Characterization and adsorption of *Lactobacillus* virulent phage P1

X. Chen,¹ Y. Xi, H. Zhang, Z. Wang, M. Fan, Y. Liu, and W. Wu

Key Laboratory of Dairy Biotechnology and Engineering, Ministry of Education, Inner Mongolia Agricultural University, Huhhot, 010018, P. R. China

ABSTRACT

Bacteriophage infection of lactic acid bacteria is considered an important problem worldwide in the food fermentation industry, as it may produce low quality or unsafe foods, cause fermentation failure, and result in economic losses. To increase current knowledge on the properties of *Lactobacillus* virulent phages, we evaluated the effect of divalent cations, temperature, pH, and chloramphenicol on the adsorption ability of *Lactobacillus* virulent phage P1. Phage P1 was isolated from the abnormal fermentation liquid of *Lactobacillus plantarum* IMAU10120. The results showed that this phage belonged to the Siphoviridae family. The latent period of this phage was 45 min, and the burst time was 90 min. Burst size was 132.88 ± 2.37 phage counts expressed per milliliter per infective center. This phage showed good tolerance at different temperatures, but incubation at 50°C only affected its adsorption. Adsorption rate reached a maximum value between 30 and 42°C. A high adsorption value of phage infectivity was obtained from pH 6 to 8. Moreover, calcium ions promoted and increased the adsorption capacity of phage P1, but magnesium ions had negative effects. Chloramphenicol had no effect on phage adsorption. This study increased current knowledge on the characterization and biological aspects of *Lactobacillus* virulent phages, and may provide some basic information that can be used to design successful antiphage strategies in the food industry.

Key words: *Lactobacillus* virulent phage, tolerance, adsorption, burst size

INTRODUCTION

Lactobacillus plantarum is a flexible and versatile species that grows in diverse environment niches, such

as fermented food products, meat, and plant materials (Ma et al., 2016), and it is frequently considered a natural inhabitant of the human gastrointestinal tract (Costa et al., 2014). It has been used in human clinical trials to promote a beneficial effect on the immune system, to alleviate intestinal disorders, and to reduce the risk of cardiovascular disease. It is also involved in many fermentation processes in the food industry and to improve the organoleptic characteristics of the final products (Costa et al., 2014).

However, the increasing use of *L. plantarum* as a starter or adjunct culture may increase the chances of phage infections in industrial environment, with adverse effects on the final products (Briggiler Marcó et al., 2012). Contamination with bacteriophage is currently considered one of the greatest problems in food fermentation industry worldwide, especially in dairy fermentation. In the modern food industry, the disruption of lactic acid fermentation by bacteriophages can lead to slow or substandard dairy fermentation. Moreover, virulent phages can lyse starter cultures, yielding low-quality products, and then lead to economic losses for the producer. Consequently, efficient control measures to minimize problems caused by phage attacks are essential. Thus, knowledge about phage population and biology is necessary to carry out successful antiphage strategies (Kodaira et al., 1997; Emond and Moineau, 2007).

Adsorption to the host cell surface is considered the first step of phage infection, and it has been studied for several species of lactic acid bacteria (Capra et al., 2006; Müller-Merbach et al., 2007; Suárez et al., 2008; Briggiler Marcó et al., 2010). To our knowledge, information related to phages infected on *L. plantarum* is very limited. *Lactobacillus plantarum* IMAU10120 was isolated from traditional fermented yogurt in Inner Mongolia, China. This strain exhibited several desirable properties, including high acid and bile tolerance, good aggregation and antibacterial activities, as well as high soy milk fermentation efficiency and strong stability upon storage. Virulent phage P1 was isolated from the fermented liquid of *L. plantarum* IMAU10120 lacking significant viable counts. The purpose of the

Received April 19, 2016.

Accepted May 25, 2016.

¹Corresponding author: chenxia8280@163.com

current work was to analyze the characteristics of *L. plantarum* phage P1 and evaluate the interaction of this phage with its host strain, especially the influence of physicochemical parameters on phage adsorption to sensitive cells.

MATERIALS AND METHODS

Bacterial Strains, Phages, and Culture Condition

Lactobacillus plantarum IMAU10120 was grown at 37°C in de Man, Rogosa, and Sharpe (MRS) broth (Difco, Becton Dickinson and Company, Franklin Lakes, NJ) and used as the host strain for phage P1. *Lactobacillus plantarum* phage P1 was isolated from the abnormal fermented liquid of *L. plantarum* IMAU10120. For phage amplification, MRS was supplemented with 10 mM CaCl₂. Phage stocks were prepared as described previously (Neviani et al., 1992) and stored as lysates at 4°C. Phage counts expressed in plaque-forming units (pfu) per milliliter were obtained using the double-layer plaque titration method (Quiberoni et al., 2011). The bacterial strain was obtained from the Lactic Acid Bacteria Collection Center in the Key Laboratory of Dairy Biotechnology and Engineering, Ministry of Education, Inner Mongolia Agricultural University.

Electron Microscope

Electron micrographs of phages were obtained according to De Antoni et al. (2010). Phage suspensions were concentrated by centrifugation (1 h, 70,000 × *g*, 5°C) and then stained using uranyl acetate (2% wt/vol, pH 4.5) or phosphotungstic acid (2% wt/vol). Electron micrographs were taken with an JEOL H-7000 electron microscope (Jeol USA Inc., Peabody, MA) operating at 75 kV. Phage morphologies and dimensions (capsid diameter, tail length and width) were recorded.

One-Step Growth Curve

The host strain, *L. plantarum* IMAU10120, was grown to exponential growth (optical density at 600 nm = 0.5) was harvested and suspended in 100 µL of MRS-Ca broth. Phages were added at a multiplicity of infection (MOI) of 2. After adsorption (15 min at 37°C), cells were harvested by centrifugation at 10,000 × *g* for 5 min at room temperature. Decimal dilutions of this suspension were carried out and incubated at 37°C. At regular intervals (15 min), 100 µL of each dilution was collected for bacteriophage counts (Capra et al., 2006). Latent period, burst time, and burst size were calculated from one-step growth curve.

Influence of Temperature on Phage and Strain Viability

Phages (10⁷ pfu/mL) or strain culture (10⁸ cfu/mL) were suspended in MRS broth, placed into Eppendorf tubes (1 mL final volume), and incubated at 0, 10, 20, 30, 37, 42, and 50°C for 30 min. The surviving phage particles or bacterial cells were immediately counted and the results were expressed as a percentage of remaining phage or cell counts to the initial phage or cell counts.

Influence of pH on Phage and Strain Viability

Phages (10⁷ pfu/mL) or strain culture (10⁸ cfu/mL) were suspended in MRS broth at various pH (from 2 to 11), placed into Eppendorf tubes (1 mL final volume) for 30 min, and used to test the effect of pH on phage particles or bacterial cells, which were immediately plated and counted as described above. The results were expressed as a percentage of remaining phage or cell counts to the initial phage or cell counts.

Influence of Temperature on Phage Adsorption

The adsorption of phages on *L. plantarum* IMAU10120 was determined at 0, 10, 20, 30, 37, 42, and 50°C. The infected (MOI = 0.5) *L. plantarum* cultures in MRS broth were incubated at different temperatures for 45 min. Results were expressed as percentages of adsorption after 45 min and plotted against temperature values.

Influence of pH on Phage Adsorption

The influence of different pH values (from 2 to 11) on cell lysis was evaluated by incubating the infected (MOI = 2) *L. plantarum* cultures in MRS broth at 37°C for 45 min. The results were expressed as percentage of adsorption after 45 min and plotted against pH values.

Influence of Divalent Cations on Phage Adsorption

The influence of Ca²⁺ and Mg²⁺ on cell lysis was checked by incubating the infected (MOI = 0.5) cells in MRS broth with and without CaCl₂ or MgCl₂ (10 mmol/L) at 37°C. At intervals (5, 15, 30, and 45 min), tubes were removed and centrifuged (10,000 × *g*, 5 min, at room temperature) to sediment the phage-adsorbed bacteria. Then, the supernatants were assayed for unadsorbed phages (double-layer plate titration), and the counts were compared with the titer of a control without cells. The results were expressed as percentages of adsorbed phage counts to the initial phage counts.

Influence of Cell Protein Synthesis Inhibitors on Phage Adsorption

The minimum concentration of chloramphenicol needed to inhibit protein synthesis in *L. plantarum* IMAU10120 was determined as described by Briggiler Marcó et al. (2012). Results were expressed as percentages of adsorption after 45 min and plotted against chloramphenicol concentration.

Statistical Analysis

All data were analyzed using the Originpro software (8.6, Originlab, Originlab Corp., Northampton, MA). Experiments were replicated 3 times. Means were compared using the one-way ANOVA procedure in SPSS (version 20.0.0, IBM Corp., Armonk, NY) at $P < 0.05$.

Figure 1. Electron micrograph of *Lactobacillus plantarum* phage P1.

RESULTS AND DISCUSSION

Electron Microscopy

An electron micrograph of *L. plantarum* phage P1 is shown in Figure 1. The phage had an isometric capsid about 71.7 ± 3.0 nm and a long noncontractile tail (about 272 ± 3.0 nm long, 11.3 ± 1.5 nm wide). Thus, based on morphology, it belonged to Siphoviridae family, as do most characterized *L. plantarum* phages (Vil-lion and Moineau, 2009; Briggiler Marcó et al., 2012).

One-Step Growth Curve

Figure 2 shows the one-step growth curve of phage P1. As can be seen, the latent period of this phage was 45 min and the burst time was 90 min. Burst size was 132.88 ± 2.37 pfu/infective center, which was higher than that of most *Lactobacillus* virulent phages (De Antoni et al., 2010; Briggiler Marcó et al., 2010).

Influence of Temperature on Phage and Strain Viability

From Figure 3a, we can conclude that the viability of phage suspensions was maintained, even at 50°C. More than 95% of initial phage particles remained viable after 30 min of treatment in MRS broth. However, the viability of *L. plantarum* IMAU10120 was influenced by temperature. From Figure 3b, we can see that *L. plantarum* IMAU10120 grew well in a temperature range from 30 to 50°C but growth was slightly inhibited at temperatures $\leq 20^\circ\text{C}$.

Influence of pH on Phage and Strain Viability

Unlike temperature, pH had a clear effect on phage infectivity (Figure 4a). High viability ($>90\%$) for P1 was observed in a pH range from 6 to 8. The infectivity of viral suspensions was inactivated completely after 30 min at pH 2 and almost completely (1.17%) at pH 3. This was in accordance with previous reports (Briggiler Marcó et al., 2010). As seen in Figure 4b, the viability of *L. plantarum* IMAU10120 was not influenced by pH. At pH 2, the viability of *L. plantarum* IMAU10120 reached 2.19×10^8 cfu/mL.

Influence of Temperature on Phage Adsorption

Figure 5 shows the influence of temperature on phage adsorption. More than 85% of phage particles were adsorbed after 45 min at all temperatures tested except at 50°C. Incubation at 50°C caused an increase in the per-

Figure 2. One-step growth curve of *Lactobacillus plantarum* phage P1. Values are the mean of 3 determinations. pfu = plaque-forming units (phage counts). Error bars represent 95% CI.

centage of unadsorbed phage particles (27.67%). Maximum adsorption was achieved between 30 and 42°C, which is similar to previous results (Briggiler Marcó et al., 2010); this may be because these temperatures are closer to the optimal temperature of growth for *L. plantarum*.

Influence of pH on Phage Adsorption

The influence of pH on phage adsorption is shown in Figure 6. Between a pH of 4 to 8, phage P1 showed a high rate of adsorption (more than 90%) on the host strain, with the highest adsorption at pH 7. At pH 9, the extent of adsorption was 50.00%, and at pH 11, adsorption decreased to 7.78%.

Influence of Divalent Cations on Phage Adsorption

Cell lysis in MRS broth occurred even without divalent cations but it was faster in the presence of Ca^{2+} (Figure 7). Moreover, plaques (i.e., lysis) were very clear in the presence of Ca^{2+} , whereas in the presence of Mg^{2+} or in the absence of cations, plaques were diffused or failed to clear.

Influence of Cell Protein Synthesis Inhibitors on Phage Adsorption

The chloramphenicol concentration used in the experiment was 20 $\mu\text{g/mL}$; in that condition, the inhibition of protein synthesis in cells was maintained after removal of chloramphenicol. Thus, phage adsorption process was carried out without antibiotics. Treatment with chloramphenicol did not affect adsorption ($P > 0.05$) on cells compared with untreated cells. After 45 min of incubation, more than 94.5% of initial phage particles were adsorbed on cells with or without chloramphenicol treatment (data not shown).

Lactobacillus plantarum phage P1 was isolated from the abnormal fermented liquid of *L. plantarum* IMAU10120, which was isolated from traditional fermented yogurt in Inner Mongolia, China. Electron microscopy showed an isometric capsid of 71.7 ± 3.0 nm and long noncontractile tails (about 272 ± 3.0 nm long, 11.3 ± 1.5 nm wide), and the phage was classified as a member of the Siphoviridae family, similar to most characterized *L. plantarum* phages. Yoon et al. (2011) isolated a *Lactobacillus* temperate phage Sha 1 from mitomycin C-induced lysate of *L. plantarum* from kimchi of a similar size (with an isometric head about $58 \times$

Figure 3. Influence of temperature on viability of (a) *Lactobacillus plantarum* phage P1, and (b) the host strain *Lactobacillus plantarum* IMAU10120 (measured by total viable count, TVC). Values were the mean of 3 determinations. Error bars represent 95% CI. Color version available online.

Figure 4. Influence of pH on viability of (a) *Lactobacillus plantarum* phage P1, and (b) the host strain *Lactobacillus plantarum* IMAU10120 (measured by total viable count, TVC). Values were the mean of 3 determinations. Error bars represent 95% CI. Color version available online.

60 nm and a long tail about 259×11 nm). De Antoni et al. (2010) isolated 2 *L. plantarum* phages, FAGK1 and FAGK2, from kefir grains, which showed isometric capsids and long noncontractile tails with transversal striations. Briggiler Marcó et al. (2012) characterized 2 *L. plantarum* virulent phages, ATCC 8014-B1 and ATCC 8014-B2, which were isolated from corn silage and anaerobic sewage sludge, and found they had long noncontractile tails and belonged to the Siphoviridae family. However, Chibani-Chennoufi et al. (2004) isolated a virulent *L. plantarum* myophage, LP65, from industrial meat fermentation, which belonged to a genus of the family Myoviridae, suggesting that lateral gene transfer may have occurred between myo- and siphophages and the *L. plantarum* host in the more distant past. Villion and Moineau (2009) analyzed 186 *Lactobacillus* phages by electron microscopy and found that 109 belonged to the Siphoviridae family, 76 to the Myoviridae family, and 1 to the Podoviridae family.

The burst size of phage P1 was 132.88 ± 2.37 pfu/infective center, which was significantly higher than that of most *Lactobacillus* virulent phages. Lu et al. (2003) reported that the average burst size for phage ϕ JL-1

Figure 5. Influence of temperature on the adsorption (after 45 min, in de Man, Rogosa, and Sharpe-Ca broth) of *Lactobacillus plantarum* phage P1. Values were the mean of 3 determinations.

Figure 6. Influence of pH value on the adsorption (after 45 min, in de Man, Rogosa, and Sharpe-Ca broth) of *Lactobacillus plantarum* phage P1. Values were the mean of 3 determinations. Error bars represent 95% CI.

at 30°C was 22 pfu/infective center. Other researchers found that the burst sizes for phages FAGK1 and FAGK2 on *L. plantarum* ATCC 8014 at 37°C were 10.8 and 12 pfu/infective center, respectively (De Antoni et al., 2010). Briggiler Marcó et al. (2010) reported burst

size at 37°C for phages B1 and B2 on *L. plantarum* ATCC 8014 were 60 and 83 pfu/infective center, respectively; however, Trevors et al. (1983) isolated a bacteriophage, *fri*, with the burst size of 200 pfu/infective center at 30°C from the *L. plantarum* portion of a commercial meat starter culture. The effect of temperature on phage propagation could be due to temperature-dependent changes in the bacterial cell wall affecting the adsorption ability of the phages, and temperatures of 30 and 37°C may be more effective (Caso et al., 1995). Although temperature had little effect on the survival rate of *L. plantarum* phage P1 (Figure 3a), 50°C significantly reduced adsorption rate on the host strain (Figure 5). Furthermore, phage P1 exhibited high infectivity at pH 6 to 8, a narrower range than the 4 phages (B1, B2, FAGK1, FAGK2) from *L. plantarum* ATCC 8014 (Briggiler Marcó et al. 2010). Our results also showed that this phage adsorbed effectively on *L. plantarum* IMAU10120 cells at pH ranging from 4 to 8. Thus, optimal adsorption conditions for the phage studied herein were pH ranging from 6 to 8 and incubation temperatures from 30 to 42°C. Thus, thermal treatments or changes in environmental pH values could be considered when designing phage-control strategies in fermentation processes involving the host bacteria.

Figure 7. Influence of divalent cations on the adsorption kinetics (at 37°C) of *Lactobacillus plantarum* phage P1. Values were the mean of 3 determinations. Error bars represent 95% CI. Color version available online.

The addition of calcium ions significantly increased the adsorption rate of phage P1, and magnesium ions expressed a negative effect on its adsorption. Unlike our results, in Briggiler Marcó et al. (2010), neither Ca^{2+} nor Mg^{2+} was necessary for adsorption or to complete the lytic cycle of the 4 phages (B1, B2, FAGK1, FAGK2). Lu et al. (2003) reported that excess Ca^{2+} (5–30 mM) in MRS medium did not affect the adsorption rate in the first 30 min but did promote rapid phage propagation and cell lysis. Those authors also suggested that the levels of calcium or other cations in MRS medium were sufficient for the initial infection steps but not for subsequent cycles. For other lactobacilli phages, the requirement of calcium for adsorption or lysis was variable (Capra et al., 2006; Suárez et al., 2008). The addition of inhibitors of cell protein synthesis (chloramphenicol) did not affect the adsorption of phage P1, which was similar to the results of Briggiler Marcó et al. (2010). As described previously, it would not be expected that phage binding is an energy-dependent process. Based on the results obtained in our research, further studies might be needed to examine the effects of high thermal, chemical, and photocatalytic treatments on inactivation. Furthermore, more studies are needed to determine if the decrease in adsorption is related to the disorganization of phage receptor sites or to the absence of host cell energy (Briggiler Marcó et al., 2010).

CONCLUSIONS

A *Lactobacillus* virulent phage P1 belonging to the Siphoviridae family was isolated from abnormal fermented liquid of *L. plantarum* IMAU10120. The latent period of this phage was 45 min, the burst time was 90 min, and the burst size was 132.88 ± 2.37 pfu/infective center. Temperature had little effect on phage and strain viability but a temperature of 50°C significantly reduced the adsorption rate of the phage. pH had a more obvious influence on phage viability and adsorption rate, and optimum pH values for phage adsorption ranged from 6 to 8. Calcium ions promoted and increased the adsorption rate of phage P1 but chloramphenicol (an inhibitor of cell protein synthesis) had no effect. Further studies should focus on the high thermal, chemical, and photocatalytic resistance of this virulent phage and designing the available strategies to reduce phage infections in industrial environments.

ACKNOWLEDGMENTS

This work was supported by Natural Science Foundation of China (Beijing; Grant No. 31301517), Natural

Science Foundation of Inner Mongolia, Huhhot, China (Grant No. 2013MS1207).

REFERENCES

- Briggiler Marcó, M., J. E. Garneau, D. Tremblay, A. Quiberoni, and S. Moineau. 2012. Characterization of two virulent phage of *Lactobacillus plantarum*. Appl. Environ. Microbiol. 78:8719–8734.
- Briggiler Marcó, M., J. A. Reinheimer, and A. Quiberoni. 2010. Phage adsorption to *Lactobacillus plantarum*: Influence of physiological and environmental factors. Int. J. Food Microbiol. 138:270–275.
- Capra, M. L., A. Quiberoni, and J. Reinheimer. 2006. Phages of *Lactobacillus casei/paracasei*: response to environmental factors and interaction with collection and commercial strains. J. Appl. Microbiol. 100:334–342.
- Caso, J. L., C. G. de los Reyes-Gavilán, M. Herrero, A. Montilla, A. Rodríguez, and J. E. Suárez. 1995. Isolation and characterization of temperate and virulent bacteriophage of *Lactobacillus plantarum*. J. Dairy Sci. 78:741–750.
- Chibani-Chennoufi, S., M. L. Dillmann, L. Marvin-Guy, S. Rami-Shojaei, and H. Brüssow. 2004. *Lactobacillus plantarum* bacteriophage LP65: A new member of the SOP1-like genus of the family Myoviridae. J. Bacteriol. 186:7069–7083.
- Costa, G. N., F. C. Marcelino-Guimarães, G. T. Vilas-Bôas, T. Matsuo, and L. H. S. Miglioranza. 2014. Potential fate of ingested *Lactobacillus plantarum* and its occurrence in human feces. Appl. Environ. Microbiol. 80:1013–1019.
- De Antoni, G., M. Zago, O. Vasek, G. Giraffa, D. Carminati, M. Briggiler Marcó, J. Reinheimer, and V. Suárez. 2010. *Lactobacillus plantarum* bacteriophages isolated from Kefir grains: Phenotypic and molecular characterization. J. Dairy Res. 77:7–12.
- Emond, E., and S. Moineau. 2007. Bacteriophages and food fermentations. Pages 93–124 in Bacteriophage: Genetics and Molecular Biology. S. McGrath and D. van Sinderen, ed. Horizon Scientific Press/Caister Academic Press, Norfolk, VA.
- Kodaira, K. I., M. Oki, M. Kakikawa, N. Watanabe, M. Hirakawa, K. Yamada, and A. Taketo. 1997. Genome structure of the *Lactobacillus* temperate phage wgle: The whole genome sequence and the putative promoter/repressor system. Gene 187:45–53.
- Lu, Z., F. Breidt, H. P. Fleming, E. Altermann, and T. R. Klaenhammer. 2003. Isolation and characterization of a *Lactobacillus plantarum* bacteriophage Ø JL-1 from a cucumber fermentation. Int. J. Food Microbiol. 84:225–235.
- Ma, C., G. Cheng, Z. Liu, G. Gong, and Z. Chen. 2016. Determination of the essential nutrients required for milk fermentation by *Lactobacillus plantarum*. LWT Food Sci. Technol. 65:884–889.
- Müller-Merbach, M., K. Kohler, and J. Hinrichs. 2007. Environmental factors for phage-induced fermentation problems: Replication and adsorption of the *Lactococcus lactis* phage P008 as influenced by temperature and pH. Food Microbiol. 24:695–702.
- Neviani, E., D. Carminati, and G. Giraffa. 1992. Selection of some bacteriophage-and lysozyme-resistant variants of *Lactobacillus helveticus* CNRZ 892. J. Dairy Sci. 75:905–913.
- Quiberoni, A., V. B. Suárez, A. G. Binetti, and J. A. Reinheimer. 2011. Bacteriophage. Pages 430–438 in Encyclopedia of Dairy Science. Vol. 1. 2nd ed. J. Fuquay, P. Fox, and P. McSweeney, ed. Academic Press, Elsevier Science, Cambridge, MA.
- Suárez, V., S. Moineau, J. Reinheimer, and A. Quiberoni. 2008. Argentinean *Lactococcus lactis* bacteriophage: Genetic characterization and adsorption studies. J. Appl. Microbiol. 104:371–379.
- Trevors, K. E., R. A. Holley, and A. G. Kempton. 1983. Isolation and characterization of a *Lactobacillus plantarum* bacteriophage isolated from a meat starter culture. J. Appl. Bacteriol. 54:281–288.
- Villion, M., and S. Moineau. 2009. Bacteriophages of lactobacillus. Front. Biosci. (Landmark Ed.) 14:1661–1683.
- Yoon, B. H., S. Hwan Jang, and H. I. Chang. 2011. Sequence analysis of the *Lactobacillus* temperate phage Sha1. Arch. Virol. 156:1681–1684.