutter E (1997). Phage therapy: bacteriophages as antibiotics. Available online as http://www.evergreen.edu/phage/phagetherapy/phagetherapy.

Laukova A, Marekova M and Javorsky P (1993). Detection and antimicrobial spectrum of a bacteriocin like substances produced by *Enterococcus faecium* CCM4231. *Lett. Appl. Microbiol.,* **16**(15): 257-260.

Rasool SA (2002). Bacterial Viruses: Basic and Applied Concepts, UGC, Pakistan, pp.38-56.

Riley M A and Wertz JE (2002). Bacteriocins: evolution, ecology and application. *Ann. Rev. Microbiol.,* **56**: 117-173.

Sarkar BL, Ghosh AN and Rodrigues DP (2004). Newly isolated *Vibrio cholerae* Non-01, Non-0319 Phages. *Emerg. Infect. Dis.,* **10**(4): 445-453.

Strelitz F, Flon H, Weiss U and Asheshov NI (1955). Aklavin, an antibiotic substance with antiphage activity. *J. Bacteriol.,* **72**(1): 90-94.

Wachsman MB, Fairas ME, Takeda E, Sesma F, de-Torres RA and Coto CE (1999). Antiviral activity of enterocin CRL35 against herpesviruses. *Int. J. Antimicrob. Agents.,* **12**: 293-299.

Wachsman M B, Castilla V, de Ruiz-Holgado A P, de Torres R A, Sesma F and Coto CE (2003). Enterocin CRL35 inhibits late stages of HSV-1 and HSV-2 replication *in vitro*. *Antiviral Res.,* **58**(1): 17-24.

Zerov U P and Ashmarin IP (1976). Identification and study of species specificity of antiphage lipopolysaccharides found in the preparations of bacterial DNA. *Journal of Hygiene, Epidemiology, Microbiology and Immunology*, **21**: 284-291.

Received: 15-06-2006 – Accepted 09-08-2006