

Host Range of *Listeria* Prophages Induced from Lysogenic *Listeria* Isolates from Foods and Food-related Environments in Thailand

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ABSTRACT

Prophages have been shown to be associated with improving the survival and fitness of Listeria spp., especially Listeria monocytogenes—a fatal foodborne pathogen. Bioinformatics has revealed the presence of many prophages in Listeria genomes. However, understanding the distribution of prophages in Listeria isolates is limited to those that have no sequences available on the database. In this study, Mitomycin C induction was used to obtain prophages in free-floating form. Among 236 isolates from various sources, prophages were induced from 13/108 (12%) isolates of L. monocytogenes and 10/128 (7.8%) isolates of Listeria spp. Of 39 induced phages obtained, most phages were originated from foods. Phenotypic characterization was performed by the host range determination against 17 hosts representing 9 major serotypes of L. monocytogenes and 4 other species of Listeria. Induced phages were classified into three groups. The majority of phages (groups A and C) were host-specific-phages with the ability to lyse one to seven (<42%) hosts. However, five phages (group B) showed broader host range than phages in other groups, which could lyse 8–10 (47–59%) hosts. Both induced and isolated Listeria phages showed

a high ability to lyse strains of L.monocytogenes serotype 4, while the induced phages showed narrow host range compared to the isolated phages. Host range data allows the prediction of particular L. monocytogenes subtypes or Listeria species that could be affected by the induced phages, leading to gene transfer upon phage infection. Information obtained here is useful to understand the diversity and role of prophages in Listeria genomes.

Keywords: *Listeria* spp., *Listeria* prophage, Food processing, Phage host range

INTRODUCTION

Listeria monocytogenes is an important foodborne pathogen causing potentially fatal listeriosis infections with a high mortality rate of up to 30% (Swaminathan and Gerner-Smidt, 2007). The genus of *Listeria* comprises 17 species, including two pathogenic species *L. monocytogenes* and *L. ivanovii*. Previous studies reported that various types of foods could be contaminated with *L. monocytogenes* (Vongkamjan et al., 2015, 2016). This pathogen has been shown the ability to be well adapted and survive longer in specific environments (Palumbo and Williams, 1991; Tolvanen et al., 2008; Burgess et al., 2016). Prophages have been shown the linkage on providing greater survival and fitness of *Listeria* spp., especially *L. monocytogenes* (Orsi et al., 2008; Verghese et al., 2011).

Prophage is a phage-related sequence which has integrated into bacterial chromosome and become part of the bacterial genome. Prophages have been reported to be commonly present in the *Listeria* genomes (Nelson et al., 2004; Kuenne et al., 2013), for example, *L. monocytogenes* strains F6854, L99, HCC23, J0161 (Kuenne et al., 2013; den Bakker et al., 2013). *L. innocua* strain CLIP11262 harbored up to six prophage (-like) elements, including 5 prophages and 1 monocolin (Nelson et al., 2004). Previous studies mostly applied bioinformatics analysis to search for prophage regions in the genome of *Listeria*. This approach restricts information on prophage diversity to those lysogens (prophage-carrying *Listeria*) that have no sequences available on the database.

Induction is a mechanism by which prophage can be induced to escape a dying host and enter the lytic cycle, producing a free-floating form called induced phage. In previous studies, the occurrence of prophages has been measured by induction from the lysogenic isolates (Jiang and Paul, 1996; Chen et al., 2006). Some antibiotics, UV radiation, sunlight, temperature and pressure have been previously reported as common inducing-agents for prophage induction (Jiang and Paul, 1996; López et al., 2014). However, mitomycin C has been regarded as the most popular agent to induce prophages and produce infective phages (Loessner et al., 1991; Verghese et al., 2011).

Phage characterization is useful for obtaining information on the diversity of both phages isolated from natural sources (freely isolated phages) and phages induced from the lysogens. Previous studies have performed a phenotypic characterization by host range determination in *Listeria* phages isolated from natural dairy farms (Vongkamjan et al., 2012) or seafood processing (Vongkamjan et al., 2017) as well as turkey processing plant environments (Kim et al., 2008). However, there is still limited information on the characterization of *Listeria* phages induced from lysogenic isolates.

In this study, mitomycin C induction was performed to examine the prevalence of prophage-carrying *Listeria* among isolates obtained from various foods and food-related environments. The newly induced *Listeria* phages were then characterized phenotypically by host range determination and compared to that of freely isolated *Listeria* phages from seafood environment samples. This information is useful to link or speculate particular sources that showed high prevalence and diversity of prophages, which is a potential factor for the bacteria to gain better survival and present in foods and food-related environments.

MATERIALS AND METHODS

L. monocytogenes and *Listeria* spp. used in this study

A total of 236 *Listeria* isolates consisting of 108 isolates of *L. monocytogenes* and 128 isolates of *Listeria* spp. (non-*monocytogenes*) (Table 1) were tested for the presence of prophage by mitomycin C induction. These isolates were previously obtained by the standard *Listeria* isolation protocols (Bacteriological Analytical Manual of the US Food and Drug Administration) and confirmed by PCR (Vongkamjan et al., 2015, 2016, 2017). *Listeria* isolates were classified into five categories based on the sources (foods and food-related environments). The food sources included raw and ready-to-eat products of animal origin (44 isolates), seafood/ aquatic origin (103 isolates) or vegetable origin (8 isolates). The food-related environments included food contact surfaces (41 isolates), for example, buckets/ trays/ baskets used to contain products, blenders, knives, gloves, tables; and non-food contact surfaces (40 isolates) such as digital scales, cleaning sponges, and drains.

Table 1. Prevalence of *Listeria* lysogens among the isolates of *L. monocytogenes* and *Listeria* spp. from various sources.

Source of isolates	<i>L. monocytogenes</i>		<i>Listeria</i> spp. (non- <i>monocytogenes</i>)	
	No. of isolates tested	No. of lysogens (%) ^a	No. of isolates tested	No. of lysogens (%) ^a
Animal origin products (Ani)	20	8 (40)	24	3 (12.5)
Seafood/ aquatic products (Sea)	39	2 (5.1)	64	6 (9.4)
Vegetable products (Veg)	3	0	5	1 (20)
Food contact surfaces (FCS)	20	2 (10)	21	0
Non-food contact surfaces (NFCS)	26	1 (3.8)	14	0
Total	108	13 (12)	128	10 (7.8)

Note: ^aPercentage of the lysogens is calculated by number of lysogens found in each source out of the total number of tested isolates from that source.

Four *L. monocytogenes* reference strains (Mack, FSL F2-695, FSL J1-208 and F2365 were obtained from Food Safety Lab (FSL), Cornell University) were used as propagating hosts for phage induction, purification and lysate preparation. Host range determination was performed using the same panel of hosts as previously studied by Vongkamjan et al. (2017). In which, 13 strains of *L. monocytogenes* representing nine major serotypes of *L. monocytogenes* included serotypes 4a, 4b, 4c (n=7), serotypes 1/2a, 1/2b, 1/2c (n=3) and serotypes 3a, 3b, 3c (n=3). Additional four strains representing other four species (*L. innocua*, *L. ivanovii*, *L. marthii*, *L. seeligeri*) were also included. All of *Listeria* strains/ isolates were kept in Brain Heart Infusion (BHI, Oxoid, UK) broth with 15% glycerol and stored at -80°C at the department of Food Technology, Prince of Songkla University (PSU), Thailand.

Induction of *Listeria* prophages by mitomycin C

Cultures of *Listeria* isolates were prepared by inoculating a single colony of each tested isolate in 5 ml of Luria Bertani (LB, Himedia, Mumbai, India) broth with a-supplement of 50 mM morpholinepropanesulfonic acid (MOPS), 1% (wt/vol) glucose, 10 mM CaCl₂, and 10 mM MgCl₂ (LB-MOPS-Glu-salts) (Vongkamjan et al., 2012). The tested isolate was incubated at 30°C with shaking (220 rpm) for 8–10 h to reach a 600 nm optical density (OD₆₀₀) of 0.4 to 0.5. The culture of each tested isolate was mixed with mitomycin C (Sigma-Aldrich, St Louis, USA)

to a final concentration of 1 µg/ml, followed by additional incubation for 7 h at the same conditions (modified from Fortier and Moineau, 2007). A quantity of 200 µl of mitomycin C-treated culture was mixed with 100 µl of each propagating host overnight culture in 2 ml of LB-MOPS-Glu-salts broth. Then, the co-culture was incubated for 18 h at 30°C with shaking (220 rpm). To isolate the induced phage, 1 ml of overnight co-culture was centrifuged at 10,000×g for 15 min at 4°C. The supernatant was used to prepare an overlay using 0.7% LB-MOPS top-agar with a 10-fold-diluted overnight culture of a given propagating host, then poured on the 1.5% LB-bottom-agar (Vongkamjan et al., 2012). After an overnight incubation at 30°C, each plate was examined for plaque-forming on the host lawn. The presence of plaque formation indicated the appearance of induced phage, the tested *Listeria* isolate was recorded as a lysogen. Each different types of plaque morphology observed (differentiated by a plaque diameter between 0.5–2mm, star/round shape, turbid/ translucent/clear zone) was recorded as a single induced phage.

Purification of induced *Listeria* phages and preparation of phage lysates

An isolated plaque of each plaque-morphology type was selected for phage purification using the double layer method. This step was conducted three times to ensure a pure phage following the protocol previously described by Vongkamjan et al. (2012). Upon the third purification, a single plaque was used to prepare a high titer phage stock using two confluent lysis plates. A volume of 7 ml of Phosphate Buffer Saline (PBS) with a pH of 7.4 was added to each plate, followed by a centrifugation at 5,000×g for 15 min at 4°C and filtration through a 0.22-µm pore-size filter. Phage titers were determined by spotting 5-µl of eight of 10-fold serial phage lysate dilutions on the prepared lawn of the propagating host, as mentioned above.

Determination of phage host range

The host range of each induced *Listeria* phage was determined with the same host panel as previously used to characterize the host range of *Listeria* phages isolated from seafood processing samples (Vongkamjan et al., 2017). A quantity of 5-µl of the diluted phage representing 100×RTD (routine test dilution) (approximately 10⁶–10⁷ PFU/ml) was spotted on the prepared lawn of each host, followed by an overnight incubation at 30°C. Each spot on the lawn was examined and recorded as lysing (+), which known as the presence of multiple or single plaque(s), turbid or confluent lysis at the spotting area or no lysing (-). The experiment was carried out in triplicate. The induced phage was considered that could lyse a tested host when plaque forming was observed in at least two replicates. Lysis ability of the induced phages against the tested hosts was used to classify these phages into clusters. Hierarchical clustering was performed using the R program version 3.1.2. with Ward's method and binary distance as previously described (Vongkamjan et al., 2012).

Comparison of host ranges between newly induced *Listeria* phages and freely isolated *Listeria* phages from seafood processing environments in Thailand

Host range data of 39 newly induced *Listeria* phages in this study and the data of 29 freely isolated *Listeria* phages from the previous study by Vongkamjan et al. (2017) were compared. These two sets of *Listeria* phages were tested against the same panel of reference hosts in order to examine the difference between lysis abilities of induced phages and isolated phages. A previously reported *Listeria* phage LP124 was used as a control for both sets of induced and isolated *Listeria* phages.

RESULTS

Occurrence of *Listeria* lysogens among the *Listeria* isolates from various sources

From total of 236 *Listeria* isolates used for determination the presence of prophages by mitomycin C induction, 23 *Listeria* isolates were lysogens (Table 1). Of these, 13/108 (12%) lysogens were *L. monocytogenes* and 10/128 (7.8%) lysogens were *Listeria* spp. Among the 108 tested *L. monocytogenes* isolates, the prevalence of lysogens was highest in the isolates from Ani (40%) followed by FCS (10%) and Sea (5.1%). Among the 128 *Listeria* spp. isolates, prevalence of lysogens was highest in the isolates from Veg (20%), followed by Ani (12.5%) and Sea (9.4%). In particular, none of *L. monocytogenes* isolates from Veg or *Listeria* spp. isolates from FCS and NFCS were lysogens.

Distribution of prophages in *Listeria* lysogens from various sources

Based on the presence of distinct types of plaque morphology observed, 39 induced *Listeria* phages were obtained from 23 lysogens (Table 2). From the 13 lysogens of *L. monocytogenes*, 28 induced *Listeria* phages were obtained upon mitomycin C induction. Of these, 20 phages (51.3%) were from *L. monocytogenes* lysogens of Ani, the remaining phages was from *L. monocytogenes* lysogens of Sea (10.2%), FCS (7.7%) and NFCS (2.6%). From the 10 lysogens of *Listeria* spp., 11 induced phages were obtained. All of which were induced from *Listeria* spp. lysogens of the food sources with Sea (17.9%), followed by Ani (7.7%) and Veg (2.6%).

Table 2. Distribution of prophages in *Listeria* lysogens from various sources.

Source of lysogens	<i>L. monocytogenes</i>		<i>Listeria</i> spp. (non- <i>monocytogenes</i>)	
	No. of lysogens	No. of induced phages (%) ^a	No. of lysogens	No. of induced phages (%) ^a
Animal origin products (Ani)	8	20 (51.3)	3	3 (7.7)
Seafood/ aquatic products (Sea)	2	4 (10.2)	6	7 (17.9)
Vegetable products (Veg)	0	0	1	1 (2.6)
Food contact surfaces (FCS)	2	3 (7.7)	0	0
Non-food contact surfaces (NFCS)	1	1 (2.6)	0	0
Total	13	28 (71.8)	10	11 (28.2)

Note: ^aPercentage of the induced phages is calculated by number of induced phages in each source out of the total number of induced phages obtained (n = 39).

Phages induced from both *L. monocytogenes* lysogens and *Listeria* spp. (non-*monocytogenes*) lysogens were able to be propagated in *L. monocytogenes* hosts. By using different serotypes of *L. monocytogenes* as propagating hosts, distribution of induced *Listeria* phages was various with between 6 and 12 induced phages obtained in each propagating host (data not shown).

Lysis ability of induced *Listeria* phages on *Listeria* hosts

All 39 induced *Listeria* phages were used for the host range determination against 17 reference strains representing nine serotypes of *L. monocytogenes* and four other *Listeria* spp. (Figure 1). Clustering analysis using the R program classified these induced phages into 22 lysis profiles, representing three host range groups, labeled A to C. Group C contained 17 host-specific-phages, which could lyse only one to three (<20%) hosts of *L. monocytogenes* serotype 4. Group A contained 17 phages with similar host range as those in group C (host-specific phages), but they could lyse more hosts of *L. monocytogenes* serotype 4 and *L. marthii*. However, the remaining five phages (group B) showed broader host range than phages in group A and C. In which, group B phages could lyse 8–10 (47–59%) hosts tested.

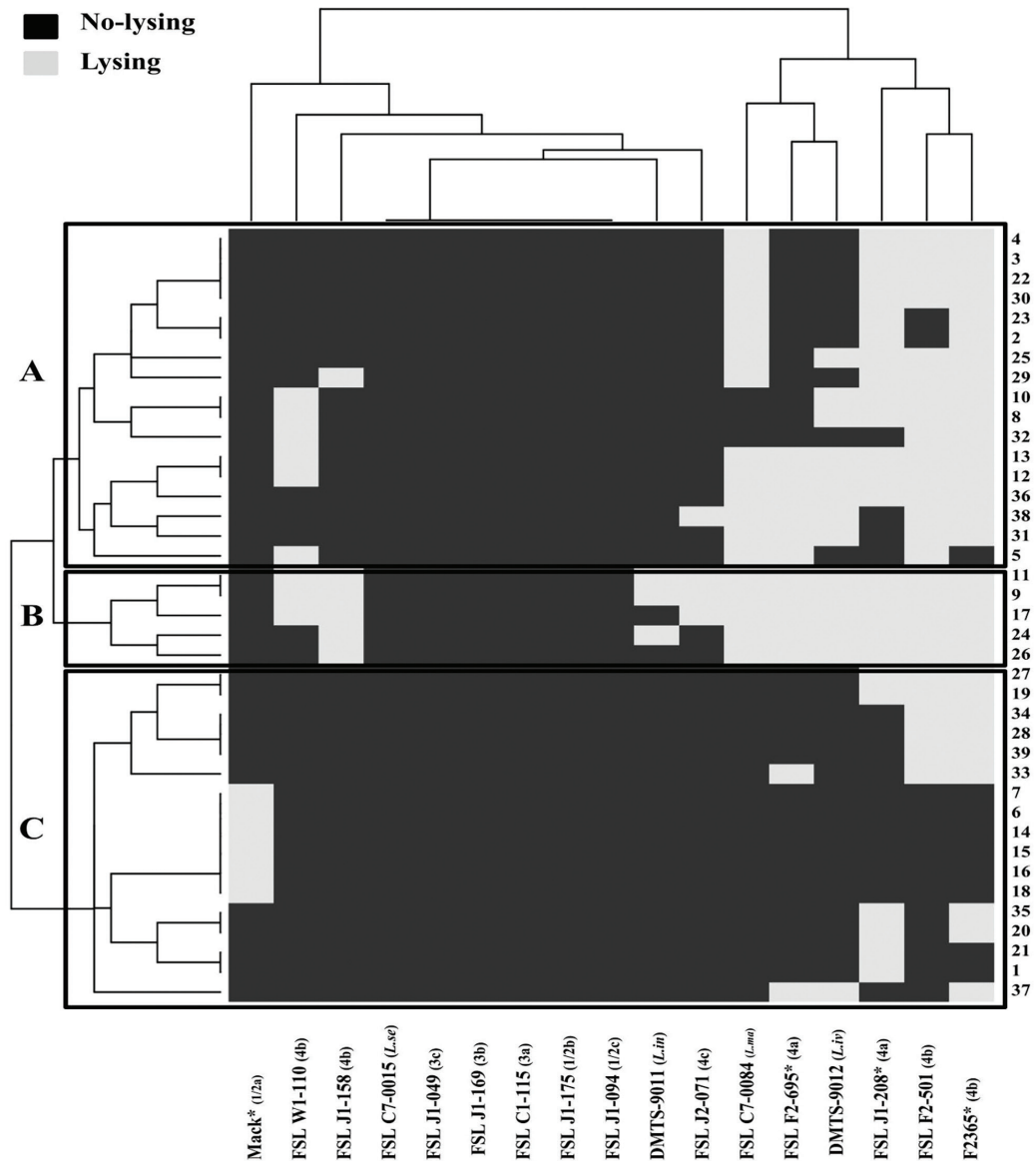


Figure 1. Clustering analysis of 39 induced *Listeria* phages against 17 *Listeria* hosts using the R program version 3.1.2. X-axis represents 13 *L. monocytogenes* and 4 *Listeria* spp. host strains. Y-axis represents 39 induced *Listeria* phages. Black represents no-lysing and light-gray represents lysing on a given host strain. Four references strains marked with “*” were used as the propagating hosts for prophage induction, phage purification and phage lysate preparation.

Of the tested hosts, *L. monocytogenes* serotype 4 strains were highly susceptible to the induced phages, which were lysed by up to 30/39 phages tested. For example, strain F2365 (serotype 4b) was lysed by 30 induced phages, strain FSL J1-208 (serotype 4a) was lysed by 24 induced phages. In addition, *L. marthii* (FSL C7-0084) was lysed by 19 induced phages. In contrast, five strains of *L. monocytogenes* serotypes 1/2 (1/2b, 1/2c) and serotypes 3 (3a, 3b, 3c) and one strain of *L. seeligeri* shown to be resistant to all of the induced phages.

Comparison of host ranges between induced *Listeria* phages and *Listeria* phages freely isolated in Thailand

By having the same set of *Listeria* hosts used in the phage host range determination, phage lysis profiles of our induced phages were compared to those of freely isolated *Listeria* phages from a previous study (Vongkamjan et al., 2017). Figure 2 showed the percentage of induced/isolated phages which were able to infect each host. In general, most of the induced phages were host-specific with the ability of lysing < 42% of the hosts, while the freely isolated phages presented both broad and narrow host ranges with the ability of lysing $\geq 50\%$ of the hosts. For instance, the lysis ability of isolated phages in each host was at least double that of the induced phages. All of the isolated phages could lyse serotype 4 strains (except F2365) and *L. marthii*. However, all of the induced phages were resistant to most hosts representing *L. monocytogenes* serotype 1/2 (except Mack), serotype 3, *L. innocua* and *L. seeligeri*. Interestingly, none of phages in both groups of induced and isolated phages could lyse two hosts of *L. monocytogenes* serotype 3b and serotype 3c.

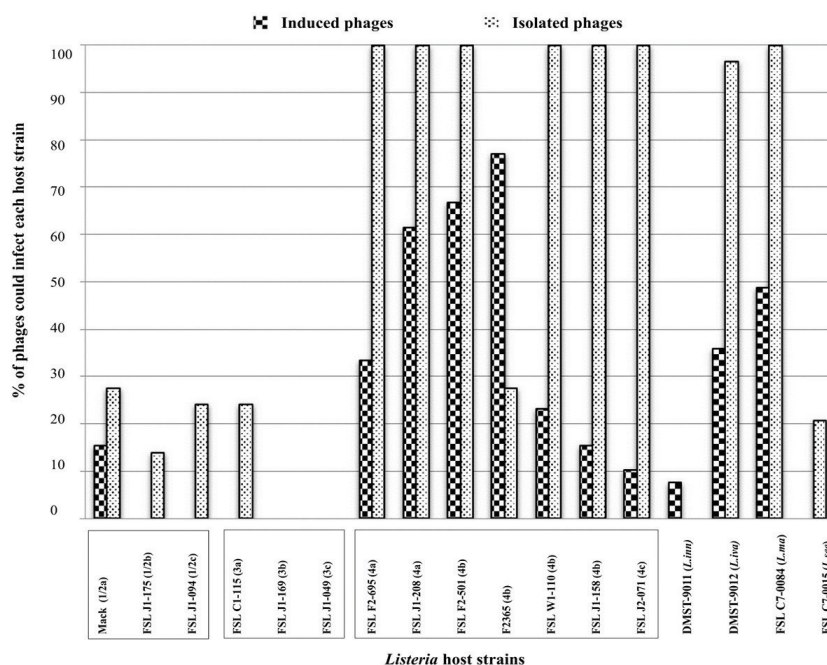


Figure 2. Lysis ability of the induced *Listeria* phages (n=39) and freely isolated *Listeria* phages (n=29) against *Listeria* hosts. X-axis represents 13 *L. monocytogenes* and 4 *Listeria* spp. host strains. Y-axis represents percentage of induced *Listeria* phages/isolated *Listeria* phages which could lyse each host.

DISCUSSION

High distribution of prophages in *Listeria* isolates from foods, especially from animal origin products

Listeria lysogens are known to be widespread among *Listeria* spp. (Loessner et al., 1991) and the induction of prophages by mitomycin C was carried out to examine the prophage prevalence. Among 236 *Listeria* isolates, 23 isolates (9.7%) were lysogens and produced plaque in *L. monocytogenes* propagating host(s). *Listeria* isolates from food sources (Ani, Sea and Veg) were more likely to carry inducible phages (12.9%) than those from non-food sources (3.7%). Of the food source, up to 51.3% *L. monocytogenes* isolates of animal origin products were lysogens. Prevalence of prophages found here were similar to the report in a previous study (Ferreira et al., 2011), in which prophages were found in 53.7% of *L. monocytogenes* isolates (n=41) from meat processing plants. Overall, findings suggest that *Listeria* isolates of animal origin or related environments are more likely to carry prophages.

Although reports relating to *Listeria* prophages are limited, in other bacteria such as *Staphylococcus aureus*, phages were obtained from meat (30%), seafood (67%) and dairy-related environments (97%), (Gutiérrez et al., 2016). Another study reported that none of the nine isolates of *Clostridium difficile* from meat carried prophages, nine out of 49 isolates of *C. difficile* from animal and human origins carried prophages (Sekulovic et al., 2014).

Induced *Listeria* phages appear to be host-specific with a higher ability to lyse *L. monocytogenes* serotype 4 than other serotypes

Majority of induced *Listeria* phages (87%) belonged to host range groups A and C which represented as host-specific phages. Similar findings have previously been reported that induced phages showed narrow host range (Horgan et al., 2010; Wright et al., 2013). The host range data pointed out that both sets of phages could lyse most strains of *L. monocytogenes* serotype 4 and *L. marthii*. Similar findings were also observed in the isolated *Listeria* phages from silage (Vongkamjan et al., 2012). In addition, another study reported that *Listeria* phages isolated from a turkey processing environment could lyse most strains of *L. monocytogenes* serotype 1/2a and serotype 4b (Kim et al., 2008). Finding suggests that not only the isolated *Listeria* phages, but also the induced *Listeria* phages showed a high ability in lysing *L. monocytogenes* serotype 4. One possible explanation for the difference of phage susceptibility between serotypes of *L. monocytogenes* is the differences in the structures of wall teichoic acids (WTA). It has been reported that *L. monocytogenes* of serotype 4 contain a WTA structure with terminal glucose and galactose residues, which are reported to be important for phage adsorption (Wendlinger et al., 1996; Eugster and Loessner, 2011).

Most of the induced *Listeria* phages could not lyse strains of serotype 1/2 (1/2b, 1/2c) and serotype 3 (3a, 3b, 3c), *L. innocua* and *L. seeligeri*. It is of interest that serotype 1/2a is linked to the increased cases of listeriosis in the last decade (Mammina et al., 2013; Marini et al., 2016). This suggests the possible relationship between resistance to phage infection and higher survival and virulence of this serotype. Alternative explanation is that these hosts may harbor certain phage-related sequences to aid their survival in the environment without being affected by phage lysis. As explained in a previous study, *Oenococcus oeni* isolates that were phage-resistant contained a sequence that was homologous to the tested phage sequence (Poblet-Icart et al., 1998). Our understanding is still limited to clarify the resistance mechanism in these host strains. Further experiment, for example, prophage induction of these *Listeria* hosts and genome sequencing analysis are needed to elucidate this phenomenon.

Induced *Listeria* phages show a narrow host range as compared to freely isolated *Listeria* phages

Overall, induced *Listeria* phages showed narrow host range as compared to the isolated *Listeria* phages. Previous studies have been reported that the isolated *Listeria* phages from turkey processing showed a wider host range (Kim et al., 2008), from silage at dairy farm showed both narrow and broad host range (Vongkamjan et al., 2012). This can be explained by the unique characteristic of induced phage as it has the ability to incorporate its genome into the host chromosome instead of only lysing the host. In previous studies, for example, Loessner and Busse (1990) also reported that the phages isolated from lysogenic strains had a narrower lytic spectrum than that of lytic phages. The host range data suggests that not only induced phages but also isolated phages were less susceptible to strains of *L. monocytogenes* serotype 1/2 and serotype 3 rather than serotype 4 strains.

CONCLUSION

In summary, this study provides new insights into the phenotypic characteristics of *Listeria* phages induced from *Listeria* isolates from various food and food-related environments. Prophage distribution data could be linked to particular sources where *Listeria* prophage-carrying isolates are prevalent. These isolates of *L. monocytogenes* or other *Listeria* spp. may gain the ability to better survive in foods and processing environments. The newly induced *Listeria* phages described herein provide a baseline for further study on prophage diversity and the role of prophages in facilitating the survival and fitness of *Listeria* hosts in foods and food-related environments.

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