Characterization of a novel Morganella morganii bacteriophage FSP1 isolated from river water

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Introduction

Morganella morganii, a motile gram-negative rod belonging to the family Enterobacteriaceae, has low pathogenicity, but compromised patients can develop diarrhea, wound infections, urinary tract infections, bacteremia, and sepsis due to M. morganii (Tucci & Isenberg, 1981; Chang et al., 2011). In addition, M. morganii is known as a major histamine producer due to its powerful histidine decarboxylase. Morganella morganii is responsible for histamine accumulation on food (López-Sabater et al., 1996; Rodtong et al., 2005), which often causes histamine poisoning (also referred to as scombroid poisoning) in decomposed fishery products (Becker et al., 2001). Morganella morganii was detected in the gill and skin of mackerel, sardine, and albacore. Moreover, the surface of conveyer belts and plastic totes contacted with mackerel and sardine is contaminated during processing although no M. morganii was found in the processing plant before processing (Kim et al., 2003). Although many cases present with mild symptoms, rare cases present with severe symptoms that can be potentially life-threatening (Sánchez-Guerrero et al., 1997; D’Aloia et al., 2011). Therefore, the inhibition of M. morganii growth in food is important to reduce the risk of histamine poisoning.

Bacteriophages (phages) are bacterial virus and a notable antimicrobial agent. The therapeutic potential of phages has not been an area of interest since the discovery of antibiotics. However, phages have recently been suggested as an alternative antibacterial agent to counteract the emergence of antibiotic-resistant bacteria (Kutateladze & Adamia, 2010). In fact, many phages, including Staphylococcus aureus phage P-27/HP (Gupta & Prasad, 2011) and Pseudomonas aeruginosa phage M4 (Fu et al., 2010), have been investigated for their ability to control human pathogenic infections. Phages are also available for food safety. The United States Department of Agriculture and Food and Drug Administration approved LISTEX™ P100 and SALMONELEX™ (Micreos Food Safety, Netherlands) to control food pathogens. The major components are Listeria monocytogenes phage P100 (LISTEX™ P100) and Salmonella phages (SALMONELEX™) and are a ‘Generally

Abstract

Morganella morganii has been identified as a causative agent of opportunistic infections and histamine poisoning. Bacteriophage is a virus and has recently been considered an alternative agent to antibiotics for the control of bacteria that have developed antibiotic resistance. In this study, a novel M. morganii bacteriophage isolated from river water was characterized. The isolated phage, termed FSP1, was purified by polyethylene glycol precipitation followed by cesium chloride density-gradient centrifugation. FSP1 has infectivity against only M. morganii and was identified as a Myoviridae bacteriophage through morphological analysis with transmission electron microscopy. According to the one-step growth curve, the FSP1 latent period, eclipse period, and burst size were 30, 20 min, and 42 PFU infected cell⁻¹, respectively. The genome size of FSP1 was estimated to be c. 45.6–49.4 kb by restriction endonuclease analyses. Moreover, challenge testing against M. morganii in vitro revealed that FSP1 had high lytic activity and that the viable cell count of M. morganii was reduced by 6.12 log CFU mL⁻¹ after inoculation with FSP1 at a multiplicity of infection (MOI) = 10. These results suggested that FSP1 could be used as a biocontrol agent against M. morganii for treatment of infectious disease treatment or food decontamination.
Recognized As Safe’ food processing aid. Moreover, phage-based biocontrol strategies to control Escherichia coli, Bacillus cereus, and Campylobacter jejuni were also developed (Atterbury et al., 2003; Viazis et al., 2011; Bandara et al., 2012). To date, no studies have focused on the use of phages to control M. morganii, although only a few studies related to M. morganii phages have been reported (Schmidt & Jeffries, 1974; Zhu et al., 2010).

In this study, we describe the novel M. morganii phage FSP1 isolated from river water, and we characterize FSP1 for use as an antimicrobial agent against M. morganii.

Materials and methods

Bacterial strains and growth conditions

The strains used in this study are listed in Table 1. All bacteria listed in Table 1 were incubated at 30 °C for 18 h in tryptic soy broth (TSB, BD, Franklin Lakes, NJ) or TSB supplemented with 0.6% yeast extract (BD).

Table 1. List of bacterial strains in this study and host range of FSP1

<table>
<thead>
<tr>
<th>Bacterial species</th>
<th>Strain</th>
<th>Plaque formation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gram-negative bacteria</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Morganella morganii ssp. morganii</td>
<td>NBRC3848T</td>
<td>+</td>
</tr>
<tr>
<td>Morganella morganii ssp. morganii</td>
<td>NBRC3168</td>
<td>+</td>
</tr>
<tr>
<td>Morganella morganii ssp. morganii</td>
<td>ATCC25829</td>
<td>+</td>
</tr>
<tr>
<td>Aeromonas hydrophila</td>
<td>NBRC3820</td>
<td>–</td>
</tr>
<tr>
<td>Citrobacter freundii</td>
<td>JCM1657T</td>
<td>–</td>
</tr>
<tr>
<td>Cronobacter sakazakii</td>
<td>ATCC51329</td>
<td>–</td>
</tr>
<tr>
<td>Enterobacter gergoviae</td>
<td>JCM1234</td>
<td>–</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>JCM1649T</td>
<td>–</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>ATCC51446</td>
<td>–</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>ATCC51813</td>
<td>–</td>
</tr>
<tr>
<td>Hafrnia alvei</td>
<td>JCM1666T</td>
<td>–</td>
</tr>
<tr>
<td>Klebsiella pneumoniae</td>
<td>ATCC13883</td>
<td>–</td>
</tr>
<tr>
<td>Proteus hauseri</td>
<td>ATCC13315</td>
<td>–</td>
</tr>
<tr>
<td>Proteus mirabilis</td>
<td>JCM1669T</td>
<td>–</td>
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<td>Proteus mirabilis</td>
<td>ATCC33583</td>
<td>–</td>
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<tr>
<td>Proteus vulgaris</td>
<td>ATCC33420</td>
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<tr>
<td>Providencia alcalifaciens</td>
<td>ATCC9886</td>
<td>–</td>
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<td>Providencia rettgeri</td>
<td>ATCC9250</td>
<td>–</td>
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<td>Providencia rustigiani</td>
<td>JCM3953T</td>
<td>–</td>
</tr>
<tr>
<td>Providencia stuartii</td>
<td>ATCC3672</td>
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<tr>
<td>Pseudomonas fluorescens</td>
<td>JCM5963T</td>
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<tr>
<td>Raoultella ornithinolytica</td>
<td>ATCC31898</td>
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<tr>
<td>Salmonella Enteritidis</td>
<td>NBRC3313</td>
<td>–</td>
</tr>
<tr>
<td>Salmonella Infantis</td>
<td>ATCC51741</td>
<td>–</td>
</tr>
<tr>
<td>Salmonella Typhimurium</td>
<td>IID1000</td>
<td>–</td>
</tr>
<tr>
<td>Yersinia enterocolitica</td>
<td>ATCC9610</td>
<td>–</td>
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<tr>
<td>Gram-positive bacteria</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacillus cereus</td>
<td>JCM2152T</td>
<td>–</td>
</tr>
<tr>
<td>Listeria monocytogenes</td>
<td>ATCC7644</td>
<td>–</td>
</tr>
<tr>
<td>Paenibacillus chibensis</td>
<td>NBRC15995T</td>
<td>–</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>NBRC14462</td>
<td>–</td>
</tr>
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</table>

+, clear plaque; –, no plaque.

Phage isolation

Phages were isolated by the method of Carvalho et al. (2010a) with modifications. Three hundred milliliters of a river water sample was added to 300 mL of double strength TSB supplemented with 400 µg mL⁻¹ CaCl₂ and 400 µg mL⁻¹ MgSO₄, and the host strains M. morganii NBRC3848T, M. morganii NBRC3168, and M. morganii ATCC25829, at the exponential phase, were inoculated. After incubation at 30 °C for 12 h, the cultures were centrifuged (3500 g, 30 min, 4 °C), and the supernatants were filtrated using 0.45-µm polyvinylidene fluoride filters (Merck Millipore, Billerica, MA). For detection of phage, filtrates were spotted on a lawn of M. morganii NBRC3848T made using the double agar overlay method and incubated at 37 °C for 24 h. Clear zones were picked and suspended in 1 mL of SM buffer (100 mM NaCl, 8 mM MgSO₄·7H₂O, 50 mM Tris-HCl, and 0.01% gelatin, pH 7.5), and single plaques were formed using the double agar overlay method. This procedure was repeated at least three times.

Propagation and purification of phage

FSP1 was propagated and purified by polyethylene glycol precipitation followed by cesium chloride density-gradient centrifugation. FSP1 was inoculated into log phase culture of M. morganii NBRC3848T. The culture was incubated at 30 °C for growth of FSP1 followed by centrifugation (10 000 g, 30 min, 4 °C) and filtration with 0.45-µm polyvinylidene fluoride filters. After DNase I and RNase A (Sigma-Alrich, St. Louis, MO) were added at 1 µg mL⁻¹ and incubated at room temperature for 1 h, the FSP1 lysate was treated with 1 M sodium chloride for 1 h on ice. Furthermore, polyethylene glycol 6000 (Wako pure chemical industries, Osaka, Japan) was added to final concentration of 10%. The mixture was incubated at 4 °C for 12 h and was then centrifuged (10 000 g, 20 min, 4 °C) to precipitate phage particles. The pellet was suspended in SM buffer, and the polyethylene glycol was removed by chloroform extraction and centrifugation (10 000 g, 10 min, 4 °C). Purification of FSP1 was carried out by cesium chloride density-gradient centrifugation (100 000 g, 2 h, 4 °C), and the sample was dialyzed against SM buffer. The purified phage was stored at 4 °C for further investigations.

Host range determination

The host range of FSP1 was determined using a spot test method. One hundred microliters of each bacterial
culture listed in Table 1 was added to 3.5 mL of 0.5% molten agar, and this mixture was overlaid onto a tryptic soy agar (BD) plate. Ten microliters of FSP1 solution (10^7 PFU mL^{-1}) was spotted onto each agar plate. Plates were incubated at 37 °C for 6 h and were then examined for clear zones on the bacterial lawn.

### Transmission electron microscopy

The morphology of FSP1 was analyzed by negative staining with 2.5% samarium triacetate, which was reported as a good negative-staining reagent (Nakakoshi et al., 2011). The FSP1 solution was dropped on a 200-mesh Cu grid (Nissin EM, Tokyo, Japan) for 3 min. The excess solution was absorbed with filter paper, and 2.5% samarium triacetate was dropped on the grid. The excess solution was absorbed with filter paper, and FSP1 was examined using a transmission electron microscope (JEM-1011, JEOL, Tokyo, Japan).

### Isolation and restriction analysis of phage DNA

FSP1 was suspended to TENS buffer (50 mM Tris-HCl, 100 mM EDTA, 100 mM NaCl, 0.3% sodium dodecyl sulfate, pH 8.0, Stenholm et al., 2008). Proteinase K was added to a final concentration of 200 μg mL^{-1}, and the mixture was incubated at 65 °C for 10 min for extraction of phage DNA. Purification of the phage DNA was carried out using the Phage DNA Isolation Kit (Norgen Biotech, Thorold, Ontario, Canada) according to the manufacturer’s instruction. For genome size estimation, isolated phage DNA was digested with the restriction endonucleases EcoRI, HindIII, and XbaI (Nippon gene, Tokyo, Japan) according to the manufacturer’s instructions. The digested DNA fragments were separated with agarose gel electrophoresis using 0.5% SeaKem gold agarose (Takara Bio, Shiga, Japan). The DNA bands were visualized by SYBR® green nucleic acid stain (Sigma-Aldrich), and the sum of each band size was calculated as the phage genome size. Gene ladder wide I (Nippon gene) and 2.5 kb DNA ladder (Takara Bio) were used as the molecular size markers.

### Temperatures and pH stability test

To determine the thermal stability of FSP1, FSP1 was suspended to give a final concentration of 10^7 PFU mL^{-1} in TSB. The mixture was incubated at 55–70 °C for 1 h, samples were collected every 10 min, and the PFU of FSP1 was determined using serial dilution and the double agar overlay method. To determine the pH stability of FSP1, FSP1 was suspended to give a final concentration of 10^7 PFU mL^{-1} in TSB (pH 1.0–12.0) and incubated at 30 °C for 24 h. The PFU of FSP1 was determined using serial dilution and the double agar overlay method.

### Adsorption assay

*Morganella morganii* NBRC3848^T was incubated at 30 °C until reaching an OD_{600 nm} = 0.1 in TSB. The FSP1 suspension was added at a multiplicity of infection (MOI) of 0.01, and the mixed culture was incubated at 30 °C. Every 5 min, samples were collected and centrifuged at 10 000 g for 2 min, followed by filtration using a 0.45-μm polyvinylidene fluoride filter. The PFU of nonadsorbed phage in the supernatant was determined using serial dilution and the double agar overlay method.

### One-step growth experiments

*Morganella morganii* NBRC3848^T was incubated at 30 °C until reaching an OD_{600 nm} = 0.1 in TSB. FSP1 was added at an MOI = 0.1 and adsorbed at 30 °C for 5 min. The free phages in the mixture were removed by centrifugation (10 000 g, 2 min, 4 °C), and the pellet was suspended in the same volume of fresh TSB. The suspension was incubated at 30 °C, and the samples were collected after 5 min. The phage titer of the sample was measured in two ways: A sample was measured either immediately after sampling or after chloroform treatment (for lysis of the bacterial cells). The burst size, latent period, and eclipse period of FSP1 were determined by changes in the phage titer.

### Challenge test

*Morganella morganii* NBRC3848^T was inoculated into fresh TSB for 6 log CFU mL^{-1}, the culture was incubated at 30 °C, and the samples were collected at 1-h intervals. The samples were serially diluted and spread onto tryptic soy agar plates. When the OD_{600 nm} reached 0.1, which corresponds to a viable cell count of c. 10^7 CFU mL^{-1}, the culture was divided into two sets of samples, and FSP1 was added to only one sample at a MOI = 1.0 or 10. Both samples were incubated and spread onto tryptic soy agar plates at 1-h intervals. The tryptic soy agar plates were incubated at 37 °C for 24 h, and the CFU were enumerated.

### Results

#### Isolation and host range of an isolated phage

In this study, we isolated a *M. morganii* phage from a river water (Odajima river in Hakodate, Japan) sample, and the isolated phage was designated as FSP1. The host
range of FSP1 was determined for 26 strains of gram-negative bacteria and 4 strains of gram-positive bacteria. FSP1 formed plaques for 3 strains: *M. morganii* NBRC3848T, *M. morganii* NBRC3168, and *M. morganii* ATCC25829, but no other strains were infected by FSP1 (Table 1). Moreover, FSP1 formed clear single plaques on the *M. morganii* strains used in this study.

**Morphology of FSP1**

The morphology of FSP1 virions was observed by negative staining. Transmission electron microscopy showed that FSP1 virions had a head measurement of $110 \pm 7.2$ nm (mean $\pm$ SD) and a contractile tail measurement of $114 \pm 5.5$ nm ($n = 8$) (Fig. 1). Therefore, it is suggested that FSP1 belongs to the *Myoviridae* family, a member of tailed phage, which has contractile tail consisting of a sheath and a central tube.

**Adsorption and one-step growth of FSP1**

For elucidation of the FSP1 growth property, we conducted an adsorption assay and one-step growth curve analyses. Forty percent of FSP1 was adsorbed to *M. morganii* NBRC3848T at 5 min. In addition, almost all of the FSP1 was adsorbed at 40 min (data not shown). According to the one-step growth experiment, the latent period of FSP1 was 30 min, the rise period was 15 min, the eclipse period was 20 min, and the burst size was 42 PFU infected cell$^{-1}$ (Fig. 2).

**Genomic size of FSP1**

To determine the FSP1 genome size, FSP1 DNA was digested with the restriction endonucleases EcoRI, HindIII, and XbaI. Agarose gel electrophoresis of the digested DNA showed that FSP1 DNA had many restriction sites for each of the restriction endonucleases (Fig. 3). The genome size of FSP1 DNA was calculated as the sum of each band and was c. 45.6–49.4 kb.

**Thermal and pH stability of FSP1**

To analyze the stability of FSP1, thermal and pH stability were evaluated. FSP1 was stable at 50°C but heating to...
55 and 60 °C caused a decrease of c. 3.5 log PFU mL⁻¹ of FSP1 virions, and no FSP1 virions were detected after heating at 65 and 70 °C (Fig. 4a). In addition, FSP1 was stable frozen at −40 °C for 24 h (data not shown). A pH stability analysis showed that FSP1 was stable at various pH levels, except strong acidic (lower than pH 3.0) and alkaline (pH 12.0) levels (Fig. 4b). This thermal and pH stability is beneficial for the use of FSP1 as a biocontrol agent against M. morganii.

Antimicrobial action of FSP1 against M. morganii

A bactericidal effect of FSP1 (MOI = 1.0 or 10) was examined by changes in the viable cell count of M. morganii NBRC3848T. At a MOI = 1.0, the viable cell count of M. morganii decreased from 7.2 log CFU mL⁻¹ to 3.7 log CFU mL⁻¹ (Fig. 5a). At a MOI = 10, the bacterial count was not detectable (< 1 log CFU mL⁻¹) after 1 h of FSP1 inoculation (Fig. 5b). These results suggest that FSP1 is expected to be an antimicrobial agent for M. morganii control and that its bactericidal effect is related to the MOI. However, the bacterial count of M. morganii began to recover after an additional incubation for 2 h at MOI = 1.0 and 5 h at MOI = 10.

Discussion

Phages, which possess a different mode of action compared to antibiotics, are expected to be an alternative to antibiotics (O’Flaherty et al., 2009). Controlling pathogens through the use of phages has been investigated for various applications such as therapeutic drugs for human, domestic animal, plants, hatchery fish, and food additives (Carvalho et al., 2010a; Gupta & Prasad, 2011; Addy et al., 2012; Chibeu et al., 2013; Madsen et al., 2013). However, studies on the growth inhibition of M. morganii by lytic phages have not been reported. The aim of this study was to isolate and characterize a phage to control M. morganii.

According to the method of Carvalho et al. (2010a) with minor modification, we isolated a M. morganii phage, named FSP1, from river water in Hakodate, Hokkaido, Japan. Next, we examined the host range of FSP1 because...
the infection specificity of phages is generally species- or strain dependent. FSP1 formed plaques for all of the strains of *M. morganii* tested in this study, but FSP1 could not form plaques against other gram-positive or gram-negative bacteria, suggesting that FSP1 was a highly specific phage to *M. morganii* and these *M. morganii* strains might have a common and conservative phage receptor among this species. Restriction analyses of FSP1 DNA and transmission electron microscopic observation showed that FSP1 had a 45.6–49.4 kb genome and belonged to the *Myoviridae* family from the virion tail structure (Figs 1 and 3). Because agarose electrophoresis showed bands digested by restriction endonucleases, FSP1 DNA was estimated to be linear, double-strand DNA. Another *M. morganii* phage, named Mmp1, is in the *Podoviridae* family and has a genome size of c. 38 kb (Zhu et al., 2010).

To examine the potential of FSP1 as an antimicrobial agent for *M. morganii*, we evaluated the adsorption rate, latent period, rise period, and burst size of FSP1 against *M. morganii* NBRC3848T. The adsorption rate, latent period, eclipse period, rise period, and burst size were determined to be 40% for 5, 30, 20, 15 min, and 42 PFU infected cell⁻¹, respectively (Fig. 2). Other *Myoviridae* phages generally have a latent period, eclipse period, and burst size of 15–50, 10–40 min, and 50–150 PFU infected cell⁻¹, respectively (Raya et al., 2006; Carey-Smith et al., 2006; Uchiyama et al., 2008; Hsieh et al., 2011; Park et al., 2012). Therefore, the growth parameters of FSP1 were similar to those of other *Myoviridae* phages. FSP1 was stable at pH 4.0–10.0, freezing at −40 °C for 24 h, and heating at 50 °C for 1 h (Fig. 4a and b). These results suggest that the growth parameters and stability of FSP1 will be suitable for use as a biocontrol agent against *M. morganii* in food, especially in ready-to-eat food.

The bactericidal action of phages is one of the most important factors to evaluate for potential use as an antimicrobial agent. The viable cell count of *M. morganii* NBRC3848T was substantially reduced to below the detection limit at a MOI = 10 in a challenge test (Fig. 5a and b). The extremely high lytic activity indicates that FSP1 can be used for the efficient control of *M. morganii* although the challenge test was performed in media. The *in vivo* challenge test will be the next step on our research. The phage therapy on the fishery products should be carried out by spraying or dipping because *M. morganii* phage, named FSP1, that inhibits histamine-producing *M. morganii*. Our results showed that the host specificity, thermal and pH stability, growth parameters, and antimicrobial activity of FSP1 were useful for controlling *M. morganii* as a food additive and therapeutic agent. To our knowledge, this is the first report investigating the growth and antimicrobial activity of a *Myoviridae* phage inhibiting *M. morganii*.

**References**


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