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Research Note

Removal of *Listeria monocytogenes* Biofilms with Bacteriophage P100†

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ABSTRACT

Listeria monocytogenes is an important foodborne pathogen with a persistent ability to form biofilm matrices in the food processing environments. In this study, we have determined the ability of bacteriophage P100 to reduce *L. monocytogenes* cell populations under biofilm conditions by using 21 *L. monocytogenes* strains representing 13 different serotypes. There were considerable differences in the ability of various strains of *L. monocytogenes* to form biofilms, with strains of serotype 1/2a showing maximum biofilm formation. Irrespective of the serotype, growth conditions, or biofilm levels, the phage P100 treatment significantly reduced *L. monocytogenes* cell populations under biofilm conditions. On the stainless steel coupon surface, there was a 3.5- to 5.4-log/cm^2 reduction in *L. monocytogenes* cells by phage treatment. These findings illustrate that phage P100 is active against a wide range of *L. monocytogenes* strains in biofilm conditions.

The persistence of *Listeria monocytogenes* biofilms on food and nonfood contact surfaces is the major attribute facilitating this pathogen's environmental spread and subsequent contamination of ready-to-eat food products *(11, 20)*. In a food processing plant, the common sites for *L. monocytogenes* isolations are floor drains, conveyor belts, stainless steel equipment surfaces, product transportation racks, and cold rooms *(37)*. Prevailing conditions on these sites such as ample water content and food residues further provide a unique opportunity for pathogenic *L. monocytogenes* to form a biofilm matrix and reside therein *(11)*.

Though the adherence strength of different *L. monocytogenes* strains varies, the majority of *L. monocytogenes* strains of different serotypes are able to produce biofilm mass under defined laboratory conditions *(11, 15)*. The genetic determinants required for *L. monocytogenes* biofilm formation are still relatively unknown *(18),* although screening of different insertion mutant libraries along with phenotypic characterization has produced some meaningful information. Some of the important genetic determinants involved in *L. monocytogenes* biofilm include surface-adhering protein BapL *(18),* flagellar protein FlaA, and DNA-binding two-component response regulator DegU *(22),* SOS-controlled protein YneA *(42),* quorum-sensing–regulated peptidase AgrD *(30),* and the ability of some strains to produce truncated InlA over full-length InlA *(12)*.

L. monocytogenes cells present in a biofilm matrix show greater resistance to antimicrobial agents than those present in planktonic environments *(26, 31)*. Examples of antimicrobial agents that have been tested for their efficacy against *L. monocytogenes* biofilms include alkaline and acidic electrolyzed water *(3),* octenidine hydrochloride *(1),* quaternary ammonium compounds, sodium hypochlorite, and hydrogen peroxide *(26, 44)*. These findings report a 2- to 4-log reduction in *L. monocytogenes* populations in biofilms and indicate that although sanitizer compounds aid in biofilm removal, the degree of biofilm removal depends on factors such as biofilm age, the surface on which biofilm is formed, and the substrate in which the biofilms were produced. In addition, repeated exposure to single disinfecting agents could also confer subsequent insensitivity to *L. monocytogenes* cells *(26)*. Apart from chemically based treatments, Zhao et al. *(46)* used a competitive-exclusion strategy and demonstrated that the use of *Lactococcus lactis* and *Enterococcus durans* in poultry processing floor drains resulted in 2- to 6-log reductions of *L. monocytogenes* compared with 8 log CFU/100 cm^2 levels in control coupons containing 28-day-old biofilm cells.

Another promising approach to control and eradicate biofilm formation is the use of bacteriophages as antibacterial agents *(4, 33, 36, 46)*. Bacteriophages (phages) are viruses that infect bacterial cells specific for a target genus, serotype, or strain. Bacteriophages are ubiquitous in nature, and as many as 10^8 phage particles can be isolated from 1 g of soil or water *(27)*. Phages have also been isolated from several food products such as meat, dairy, and vegetable products *(2, 5, 16, 19, 43)*. All phages are obligate parasites, meaning that they rely on a specific host for propagation. For biocontrol strategies, lytic phages that have the ability to

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TABLE 1. *Details of* L. monocytogenes *strains representing 13 serotypes used in this study*[a]

ID	Strain	Serotype	Lineage	Source
1	V7	1/2a	II	Raw milk (FDA)
2	EGD	1/2a	II	Institut Pasteur, Paris, France
3	F4260	1/2b	I	Human cerebrospinal fluid and blood (CDC)
4	V2	1/2c	II	Human cerebrospinal fluid (VICAM)
5	ATCC 19113	3a	II	Human (ATCC)
6	SLCC 2540	3b	I	Human cerebrospinal fluid (SLCC)
7	SLCC 2479	3c	I	Unknown (SLCC)
8	ATCC 19114	4a	III	Bovine brain (ATCC)
9	ScottA	4b	I	Human clinical (FDA)
10	F4393	4b	I	Cheese (CDC)
11	F5069	4b	I	Milk (CDC)
12	F1057	4b	I	Milk (CDC)
13	F1109	4b	I	Milk (CDC)
14	ATCC 43257	4b	I	Mexican-style cheese (ATCC)
15	F2385	4b	I	Cheese, epidemic strain, California (1985)
16	NRRL 33083	4b	I	Animal origin (USDA-ARS)
17	Murry B	4ab	II	FDA
18	ATCC 19116	4c	III	Chicken (ATCC)
19	ATCC 19117	4d	II	Sheep (ATCC)
20	ATCC 19118	4e	II	Chicken (ATCC)
21	SLCC 2482	7	II	Human feces (SLCC)

[a] Lineage information is based on *L. monocytogenes* phylogenetic classification *(10, 28)*. FDA, U.S. Food and Drug Administration, Washington, DC; CDC, Centers for Disease Control and Prevention, Atlanta, GA; VICAM, VICAM Inc., Watertown, MA; ATCC, American Type Culture Collection, Rockville, MD; SLCC, Special *Listeria* Culture Collection, Wurzburg, Germany; USDA-ARS, U.S. Department of Agriculture, Agricultural Research Service, Washington, DC.

rapidly lyse bacterial cells without integration into bacterial DNA are recommended *(13, 17, 29, 32)*. Some successful examples of the use of bacteriophages that target biofilms are the killing of *Pseudomonas fluorescens* cells present in biofilm mass, using phage phiIBB-PF7A *(33)*; inhibition of *Pseudomonas aeruginosa* biofilm formation with four different lytic phages *(21)*; and inhibition of *Staphylococcus epidermidis* biofilm with phage K *(9)*.

Recently, the U.S. Food and Drug Administration approved two bacteriophage preparations, using phages P100 and LMP-102, as food ingredients to control *L. monocytogenes* contamination *(39–41)*. Currently, phage P100 is approved for ''food in general'' at levels not to exceed 10^9 PFU/g and LMP-102 is approved for ready-to-eat meat and poultry products as a surface treatment at level of 10^9 PFU/500 cm^2. In previous research studies, the efficacy of phage P100 was evaluated in soft cheese, ready-to eat-foods, cold-smoked salmon, and frankfurters *(7, 8, 14)*, whereas LMP-102 was tested on honeydews and apples *(23)*. We have recently demonstrated that generally recognized as safe phage P100 is an effective antilisterial agent on both fresh catfish and fresh salmon fillets *(34, 35)*. However, there are currently no reports on the efficacy of this approved phage on *L. monocytogenes* cells present in a biofilm matrix. In this study, we evaluated the efficacy of phage P100 against 21 different *L. monocytogenes* strains that represent 13 serotypes. Phage P100 efficacy was tested against (i) *L. monocytogenes* biofilms formed in polystyrene microtiter wells and (ii) *L. monocytogenes* cells firmly attached or grown as biofilms on the stainless steel coupon surfaces.

MATERIALS AND METHODS

***L. monocytogenes* strains.** Twenty-one different *L. monocytogenes* strains representing all 13 *L. monocytogenes* serotypes were used in this study. These included two strains of serotype 1/2a, eight strains of serotype 4b, and one strain of each of the following serotypes: 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4c, 4d, 4e, and 7. The identification, lineage, and source information of these strains are provided in Table 1. The lineage information for different serotypes is based on earlier *L. monocytogenes* phylogenetic classification *(10, 28)*. All strains were maintained in tryptic soy agar (TSA) slants at 4°C for up to 2 months and cultured in 10 ml of tryptic soy broth (TSB) at 37°C for 18 to 24 h.

Commercial bacteriophage preparation. U.S. Food and Drug Administration–approved *(38, 39, 41)* single-bacteriophage preparation Listex P100 (phage P100) was obtained from EBI Food Safety, Wageningen, The Netherlands. Phage P100 stock concentrations of 11 $\pm$ 0.2 log PFU/ml were obtained in buffered saline.

Sensitivity of different *L. monocytogenes* strains to phage P100. The sensitivity of phage P100 to different *L. monocytogenes* strains was assayed using a plaque-forming bioassay as described earlier *(34, 35)*. The bacteriophage suspension was serially diluted in a sterile buffer (100 mM NaCl, 10 mM $MgSO_4$, 50 mM Tris-HCl, pH 7.5), and 100 μl of the diluted phage suspensions were mixed with 150 μl of different *L. monocytogenes* strain cells (optical density at 600 nm [OD_{600}], ~1.2) in 4 ml of sterile soft agar (TSB containing 0.4% agar) at 42°C. The soft agar mixture was gently vortexed, poured onto a TSA plate and distributed evenly by gentle rotation of the TSA plate. The soft agar was allowed to solidify for 30 min at room temperature, and plates were subsequently incubated in an inverted position for 18 to 24 h at

30°C. Following the incubation period, the ability of phage P100 to form plaques on different *L. monocytogenes* strains was recorded.

Effect of phage P100 against *L. monocytogenes* biofilms. The efficacy of the phage against *L. monocytogenes* biofilms of different strains was determined by a quantitative crystal violet staining assay in a 96-well polystyrene microtiter plate (Midsci, St. Louis, MO). Stationary-phase cultures of each strain were grown in TSB for 18 to 24 h and diluted (1:100) to attain an inoculum level of 10^7 CFU/ml. For each strain, 200 μl of prepared inoculum was added to each of 12 wells of microtiter plates by using a multichannel pipettor and incubated for 48 h at 37°C without agitation. Additionally, negative control wells that contained 200 μl of sterile TSB only were included. Following the incubation period, nonadherent bacteria were removed by pipetting out the media content, and wells were washed twice to remove loosely bound cells. The washing step consisted of adding and removing 200 μl of sterile saline solution in each well by a multichannel pipettor. The wells were then treated with 200 μl of phage P100 solution at a titer of 10^9 PFU/ml to each of six wells per strain. Alternatively, for the control treatment 200 μl of saline was added, using six wells per strain. Phage or saline solutions were also added in negative control wells that originally contained sterile TSB only. After 24 h of incubation at 37°C, the 96-well plates were subjected to crystal violet staining steps to quantify the biofilms. All wells were washed twice with 200 μl of physiological saline, and then 200 μl of 1% (wt/vol) crystal violet solution was added to the microtiter wells for 15 min. A similar staining procedure was employed with 12 empty wells to ensure that crystal violet stain did not attach to the 96-well plate surface. The unbound crystal violet stain was removed by washing six times by adding and removing 200 μl of physiological saline. A 200-μl volume of ethanol-acetone (80:20) was added in each well to solubilize the bound crystal violet from the stained *Listeria* biofilms, to be quantified as OD_{562} by use of a Bio-Tek (model- EL_X800_{NB}) plate reader.

Effect of phage P100 against biofilms cells of *L. monocytogenes* on stainless steel surfaces. Phage P100 was tested for its ability to remove *L. monocytogenes* cells from stainless steel coupons (1 by 1 cm; 2B finish) after inoculation with five strains of *L. monocytogenes* cells. A five-strain mixture of *L. monocytogenes* cells prepared by mixing equal 2-ml volumes of overnight grown cultures (OD_{600}, ~1.2) of two strains of serotypes 1/2a (strain ID nos. 1 and 2) and three strains of serotype 4b (strain ID nos. 10, 11, and 14) was used for inoculation.

Two different types of cell adherence assays on stainless steel coupons were performed with *L. monocytogenes*. In the first assay, a high concentration of the five-strain mixture of *L. monocytogenes* cell suspension was directly added onto the stainless steel surface and cells were allowed to adhere firmly for 48 h at room temperature (22 ± 1°C) *(33)*. For this purpose, 10 ml of a five-strain mixture of 10^9 CFU of *L. monocytogenes* per ml was centrifuged and the pellet was resuspended in 1 ml of saline to yield a concentration of 10^{10} CFU of cells per ml. Fifty microliters of 10^{10}-CFU/ml cell suspension was spotted onto each 1-cm^2 sterile stainless steel coupon (equivalent to 8.5 log CFU/cm^2). The coupons were kept in a 24-well plate and incubated at room temperature for 48 h. In a second assay, *L. monocytogenes* cells were allowed to grow on the stainless steel coupon surface at room temperature (22 ± 1°C) for 7 days to generate an *L. monocytogenes* biofilm *(45)*. The five-strain mixture of *L. monocytogenes* was serially diluted in sterile TSB at 10^4 CFU/ml, and 50-μl aliquots of these serial dilutions were added onto a stainless steel coupon surface placed in a 24-well plate. On days 1, 3, and 5, the residual media contents present on coupons were carefully pipetted out and replaced with fresh TSB to facilitate the *L. monocytogenes* cell growth on stainless steel coupons.

After the preparation of cells, either firmly attached or grown as biofilm, as described above, each stainless steel coupon was washed twice by dipping in 1 ml of saline (for 15 s) to remove the unbound *L. monocytogenes* cells. The coupon was then placed in a 24-well polystyrene plate (Midsci, St. Louis, MO) for treatment with 1 ml of 10^9 PFU of phage P100 per ml for 24 h at room temperature. Alternatively, the coupon was placed in a 1-ml saline solution for 24 h for untreated control. The 1-ml volume of phage or saline solution in a 24-well plate was sufficient to completely cover the stainless steel coupon surface. After the phage treatment step, the stainless steel coupons were washed three times in sterile saline solution to remove phage particles. In the washing step, each stainless steel coupon was lifted by sterile forceps and tapped two or three times on the edge of the well surface to drain excessive liquid and washed by gently shaking for 30 s in 1 ml of sterile saline solution placed in a separate 24-well plate. Subsequently, coupons were vortexed vigorously in 10 ml of peptone water containing 0.02% Tween 80 for 5 min, and suspensions were serially diluted if required and 0.1-ml sample aliquots were spread plated on PALCAM (Fisher Scientific, Hampton, NH) agar plates containing 6 mg of ceftazidime per liter for incubation at 37°C for 48 h.

Statistical analysis. All experiments were performed twice by maintaining three replicates for plaque assays, six replicates for microtiter well crystal violet biofilm assays, and three replicates for stainless steel coupon assays. Mean values and standard errors for OD_{562} and log CFU per square centimeter viability counts were calculated using an Excel spreadsheet. When desired, paired *t*-test analyses were performed using SPPS statistical analysis software package (version 12.0, SPSS, Chicago, IL) to determine the mean significant differences between control and phage treatments.

RESULTS AND DISCUSSION

In the present study, we tested antilisterial bacteriophage preparation phage P100 for its ability to reduce the *L. monocytogenes* cells present in a biofilm mass in 96-well plates and on stainless steel coupon surfaces. Prior to determining the effect of phage P100 on biofilm cells, the sensitivity of different *L. monocytogenes* strains to phage P100 was evaluated by determining plaque-forming ability on soft-agar overlay assay. Phage P100 was able to produce plaques on all 21 *L. monocytogenes* strains tested. Of critical importance, the sizes of the plaques were 3 to 4 mm for all strains except for strains no. 3 (serotype 1/2b) and no. 21 (serotype 7), which produced considerably smaller plaques, of ~0.2 to 0.4 mm.

Figure 1 shows the levels of biofilm that are produced by different strains of *L. monocytogenes* and the reductions in these biofilm levels after phage P100 treatment. Under these conditions, the biofilms were generated in stationary phase for 48 h in a 96-well microtiter plate and then treated with phage P100 or physiological saline (control) for 24 h, and after that reductions in biofilm mass were determined by crystal violet staining assay. The crystal violet staining assay is a good indicator for quickly screening the biofilm mass in laboratory models and has been frequently used by

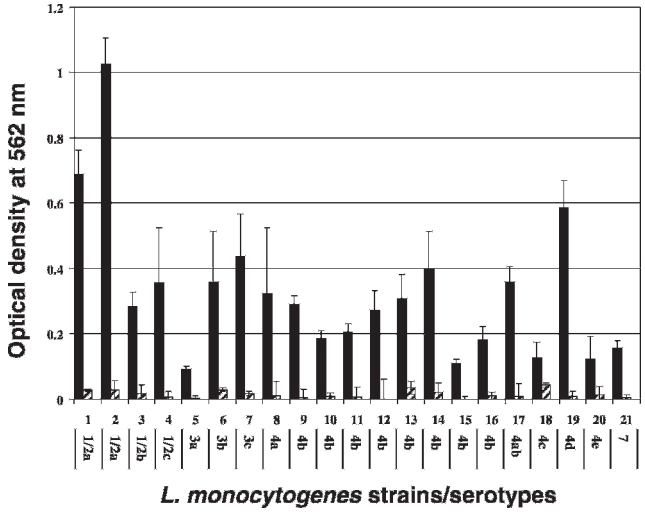

FIGURE 1. *Reductions of* L. monocytogenes *biofilm produced in 24-well polystyrene plates with phage P100 treatment based on* OD_{562} *measurements after crystal violet staining. The* x *axis shows the numbers of* L. monocytogenes *strains described in Table 1, and the* y *axis represents* OD_{562} *measurements obtained in the crystal violet staining assay. Solid bars represent the control (no phage), and bars with hatched lines represent the 24-h phage treatment. Data presented here are 24 h after the phage infection period. The decreases in* OD_{562} *between phage and control treatments were statistically different for different* L. monocytogenes *strains based on paired* t *test (*$P < 0.05$*).*

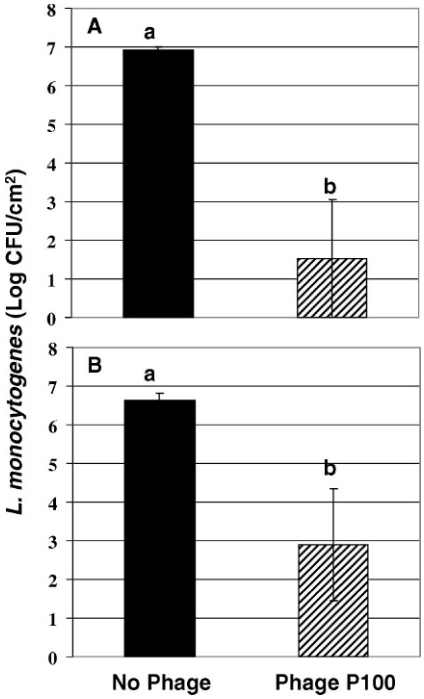

FIGURE 2. *Reduction of* L. monocytogenes *cells from stainless steel surfaces after phage P100 treatment. (A) Stainless steel coupon assay 1: a five-strain mixture of* L. monocytogenes *cells were firmly attached in nutrient-limiting conditions on the stainless steel coupon for 2 days prior to 24-h phage P100 treatment. (B) Stainless steel coupon assay 2: reduction of five-strain mixture of* L. monocytogenes *multilayered biofilm grown on the stainless steel coupon for 7 days prior to 24-h phage P100 treatment. The data (given in log CFU per square centimeter) presented on the* y *axis are based on viable* L. monocytogenes *cells recovered from stainless steel coupon surfaces following 24 h of phage or control (saline instead of phage) treatment. Solid bars represent the control (no phage), and bars with hatched lines represent the phage treatment. Bars with different lowercase letters show significant differences based on a paired* t *test (*$P < 0.05$*) within the control and 24-h phage treatment.*

several research studies for evaluating the biofilm-forming capabilities and control measures for different bacteria *(9, 11, 21)*. All strains of *L. monocytogenes* tested in this study produced biofilms in microtiter plate wells with the levels of biofilm formation varying among strains. As additional experimental controls, the surfaces of 96-well plate wells and phage and saline solutions were also tested to ensure that they do not have any effect on retention of crystal violet stain. For all these, OD_{562} readings were negligible, yielding ~0.05 units, and these values were subtracted from OD_{562} data of different *L. monocytogenes* biofilms treated with phage P100 or saline.

The two strains of *L. monocytogenes* belonging to serotypes 1/2a showed the highest levels of biofilm formation (OD_{562} of 0.7 for strain no. 1 and of 1.0 for strain no. 2). On the other hand, the nine strains of *L. monocytogenes* belonging to serotype 4b resulted in a weak to medium biofilm formation with OD_{562} ranging from 0.1 to 0.4 (strains no. 9 to 16). Among the other strains, those belonging to serotypes 3a, 4c, 4e, and 7 also formed weak biofilms. Strains of *L. monocytogenes* serotypes 1/2a and 4b that belong to lineages I or II are the most important for clinical and food safety risks *(6, 25, 28)*. Previously, in agreement with the results of our study, it has been reported that a group of 1/2a strains were more efficient in biofilm formation than were the strains of serotype 4b *(6, 15)*. Phage P100 treatment of preformed *L. monocytogenes* biofilms in microtiter wells yielded reductions (based on OD_{562} measurement of crystal violet–stained cells) in biofilm mass compared with the control samples treated with saline ($P < 0.05$). Irrespective of serotypes or initial biofilm formation levels, the phage treatment resulted in a near elimination of biofilm mass in all *L. monocytogenes* strains tested. Similarly, with other phages, significant reductions in biofilm mass were achieved within 24 h of phage treatment against *P. aeruginosa (21)* and *S. epidermidis (9)*.

Figure 2 shows the effect of phage P100 on reductions of *L. monocytogenes* biofilm cells from the stainless steel coupons. For the enumeration of *L. monocytogenes* cells from stainless steel coupons, three extraction methods, i.e., sonication, vortexing, and shaking with beads, were found to be equally effective in recovering the firmly attached bacterial cells from a biofilm matrix *(24)*. In our assay, *L. monocytogenes* cells from stainless steel coupons were recovered by 5 min of vortexing in 10 ml of peptone water containing 0.02% Tween 80. When the stainless steel coupons were inoculated with an initial level of 8.5 log CFU/cm^2, viable *L. monocytogenes* biofilm cells recovered were approximately 7 log CFU/cm^2 after 2 days of attachment (Fig. 2A). This may be attributed to the loss of unbound cells during the washing step and/or lost viability of some cells due to nutrient-limiting conditions (cells

suspended in saline) maintained on stainless steel coupons for *L. monocytogenes*. The phage P100 treatment resulted in a significant 5.4-log/cm^2 reduction of *L. monocytogenes* cells from these coupons compared with the untreated controls ($P < 0.05$) (Fig. 2A). Alternatively, in another assay with 1-week-old multilayer biofilms, the *L. monocytogenes* cell numbers increased from the initial inoculum level of ~1.7 to ~6.6 log CFU/cm^2. Phage P100 treatment of these multilayer biofilms reduced the *L. monocytogenes* population by 3.5 log CFU/cm^2. Overall, the decline in *L. monocytogenes* cell counts following phage P100 treatment was approximately 2 log CFU/cm^2 less on stainless steel coupons for multilayered *L. monocytogenes* biofilm cells (Fig. 2B) than for those cells that were firmly attached for 2 days (Fig 2A). The observed difference in *L. monocytogenes* reduction could be attributed to the fact that bacterial adherence strength is proportional to the time allowed for attachment and adherence *(45)*.

In conclusion, the work performed reveals the potential of phage treatment as an alternative strategy for *L. monocytogenes* biofilm control. In real environmental conditions, the biofilm matrix could consist of diverse bacterial species, and also some *L. monocytogenes* cells may either lack the phage binding receptors or be inaccessible under some conditions. The successful phage application in any environment will depend on the level of polysaccharide-degrading enzymatic activity by which the integrity of the biofilm may be destroyed *(6, 36)*. Also, random mutations, if occurring in specific regions (e.g., in a phage receptor binding gene or in a gene affecting exopolysaccharide production) of *L. monocytogenes,* may lead to development of resistance against the phage. In such scenarios, a cocktail of different phages may be essential for removing *L. monocytogenes* biofilms. Our future experiments will therefore determine the ability of phage cocktails to remove mixed *L. monocytogenes* biofilms that occur with other normal microflora on food contact surfaces.

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