

## EFFECTS OF P22 BACTERIOPHAGE ON *SALMONELLA ENTERICA* SUBSP. *ENTERICA* SEROVAR TYPHIMURIUM DMC4 STRAIN BIOFILM FORMATION AND ERADICATION

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**Abstract:** Over the last decades, several antimicrobial agents have been made available. Due to increasing antimicrobial resistance, bacteriophages were rediscovered for their potential applications against bacterial infections. In the present study, biofilm inhibition and eradication of *Salmonella enterica* subsp. *enterica* serovar Typhimurium DMC4 strain (*S. Typhimurium*) was evaluated with respect to different incubation periods at different P22 phage titrations. The efficacy of P22 phage on biofilm formation and eradication of *S. Typhimurium* DMC4 strain was screened *in vitro* on polystyrene and stainless steel surfaces. The biofilm forming capacity of *S. Typhimurium* was significantly reduced at higher phage titrations ( $10^6$  pfu/mL  $\leq$ ). All phage titers ( $10^4$ - $10^8$  pfu/mL) were found to be effective at the end of the 24 h-incubation period whereas higher phage titrations were found to be effective at the end of the 48 h and 72 h of incubation. P22 phage has less efficacy on already formed, especially mature biofilms (72 h-old biofilm). Notable results of P22 phage treatment on *S. Typhimurium* biofilm suggest that P22 phage has potential uses in food systems.

**Key words:** *Salmonella*; biofilm; bacterial adhesion; bacteriophage; eradication

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### INTRODUCTION

The enteric pathogen *Salmonella* Typhimurium causes gastroenteritis in humans and other mammals. *S. Typhimurium* serovar, along with Enteritidis, Virchow and Hadar serovars, is one of the most common serovars isolated from humans in developing countries. It has a zoonotic origin and can exhibit resistance to commonly used antibiotics (Philips et al., 2004). Moreover, many *Salmonella* strains are able to form complex surface-associated communities, called biofilms. Biofilms contribute to the resistance and persistence of *Salmonella* in both host and non-host environments and are especially important in food processing environments and poultry (Steenackers et al., 2012; Diez-Garcia et al., 2012). Biofilms are multicellular cell aggregates that are encased in a self-produced extracellular matrix (Branda et al., 2005; Hall-Stoodley and Stoodley, 2009). Furthermore, it is known that approximately 80% of all bac-

terial infections are related to biofilms (Davies, 2003; Hall-Stoodley and Stoodley, 2009).

Many scientists in the last decades have focused their research on designing novel biofilm treatment strategies. Well-known antimicrobial treatment strategies may be mostly inefficient in eradicating biofilms (Davies, 2003). There are three major strategies to treat biofilms: prevention of biofilm formation, removal/killing of biofilm and weakening of biofilm (Alhede et al., 2011).

Bacteriophages, as an antimicrobial tool against biofilms, are preferred tools for treating biofilm infections *in vivo* and eradicating existing biofilms in non-host environments. Although application of bacteriophages against bacterial infections is an old approach used prior to the discovery and widespread use of antibiotics. However, it has a limited clinical use. Over the last decades, due to the emergence of pathogenic

bacteria resistant to the most currently available antimicrobial agents, bacteriophages were rediscovered for their potential applications in the eradication of bacterial infections (Sulakvelidze et al., 2001).

The utilization of bacteriophages to fight against bacterial biofilms has become a novel strategy. Bacteriophages can be used efficiently against biofilm infections and in *in vitro* biofilm setups. Many reports have shown that bacteriophages have a widespread use to reduce biofilm formation and to eradicate already formed biofilms. It has been also proved that bacteriophages can reduce biofilm formation *in vivo*. *In vitro* biofilms of *Streptococcus epidermidis* on silicone catheters can be reduced effectively when the catheter is pretreated with a lytic bacteriophage (Curtin and Donlan, 2006). Lytic bacteriophage treatments showed hopeful results on septicemic mouse infected with the multi-drug-resistant *Pseudomonas aeruginosa* and *Klebsiella pneumonia* (Vinodkumar et al., 2005; Vinodkumar et al., 2008). There are also several reports about phage and biofilm interactions for many bacteria such as *Bacillus subtilis* (O'Flaherty et al., 2009), *Escherichia coli* (Corbin et al., 2001), *Enterococcus faecalis* (Teng et al., 2009), *Lactococcus lactis* (Briandet et al., 2008), *Listeria monocytogenes* (Hibma et al., 1997), *Pseudomonas fluorescens* (Del Pozo and Patel, 2007) and *Staphylococcus aureus* (Resch et al., 2005).

P22 phage infects *S. Typhimurium* via binding to the O-antigen lipopolysaccharide on the surface of the host. The phage's tail fiber protein has endorhamnosidase activity, which cleaves the O-antigen chain (Prevelige, 2006). P22 is a temperent phage and it can be used as a perfect transduction tool for genetic studies of *S. Typhimurium*. In this study, we aimed to evaluate the prevention and eradication potentials of P22 phage on *S. Typhimurium* biofilm.

## MATERIALS AND METHODS

### Bacterial strains and growth conditions

*S. Typhimurium* DMC4 strain isolated from a beef product in Turkey was obtained from the Ankara University Prokaryotic Genetics Laboratory culture

collection. The culture was inoculated into Luria-Bertani broth and incubated at 37°C for 18 h with shaking at 200 rpm. LB without NaCl (LB<sup>wo</sup>/NaCl; bacto-tryptone 10 g/L (Fluka, Taufkirchen, Germany), yeast extract 5 g/L (Merck, Darmstadt, Germany)) was used to maintain optimum conditions for biofilm assays (Römling et al., 1998).

### Preparation of P22 phage lysates

Preparation and titration of P22 phage were performed as described by Davis et al. (1980). Briefly, 1 mL overnight culture of *S. Typhimurium* DMC4 strain was added to 4 mL P22 transduction broth (transduction broth; 2 mL 50x medium (sterilized with CHCl<sub>3</sub>), 1 mL sterile 20% glucose, 0.1 mL P22 lysate (sterilized with CHCl<sub>3</sub>); the final volume was adjusted to 100 mL by the addition of sterile LB broth. This suspension was incubated for 18 h at 37°C with shaking at 200 rpm. The culture was transferred into microfuge tube and centrifuged for 2 min at 12000 rpm to pellet the cell debris. Finally, several drops of CHCl<sub>3</sub> (Merck, Darmstadt, Germany) were added to the lysate. The lysate was stored for a long time at 4°C. To check the titrations of phage lysates, LB soft agar was melted in a microwave and cooled in a 50°C heating block. Then 0.1 mL of overnight culture of *S. Typhimurium* DMC4 strain was added to melted soft agar and poured immediately onto LB agar plates. Finally, 20 µL of appropriate phage dilutions were spotted onto the culture lawn (from 10<sup>4</sup> to 10<sup>9</sup> phage dilutions in sterile 0.85% NaCl).

### Biofilm sampling for microtiter plate assay and stainless steel application

The microtiter plate assay was based on the method described by Woodward et al. (2002) with some modifications of Stepanovic et al. (2000). The cultures were diluted in LB without NaCl broth to OD<sub>595nm</sub> = 0.2 (~10<sup>8</sup> CFU/mL), and 30 µL of this suspension was transferred to each well of 96-well polystyrene microtiter plates filled with 100 µL LB without NaCl broth, and then plates were incubated statically for 24 h, 48 h and 72 h at 20°C. Control wells were filled with

the test broth only. Following the incubation, plates were washed twice with sterile 0.85% NaCl solution to remove planktonic cells. The plates were allowed to dry under a safety hood. Following this step, 130  $\mu$ L of 98% methanol was added (Merck, Darmstadt, Germany) and the plates were incubated for 10 min. The methanol was removed and plates were dried again. One hundred thirty  $\mu$ L of 1% crystal violet (Merck, Darmstadt, Germany) was transferred into the wells and stained for 30 min. After staining, plates were washed under running tap water. Finally, 130  $\mu$ L of 33% glacial acetic acid (Sigma-Aldrich, Taufkirchen, Germany) was added to each well and bound dye was solubilized. The OD of each well was measured at 595 nm (Bio-Rad, USA). The results for the tested strain were calculated by subtracting the median OD<sub>595nm</sub> of the three parallels of the control (LB without NaCl broth only) from the median OD<sub>595nm</sub> of the three parallels of sample.

Stainless steel coupons (type 304) were chosen for screening biofilm production since stainless steel is frequently used for food-processing environments. The coupons (0.1, 0.8 and 2.5 cm) were prepared for treatment assays as previously described by Giaouris et al. (2005). Coupons were placed individually in 3.5 mL of LB without NaCl broth media, and test tubes with the coupons were autoclaved at 121°C for 15 min. Overnight cultures were adjusted to OD<sub>595nm</sub> of 0.2 ( $\sim 10^8$  CFU/mL), and 1 mL of these suspensions were inoculated into test tubes. The test tubes were incubated for 24, 48 and 72 h at 20°C. Following incubation, each coupon was taken with sterile forceps, transferred to an empty sterile tube and allowed to dry at room temperature for 5 min. The coupons were rinsed twice with 5 mL of 0.85% sterile physiological saline solution to remove planktonic counterparts. After washing, each coupon was transferred to a new tube containing 4.5 mL of sterile physiological saline solution and five sterile glass beads (3 mm in diameter). The coupons were then vortexed for 1 min at maximum intensity (IKA Genius Vortex 3, Staufen, Germany).

Quantification of biofilm cells was performed using the agar plating method. Colonies on LB agar plates were counted after incubation at 37°C for 18 h.

The count of CFU (colony forming unit) per square centimeter was transformed to a logarithmic value.

### **Evaluation of P22 phage on microbial adhesion and biofilm formation (phage treatment)**

*S. Typhimurium* DMC4 strain was cultured with P22 phage to assess the inhibition potential of P22 phage on biofilm formation. The 96-well polystyrene microtiter plates were filled with the appropriate phage suspensions and control wells were filled with the test broth and inoculum only. Titration of phage suspensions were adjusted to  $10^4$ - $10^8$  PFU/mL using test medium (LB without NaCl) to maintain the same experimental conditions of biofilm sampling. The assay was performed as described above. The plates were incubated for 24, 48 and 72 at 20°C. The biofilm reduction percentages were calculated according to the following formula  $[(C-B)-(T-B)] / [(C-B)] \times 100$  where C = control wells containing only strain inoculum, B = blank wells containing test medium and appropriate phage dilution without strain inoculum, and T = treatment wells containing strain inoculum and appropriate phage dilution.

To perform stainless steel application, test tubes containing sterile stainless steel coupons were filled with appropriate phage suspensions adjusted to  $10^4$ - $10^8$  PFU/mL in test medium. The control tubes were filled with only test medium. The test tubes were incubated for 24, 48 and 72 h at 20°C. The assay was performed as described previously. The logarithmic reduction of biofilm cells was calculated according to the following formula:  $[PK: (1 - 10^{-LR}) \times 100\%]$  where PK is the percentage killing, LR is the log reduction  $[\log_{10}(\text{untreated viable cell density}) - \log_{10}(\text{treated viable cell density})]$ .

### **Evaluation of P22 phage on already formed biofilms (phage post-treatment)**

Twenty-four-h, 48-h and 72-h-old biofilm samples were prepared according to the method described above to assess the eradication potential of P22 phage. Already formed biofilm samples on microtiter plate

wells and stainless steel coupons were treated with appropriate phage dilutions ( $10^4$ - $10^8$  PFU/mL). The negative control samples were prepared only with test medium. Following incubation at 37°C for 24 h to maintain the optimum phage infection conditions, experimental data were obtained as described above. Finally, the values obtained from experiments were compared to evaluate the biofilm eradication potential of P22 phage. The percentage of biofilm eradication was calculated by the same formulas given above for both microtiter plate and stainless steel assays.

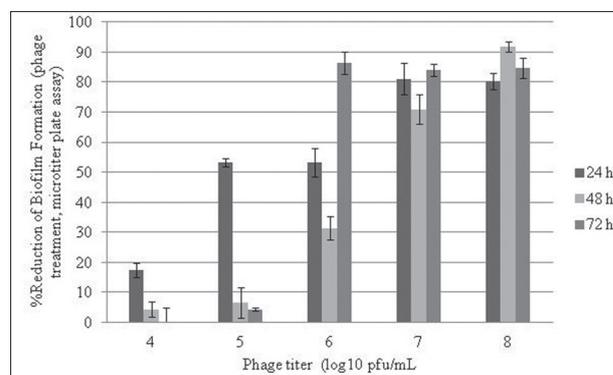
### Statistical analysis

All statistical analyses were performed by Minitab statistics software, version 15 (USA). Experiments were performed in triplicate. Bars were given as mean standard deviation values. The one-way ANOVA test was preferred for all mean values associated with data from the microtiter plate and stainless steel assays. Pearson's correlation was used to analyze the differences in biofilm production on polystyrene and stainless steel surfaces.

## RESULTS

### Effect of P22 phage on bacterial adhesion and biofilm forming (phage treatment)

To evaluate the efficacy of P22 phage on the inhibition of bacterial adhesion and biofilm formation, phage treatment assays were performed using *S. Typhimurium* DMC4 strain for different incubation periods (24, 48 and 72 h) and two types of abiotic surface (polystyrene and stainless steel). Many of the *S. Typhimurium* strains were screened for their biofilm formation capabilities, and the DMC4 strain was selected as a good biofilm producer in further experiments (data not shown). Ninety-six-well polystyrene microtiter plates and stainless steel surfaces to evaluate the biofilm-producing abilities of *S. Typhimurium* DMC4 strain were preferred due to their common usage in food manufacturing and processing environments. To detect the effects of P22 phage on the biofilm-producing cells of *S. Typhimurium* DMC4 strain, microtiter plates



**Fig. 1.** Effect of phage treatment on biofilm formation during 24-, 48- and 72-h incubation periods and the reduction percentages of biofilm formation (microtiter plate assay) (Bars represent standard deviation).

were incubated with appropriate phage dilutions for 24, 48 and 72 h. The effects of phage treatment on biofilm formation are summarized as a percentage of biofilm reduction in Fig. 1. All phage titrations ( $10^4$ - $10^8$  PFU/mL) were found only to be effective at the end of the 24-h and 48-h incubation periods, whereas higher phage titrations were only found to be effective at the end of the 72-h incubation period ( $10^6$  PFU/mL  $\leq$ ). Means of biofilm quantities were compared by one-way ANOVA;  $p < 0.05$ . As regards the microtiter plate assay, the 48-h incubation period and the highest phage titrations ( $10^8$  PFU/mL) were detected as the most effective conditions for the prevention of biofilm formation (Fig. 1).

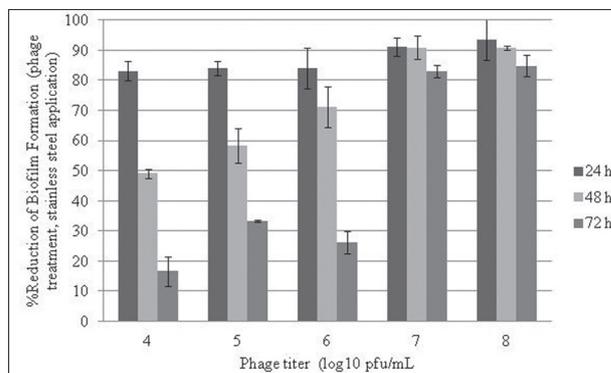
Results obtained from the stainless steel assay were not similar with microtiter plate assay results. Results are shown in Fig. 2. The effect of P22 phage on biofilm reduction of the tested strain on stainless steel surfaces was found to be much more effective in comparison to the microtiter plate assay results. A negative correlation was detected between the rates of biofilm quantity on polystyrene microtiter plates and stainless steel surfaces ( $r = -0.627$  by Pearson correlation,  $p < 0.01$ ). However, due to the long incubation periods, the phage effect was significantly reduced, similarly to the microtiter plate assay. Significant biofilm reduction was detected only for higher phage titrations ( $10^7 \leq$  PFU/mL) at the end of 72-h incubation period. The values obtained from these experiments

were converted to log reduction percentage (Fig. 2). P22 phage could not easily prevent biofilm formation after the 24-h and 48-h-old incubation periods and at lower phage titration (Fig. 2).

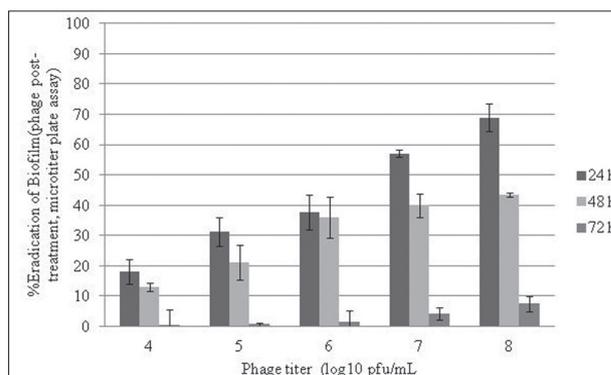
### Effect of P22 phage on already formed biofilms (phage post-treatment)

The removal activity of P22 phage on already formed 24-, 48-, and 72-h-old biofilms was evaluated by microtiter plate assay and stainless steel application. First the microtiter plate assay was performed to detect the removal of biomass. The 24-, 48-, and 72-h-old biofilms in plate wells were post-treated with appropriate phage dilutions ( $10^4$ - $10^8$  PFU/mL) and incubated at 37°C for 24 h. The results are given in Fig. 3. Biomass removal activities of 57.14% and 68.99% of P22 phage were detected for the 24-h-old biofilm at phage titrations of  $10^7$  and  $10^8$  PFU/mL, respectively. In the 48-h-old biofilms, the phage titrations of  $10^7$  and  $10^8$  PFU/mL resulted in 39.97% and 43.36% of biomass removal activity, respectively (one-way ANOVA,  $p < 0.05$ ). Apart from higher phage titrations such as  $10^7$  and  $10^8$  pfu/mL, the eradication capability of P22 phage was found to be less effective on the 48-h-old biofilm structure (the samples on polystyrene surfaces) at lower phage titrations (Fig. 3). The least removal efficiency was detected for the 72-h-old biofilm structure by phage post-treatment (0.54%, 0.78%, 1.5%, 4.37% and 7.56% eradication for  $10^4$ ,  $10^5$ ,  $10^6$ ,  $10^7$  and  $10^8$  PFU/mL phage titrations, respectively) (one-way ANOVA,  $p < 0.05$ ).

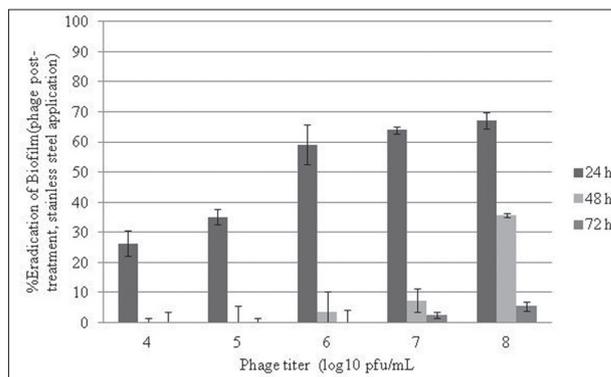
P22 phage showed less eradication capacity against 24- and 48-h-old biofilms on stainless steel coupons compared to microtiter plate surfaces. Similar to microtiter plate assay results, phage efficiency was dramatically decreased for the 72-h-old biofilm samples (samples on stainless steel surfaces) (no eradication; 0% was detected for  $10^4$ ,  $10^5$ ,  $10^6$  PFU/mL titrations) (Fig. 4). Crystal violet staining was also performed for stainless steel surfaces to confirm the removal activity of P22 phage. As a result, decreased crystal violet staining was observed on stainless steel coupons, indicating that P22 phage has biofilm eradication activity.



**Fig. 2.** Effect of phage treatment on biofilm formation during 24-, 48- and 72-h incubation periods and the reduction percentages of biofilm formation (stainless steel application) (Bars represent standard deviation).



**Fig. 3.** Effect of phage post-treatment on already formed 24-, 48- and 72-h-old biofilm samples and the eradication percentages of biofilm (microtiter plate assay) (Bars represent standard deviation).



**Fig. 4.** Effect of phage post-treatment on already formed 24-, 48-, 72-h-old biofilm samples and the eradication percentages of biofilm (stainless steel application) (Bars represent standard deviation).

## DISCUSSION

This study has shown that P22 phage is able to infect *S. Typhimurium* biofilm and has an eradication potential on already formed biofilm of tested *S. Typhimurium* strain. P22 infection begins via binding of the gp9 tail spike protein of phage to the O-antigen lipopolysaccharide exposed on the surface of the host. This tail fiber protein has endorhamnosidase activity, which cleaves to the O-antigen chain (Prevelige, 2006). It is well known that *Salmonella* produces an O-antigen capsule coregulated with the fimbria and cellulose-associated extracellular matrix (Gibson et al., 2006). Gram-negative bacteria typically consist of surface exposed O-antigen, a core structure that is embedded in the outer membrane lipid bilayer. This study may reveal that the infection of *S. Typhimurium* biofilm by P22 phage begins via the trapping of phages by cells that are in the outer layers of the biofilm and also phages can infect planktonic bacteria that could go on to form a biofilm. The results of the present study suggest that although the phage treatment of two abiotic surfaces can effectively reduce the formation of biofilm, it is less effective in eradicating already formed biofilms. This phenomenon indicates that P22 phage may have a notable potential to inhibit *S. Typhimurium* biofilm formation, especially in food and clinical environments.

Many researches showed that significant biofilm reductions (ranging from 1 to 6 log), depend upon the constituents of the biofilm, biofilm age, phage selection and duration of treatment (Corbin et al., 2001; Sharma et al., 2005). It was found that treatment with P22 phage was much more effective in inhibiting biofilm formation (ranging from 0.08 to 1.2 log<sub>10</sub>) than eradicating post-treated mature biofilm (from 0 to 0.5 log<sub>10</sub>). The duration of the phage treatment and the age of biofilm are critical to the inhibition of biofilm formation and eradication of mature biofilms of *S. Typhimurium*. When phage treatment is extended, biofilm formation is completed in the late incubation period due to the decrement of phage titration or a possible increasing of phage resistance. We also checked the effectiveness of phage infection capability on planktonic cultures of the strain in the

exponential phase. Therefore, phage resistance may increase or there might be a lack of phage penetration due to the long-term incubation periods and biofilm matrix (data not shown). This phenomenon should be examined to illuminate the phage resistance in *S. Typhimurium* biofilms for further studies. P22 phage has some potential to develop a control strategy for the biofilm formation of *S. Typhimurium*. However, it has less effectiveness in the eradication of mature biofilms. Hence, it might be used in future studies as a contributing agent for effective phage cocktails that can eradicate mixed biofilm formations and can be combined with different antimicrobial agents to treat biofilm formations.

There are also some reports showing that phage predation can enhance the formation of the biofilm. Hosseinidoust et al. (2013) discovered that single-species biofilm formation of *Pseudomonas aeruginosa* can be enhanced with the treatment of different phages. However, in our study, the induction of biofilm formation after the P22 phage challenge was not observed. Furthermore, P22 phage can be a good candidate to study phage and *S. Typhimurium* biofilm interactions. In this respect, phage-induced biofilm dispersion models should be studied to reveal the roles of lysogens in biofilms.

*Salmonella* strains can cause serious problems due to cross contamination in food environments. Discovering novel antimicrobial agents, such as bacteriophages, for the foodborne pathogens, especially for disinfectant- and antibiotic-resistant strains, is a current topic. P22 phage could be a potential alternative for eliminating *S. Typhimurium* biofilms in food systems, and it might be used as a supplement with other effective phage cocktails and disinfectants. The results of this study are also important for providing initial data to unveil P22 phage/*S. Typhimurium* biofilm interactions. Finally, it was concluded that P22 phage might have possible critical characteristics to control *S. Typhimurium* biofilms.

**Authors' contribution:** Basar Karaca designed the study, performed experiments and wrote the manuscript. Nefise

Akcelik and Mustafa Akcelik analyzed the data and provided the reagents, material and all laboratory equipment.

**Conflict of interest disclosure:** The authors declare that they have no competing interests.

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