Isolation and identification of a natural bacteriophage as a biocontrol agent against *Proteus vulgaris*

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Abstract
Proteus vulgaris as an opportunistic microorganism in human infections especially in immunodeficient individuals is of medical importance. While the potential of bacteriophages as efficient, safe and cost-effective antimicrobial agents to combat resistant bacteria was well recognized, lytic phage activity against P. vulgaris has not been investigated since 1967. Here we describe the isolation and characterization of a natural lytic phage capable of infecting P. vulgaris strain, which is isolated from clinical samples. Following the primary screening, the most efficient phage was chosen for further characterization. The morphology, killing efficiency and bacterial host range were determined under controlled conditions in the laboratory. Isolated phage was shown to have broad host range and infected Bacillus subtilis, Pseudomonas aeruginosa and Staphylococcus epidermidis as well as P. vulgaris. The virion was designated as gP0Bh-MGP1 and characterized by a head diameter of about 84 nm and a tail size of about 28 × 126 nm. It belongs morphologically to the myoviridae family as analyzed by transmission electron microscopy (TEM). The result of this investigation indicated that the isolated phage, gP0Bh-MGP1, is efficient in lysing different pathogenic agents and may be a good candidate to be used as an agent to combat infections caused by resistant strains.

Keywords: Phage therapy, *Proteus vulgaris*, antibiotic resistance, lytic phage.

Introduction

The environmentally widespread genus *Proteus* makes up a part of the normal flora of the human and animal gastrointestinal tract.[1] This genus currently consists of five named species (*P. mirabilis, P. penneri, P. vulgaris, P. myxofaciens and P. hauseri*)[2]. *P. vulgaris* is rod-shaped gram-negative *Bacillus* and is classified in Enterobacteriaceae. Based on the biochemical tests, it is a nitrate-reducing, indole and catalase-positive and hydrogen sulfide-producing bacterium[3]. *P. vulgaris* is distinguished from *P. mirabilis*, the most frequently isolated member of the genus according to laboratory fermentation tests. Glucose, sucrose and maltose are readily fermented by *P. vulgaris*, while *P. mirabilis* metabolizes glucose readily and sucrose slowly and does not ferment maltose[2].

Although *Escherichia coli* accounts for the majority of cases of urinary tract infections, *Proteus* spp. ranks third as the cause of uncomplicated cystitis, pyelonephritis and prostatitis, particularly in hospital-acquired cases[1]. The mortality rate in *Proteus*-related bacteremia infections is 25%[4]. *P. vulgaris* is a urease positive bacterium, which increases the pH of urine to above 8.3 by producing ammonia from urea and elevate the pH above 8.3 of urine[5]. It initiates crystallization of calcium and magnesium phosphates in the urine that results in the creation of crystalline biofilms and the encrustation of the patient catheter[6]. There are also some reports of life-threatening infection by *P. vulgaris*. In a case report, an ecthyma gangrenosum was described in a 57-year-old woman caused by *P. vulgaris* and *Candida albicans*[7].

Most strains of *Proteus mirabilis* are sensitive to ampicillin and cephalosporins, while *P. vulgaris* is resistant to these antibiotics. However, this organism is isolated less often in the laboratory and usually targets immunosuppressed individuals. In 2013 five urinary tract associated isolates including *Proteus*, responsible for causing infection, were evaluated for their antibiotic sensitivity and most of the strains showed the resistance against the broad spectrum antibiotics: Ceftazidime (30µg), Ofloxacin (50µg), Norfloxacin (30µg), Tetracycline (30µg), Ampicillin (30µg), Chloramphenicol (25µg) and Gentamycin (20µg)[8].

The extensive and frequently unreasonable use of antibiotics has resulted in the global problem of antibiotic resistance and the creation of multidrug-resistant bacteria that lowers the efficacy of antimicrobial therapy and increases the mortality rate of infectious diseases. This crisis has prompted extensive research aiming at discovering of new classes of antibacterial agents or alternative antibacterial therapies. Bacteriophages, bacteria's natural predators, have attracted inclusive attention as biological antibacterial agents in recent years[9]. There are several studies on isolation and characterization of *P. mirabilis* lytic phages[10,11].

However, reports related to screening and investigation of lytic phages affecting *P. vulgaris* have not been documented since 1967 based on literature review done in this study[12]. In the present study, a lytic phage against *P. vulgaris* was isolated from water resources and characterized and its effectiveness to inhibit bacterial growth was evaluated. This phage is also effective on *Pseudomonas aeruginosa* and *Staphylococcus aureus* which are the other microorganisms associated with *Proteus* infections.

Material and Methods

Bacterial strains and culture conditions: *P. vulgaris* was used for screening the samples and determining the phage titer. All bacterial species (Table 1) used for determining the host range of phage were purchased from PTCC (Persian Type Culture Collection). Cultivation of bacteria was performed by harvesting an isolated colony from streak Luria-Bertani (LB) agar plate and inoculating into LB broth (0.5% yeast extracts, 1% peptone and 5% NaCl, pH 7.2). 1.5% LB agar plates were utilized for solid media tests. The bacteria were grown overnight at 37°C under aerobic conditions with shaking at 120 RPM.

Phage isolation, propagation and titration: Bacteriophages were isolated from river water samples near to rural region where it is polluted with sewage or fecal matters of livestock as previously described with some modifications[13]. Briefly, the samples were put in a flask containing 50 ml of LB medium and 50 µl of the P. vulgaris suspension with an optical density of 0.6. The solution was incubated for 24 h in a rotary shaker, 150 rpm at 37 °C and afterward centrifuged for 10 min at 5000 × g. The supernatant was filter sterilized, precipitated by the addition of 20% polyethylene glycol 8000 and 2.5 M NaCl, centrifuged at 12000 × g and then used to test for antibacterial activity.

Screening of the isolated bacteriophages was carried out through plaque assay on *P. vulgaris* culture using double layer agar plate[14]. The clear plaques formed on the lawn of bacteria were stabbed with a needle and eluted with a small volume of SM buffer (0.05% NaCl, 0.2% $MgSO_4 \cdot 7H_2O$, 0.005 M Tris-HCl, pH 7.5). The phage suspensions were serially propagated twice on the same strain. The most efficient bacteriophage with lytic (clear) plaques was selected for further study and designated gP0Bh-MGP1. The isolated phage was incubated with *P. vulgaris* for 10 min without agitating and then with shaking (120rpm) until visible lysis was begun. The lysate was centrifuged at 5000 × g for 20 min and the phages precipitated by supplementing NaCl and PEG8000 (0.5 M and 20%, respectively) as mentioned before. The phage pellet was again dissolved in SM buffer.

Determination of the host range of the phage gP0Bh-MGP1
Isolated phage was tested for its ability to form plaque on a range of gram-positive and gram-negative bacterial species (Table 1). The sensitivity of each bacterial species to *P. vulgaris* infective phage was determined using the spot assay as described above. As a control, bacterial strains were also mock infected with sterile phage buffer. All experiments were performed in triplicate fashion to validate results. Results were evaluated based on observation of the clear zone and further dilutions were tested for single plaques to confirm phage lysis rather than bacteriocin induced lysis.

Electron microscopy: The isolated phage of 10 ml of the mixture from large-scale production containing $\approx 10^9$ PFU/ml, precipitated by centrifugation at 40,000× g for 30 minutes, followed by resuspension of the pellet in 200 µl of SM buffer (pH = 7.4), was used for electron microscopy analysis. Preparations of bacteriophages particles were made by standard negative staining procedure. The concentrated bacteriophage suspension was adsorbed on a copper grid with carbon coated formvar films and then stained with 2% uranyl acetate for 20 seconds. The grid was then washed twice with ddH_2O and air dried. The grids were observed with an electron microscope (Philips, UK).

Proteome analysis of the phage gP0Bh-MGP1: The phage proteins were analyzed using SDS-PAGE according to the Laemmli method. Purified phage suspension was mixed with trichloroacetic acid and ice-cold acetone and maintained at -20°C for 1.5 h. Proteins were precipitated by centrifugation (16,000×g 20 min, 4°C) followed by re-suspending in the sample buffer. The sample was then loaded onto 12.5% SDS-PAGE slab gel and electrophoresed with Tris-glycine buffer.

Results and Discussion

Phage antibacterial action: Collected samples were screened by spotting on a soft agar overlay and then to assess isolated phage impact on *P. vulgaris.* Different dilutions of phage were mixed with host suspension and distinct inhibition zones were counted (figure 1). Several plaques with different size were seen on the lawn of *P. vulgaris*, some of them surrounded by a hazy halo zone. Clear appearance of plaques indicated strong lytic activity of phages and their ability to the efficient killing of the bacterial strain. The observation of halo zone around the plaques suggests the ability of the isolated phage to produce soluble polysaccharide-degrading enzymes[15]. These enzymes are very useful tools for solubilizing biofilms of bacterial strains. A plaque with the largest plaque and halo zone was purified and amplified for further analysis.

Morphological characterization and host range of phage gP0Bh-MGP1: Results of the transmission electron microscopy (TEM) indicated that the phage has morphological characteristics of the myoviridae family, featured by a head of icosahedral symmetry and a long- tail. The gP0Bh-MGP1 has a head of ≈ 85 nm in diameter with a long tail ≈125 nm length (figure 2). This result is in accordance with the features of myoviridae family of bacteriophages[16,17].

To determine the host range of identified phage against different species of bacteria, the lysis ability was examined by spot assay technique and the results were summarized in table 1. Accordingly, gP0Bh-MGP1 had a lytic activity towards *Bacillus subtilis*, *Pseudomonas aeruginosa* and *Staphylococcus epidermidis* in addition to *P. vulgaris* and therefore showed a quite wide host range from gram-positive to gram-negative bacterial family. The effectiveness of isolated phage against *P. aeruginosa* and *S. epidermidis* is an advantage for some clinical conditions where multiple pathogens are responsible for the infection, especially in the

case of immunocompromised patients. Broad-host range is another character of some subfamilies of myoviridae[16,18].

For further characterization of the isolated phage, analysis of proteome was performed. According to SDS-PAGE result, the protein composition of phage gP0Bh-MGP1 includes at least 9 distinct bands. The most predominant band could be accounted for major capsid protein, which has a size of approximately 37 kDa. Another abundant protein has a molecular weight of 65 kDa that may be the tail sheath protein of the phage gP0Bh-MGP1 (Fig. 3). These results were also reported in characterization of myoviridae family[19].

This study represents example of a lytic phage that targets *P. vulgaris*. Growing extent of bacterial strains resistant to antibiotics, promising antimicrobial effect of isolated phage and meanwhile very cost-effective methods for identification and production, suggest this agent as a potential candidate for further examinations against *P. vulgaris*. Production of polysaccharide-degrading enzymes that can disrupt the exopolysaccharide and the lytic ability of phage results in a powerful tool for eradication of the infections by vulnerable bacterial strains even if they develop biofilm.

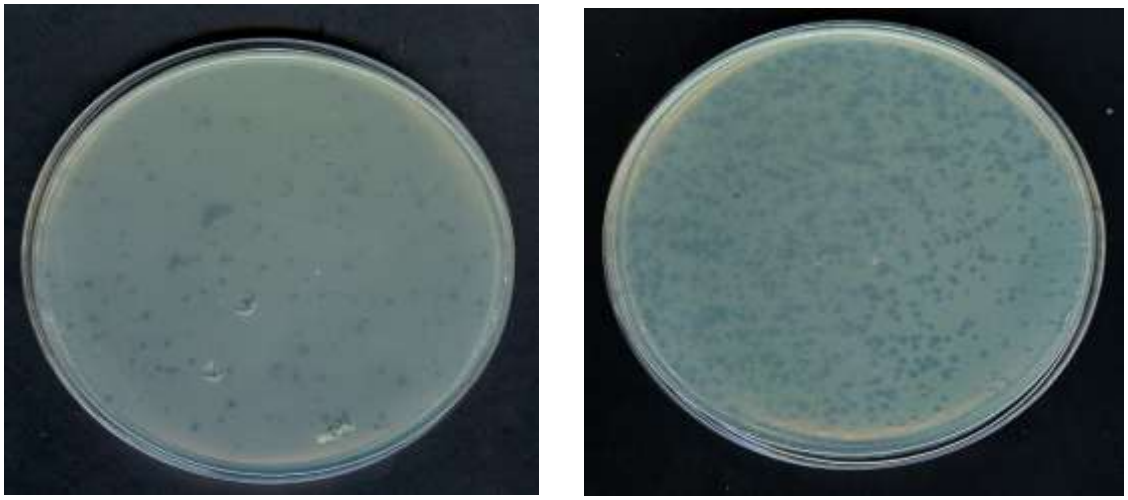

Figure 1: Plaques of phage gP0Bh-MGP1 on the lawn of *P. vulgaris*. A hazy halo zone is seen around some plaques.

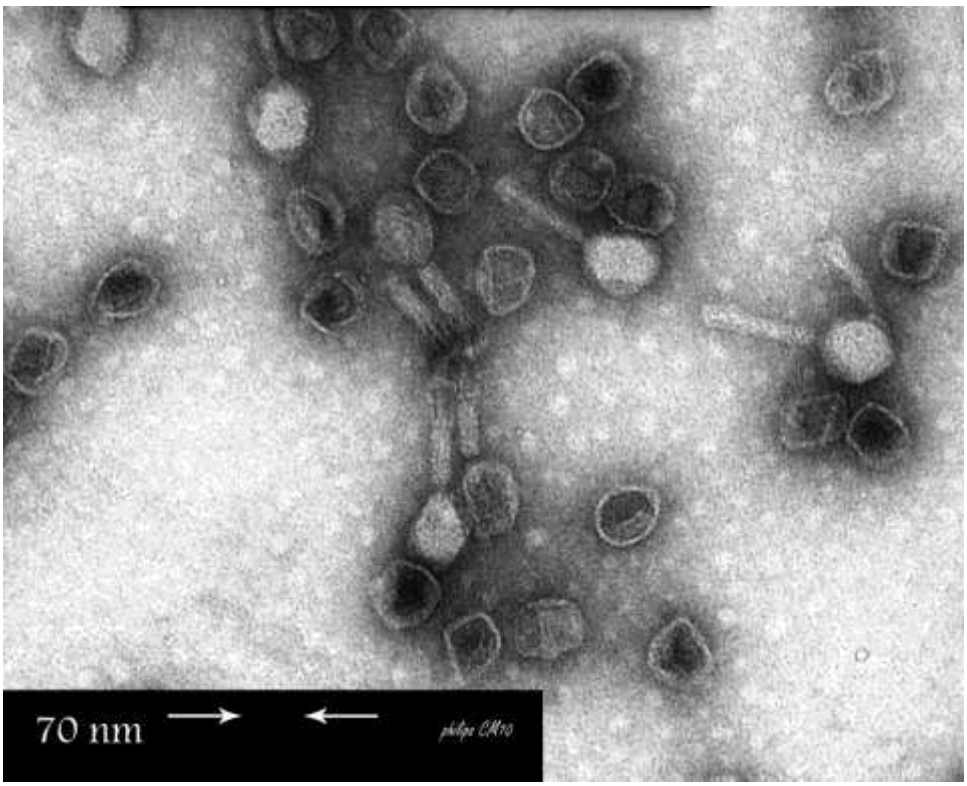

Figure 2: Electron micrograph of phage gP0Bh-MGP1 particles under transmission electron microscope. Scale bar represents 70 nm. Viruses were stained negatively with uranyl acetate.

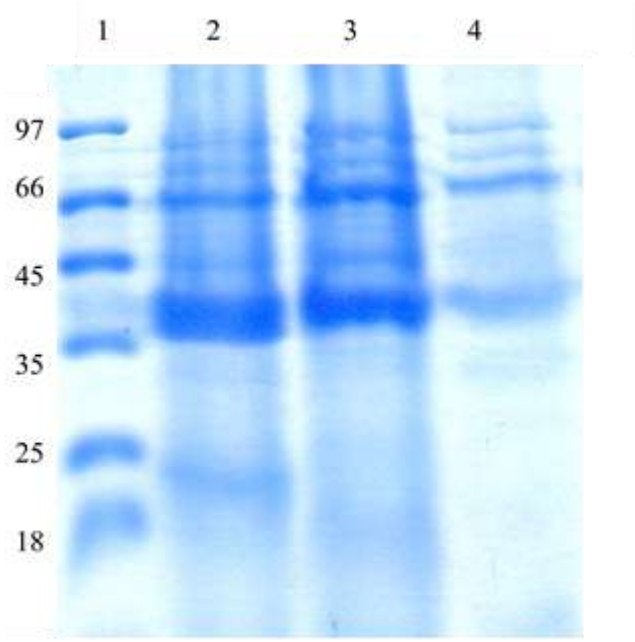

Figure 3: Proteome of the phage gP0Bh-MGP1. Lane 1, size marker, lane 2 TCA precipitated phage proteins, lane 3, Acetone precipitated phage protein, lane 4 diluted acetone precipitated phage proteins.

Table 1
Host range of the phage gP0Bh-MGP1. The symbol + shows that phage produces lysis while symbol – indicates that lysis was not observed.

Bacteria	PTCC code	Spot assay result
Bacillus subtilis	1023	+
Escherichia coli	1329	-
Pseudomonas aeruginosa	1074	+
Salmonella typhi	1609	-
Staphylococcus aureus	1112	-
Staphylococus epidermidis	1114	+
Bacillus stearothermophilus	1353	-
Shigella dysenteriae	1188	-
Shigella flexneri	1234	-
Vibrio cholera	1611	-
Streptococus pyrogenus	1447	-
Pseudomonas aeroginosa	1347	+
E. coli O157	Clinically isolated	-

Conclusion

The worldwide spreading of multi-drug resistant bacterial strains which result in the failure of antibiotic therapy of infections, has reintroduced the interest in phage therapy. Due to different mechanisms of action, phage therapy is effective against antibiotic-resistant bacterial strains and target desired virulent pathogens with negligible effect on the normal flora.

The gP0Bh-MGP1 phage is a newly isolated lytic bacteriophage of the myoviride family able to effectively lyse *P. vulgaris* as well as *B. subtilis*, *P. aeruginosa* and *S. epidermidis*. The phage gP0Bh-MGP1 is characterized by electron microscopy and proteomic study. However, more characterization is required to introduce it as a clinical weapon against pathogens. The broad-host range or polyvalence character of the phage may result in its activity on infections with multiple bacterial sources.

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References

1. Root R.K., Clinical infectious diseases: a practical approach, Oxford University Press, New York, Oxford, 1013 **(1999)**

2. O 'Hara C.M., Brenner F.W. and Miller J.M., Classification, identification and clinical significance of Proteus, *Providencia and Morganella*, *Clin Microbiol Rev.*, **13(4)**, 534-46 **(2000)**

3. Pal N., Sharma N., Sharma R., Hooja S. and Maheshwari R.K., Prevalence of multidrug (MDR) and extensively drug resistant

(XDR) proteus species in a tertiary care hospital, India, *Int J Curr Microbiol Appl Sci*., **3**, 243-52 **(2014)**

4. Kim B.N., Kim N.J., Kim M.N., Kim Y.S., Woo J.H. and Ryu J., Bacteraemia due to tribe Proteeae: a review of 132 cases during a decade (1991-2000), *Scandinavian Journal of Infectious Diseases*, **35(2)**, 98-103 **(2003)**

5. Stickler D., Morris N., Moreno M.C. and Sabbuba N., Studies on the formation of crystalline bacterial biofilms on urethral catheters, *Eur J Clin Microbiol Infect Dis.*, **17(9)**, 649-52 **(1998)**

6. Broomfield R.J., Morgan S.D., Khan A. and Stickler D.J., Crystalline bacterial biofilm formation on urinary catheters by urease-producing urinary tract pathogens: a simple method of control, *J Med Microbiol.*, **58(Pt 10)**, 1367-75 **(2009)**

7. Lee Y.J., Jung I.O. and Oh D.Y., A Rare Case of Ecthyma Gangrenosum Caused by Proteus vulgaris and Candida albicans in a Patient with Castleman Disease, *Arch Plast Surg*., **42(6)**, 805-7 **(2015)**

8. Tariq A.L. and Reyaz A.L., Terminalia chebula: A treatment against pathogenic proteus vulgaris strains associated with urinary tract infection, *International Journal of Research in Ayurveda and Pharmacy*, **4(4)**, 560-563 **(2013)**

9. Moradpour Z. and Ghasemian A., Modified phages: novel antimicrobial agents to combat infectious diseases, *Biotechnol Adv.*, **29(6)**, 732-8 **(2011)**

10. Melo L.D.R., Veiga P., Cerca N., Kropinski A.M., Almeida C., Azeredo J. and Sillankorva S., Development of a Phage Cocktail to Control Proteus mirabilis Catheter-associated Urinary Tract Infections, *Frontiers in Microbiology*, **7**, 1024 **(2016)**

11. Morozova V. et al, Lytic bacteriophage PM16 specific for Proteus mirabilis: a novel member of the genus Phikmvvirus, *Archives of Virology*, **161(9)**, 2457-2472 **(2016)**

12. Coetzee J.N., de Klerk H.C. and Smit J.A., A transducing bacteriophage for *Proteus vulgaris*, *J Gen Virol*., **1(4)**, 561-3 **(1967)**

13. Ghasemian A., Bavand M. and Moradpour Z., A broad-host range coliphage against a clinically isolated E. coli O157: Isolation and characterization, *Journal of Applied Pharmaceutical Science*, **7(3)**, 123-128 **(2017)**

14. Moradpour Z., Sepehrizadeh Z., Rahbarizadeh F., Ghasemian A., Yazdi M.T. and Shahverdi A.R., Genetically engineered phage harbouring the lethal catabolite gene activator protein gene with an inducer-independent promoter for biocontrol of *Escherichia coli.*, *FEMS Microbiol Lett.*, **296(1)**, 67-71 **(2009)**

15. Hughes K.A., Sutherland I.W. and Jones M.V., Biofilm susceptibility to bacteriophage attack: the role of phage-borne polysaccharide depolymerase, *Microbiology*, **144(11)**, 3039-3047 **(1998)**

16. Lavigne R., Darius P., Summer E.J., Seto D., Mahadevan P., Nilsson A.S., Ackermann H.W. and Kropinski A.M., Classification of Myoviridae bacteriophages using protein sequence similarity, *BMC Microbiology*, **9(1)**, 224 **(2009)**

17. Pitaksutheepong C., Abhisingha M., Dumnin J. and Visessanguan W., Isolation, detection and inactivation of a Myoviridae bacteriophage infecting Bacillus amyloliquefaciens FB11, *Annals of Microbiology*, **65(4)**, 1841-1846 **(2015)**

18. Ryu K. and Ahn J., Changes in physiological properties of bacteriophage-insensitive Staphylococcus aureus, *Annals of Microbiology*, **65(4)**, 1879-1884 **(2015)**

19. Klumpp J., Dorscht J., Lurz R., Bielmann R., Wieland M., Zimmer M., Calendar R. and Loessner M.J., The terminally redundant, nonpermuted genome of Listeria bacteriophage A511: a model for the SPO1-like myoviruses of gram-positive bacteria, *Journal of Bacteriology*, **190(17)**, 5753-5765 **(2008)**.

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