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Research Note

In Vitro Assessment of the Susceptibility of Planktonic and Attached Cells of Foodborne Pathogens to Bacteriophage P22-Mediated *Salmonella* Lysates

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ABSTRACT

This study was designed to evaluate the lytic activity of bacteriophage P22 against *Salmonella* Typhimurium ATCC 19585 (*Salmonella* Typhimurium P22$^-$) at various multiplicities of infections (MOIs), the susceptibility of preattached *Salmonella* cells against bacteriophage P22, and the effect of P22-mediated bacterial lysates (extracellular DNA) on the attachment ability of *Listeria monocytogenes* ATCC 7644 and enterohemorrhagic *Escherichia coli* ATCC 700927 to surfaces. The numbers of attached *Salmonella* Typhimurium P22$^-$ cells were effectively reduced to below the detection limit (1 log CFU/ml) at the fixed inoculum levels of 3×10^2 CFU/ml (MOI = 3.12) and 3×10^3 CFU/ml (MOI = 4.12) by bacteriophage P22. The attached *Salmonella* Typhimurium P22$^-$ cells remained more than 2 log CFU/ml, with increasing inoculum levels from 3×10^4 to 3×10^7 CFU/ml infected with 4×10^8 PFU/ml of P22. The number of preattached *Salmonella* Typhimurium P22$^-$ cells was noticeably reduced by 2.72 log in the presence of P22. The highest specific attachment ability values for *Salmonella* Typhimurium P22$^-$, *Salmonella* Typhimurium ATCC 23555 carrying P22 prophage (*Salmonella* Typhimurium P22$^+$), *L. monocytogenes,* and enterohemorrhagic *E. coli* were 2.09, 1.06, 1.86, and 1.08, respectively, in the bacteriophage-mediated cell-free supernatants (CFS) containing high amounts of extracellular DNA. These results suggest that bacteriophages could potentially be used to effectively eliminate planktonic and preattached *Salmonella* Typhimurium P22$^-$ cells with increasing MOI. However, further research is needed to understand the role of bacteriophage-induced lysates in bacterial attachment, which can provide useful information for the therapeutic use of bacteriophage in the food system.

Bacterial biofilms are commonly found in the natural ecosystem and frequently formed on implanted medical devices and surfaces in food processing *(27, 32)*. The formation of biofilms is well recognized as a bacterial survival strategy against unfavorable conditions, including environmental and metabolic stresses. These surface-attached bacterial communities are highly structured within a matrix of an extracellular polymeric substance composed of polysaccharides, lipids, nucleic acids, and proteins *(7, 9, 12, 33)*. The outer layers of biofilm provide a barrier to penetration of antimicrobial molecules, and the inner layers of biofilms can enter a dormant state of low metabolic activity, leading to significantly increased antibiotic resistance *(7, 15, 31)*. The biofilms formed in food processing facilities and on food products can require the frequent use of antimicrobials or antibiotics, resulting in a significant loss of revenue. In the last few decades, biofilm-associated foodborne diseases have become important emerging public health concerns *(2, 6, 21, 29)*.

Bacteriophage therapy has received much attention with the increased appearance of multidrug-resistant bacteria *(4, 16, 17, 26)*. A bacteriophage can be a potential alternative for the treatment of stress-resistant biofilms because of its specific target-binding property with no side effects on beneficial bacteria or eukaryotic cells *(20, 24, 28, 30)*. Lytic bacteriophage can specifically and effectively lyse target bacteria regardless of their stress resistance *(14)*. The bacteriophage-mediated lysis can cause the release of bacterial cell components such as extracellular DNA (eDNA) *(5, 12, 14, 22)*. The extracellular polymeric substance is known to be a major biofilm-promoting factor. This raises the question as to whether the release of eDNA resulting from lytic phage-mediated lysis plays a key role in biofilm formation by different bacterial species. Relatively few studies have directly focused on the effect of bacteriophage-mediated bacterial lysates on biofilm formation by host and nonhost pathogens. Understanding bacteriophage-biofilm interactions is essential for the development of effective phage therapy for biofilm-associated contamination in the food system. Therefore, the objectives of this study were to evaluate (i) bacteriophage P22-mediated lysis of *Salmonella enterica* serovar Typhimurium ATCC 19585 (*Salmonella* Typhimurium P22$^-$) at different multiplicities of infection (MOIs); (ii) bacteriophage P22 susceptibility of preformed *Salmonella* Typhimurium P22$^-$ and *Salmonella*

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Typhimurium ATCC 23555 carrying P22 prophage (*Salmonella* Typhimurium P22$^+$) attached cells; and (iii) the effect of P22-mediated bacterial lysates on the ability of nonhost bacterial pathogens (*L. monocytogenes* ATCC 7644 and enterohemorrhagic *Escherichia coli* ATCC 700927) to attach to surfaces.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Strains of *Salmonella enterica* serovar Typhimurium ATCC 19585 (*Salmonella* Typhimurium P22$^-$), *Salmonella* Typhimurium ATCC 23555 carrying P22 prophage (*Salmonella* Typhimurium P22$^+$), *L. monocytogenes* ATCC 7644, and enterohemorrhagic *E. coli* EDL 933 ATCC 700927 were purchased from the American Type Culture Collection (Manassas, VA). The strains were cultured in Trypticase soy broth (TSB; BD, Sparks, MD) at 37°C for 20 h. All cultures were centrifuged at 3,000 × *g* for 20 min at 4°C. The pellet was resuspended and diluted to 10^6 CFU/ml with 0.1% sterile buffered peptone water (BPW, HiMedia Laboratory, Mumbai, India).

Bacteriophage propagation and plaque assay. Bacteriophage P22 (ATCC 97540) was propagated in TSB containing its host strain (*Salmonella* Typhimurium ATCC 19585). After 24 h incubation at 37°C, the culture was centrifuged at 3,000 × *g* for 20 min and filtered using a 0.2-μm-pore-size sterilized filter to remove remaining bacterial cells and debris. The phage titer of supernatant was determined by a soft-agar overlay method *(1)*. The filtrate was serially diluted (1:10) with phosphate-buffered saline (PBS). Each decimal dilution was transferred onto the surface of prewarmed base agar (37°C) and mixed with 5 ml of 0.7% soft agar (45°C) in TSB containing the host *Salmonella* Typhimurium ATCC P22$^-$. After the soft agar solidified, the plates were incubated for 24 h at 37°C to enumerate free bacteriophage P22 expressed as PFU. The bacteriophage stock (10^{10} PFU/ml) was stored at 4°C prior to use.

Attachment ability assay. The assay for attachment ability was conducted in a static model system using a 96-well flat-bottomed polystyrene microtiter plate (BD Falcon; Durham, NC). *Salmonella* Typhimurium ATCC 19585 cells were inoculated into TSB at 3×10^2, 3×10^3, 3×10^4, 3×10^5, 3×10^6, and 3×10^7 CFU/ml, each of which was infected with bacteriophage P22 at the levels of 4×10^3, 4×10^4, 4×10^5, 4×10^6, 4×10^7, and 4×10^8 PFU/ml, showing different MOIs. MOIs were calculated by dividing the number of bacteriophages P22 infected (N_P) by the number of *Salmonella* Typhimurium ATCC 19585 cells (N_H). After 24 h of incubation at 37°C, the cell suspensions were collected to determine the planktonic growth at 570 nm using a Multiskan FC microplate reader (Thermo Scientific Inc., Rochester, NY). The surface-attached cells were estimated to assess the attachment ability of *Salmonella* Typhimurium ATCC 19585.

Enumeration of attached cells. The attached cells were counted by using the spread plate method. Each well of 96-well microtiter plates was washed twice with 0.1% buffered peptone water to eliminate loosely attached cells. The attached cell layer was rubbed with a sterile cotton swab, which was immersed and vortexed vigorously with 1 ml of 0.1% buffered peptone water. Each decimal dilution (0.1 ml) was spread plated in duplicate on Trypticase soy agar and incubated at 37°C for 48 h.

Bacteriophage susceptibility of preattached cells. *Salmonella* Typhimurium P22$^-$ and *Salmonella* Typhimurium P22$^+$ attached cells were prepared using a 24-well flat-bottomed polystyrene microtiter plate (BD Falcon) containing TSB. Each culture was inoculated at approximately 10^5 CFU/ml and incubated at 37°C until the attached cell count was reached to more than 10^5 CFU/cm^2. After incubation, each well was rinsed to remove the nonattached cells. The preattached cells were infected with 10^8 PFU/ml of bacteriophage P22 and further incubated at 37°C for 24 h.

Preparation of bacteriophage-mediated cell lysate. *Salmonella* Typhimurium ATCC 19585 cells (10^7 CFU/ml) were incubated in TSB with and without bacteriophage P22 (10^8 PFU/ml) at 37°C for 24 h. After incubation, the cultures were centrifuged at 3,000 × *g* for 20 min and filtered using a 0.2-μm-pore-size filter to obtain CFS. Each CFS was divided into four groups, including (i) nontreatment, (ii) DNase (Fisher Scientific, Pittsburgh, PA) treatment (50 U), (iii) heat treatment (80°C for 30 min), and (iv) DNase and heat treatment. The total amount of eDNA in the CFS treatments was extracted by ethanol precipitation and quantified using a NanoDrop spectrophotometer (Thermo Scientific Inc., Pittsburg, PA). The total protein content was measured by using a bicinchoninic acid assay.

Evaluation of attachment ability. The attachment ability of selected foodborne bacterial pathogens to a 96-well plate (BD Falcon) was evaluated in the prepared CFS treatments by crystal violet (CV) assay. *Salmonella* Typhimurium P22$^-$, *Salmonella* Typhimurium P22$^+$, *L. monocytogenes,* and enterohemorrhagic *E. coli* were cultured at 37°C for 36 h in a 96-well plate (BD Falcon) containing CFS of *Salmonella* Typhimurium P22$^-$ cells cultured with and without bacteriophage P22. After incubation, the suspended cells were transferred to a new 96-well plate for measuring the absorbance at 570 nm (*G*). The attached cell layer in each well was rinsed twice with sterile distilled water, air dried for 30 min, and then stained with 1% CV solution (160 μl). After 30 min of incubation at 37°C, the wells were rinsed with sterile distilled water to remove unbound CV and then air died for 30 min. The CV-stained attached cells were destained with 95% ethanol (150 μl). The absorbance of the destained CV was measured at 570 nm ($CV_{attachment}$). Blank wells were stained as described above and used as negative control ($CV_{control}$). The attachment ability was expressed by calculating the specific attachment ability (SAA) index: $SAA = (CV_{attachment} - CV_{control})/G$. The attachment ability of the strains used in this study was divided into three categories, including strong ($SAA > 1$), moderate ($0.5 < SAA \leq 1$), and weak ($SAA \leq 1$) *(23, 25)*.

Statistical analysis. All experiments were conducted in duplicate on three replicates. Data were analyzed using the Statistical Analysis System software (SAS, Cary, NC). The general linear model and least significant difference procedures were used to evaluate significant mean differences at the level of 5% ($P < 0.05$).

RESULTS AND DISCUSSION

Bacteriophage P22-induced susceptibility of planktonic and attached cells of *Salmonella* Typhimurium. The influence of bacteriophage P22 on the inactivation and attachment of planktonic *Salmonella* Typhimurium P22$^-$ cells was evaluated under different MOIs, as shown in Figure 1. The growth of planktonic *Salmonella* Typhimurium P22$^-$ cells infected with bacteriophage P22 was decreased with increasing the MOI with the exception of inoculum level of 3×10^7 CFU/ml (Fig. 1F). The numbers

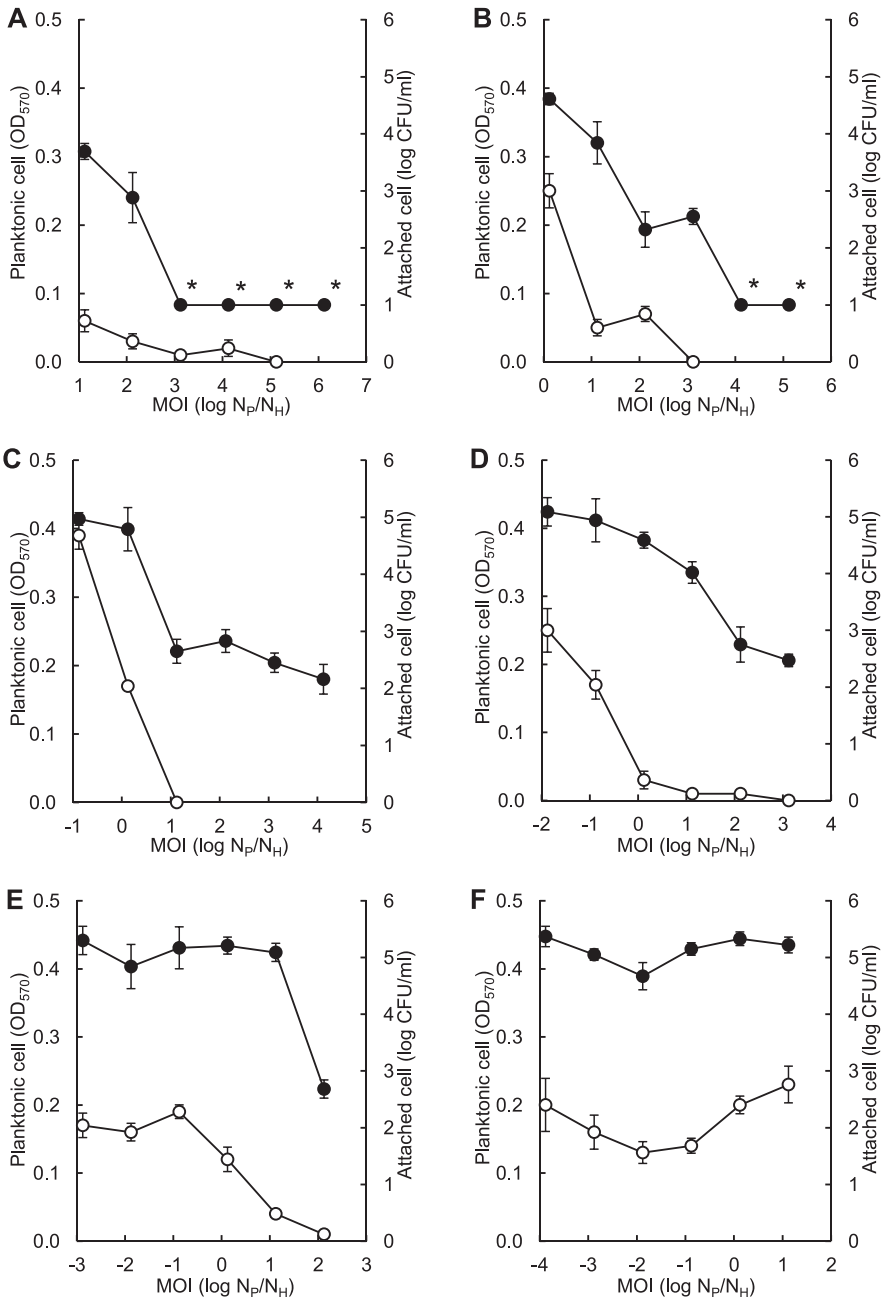

FIGURE 1. *Planktonic survival* (○) *and attachment ability* (●) *of* Salmonella *Typhimurium P22*$^-$ *at fixed inoculum levels of* 3×10^2 *CFU/ml (A),* 3×10^3 *CFU/ml (B),* 3×10^4 *CFU/ml (C),* 3×10^5 *CFU/ml (D),* 3×10^6 *CFU/ml (E), and* 3×10^7 *CFU/ml (F) with different multiplicities of infection (MOIs) of bacteriophage P22.* N_P *and* N_H *represent the number of bacteriophages P22 and the number of* Salmonella *Typhimurium P22*$^-$ *cells, respectively. * Indicates below the detection limit (1 log CFU/ml).*

of attached *Salmonella* Typhimurium P22$^-$ cells were below the detection limit at the MOIs of above 3.12 and 4.12, with low inoculum levels of 3×10^2 CFU/ml (Fig. 1A) and 3×10^3 CFU/ml (Fig. 1B), respectively. The attached *Salmonella* Typhimurium P22$^-$ cells still remained constant at 5 log CFU/ml at high inoculum level of 3×10^7 CFU/ml regardless of MOIs (Fig. 1F). The planktonic *Salmonella* Typhimurium P22$^-$ cells at 4.12 (Fig. 1C), 3.12 (Fig. 1D), and 2.12 (Fig. 1E) of MOIs were still involved in the attachment at the inoculum levels, showing more than 2 log CFU/ml of attached cells. The results suggest that the lytic activity and attachment ability varied, depending on the initial concentrations of host cells and bacteriophages, which can be essential factors for bacterial cell attachment. The enhanced binding property of bacteriophage to host bacterial ligands depends much more on the number of bacteriophages applied than on the self-replication at the early stage of infection *(13)*. In this study, the lytic response of bacteriophage P22 was increased with increasing MOI at the fixed inoculum levels of *Salmonella* Typhimurium P22$^-$ cells (Fig. 1A through 1E), while the lysogenic response was observed at the inoculum levels of 3×10^7 CFU/ml (MOI > -2.12) and mainly caused an increase in the number of attached cells (Fig. 1F). The lysogenized cells can enter into a biofilm state to maintain a balanced ratio of infected host cells and infectious bacteriophages in microbial communities *(5, 18)*.

The effect of bacteriophage P22 on the inhibition of preattached cells was evaluated in *Salmonella* Typhimurium P22$^-$ and *Salmonella* Typhimurium P22$^+$ (Fig. 2). The number of preattached *Salmonella* Typhimurium P22$^-$ cells was significantly reduced by more than 2 log CFU/cm^2 in the presence of bacteriophage P22 ($P < 0.05$). This observation suggested that bacteriophage P22 was effective

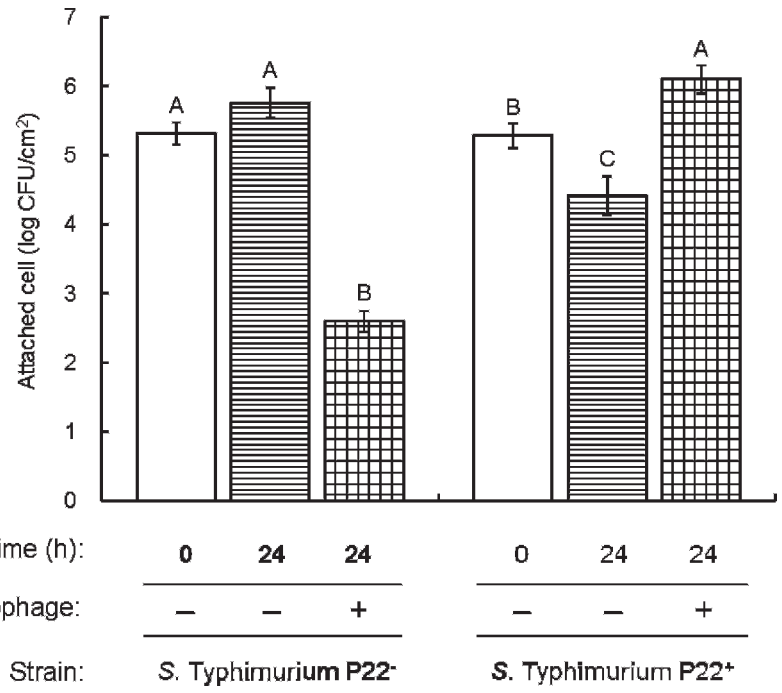

FIGURE 2. *Inhibitory effect of bacteriophage P22 on the preattached* Salmonella *Typhimurium ATCC 19585 (*Salmonella *Typhimurium P22*$^-$*) and* Salmonella *Typhimurium ATCC 23555 carrying P22 prophage (*Salmonella *Typhimurium P22*$^+$*) cells. Bars with different letters (A through C) within each strain are significantly different at* $P < 0.05$.

in inhibiting the preattached *Salmonella* Typhimurium P22$^-$ cells. The enhanced susceptibility of preattached cells to the bacteriophage was mainly attributed to the availability of specific binding sites *(33)*. The number of preattached *Salmonella* Typhimurium P22$^+$ cells was reduced by 0.87 log CFU/cm^2 in the absence of bacteriophage P22 compared with the initial number of preattached cells (5.28 log CFU/cm^2), which implies that the cryptic prophages were spontaneously released from the *Salmonella* Typhimurium P22$^+$ cells under stress conditions, such as nutrient depletion and high cell density, known as spontaneous phage induction *(5, 11, 19, 27, 31, 35)*. The number of preattached *Salmonella* Typhimurium P22$^+$ cells was increased up to 6.1 log CFU/cm^2 in the presence of bacteriophage P22. This result might be associated with superinfection exclusion, which promotes the survival and resistance of preattached cells in the presence of bacteriophages *(5)*.

Influence of bacteriophage-mediated *Salmonella* Typhimurium lysates on the bacterial attachment of various strains. The ability of selected pathogens to attach to surfaces was evaluated in the CFS of *Salmonella* Typhimurium P22$^-$ and bacteriophage P22-infected *Salmonella* Typhimurium P22$^-$ cultures. As shown in Table 1, the highest amount of eDNA was observed in the phage-mediated CFS (P-CFS), followed by the phage-mediated CFS treated with heat (PH-CFS) and DNase I (PD-CFS). Protein content showed a similar pattern of eDNA. The least amount of eDNA and proteins were 1.83 μg/ml and 9.62 mg/ml, respectively, in the CFS of *Salmonella* Typhimurium P22$^-$ cultured without bacteriophage P22 (S-CFS). The highest SAA index values were observed in *Salmonella* Typhimurium P22$^-$ (2.09) and *Salmonella* Typhimurium P22$^+$ (1.06) at the P-CFS treatment, indicating the strong attachment abilities of these strains (Fig. 3). The least attachment abilities were observed in fresh Trypticase soy broth alone, showing SAA indices <1 in all cases. This result implies that bacterial attachment might be influenced by eDNA and proteins in a concentration-dependent manner *(5)*. The lysis of the bacterial host could provide a structural component of the attached cell matrix, including polysaccharides, proteins, and nucleic acids. This observation is in good agreement with the previous report that bacterial attachment was significantly decreased in the presence of DNase I ($P < 0.05$) *(22)*. The strong attachment abilities of *Salmonella* Typhimurium P22$^-$ cells were observed in the P-CFS and PD-CFS compared with Trypticase soy broth alone the T, S-CFS, or PH-CFS. This might be due to the presence of bacteriophage P22 in the P-CFS and PD-CFS, causing an increased lysis of susceptible cells and attachment by persistent cells *(3, 27)*. In this study, the attachment ability of *Salmonella* Typhimurium P22$^+$ was relatively low compared with other pathogens. The superinfecting bacteriophages can inhibit the lysis of lysogenic *Salmonella* Typhimurium cells *(8),* resulting in

TABLE 1. *Extracellular DNA (eDNA) and protein content in cell-free supernatant (CFS) obtained from the bacteriophage-infected* Salmonella *Typhimurium P22*$^-$ *culture*

Treatment[a]	eDNA (μg/ml)	Protein (mg/ml)
S-CFS	1.83 ± 0.23 C[b]	9.62 ± 1.73 C
P-CFS	4.24 ± 0.58 A	22.51 ± 1.27 A
PD-CFS	2.91 ± 0.37 B	13.82 ± 0.83 BC
PH-CFS	3.93 ± 0.27 AB	17.44 ± 3.44 AB

[a] S-CFS, *Salmonella* Typhimurium cultured without bacteriophage P22; P-CFS, phage-mediated CFS; PD-CFS, phage-mediated CFS treated with DNase I; PH-CFS, phage-mediated CFS treated with heat.

[b] Means with different letters (A through C) within a column are significantly different at $P < 0.05$.

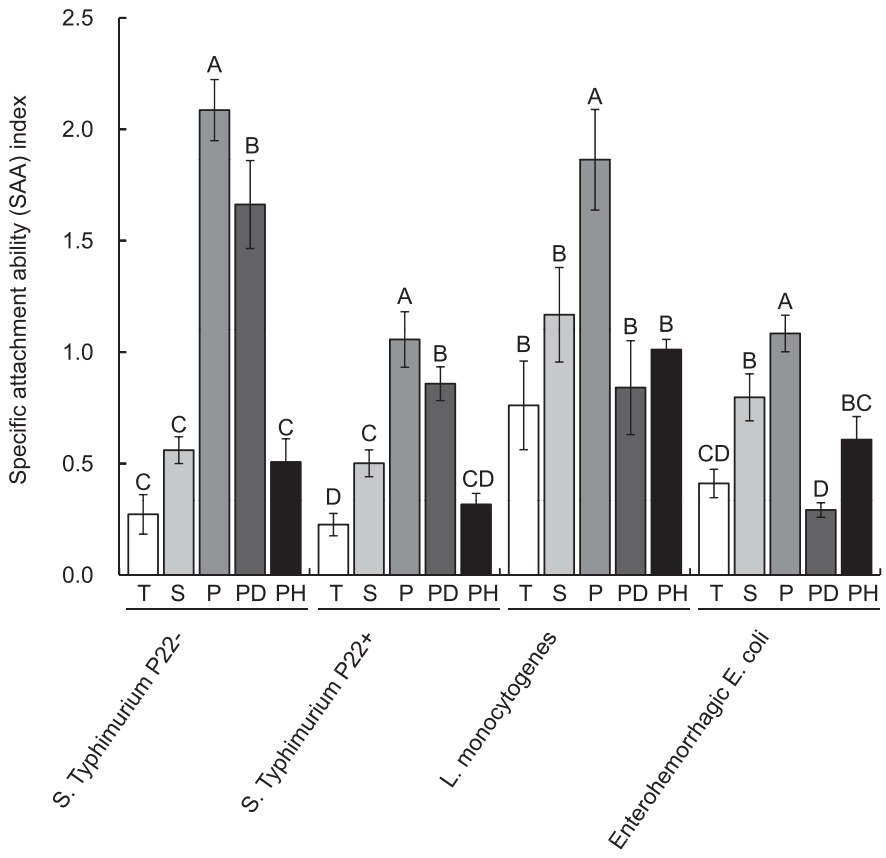

FIGURE 3. *Specific attachment ability (SAA) indices of selected pathogens cultured at 37°C for 24 h in fresh Trypticase soy broth (T; □).* Salmonella *Typhimurium-cultured cell-free supernatant (S; ■). Phage-mediated CFS (P; ■). Phage-mediated CFS treated with DNase I (PD; ■). Phage-mediated CFS treated with heat (PH; ■). Bars with different letters (A through D) within each strain are significantly different at* $P < 0.05$.

delayed bacterial attachment. Lysogenic conversion can produce attachment-promoting factors *(12).* Therefore, the spontaneous phage induction of lysogenic bacteria, although not as frequent as the lytic phage, can play an important role in bacterial attachment in natural microbial communities *(31).* The highest SAA values for *L. monocytogenes* and enterohemorrhagic *E. coli* were 1.86, and 1.08, respectively. This result indicates that bacteriophage-mediated lysis can influence attachment by other bacteria in the surrounding natural microbial communities. Compared with the P-CFS, all pathogens had low SAA values in the PH-CFS. The released enzymes might be involved in inhibition and/or stimulation of bacterial attachment *(10, 27, 34).* However, the results obtained in this study could not conclude whether extracellular enzymes directly affected the bacterial attachment to surfaces. Accordingly, further studies are needed to identify bacterial attachment-promoting factors released from bacteriophage-mediated lysis.

In conclusion, this study demonstrates the effects of virulent bacteriophage P22 on the inhibition of *Salmonella* Typhimurium $P22^-$ planktonic and attached cells at varying MOIs and bacteriophage-mediated bacterial lysates on the attachment abilities of nonhost bacterial pathogens. The most significant findings were that the initial populations of host cells and bacteriophages are crucial factors leading to the bacterial attachment. The lysogenic activity occurring at high MOIs resulted in enhanced attachment ability, superinfection exclusion increased the survival and resistance of attached *Salmonella* Typhimurium $P22^+$ cells, and bacteriophage-mediated lysates containing eDNA influenced the attachment ability of *L. monocytogenes* and enterohemorrhagic *E. coli* to surfaces. However, further study is needed to understand the mechanism of bacteriophage-induced lysis and the influence of bacteriophage-mediated bacterial lysates against diverse foodborne pathogens. Valuable information for a better understanding of the dynamic behavior of bacterial attachment in natural microbial systems and in designing effective bacteriophage-mediated control of attached cells in the food system may be provided.

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