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Note

A colorimetric microtiter plate method for assessment of phage effect on *Pseudomonas aeruginosa* biofilm

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ABSTRACT

Bacteriophages have a potential in biofilm control. The aim of the study was to develop a method for selection of the most effective *Pseudomonas aeruginosa* phages for inhibition of biofilm formation and its eradication. The microtiter plate method is based on crystal violet staining and measuring of optical density.

Attached, densely packed bacterial cells embedded in extracellular polysaccharide matrix on biotic or abiotic surfaces form a structure known as biofilm, showing many specific morphological and physiological characteristics in comparison to planktonic cells (O'Toole et al., 2000). One of the most important specificities of the attached cells is up to 1000 times higher resistance to antimicrobial agents, primarily as the result of slow growth and presence of impermeable exopolysaccharides on bacterial surface (Gilbert et al., 1997).

P. aeruginosa is a species able to form biofilms on different abiotic surfaces, including artificial implants, contact lenses, urinary catheters and endotracheal tubes (reviewed in Davey and O'Toole, 2000). On the other hand, as an opportunistic pathogen, *P. aeruginosa* can cause various infections, which are difficult to treat with conventional antibiotics. Some of the infections, such as periodonitis, prostatitis and infections of patients with cystic fibrosis are considered to be in connection with bacterial attached mode of growth on the biotic surfaces (Hanlon, 2007; Lyczak et al., 2002). The biofilm forming *P. aeruginosa* produces a great amount of alginate — a linear polymanuronic–polyguluronic acid heteropolysaccharide (Linker and Johns, 1966) which binds bacteria together, plays a role in forming microcolonies (Lam et al., 1980), and provides protection against unfavorable environmental factors, such as antimicrobials and humoral and cellular host defenses (Allison and Matthews, 1992; Abdi-Ali et al., 2006). Considering these facts, it seems useful to examine alternative solutions for degrading EPSs and controlling *P. aeruginosa* biofilms.

In the last few years, the emergence of antibiotic resistant bacteria has increased interest in phage therapy. Phages have been examined to a lesser extent as potential agents for biofilm control (Doolittle et al., 1995; Hughes et al., 1998a; Hughes et al., 1998b; Hanlon et al., 2001; Sutherland et al., 2004; Hanlon, 2007). The main advantage of phage application is their ability to degrade exopolysacharides by enzymes attached to their baseplate — polysaccharide depolymerases and lyases, important for penetration into a host cell (Sutherland et al., 2004; Hughes et al., 1998b). The starting point for studying phages and/or their enzymes in biofilm control is their isolation and determination of potency to eliminate or inhibit biofilm formation. At the moment, there is only one method that allows comparison of different phages effect on bacterial growth in microplate format (McLaughlin, 2007), several microtiter plate methods for biofilm quantification (Christensen et al., 1985; O'Toole and Kolter, 1998; Stepanovic et al., 2000) and no microtiter plate method providing comparison of phages' effect on biofilm. The primary aim of the study is to establish a microtiter plate method for evaluation of phage effect on *P. aeruginosa* biofilm formation and removal, based on a combination of the above methods. The second aim is to apply the tests for assessing of isolated *P. aeruginosa* phages efficacy on biofilm removal, as well as to modify McLaughlin method (2007) for pseudomonas phage testing and

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compare the results with the results of biofilm inhibition and eradication tests.

Two different strains of *P. aeruginosa* were used in the study. The first strain, designated as PA-4u, was isolated from environmental biofilm using a selective medium for *P. aeruginosa* (Cetrimide agar, Serva Co., Feinbiochemica, Heidelberg, Germany). Confirmation of the species was based on morphological, tinctorial and biochemical characteristics (Gram staining, oxidase test, gelatin liquefaction, production of soluble pigment and nitrate utilization). The second strain was the reference strain *P. aeruginosa* ATCC 9027. Both strains were able to form a thick, compact biofilm when cultivated in Luria Bertain (LB) broth with glucose addition (Tryptone 10 g/l; yeast extract 5 g/l; NaCl 10 g/l; glucose 5 g/l; pH 7.5).

Four lytic bacteriophages were isolated from different samples of municipal wastewater and designated as π-1, π-2, π-3 and π-4. Briefly, the samples were filtered through membrane filters (0.45 μm, Sartorius Co., Goettingen, Germany) and filtrate was added in double strengthen LB broth (v/v 1:1) inoculated with 100 μl of host strain PA-4u. After enrichment (24 h at 35 °C and 120 rpm), the phages were isolated by the overlay agar method, picking a plaque by a sterile loop (Carlson, 2005). Each phage was reisolated three times and phage stocks were prepared by plate lysis and elution using SM buffer (50 mM Tris HCl [pH 7.5]; 0.1 M NaCl; 8 mM $MgSO_4$; 0.01% w/v gelatin). The phage suspension was purified and concentrated by centrifugation (11,000×g for 10 min at 4 °C), filtration (Minisart 0.2 μm, Sartorius Biotech BmbH, Goettingen, Germany), precipitation in NaCl and PEG6000 (Merck, Hohenbrunn, Germany) and ultracentrifugation in discontinuous glycerol gradient (110,000×g for 1 h at 4 °C) (Sambrook and Russell, 2001). Plaque forming units per milliliter (PFU/ml) in the stocks were preliminary determined by the spot method, followed by overlay agar method for more precise count assessment (Carlson, 2005). The phage stocks were stored at 4±1 °C and all dilutions of the original stocks were made in SM buffer with gelatin.

Prior to the experiments, *P. aeruginosa* PA-4u and ATCC 9027 lawn were prepared by the overlay agar method, and 10 μl of each phage suspension (1×10^8 PFU/ml) was placed on the surface of semisolid medium in order to determine lytic spectra of the phages.

Bacterial growth inhibition assay was carried out using a modified method developed by McLaughlin (2007). The modification included the following: triplicate application of each phage and bacterial count combination at the same microtiter plate, the reduction of incubation period after 2,3,5-triphenyltetrazolium chloride (TTC) addition, the use of double strengthen LB broth, and the reading absorbance at different wavelength. Briefly, bacterial cultures in LB broth were incubated until reached OD_{650} 0.4 (approximately 2×10^9 CFU/ml) and used to inoculate double strengthen LB broth supplemented with 1% of glucose. Sterile 96-well microtiter plates with flat bottom (Spektar Cacak, Serbia) were filled with 100 μl of inoculated medium in such a way to get 1×10^2 CFU/well in the columns 1–3, 1×10^4 in columns 4–6, 1×10^6 in columns 7–9 and 1×10^8 CFU/well in the last three columns (10–12), with the exception of the wells H1, H4, H7 and H10 (Fig. 1A). These four wells did not contain bacteria and were filled with 100 μl of sterile double strengthen LB broth with glucose and 100 μl of sterile SM buffer. The other wells of the row H were filled additionally with 100 μl of sterile SM buffer (controls with appropriate bacterial CFU/well without phage). Appropriate phage dilutions in SM buffer (100 μl) were added into the wells of rows from A to G in such a way to get 1×10^1–1×10^7 PFU/well, respectively. The microtiter plates were incubated in a thermostat with a water container (to elevate moisture level) at 35 °C overnight with shaking (120 rpm). After incubation, 50 μl of 0.1% filter-sterilized TTC (Sigma Chemical Co., St. Louis, MO, USA) was added into each well (final concentration 200 μg/ml, i.e. 50 μg/well) and incubated for additional 3 h. The absorbance was read at 540 nm using a microtiter plate reader (Multiskan EX, Thermo-Labsystem, Vantaa, Finland).

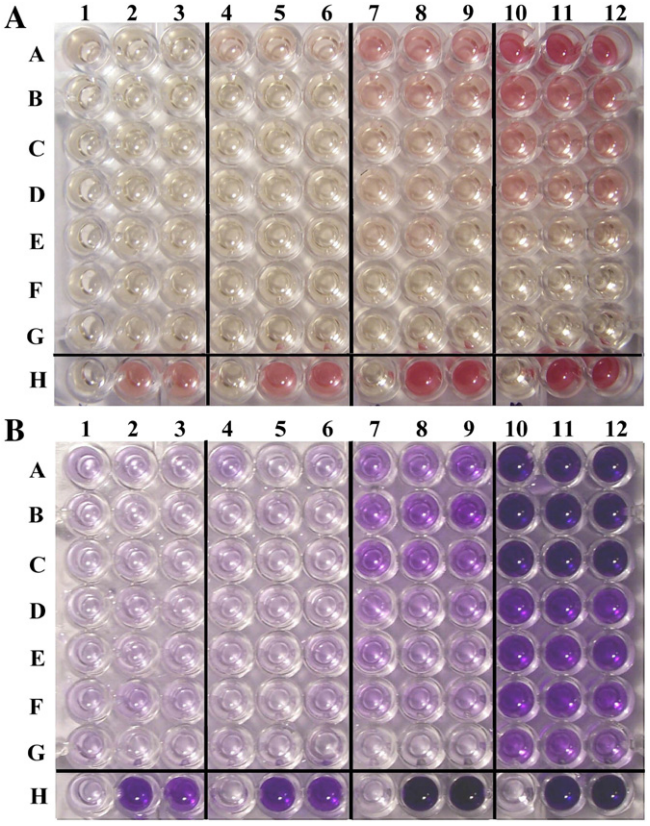

Fig. 1. Colorimetric microplate test of phage π-4 effect on *P. aeruginosa* PA-4u growth (A) and biofilm formation (B). Row H is a control (H1, H4, H7 and H10 are controls without bacteria and phages; the others are controls without phages — H2 and H3 contains 1×10^2 CFU/well of bacteria; H5 and H6 1×10^4 CFU/well; H8 and H9 1×10^6 CFU/well and H11 and H12 1×10^8 CFU/well). Rows from A to G contain from 1×10^1 to 1×10^7 PFU/well of phage π-4, respectively. Columns 1–3 contain 1×10^2 CFU/well of PA-4u; columns 4–6 1×10^4 CFU/well; 7–9 1×10^6 CFU/well and 10–12 1×10^8 CFU/well.

For the study of bacteriophage effect on biofilm formation, the plates were filled and incubated in the same way as in the previous experiment (Fig. 1B). After overnight incubation, liquid content with planktonic cells was removed from the wells and each well was washed twice with 250 μl of PBS and left to dry. Attached bacterial cells were fixed with 250 μl of absolute methanol for 15 min. The fixative was removed and the plates were air-dried. Into each well, 200 μl of 0.4% crystal violet was added and after 15 min stain was removed. The plates were washed by stream of tap water in order to remove excessive amount of the stain and left to dry. Into each well of dried plates, 250 μl of 33% acetic acid was added and left for 20 min to allow stain to dissolve. The absorbance was measured at 595 nm using the microtiter plate reader.

In order to determine phage effect on already formed biofilm, i.e. on its removal, the microtiter plates were filled with 100 μl of inoculated double strengthen medium as in the previous experiment, but instead of phages suspension in SM buffer, 100 μl of sterile SM buffer was added into each well. Consequently, the volume in each well, composition of the medium and bacterial CFU/well were the same as in the previous two experiments. The plates were incubated overnight to allow biofilm formation. When biofilm was formed after 24 hours, planktonic bacteria were removed and plates were washed once with PBS. The plates were left to dry for 10 min and then the wells were filled with 100 μl of appropriate phage dilution (as in previous cases) and with 100 μl of sterile double strengthen LB broth with glucose (to maintain the same experimental conditions). After incubation (for 24 h at 35 °C and 120 rpm), the plates were washed and stained as described above.