Original paper

Biocontrol of *Morganella morganii* subsp. *morganii* and Histamine Accumulation in Tuna Meat by Treatment with a Lytic Bacteriophage

Shogo YAMAKI*, Yuji KAWAI and Koji YAMAZAKI

Laboratory of Marine Food Science and Technology, Faculty of Fisheries Sciences, Hokkaido University, Hakodate 041-8611, Japan

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Histamine poisoning is a common seafood-borne illness worldwide, and *Morganella morganii* subsp. *morganii* is one of the most probable histamine producers. Bacteriophages are bacterial viruses that kill the host cell after infection. In this study, we evaluated the effect of *M. morganii* phage FSP1 treatment on histamine accumulation by *M. morganii* in raw tuna meat. *M. morganii* was inoculated to tuna meat and incubated at 4, 12 and 20°C to evaluate the inhibitory effects of FSP1 on the growth and histamine accumulation of *M. morganii*. Viable *M. morganii* counts were decreased significantly in response to FSP1 treatment under all conditions. Furthermore, histamine concentrations in phage-treated samples were significantly lower than those of control samples. FSP1-resistant *M. morganii* did not develop after FSP1 treatment. These results suggest that phage treatment might be an effective tool to reduce the risks of histamine poisoning by *M. morganii* in fisheries products.

Keywords: histamine poisoning, bacteriophage, bacteriophage treatment, seafood, *Morganella morganii*, food safety

Introduction

Histamine poisoning (or scombroid poisoning) is a common seafood-borne illness worldwide that results from the consumption of histamine-accumulated seafood. Bacterial histidine decarboxylases convert free histidine in fish meat into histamine. Many species of histamine-producing bacteria associated with seafood have been described, such as *Morganella morganii* subsp. *morganii*, *Enterobacter aerogenes*, *Photobacterium damsela* subsp. *damsela*, *P. phosphoreum*, *Proteus vulgaris*, and *Raoultella planticola* (Bermejo *et al.*, 2003; Ferrario *et al.*, 2012; Kanki *et al.*, 2004; Rodtong *et al.*, 2005). The major symptoms of this illness are allergic-like reactions, such as hives, rash, flushing, and facial swelling (Hungerford, 2010). However, in the most serious cases, there have been reports that histamine poisoning produces severe life-threatening symptoms (D'Aloia *et al.*, 2011; Sánchez-Guerrero *et al.*, 1997). Histamine removal from seafood is very difficult because it is stable after heating and freezing. Therefore, microbial

control of histamine-producing bacteria in seafood is the best strategy to prevent histamine poisoning.

M. morganii subsp. *morganii* is a Gram-negative bacterium in the family *Enterobacteriaceae* and a known major histamine producer associated with seafood. A polymerase chain reaction assay coupled with Southern hybridization showed that *M. morganii* contaminates the gills and skin of mackerel, sardine, and albacore as well as the processing plant environment (Kim *et al.*, 2003), suggesting that *M. morganii* is widely distributed in fish and fish processing environments. Ferrario *et al.* (2012) reported that *M. morganii* is the most active histamine-producing species among the strains isolated from tuna meat fillets. These reports suggest that *M. morganii* is one of the most likely causative agents of histamine poisoning.

Bacteriophages (phages) are bacterial viruses that infect and lyse bacterial cells. The antimicrobial potential of phages is considerable in medicine, where the emergence of antibiotic-

*To whom correspondence should be addressed.

E-mail: yamaki@fish.hokudai.ac.jp

resistant bacteria is common (Kutateladze and Adamia, 2010), although their use has not been reported except in some Eastern European countries. However, some phage products have already been approved as antimicrobial agents to control foodborne pathogens, such as *Listeria monocytogenes* and *Escherichia coli* O157:H7 (Sulakvelidze, 2013). We previously investigated the use of phages to inhibit histamine-producing bacteria and reported the characterization of the *M. morganii* subsp. *morganii* phage FSP1 and *P. damsela* subsp. *damsela* phage Phda1 (Yamaki *et al.*, 2014; Yamaki *et al.*, 2015). The *M. morganii* phage FSP1 belongs to the family *Myoviridae* and has a strong anti-*M. morganii* effect in liquid media. In this study, we evaluated the effect of FSP1 treatment on the growth and histamine accumulation of *M. morganii* subsp. *morganii* in raw tuna meat.

Materials and Methods

Bacterial strains and phage *M. morganii* subsp. *morganii* NBRC3848^T was used for antimicrobial testing in the culture medium after being grown for 18 h at 30°C in tryptic soy broth (TSB; Becton Dickinson, Sparks, MD, USA). Chloramphenicol-resistant *M. morganii* subsp. *morganii* NBRC3848^{cr} was used for antimicrobial testing on tuna meat and grown at 30°C for 18 h in TSB supplemented with chloramphenicol and 1% L-histidine (pH 6.5). *M. morganii* phage FSP1 (Yamaki *et al.*, 2014) was propagated using *M. morganii* subsp. *morganii* NBRC3848^T and concentrated by polyethylene glycol precipitation. FSP1 was purified by CsCl density-gradient ultracentrifugation (100,000 × *g*, 3 h, 4°C, *d* = 1.3, 1.5, and 1.7) and stored at 4°C until use.

FSP1 antimicrobial tests in liquid medium *M. morganii* NBRC3848^T was inoculated at 1×10^3 colony forming units (CFU) mL⁻¹ into TSB, and FSP1 was inoculated at 1×10^3 , 1×10^5 , or 1×10^7 plaque forming units (PFU) mL⁻¹. The samples were mixed and incubated at 4, 12, or 20°C. The culture media were serially diluted with saline solution supplemented with 0.1% peptone and spread onto tryptic soy agar (TSA; Becton Dickinson) to determine viable *M. morganii* cell counts. TSA plates were incubated at 30°C for 24 h, and CFUs were enumerated.

Determination of optimum phage concentration for treatment of tuna meat Raw tuna meat was purchased at a local supermarket and cut into 5 g pieces using a sterile knife. Each piece of tuna meat was placed in a sterile Petri dish. A fresh culture of *M. morganii* subsp. *morganii* NBRC3848^{cr} was centrifuged (10,000 × *g*, 4°C, 2 min) and washed with saline solution supplemented with 0.1% peptone. After serial dilution, 50 µL of the *M. morganii* subsp. *morganii* NBRC3848^{cr} suspension was inoculated at 3×10^3 CFU g⁻¹ by spotting with a pipette on the tuna meat. The inoculated samples were maintained at room temperature for 30 min, and then 100 µL of FSP1 solution was added by spotting at various concentrations (3×10^5 , 3×10^6 , 3×10^7 or 3×10^8 PFU g⁻¹). After phage treatment, the prepared samples were covered with the Petri dish's lid and stored at 20°C for various durations. Viable *M.*

morganii counts were determined as described below.

Determination of viable bacterial cell counts of tuna meats The incubated tuna meat samples were homogenized with 45 mL of phosphate buffered saline (pH 7.2), and the sample solutions were used to determine total plate counts and *M. morganii* counts. The homogenized solution for bacterial counts was serially diluted with saline solution supplemented with 0.1% peptone. Total plate counts were measured by spreading the bacteria on a TSA plate, and CFUs were counted after a 48 h incubation at 30°C. *M. morganii* counts were also measured by spreading onto modified Niven's agar (5.0 g L⁻¹ tryptone, 5.0 g L⁻¹ yeast extract, 1.0 g L⁻¹ CaCO₃, 25.0 g L⁻¹ L-histidine hydrochloride monohydrate, 5.0 g L⁻¹ NaCl, 0.06 g L⁻¹ bromocresol purple, and 20.0 g L⁻¹ agar, pH 5.1; Niven *et al.*, 1981) supplemented with 25 µg/mL chloramphenicol, and CFUs were enumerated after a 24–48 h incubation at 30°C.

Analysis of inhibitory effect of FSP1 treatment on histamine accumulation The inhibition of histamine accumulation by *M. morganii* was evaluated under two sets of inoculum conditions: a low contamination model (3×10^3 CFU g⁻¹) and a high contamination model (3×10^5 CFU g⁻¹). The tuna meat was contaminated with *M. morganii* subsp. *morganii* NBRC3848^{cr} at 3×10^3 (low contamination model) or 3×10^5 (high contamination model) CFU g⁻¹. Then, the samples were treated with FSP1 at 3×10^8 PFU g⁻¹ and incubated at 4, 12, or 20°C. Total plate counts, viable *M. morganii* cell counts, FSP1 counts, and histamine concentrations were determined. Sample preparation, contamination, treatment, and the measurement of viable cell counts were conducted as described above. Measurements of FSP1 counts and histamine concentration were conducted as described below.

Determination of phage counts in tuna meats The homogenized solution for the infectious FSP1 counts was filtered through a 0.45 µm membrane filter (polyether sulfone) and serially diluted with SM buffer (100 mM NaCl, 8 mM MgSO₄·7H₂O, 50 mM Tris-HCl, and 0.01% gelatin, pH 7.5). The PFUs of the infectious phages were measured by the plaque assay method using TSA as the bottom agar and 0.5% molten soft agar inoculated with *M. morganii* NBRC3848^T as the top agar. The number of PFUs was counted after a 24 h incubation at 30°C.

Determination of histamine concentration in tuna meats Histamine concentration in the tuna meat was analyzed by high-performance liquid chromatography (HPLC; Mett and Sturgeon, 1982). Homogenized samples were mixed with an equal volume of 10% trichloroacetic acid, and filtered through a 0.2 µm polytetrafluoroethylene filter (Merck Millipore, Billerica, MA, USA). The filtrates were subjected to HPLC analysis, and histamine concentrations were calculated using the absolute calibration curve method.

Confirmation of FSP1-resistant strains in tuna meats In the experiments on tuna meats described in the above section, *M. morganii* colonies that formed on Niven's agar from samples

incubated at 20°C for 60 h were isolated randomly after counting CFUs. The isolated strains were purified by streaking to remove the contaminating phages, and susceptibility to FSP1 was analyzed based on the plaque forming ability of FSP1.

Statistical analysis Bacterial counts were determined by duplicate plating. The results were the mean value from three independent experiments, and error bars indicate standard deviation. Analysis of variance and the Games–Howell test were conducted to detect significant differences among the control and phage treatments at various concentrations. A student's *t*-test (unpaired, two-tailed, and heteroscedastic) was conducted to analyze significant differences between viable cell counts of the control and phage-treated samples. The data were analyzed separately for each time point, and significance was based on a 5% level ($P < 0.05$). All statistical tests were conducted with R ver. 3.3.1 (R Core Team (2016). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL <https://www.R-project.org/>).

Results

Effects of temperature and phage concentration on antimicrobial activity of FSP1 in liquid medium Growth of *M. morganii* NBRC3848^T in liquid medium is shown in Fig. 1. Viable *M. morganii* cell counts of the samples treated with 10^3 or 10^5 PFU mL⁻¹ FSP1 were almost unchanged on all days at 4°C, and no significant differences were detected between control and treatment samples. However, the 10^7 PFU mL⁻¹ FSP1 treatment significantly decreased *M. morganii* about 1.5 log CFU mL⁻¹ after 6 h, viable cell counts were undetectable (< 1 log CFU mL⁻¹) at 24 h, and *M. morganii* did not grow throughout the incubation period (Fig. 1A).

M. morganii incubated at 12°C without FSP1 increased to 8.5 log CFU mL⁻¹ after 120 h. Growth of *M. morganii* was not different from that of control during the first 24 h in samples treated with 10^3 or 10^5 PFU mL⁻¹ FSP1, but viable counts under both concentrations decreased to < 1 log CFU mL⁻¹ after 72 h. In addition, in samples treated with 10^7 PFU mL⁻¹ FSP1, viable *M. morganii* cell counts decreased more rapidly than those of samples treated with 10^3 or 10^5 PFU mL⁻¹ FSP1 and were undetectable after 48 h (Fig. 1B).

Viable *M. morganii* cell counts in control samples at 20°C increased to 9 log CFU mL⁻¹ after 36 h. In contrast, *M. morganii* treated with 10^7 PFU mL⁻¹ FSP1 decreased immediately to 1.1 log CFU mL⁻¹ after 3 h. Then, *M. morganii* counts were suppressed between 1.0–1.5 log CFU mL⁻¹. Viable *M. morganii* cell counts increased to 5 log CFU mL⁻¹ during the first 12 h in the 10^3 and 10^5 PFU mL⁻¹ FSP1-treated samples, but decreased to almost 1 log CFU mL⁻¹ after 18 h. However, *M. morganii* regrew slowly in samples treated with 10^3 and 10^7 PFU mL⁻¹ FSP1 (Fig. 1C), suggesting the emergence of FSP1-resistant strains.

Effect of phage concentration on the antimicrobial effect of FSP1 on tuna meat We inoculated various concentrations of FSP1

and analyzed the changes in viable *M. morganii* cell counts to investigate the effect of phage concentration on FSP1-inhibited growth of *M. morganii* in tuna meat at 20°C (Fig. 2). Viable *M. morganii* cell counts increased to 7.6 log CFU g⁻¹ after 48 h in control samples (without FSP1 treatment), but this number was lower than that in TSB. It is thought that the difference in maximum population is due to the presence of contaminating bacteria in tuna meats other than *M. morganii*. FSP1 reduced the number of viable *M. morganii* cells; however, the antimicrobial effect of FSP1 was strongly influenced by phage concentration. Treatment with 10^5 or 10^6 PFU g⁻¹ FSP1 significantly decreased viable *M. morganii* cell counts after 12, 36, and 48 h ($P < 0.05$ vs. control samples), but this antimicrobial effect was less than that observed at the higher concentration treatments, as treatment with higher concentrations (10^7 or 10^8 PFU g⁻¹) was immediately inhibitory ($P < 0.01$ vs. control sample). FSP1 could not prevent the growth of *M. morganii* after *M. morganii* began to increase. The most effective concentration was 10^8 PFU g⁻¹ FSP1, which had a significant inhibitory effect during the entire incubation period ($P < 0.01$ at 0–36 h and $P < 0.05$ at 48 h). Based on these results, the optimum FSP1 concentration was 10^8 PFU g⁻¹, and this treatment was used in further investigations.

Inhibitory effects of FSP1 treatment on growth and histamine accumulation of *M. morganii* on tuna meat We investigated changes in the growth and histamine accumulation of *M. morganii* NBRC3848^{cr} on raw tuna meat with or without 10^8 PFU g⁻¹ FSP1 at incubation temperatures of 4, 12, or 20°C to evaluate the inhibitory effect of FSP1. Moreover, we used the low contamination (10^3 CFU g⁻¹) and high contamination (10^5 CFU g⁻¹) models to evaluate the effect of the initial concentration on *M. morganii* contamination.

The growth and histamine accumulation of *M. morganii* on low contaminated tuna meat are shown in Fig. 3. Total plate counts were almost unaffected by FSP1 because the antimicrobial effects of phages are host-specific (Fig. 3A, C, E). *M. morganii* did not increase during storage at 4°C in the low contaminated samples, and the FSP1 treatment significantly reduced viable *M. morganii* cell counts to undetectable (< 2 log CFU g⁻¹) at all sampling times ($P < 0.001$ at 0 day and $P < 0.01$ at 2–6 days; Fig. 3A). Histamine was not detected in any sample (Fig. 3B). *M. morganii* grew slowly in the low contamination model up to 5.1 log CFU g⁻¹ during the 12°C storage period. FSP1 treatment decreased viable *M. morganii* cell counts at 0 h storage (immediately after phage treatment; $P < 0.01$) and significant reductions were observed from 24 to 120 h ($P < 0.05$) except at 48 h compared with the control (Fig. 3C). Histamine was detected in control samples at 72, 96, and 120 h (121, 137, and 163 mg kg⁻¹, respectively), but histamine accumulated only in the FSP1-treated samples stored for 120 h (108 mg kg⁻¹, $P < 0.05$; Fig. 3D). *M. morganii* decreased significantly in response to the FSP1 treatment at 20°C for all experimental durations ($P < 0.001$ at 12 and 24 h, and $P < 0.01$ at 0 and 36–60 h; Fig. 3E). Histamine began to accumulate in the

Fig. 1. Growth of *Morganella morganii* NBRC3848^T in liquid medium treated with various concentrations of FSP1 at 4°C (A), 12°C (B), and 20°C (C). Symbols represent the viable cell counts of *M. morganii* of control without FSP1 treatment (◆), 10³ PFU mL⁻¹ FSP1 treatment (▲), 10⁵ PFU mL⁻¹ FSP1 treatment (■), and 10⁷ PFU mL⁻¹ FSP1 treatment (●). Dagger (†) represents undetectable viable cell count (< 1 log CFU mL⁻¹). The results are mean $\pm$ standard deviation of three independent experiments.

Fig. 2. Growth of *Morganella morganii* NBRC3848^T on tuna meat treated with various concentrations of FSP1 at 20°C. Columns represent counts of viable *M. morganii* NBRC3848^T cells treated with 10⁵ (gray), 10⁶ (slashed), 10⁷ (dotted), 10⁸ PFU g⁻¹ (white), or without (black) FSP1. Dagger (†) represents undetectable viable cell count (< 2 log CFU g⁻¹). Means with different letter are significantly different from each other ($P < 0.05$). The results are mean $\pm$ standard deviation of three independent experiments.

Fig. 3. Growth of (A, C and E) and histamine accumulation (B, D and F) by *Morganella morganii* NBRC3848^{cr} inoculated on raw tuna meat at the low contamination level (10^3 CFU g⁻¹) and incubated at 4°C (A and B), 12°C (C and D), and 20°C (E and F). Symbols represents total plate counts of control (◆), total plate count of FSP1 treatment (◇), *M. morganii* counts of control (●) and *M. morganii* counts of FSP1 treatment (○). Columns represents histamine concentration of control (black) and histamine concentration of FSP1 treatment (white). Dagger (†) represents undetectable viable cell count (< 2 log CFU g⁻¹) or histamine concentration (< 20 mg kg⁻¹). Symbols and columns labeled with an asterisk indicate significant differences compared with control samples at *P* < 0.05 (*), *P* < 0.01 (**) and *P* < 0.001 (***). The results are mean ± standard deviation from three independent experiments.

control samples after 24 h and reached 208, 2,188, 2,835, and 3,629 mg kg⁻¹ after 24, 36, 48, and 60 h storage, respectively. Histamine accumulation in the FSP1-treated tuna meat lagged about 24 h due to the inhibition of *M. morganii*, and the FSP1

treatment showed significant inhibitory effects even after 60 h storage (750 mg kg⁻¹, *P* < 0.001; Fig. 3F).

Changes in viable cell counts and histamine concentrations in the highly contaminated tuna meat are shown in Fig. 4. FSP1

Fig. 4. Growth of (A, C and E) and histamine accumulation (B, D and F) by *Morganella morganii* NBRC3848^T inoculated on raw tuna meat at the high contamination level (10^5 CFU g⁻¹) and incubated at 4°C (A and B), 12°C (C and D), and 20°C (E and F). Symbols represents total plate counts of control (◆), total plate count of FSP1 treatment (◇), *M. morganii* counts of control (●) and *M. morganii* counts of FSP1 treatment (○). Columns represents histamine concentration of control (black) and that of the FSP1 treatment (white). Dagger (†) represents undetectable viable cell count (< 2 log CFU g⁻¹) or histamine concentration (< 20 mg kg⁻¹). Symbols and columns labeled with an asterisk indicate significant differences from control samples at *P* < 0.05 (*), *P* < 0.01 (**) and *P* < 0.001 (***). The results are mean ± standard deviation of three independent experiments.

decreased about 2.5 log CFU g⁻¹ *M. morganii* in the tuna meat stored at 4°C, compared with that in the control samples during storage (*P* < 0.001 for 0 and 4 days, *P* < 0.01 for 2 and 6 days), and total plate counts decreased significantly at 0 h because of the

reduction in *M. morganii* by FSP1 (*P* < 0.05; Fig. 4A). Histamine was undetectable (< 20 mg kg⁻¹) during storage at 4°C (Fig. 4B). Viable *M. morganii* cell counts in the control samples at 12°C increased to about 7 log CFU g⁻¹ during storage, but the

populations of *M. morganii* in the FSP1-treated tuna meat decreased significantly to 3.5–4.5 log CFU g⁻¹ (Fig. 4C). Nevertheless, a significant effect of FSP1 on histamine accumulation was observed in samples only after 48 h ($P < 0.05$), whereas the histamine concentration tended to decrease in samples from the other time points ($P > 0.05$; Fig. 4D). *M. morganii* increased to 8.9 log CFU g⁻¹ in the control samples during storage at 20°C, and viable *M. morganii* cell counts in the FSP1-treated tuna samples were significantly lower by about 2 log CFU g⁻¹ than those in the control samples ($P < 0.05$). Total plate counts at the initial storage time period were significantly lower compared with those of the control because of the FSP1 infection-induced reduction in *M. morganii* ($P < 0.05$; Fig. 4E). Histamine accumulated rapidly in the control meat and reached about 3,500 mg kg⁻¹ after 36 h. Histamine concentrations of the FSP1-treated tuna samples were significantly lower than those of control samples at 12, 24, 36, and 60 h storage (< 20, 201, 1,479, and 2,103 mg kg⁻¹, respectively, $P < 0.05$; Fig. 4F). These results suggest that FSP1 treatment might be an effective way to prevent histamine poisoning by *M. morganii* in fisheries products.

We investigated the changes in viable FSP1 by the plaque assay method using *M. morganii* NBRC3848^T for all experimental durations. FSP1 was stable during the experimental period. Phage counts of each initial time point were 8.2–8.4 log PFU g⁻¹ and of each final time point were 7.6–8.3 log PFU g⁻¹.

No emergence of FSP1-resistant *M. morganii* We randomly isolated 37 and 77 colonies from low and high *M. morganii* contaminated tuna meat samples stored at 20°C for 60 h, respectively, to confirm the emergence of FSP1-resistant *M. morganii*. Based on the results of spot testing for infectivity, all isolated *M. morganii* strains showed susceptibility to FSP1 (data not shown), indicating no development of an FSP1-resistant strain in response to the FSP1 treatment during the experiment.

Discussion

Phages are remarkable as new and alternative antimicrobial agents. While no study has investigated reducing the risks of histamine in fish meat using phages, phages have been used to inhibit tyramine-producing *Enterococcus faecalis* in cheese (Ladero *et al.*, 2016). In this study, we evaluated the potential of using a phage treatment to prevent histamine accumulation by *M. morganii* subsp. *morganii* using the *M. morganii* phage FSP1, which was isolated and characterized in our previous study (Yamaki *et al.*, 2014). First, we evaluated the effects of temperature and phage concentration on the anti-*M. morganii* potential of FSP1 in a liquid medium (Fig. 1). Our results suggest that temperature and phage concentration are very important for maximizing the antimicrobial potential of FSP1. Among our experimental temperatures (4, 12, or 20°C), *M. morganii* did not grow at 4°C. FSP1 inoculated at 10³ or 10⁵ PFU mL⁻¹ showed significant antimicrobial effects against growing *M. morganii* cells at 12 and 20°C, suggesting that FSP1

infected the host cells and propagated at 12 and 20°C. However, 10⁷ PFU mL⁻¹ FSP1 immediately reduced viable *M. morganii* cell counts at all temperatures, including 4°C. This finding indicates that a high concentration of FSP1 can kill host cells even at a low temperature, since a high concentration of phages is thought to be adsorbed efficiently even at low bacterial concentrations. Moreover, 10³ and 10⁵ PFU mL⁻¹ FSP1 did not suppress viable *M. morganii* cell counts at 4°C, possibly due to low adsorption efficiency, indicating that the initial phage concentration is very important when evaluating the antimicrobial effect of phages at temperatures too low for host cell growth and low host bacterial density.

Phage concentration and type of food matrix affect growth suppression in response to phage treatment. The efficacy of phage treatment on honeydew melon, lettuce, smoked salmon, hot dogs, chocolate milk, cabbage, and meatballs has been reported to behave in a phage concentration-dependent manner (Gencay *et al.*, 2015; Guenther *et al.*, 2009; Leverentz *et al.*, 2004; Perera *et al.*, 2015). This concentration-dependency is attributed to the low bacterial cell densities and contact rates between phages and host cells in food matrices. Diffusion of phages on a food surface or in a food matrix is limited because phages are immobile. A high concentration of phages is necessary to maintain a high contact rate and attack against a low density of target host cells in foods (Hagens and Loessner, 2010). Phage treatments in liquid food are more efficient for suppressing target bacteria than those on solid food because of differences in diffusion potential of the phages. In this study, the differences in trends of inhibitory curves between liquid medium (easy to diffuse, Fig. 1) and the surface of tuna meat (difficult to diffuse, Fig. 2) support this hypothesis and a previous report (Hagens and Loessner, 2010). In addition, various phage concentrations lead to differences in the antimicrobial effects of FSP1 (Fig. 2), and the largest FSP1 inoculum size (10⁸ PFU g⁻¹) was optimal for practical use.

FSP1 treatment also significantly inhibited histamine accumulation in raw tuna meat, regardless of the initial contamination rate of *M. morganii* (Figs. 3 and 4). However, FSP1 treatment showed less of a histamine-inhibitory effect in samples stored at 12°C with a high *M. morganii* concentration, although the viable *M. morganii* cell counts were about 4 log CFU g⁻¹ during storage, compared to the other inoculum sizes and storage conditions (Fig. 4C and D). This result was assumed to be due to the presence of extracellular histidine decarboxylases released from host cells lysed by phage infection. Because phage infection could not inactivate histidine decarboxylases, the release of intracellular histidine decarboxylases to the extracellular space might continue to produce histamine in tuna meats. The *M. morganii* histidine decarboxylase is active at 5–50°C, and this activity remains for several days or weeks at –20–40°C. In addition, the histidine decarboxylases of *P. phosphoreum*, *P. damsela*, and *R. planticola* accumulated histamine in tuna and dried saury independent of

bacterial cells (Kanki *et al.*, 2007). This finding indicates that the extracellular histidine decarboxylases released from cells lysed by phages increase the risk for histamine poisoning. Therefore, although phage treatment is effective for preventing *M. morganii* growth and histamine accumulation, general hygienic control before phage treatment is the most important factor to prevent growth and production of abundant histidine decarboxylases by *M. morganii* at high contamination rates.

In this study, we chose the spotting (pipetting) method as the phage inoculation procedure due to its simplicity. In general, spraying onto a food surface is a successful and cost-effective method for practical use, rather than pipetting. Leverentz *et al.* (2003) reported differences in the antimicrobial effect between application methods (pipetting and spraying), and the antimicrobial effect of spraying tended to be greater than that of pipetting, but not significantly so. Therefore, spraying FSP1 would be the best method to enhance the antimicrobial effects on tuna meat, and a comparison of application methods is a possible next step. Moreover, we used a single strain, *M. morganii* NBRC3848^T and its chloramphenicol resistant strain, for antimicrobial testing in this study. Although FSP1 was able to infect all tested strains of *M. morganii* subsp. *morganii* (Yamaki *et al.*, 2014), it would be preferable to use a mixture or cocktail of different strains of *M. morganii* for the most broadly applicable evaluation. Our next steps include the validation of the antimicrobial effects of FSP1 on histamine accumulation of diverse strains and the construction of a *M. morganii* phage cocktail for more broad and effective control of *M. morganii* in tuna meats.

Histamine-producing bacteria associated with fish meats are not limited to *M. morganii*, although FSP1 treatment can effectively inhibit only *M. morganii*-associated histamine accumulation. However, *M. morganii* is one of the most likely histamine producers associated with outbreaks of histamine poisonings, and the inhibition of *M. morganii* is thought to reduce the risks of histamine poisoning in fisheries products. Currently, we are attempting to investigate phages infecting other histamine producers, such as *P. damsela* subsp. *damsela* (Yamaki *et al.*, 2015), *P. phosphoreum*, and *M. psychrotolerans*, for a more broad-spectrum control of histamine accumulation in fisheries products.

The emergence of phage-resistant bacteria is thought to be one of the problems facing practical phage treatment in foods and medicine. Some studies have reported the emergence of phage-resistant bacteria in food. Guenther *et al.* (2012) reported that all colonies that recovered from phage-treated liquid foods (chocolate milk and egg yolk) were phage-resistant, and Tomat *et al.* (2013) found that 25% of colonies (two of eight colonies) recovered from meat were phage resistant. Phage-resistant bacteria have not been isolated from hot dogs, sliced turkey breast, smoked salmon, mixed seafood, cabbage, or lettuce (Guenther *et al.*, 2009). These results suggest that the type of food matrix (liquid or solid) may be a factor in the emergence of phage-resistant bacteria, attributable to

the large differences in contact rates of phages and host bacteria between liquid and solid foods. In this study, we isolated 114 colonies and confirmed that there was no development of phage-resistant bacteria after phage treatment at 20°C for 60 h, suggesting that regrowth of *M. morganii* after phage treatment in tuna meat is not caused by FSP1-resistant *M. morganii*, and might be attributable to cells that escaped the phage attack due to the limited phage diffusion on solid surfaces.

Conclusion

In this study, we evaluated the effects of phage treatment on the growth and histamine accumulation of *M. morganii* in tuna meat. The antimicrobial effect of the *M. morganii* phage FSP1 was dose-dependent, and the optimal FSP1 concentration for treatment was 10⁸ PFU g⁻¹. FSP1 treatment of tuna meat showed significant inhibitory effects on the viable cell counts and histamine accumulation of *M. morganii* at 4, 12, and 20°C, and no emergence of FSP1-resistant *M. morganii* was observed. These results suggest that phage treatment might be an effective tool to decrease the risks of histamine poisoning in fisheries products.

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Conflicts of Interest

None declared.

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