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Isolation and Characterization of Lytic *Pseudomonas fluorescens* Bacteriophage isolated from milk

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Abstract

Phage-based biocontrol is an alternative method for preventing and controlling the occurrence of Pseudomonas spp. in food products. However, the use of bacteriophages to control heat-stable protease Pseudomonas fluorescens is still rare. The objectives of this study were to isolate lytic bacteriophages of P. *fluorescens*, and to evaluate their efficacy in controlling P. *fluorescens* at the laboratory level. Among these strains, only one phage, ΦTIS1, was isolated by using P. *fluorescens* TISTR 1887 as the host. Further analysis using electron microscopy indicated that ΦTIS1 belongs to the Myoviridae family. This phage ΦTIS1 was relatively stable at pH and temperature ranges of 4.0 to 12.0 and 4, 30, 37, and 45°C, respectively, after 1.5 h incubation. The partial ΦTIS1 genome was a linear double-stranded with a total length of 87,646 bp and a G + C content of 54.71%. *In vitro* studies of the effect of bacteriophages against P. fluorescens TISTR 1887 with phage ΦTIS1 using various multiplicity of infection (MOI) values showed a significant decrease in bacterial numbers during 6-12 hours of incubation, followed by bacterial regrowth. However, the phage was still able to significantly reduce the bacterial numbers compared to the control without phage. These findings suggest that phage ΦTIS1 has the potential to be an effective method for controlling the prevalence of spoilage-causing P. fluorescens strains in dairy industries.

Keywords*: *P. fluorescens; bacteriophage; biocontrol.

1. Introduction

Pseudomonas fluorescens and other members of the *P. fluorescens* group are commonly found in raw milk and dairy products (Carrascosa et al., 2015; Martin et al., 2011; Wang, & Jayarao, 2001), and they can cause spoilage and reduce shelf life in pasteurized milk (Reichler et al., 2018). These bacteria are psychrotrophic, which means

they can grow at low temperatures, such as those found in refrigerated milk. *P. fluorescens* can also produce heat-resistant proteases, which can cause milk proteins to break down and result in off-flavors and texture changes in dairy products (Fricker et al., 2011; Marchand et al., 2012; 2009; Martins, et al., 2015; Meng et al., 2017; von Neubeck et al., 2015; Zhang et al., 2020). The production of heat-resistant extracellular proteases is a common trait among *P. fluorescens* group bacterial strains, and this makes them particularly problematic in the dairy industry (Andreani et al., 2016; Baur et al., 2015; Glück et al., 2016). Most *Pseudomonas* strains, including *P. fluorescens*, are known to possess the *apr*X gene and can produce an alkaline metalloprotease called AprX. This enzyme is a major contributor to the spoilage of ultra-high temperature (UHT) milk due to its heat stability (Glück et al., 2016; Machado et al., 2017). Because of their ability to survive and even thrive at low temperatures, *P. fluorescens* group are particularly difficult to control in dairy products. Therefore, it is important to implement effective control measures, such as proper storage temperatures and sanitation practices, to prevent their growth and reduce the risk of spoilage (Guerreiro et al., 2005).

Lytic phages have recently regained attention as a promising microbicidal therapy, as they infect and destroy bacteria through cell lysis and may be effective against multidrug-resistant bacterial strains (Sulakvelidze, Alavidze, & Morris, 2001; Thiel, 2004). Lytic phages infect bacterial cells and then multiply over 10-fold in a very short period of time, with a goal of lysing the host bacterial cells to release their progenies, which then repeat the infection and lysis cycle until the host population is severely reduced (Guttman, Raya, & Kutter, 2005). One advantage of lytic phages is that they can be highly specific to certain bacterial strains, while antibiotics may have a broader spectrum of activity and can also affect beneficial bacteria in the body (Loc-Carrillo, & Abedon, 2011).

Among the phages that infect *Pseudomonas* species, those that infect *P. aeruginosa* and *P. syringae* were isolated and characterized, whereas only some phages that infect *P. fluorescens* were isolated and characterized. Phages that infect *P. fluorescens* were isolated from sewage treatment plants and wastewater of dairy industries (Sillankorva, Neubauer, & Azeredo, 2008; Eller et al., 2014), and some were able to infect a variety of *P. fluorescens* strains isolated from dairy industries (Sillankorva et al., 2008). However, complete genomes of *P. fluorescens* phages are rare, so it is important to study them before using phages for any intended purpose. Therefore, the aim of this study was to characterize a phage type of *P. fluorescens* isolated from water and milk samples, including whole genome analysis of this phage. Potential treatments for inactivating this phage were also evaluated.

2. Objectives

The aims of this study were to isolate and characterize lytic bacteriophages of *P. fluorescens* and to evaluate the efficacy of these phages in controlling *P. fluorescens* at the laboratory level.

3. Materials and Methods

3.1 Bacterial strain

Pseudomonas reference strains, including *P. aeruginosa* TISTR 2370, *P. fluorescens* TISTR 358, *P. fluorescens* TISTR 2237, *P. fluorescens* TISTR 1887, *P. fluorescens* TISTR 903, *P. fluorescens* TISTR 904, *P. acidovorans* TISTR 356, *P. otitidis* TISTR 2468, and *P. putida* TISTR 1521, were obtained from the Thailand Institute of Scientific and Technological Research (TISTR) culture collection and screened for extracellular protease activity. Stock cultures of all *Pseudomonas* spp. strains were stored at -20°C in Nutrient Broth (NB) (HiMedia, India) containing 20% (v/v) glycerol.

3.2 Screening of extracellular protease from *Pseudomonas* spp.

The *Pseudomonas* spp. strains were spotted on skim milk plate count agar plates (Himedia, India). After incubation at 37°C for 24 hours, the presence of a zone of hydrolysis around the inoculated spot indicated protease synthesis. Only the *Pseudomonas* strains that displayed a clear zone exceeding 2 millimeters in diameter were selected for further experiments.

3.3 Identification of *apr*X gene

The protease producing *Pseudomonas* spp. which were preliminarily screened from Section 3.2 were subjected to detect a specific region of the alkaline protease gene (*apr*) responsible for AprX production by *Pseudomonas* spp. Genomic DNA was isolated using traditional phenol/chloroform DNA extraction (Sambrook, et al., 1989). PCR amplification

was carried out with the primers Pse_AprXF1 CAGACCCTGACCCACGARATCGG and Pse_ AprXR1 TGAGGTTGATCTTCTGGTTCTGGG, following the protocol described by Andreani et al. (2016). PCR amplifications were carried out in a Thermal Cycler Gradient TC1000-S (Scilogex, USA) using a final volume of 50 µL of amplification mix. The mix contained 1X of 2X TopTaq Master Mix Kit (QIAGEN, USA), 0.2 µM of each primer, and 200 ng of genomic DNA as a template. The thermal cycling protocol involved an initial step at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 20 seconds, annealing of the primers at 60°C for 30 seconds, and extension at 72°C for 1 minute. The final extension step was set at 72°C for 7 min. The presence of an approximately 450 bp amplicon confirmed the identification of the *Apr*X gene.

3.4 Zymographic analysis

To determine the molecular mass of bacterial proteases produced by *Pseudomonas* strains, the 10% skim milk zymography gel method was used. The 27 µL of supernatant from overnight cultures in NB medium was mixed with 3 µL of 10X zymography sample buffer, which contained 250 mM TrisHCl (pH 6.8), 40% (w/v) glycerol, 8% (w/v) sodium dodecyl sulfate (SDS), and 0.02% (w/v) bromophenol blue (ThermoFisher Scientific, USA). Samples (30 µL) were prepared and subjected to SDS-PAGE analysis using a 15% polyacrylamide gel, following the same conditions described by Zhang et al. (2020). The gels were then stained with a staining solution containing 0.1% (w/v) Coomassie Brilliant Blue R-250 (Bio Basic Inc., Canada) in 40% (v/v) methanol and 10% (v/v) acetic acid, followed by destaining in 40% (v/v) ethanol and 10% (v/v) acetic acid. A PageRuler Prestained Protein Ladder (SMOBIO Technology Inc., China) was used as the molecular standard.

3.5 Bacteriophage isolation

Three strains, including *P. fluorescens* TISTR 1887, *P. fluorescens* TISTR 2237, and *P. aeruginosa* TISTR 2370 that produce extracellular proteases were used for bacteriophage isolation, following the protocol described by Pringsulaka et al., (2011). Phage counts were determined using the double-layer plaque method with nutrient agar, and expressed as plaque-forming units per milliliter (PFU/mL), as described by Svensson, & Christiansson (1991). To ensure isolation of a pure phage, phage purification was carried out in three consecutive rounds. Phage stocks were prepared in nutrient broth and stored at 4°C.

3.6 Electron microscopy

Transmission electron microscopy (TEM) was used to visualize the phage morphology. First, carbon-coated grids were gently placed on top of fresh overnight plaques. The grids were then negatively stained with 1% (w/v) uranyl acetate (pH 4.5). The phage morphology was examined using a TECNAI 20 TWIN transmission electron microscope, which was operated at 120 kV and a magnification of x120,000.

3.7 Host-range determination

The spot test method was employed to determine the host range of the phages (Chopin, Chopin & Roux, 1976). To test for susceptibility to the isolated phages, reference strains such as *P. fluorescens* TISTR 358, *P. fluorescens* TISTR 1887, *P. fluorescens* TISTR 2237, *P. fluorescens* TISTR 903, and *P. aeruginosa* TISTR 2370 were used. Bacterial susceptibility to a phage was determined by the presence of a plaque at the spot where the phage suspension was applied.

3.8 One-step growth curve experiments

One-step growth curve experiments were performed according to the protocol described by Sunthornthummas et al., (2017). Mid-exponential *Pseudomonas* cultures were infected with phages at a multiplicity of infection (MOI) of 0.1 and incubated at 30°C for 10 minutes. The suspension was then filtered through a 0.45 µm pore-size syringe filter to remove unadsorbed phages. The filter membrane was washed several times with NB broth to eliminate any remaining unadsorbed phages. The adsorbed phages on the filter were resuspended in 20 mL of NB broth B and incubated at 30°C and 150 rpm with shaking. Phage titers were determined by collecting samples at 5- or 10-minute intervals and using the double-layer agar method. The latent period, rise period, and burst size were calculated from the one-step growth curve, as described by Adams (1959).

3.9 Determination of optimal multiplicity of infection (MOI) of phage

To determine the MOI (multiplicity of infection), *P. fluorescens* TISTR 1887 was prepared at a concentration of 10^8–10^{10} CFU/mL. Next, 1 mL

of phage was added to 1 mL of the bacterial cells at varying ratios of 100, 10, 1, 0.1, 0.01, and 0.001. The mixture of phage and host bacteria was then incubated at 30°C for 2 hours. Following this, the culture was centrifuged, filtered, and assayed to determine the phage titer.

3.10 Effects of pH and temperature on phage stability

In order to assess the pH stability of the phage, the NB broth was adjusted to various pH levels (pH 2.0, 3.0, 4.0, 5.0, 8.0, 9.0, 10.0, 11.0, and 12.0) using 1 N HCl and 1 N NaOH. Next, the phage suspension, which contained approximately 10^7 PFU/mL, was added and incubated for 30 min at 30°C. The samples were withdrawn at time intervals and the phage titers were determined using the double-layer agar plate method. To investigate temperature stability, the phage lysates were subjected to different temperatures (4, 25, 30, 37, 45, 65, 75, and 100 °C) in NB medium at pH 7. At regular intervals, the tubes were removed and cooled in an ice-water bath. The phage titer was measured by the double-layer plate method.

3.11 Whole Genome Analyses

3.11.1 DNA extraction and sequencing

The genomic DNA of phage was extracted using the method previously described by Sambrook et al., (1989). Subsequently, the extracted DNA was assessed for its concentration and quality using a NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific, USA). The purified genomic DNA was then sent to the Beijing Genomics Institute (BGI) in China for short-read sequencing.

3.11.2 Genome assembly and annotation

Whole-genome sequencing (WGS) of phages was carried out at the Beijing Genomics Institute (BGI) in China for short-read WGS. Read quality was investigated using FASTQC. The resulting reads were *de novo* assembled into contigs using SPAdes v3.12 (Bankevich et al., 2012). The assembled genome was annotated using Prokka v1.15 (https://github.com/tseemann/prokka). Functional protein annotation was predicted using BLASTP against the NR database. The potential presence of antibiotic resistance genes and gene of virulent factor was search against the comprehensive antibiotic resistance database (CARD) and virulence factor database (VFDB) respectively. The comprised of transfer RNA genes was determined using tRNAscan-SE. The genomes of phage were visualised using a CG-view server (https://beta.proksee.ca/) (Grant, & Stothard, 2008).

3.12 *In vitro* lytic activity of the phage

In vitro lytic activity of the phage was performed to reduce the number of *P. fluorescens*. The experiments were carried out at various MOIs (10, 1, 0.1, 0.01, and 0.001). To investigate the therapeutic effects of phage on the respective hosts, two separate experiments were conducted. The first experiment involved the addition of overnight cultures of *P. fluorescens* hosts, followed by simultaneous inoculation with phages at different MOIs (prevention experiment). In the second experiment, *P. fluorescens* hosts were added and incubated for 3 hours before being inoculated with phage suspension at various MOIs (treatment experiment). For each assay, two control samples were included: the bacterial control (BC), which was inoculated with *P. fluorescens* but not with phages, and the phage control (PC), which was inoculated with phages but not with bacteria. Both the controls and the test samples were incubated under same conditions. Samples were collected from the test samples, bacterial controls, and phage controls after 0, 6, 12, 24, and 48 hours of incubation at 37 °C with shaking at 100 rpm. The phage titer was determined using the double agar layer method in each experiment. The growth of *P. fluorescens* under each condition was determined in NA medium. Three independent experiments were performed for each condition.

3.13 Statistical Analysis

All experiments were performed in triplicate. The data were expressed as mean±SD. Statistical analyses were performed using Statistical Package for Social Sciences (SPSS) version 20. Statistically significant differences among groups were analyzed using one-way ANOVA with Tukey's post-hoc test. Statistical significance was set at $P \leq 0.05$.

4. Results and Discussion

4.1. Screening of extracellular protease from *Pseudomonas* spp.

The proteolytic activities of all *Pseudomonas* strains were assessed using skim milk agar and presented as the diameter of the clear zone. Results showed that *P. aeruginosa* TISTR

2370 and *P. fluorescens* TISTR 2237 produced protease in skim milk medium with a diameter of 0.2 cm and were positive for the *apr*X gene upon testing (Figure 1). Furthermore, *P. fluorescens* TISTR 1887 produced a wider clear zone (0.5 cm) in the same medium, although it did not yield positive results for the *apr*X gene. Zymography was performed to confirm the production of protease by the selected strains.

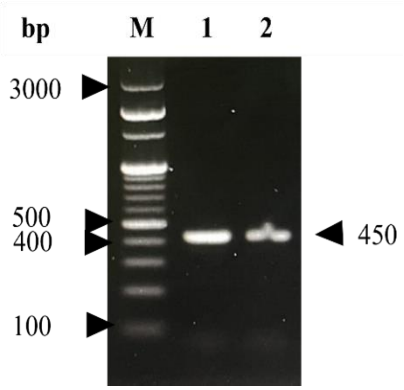

Figure 1 Detection of the *apr*X gene produced by different strains of *Pseudomonas* spp. Lane M: 100 bp marker, Lane 1: *P. aeruginosa* TISTR 2370, and Lane 2: *P. fluorescens* TISTR 2237. The resulting *apr*X gene band was observed at 450 bp, as indicated by the arrow pointing to the right.

4.2 Zymographic analysis

Zymographic analysis using skim milk as a substrate was conducted to confirm the presence of the protease. The results showed that *P. fluorescens* TISTR 1887 produced extracellular proteases with a molecular weight of approximately 180 kDa (Figure 2A), while *P. fluorescens* TISTR 2237 produced an extracellular protease with a molecular weight of approximately 47 kDa, which is identified as the AprX protease (Figure 2B). While *Pseudomonas* is known to produce a variety of proteases, including alkaline protease, metalloprotease, elastase, and serine protease (Caballero et al., 2001), the high molecular weight of the protease obtained from *P. fluorescens* TISTR 1887 is uncommon, as no previous reports have indicated that *Pseudomonas* spp. can produce a protease exceeding 180 kDa. However, recent observations indicate the presence of such a protease with a high molecular weight, which highlights the need for further investigation to confirm its existence and better understand its properties.

4.3 Phage isolation

The protease-producing strains, *P. aeruginosa* TISTR 2370 *P. fluorescens* TISTR 1887, and *P. fluorescens* TISTR 2237 were selected as host strains for the isolation of bacteriophages. Among these strains, only phage ΦTIS1 was obtained from *P. fluorescens* TISTR 1887. The plaques produced by this phage had average diameters of 0.47±0.082 cm, as shown in Figure 3. The phage isolate was further purified through three consecutive isolation steps.

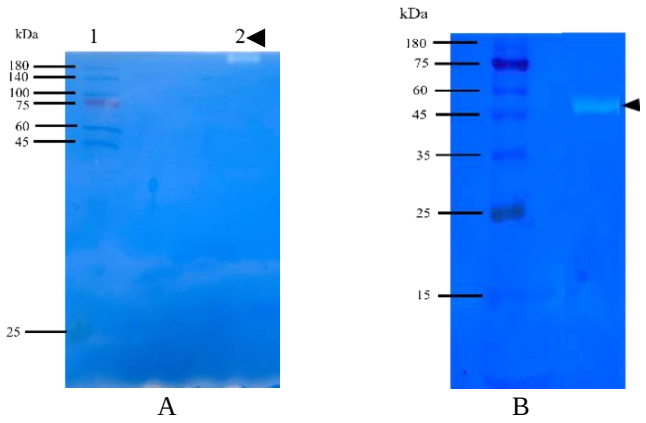

Figure 2 Skim milk Zymography of cell-free culture supernatant of *P. fluorescens* TISTR 1887 (A) and *P. fluorescens* TISTR 2237 (B); Arrow indicates protease bands.

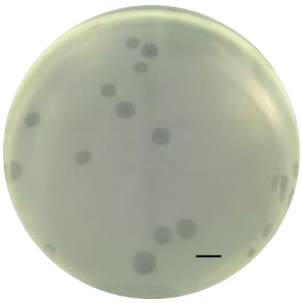

Figure 3 Plaque morphology of phage ΦTIS1 (C) against *P. fluorescens* TISTR 1887. Scale bar = 1 cm.

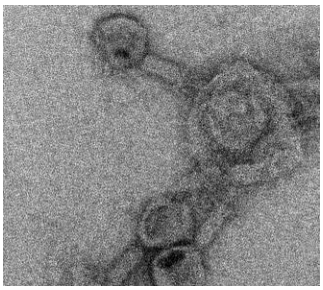

Figure 4 Electron micrograph of *P. fluorescens* phage ΦTIS1 (C). The arrows indicate the phage particles.

4.4 Phage morphology

Transmission electron micrographs revealed that ΦTIS1 virions exhibit an isometric head measuring 69.24 ± 0.34 nm in diameter, along with a tail measuring 122.20 ± 0.48 nm in length (Figure 4). Based on the morphological characteristics and criteria established by the International Committee on Taxonomy of Viruses (ICTV), ΦTIS1 was classified as a member of the *Myoviridae* family. The majority of reported phages belong to *Caudovirales* or tailed phages (Amarillas et al., 2017; Ackermann, 1999). However, phages that infect *P. fluorescens* exhibit diverse morphologies, with many belonging to the *Podoviridae* family, such as ϕIBB-PF7A (Sillankorva et al., 2008), UFJF_PfSW6, and UFJF_PfDIW6 (do Nascimento et al., 2022). Only a few phages, including phage UFJF_PfDIW6 (Hungaro et al., 2022), are classified as *Myoviridae*. Therefore, this study provides additional information on *Myoviridae* phages of *P. fluorescens.*

4.5 Host-range determination

The host range of phage ΦTIS1 was determined by spotting them on a lawn of the *Pseudomonas* spp. strains (Table 1). The protease producing strains of *Pseudomonas* including *P. aeruginosa* TISTR 2370 and *P. fluorescens* TISTR 2237, and *P. fluorescens* TISTR 1887 was used for this study. Phage ΦTIS1 was found to be able to lyse two different species, *P. fluorescens* TISTR 1887 and *P. aeruginosa* TISTR 2370, indicating the host specificity of this phage.

4.6 Optimal multiplicity of infection (MOI) and one-step growth curve

The optimal MOIs of ΦTIS1 which generated the maximum titers was between 10-100 (Table 2). The infection cycle of ΦTIS1 was characterized through the one-step growth curve experiments. The results indicate that the phage ΦTIS1 has a latent period of 10 min, a rise period of 15 min, and a burst size of about 7,000 phage particles per infected cell (Figure 5)

Table 1 Host range determination of the isolated phages

Bacterial strains	**Phages**		
	ΦF18	**ΦPF**	**ΦTIS1**
P. fluorescens PF01*	+	+	+
P. fluorescens PJRS20	-	-	-
P. fluorescens TISTR 358	+	-	-
P. fluorescens TISTR 1887*	-	-	+
P. fluorescens TISTR 2237*	-	-	-
P. fluorescens TISTR 903	+	-	+
P. aeruginosa TISTR 2370*	-	-	+
P. aeruginosa PA	-	-	-
P. entomophila PE01	-	-	-
P. mosselii PI01*	-	-	-
P. taiwanensis PT	-	-	-
P. mosselii N1	-	-	-
Total number of strains lysed	3	1	4

**Protease- producing strains*

Table 2 The optimal MOI of phage ΦTIS1

Bacteria (log_{10} CFU/mL)	Phages (log_{10} PFU/mL)	MOI	Phage titer after 2 h (log_{10} PFU/mL)
8	10	100	8.68 ± 0.23
9	10	10	8.60 ± 0.06
10	10	1	7.30 ± 0.28
10	9	0.1	6.17 ± 0.10
10	8	0.01	6.65 ± 0.66
10	7	0.001	5.69 ± 0.14

Data are expressed as average values ± standard deviation (SD) with three replicates.

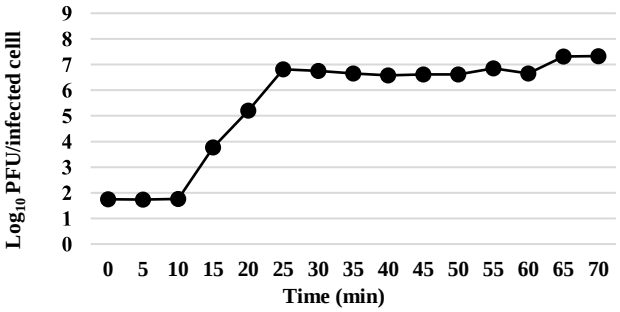

Figure 5 One-step growth curve of ΦTIS1.
(Data are the mean of triplicate independent experiments with standard deviation)

4.7 Thermal and pH stability

The stability of phages is a crucial factor to consider when developing phage-based biocontrol treatments. It has been reported that the stability of bacteriophages varies depending on the type of phage. Therefore, before any advanced application, it is essential to test the stability of the candidate bacteriophage (Vandenheuvel et al., 2013; Chumsen et al., 2020). Previous studies have shown that factors such as temperature and pH can significantly affect the survival and efficacy of phages (Pirisi, 2000; Jepson, & March, 2004; Silva et al., 2014). The study found that the bacteriophage ΦTIS1 was stable in a wide pH range of 4.0 to 12.0 (Figure 6A). However, at pH 3.0, the number of ΦTIS1 phages decreased by about 1.7 log PFU/mL compared to pH 7.0. To test their thermal stability, phage ΦTIS1 remained stable at 4 to 37°C but showed lower activity at the temperature higher than 45°C (Figure 6B). These results suggest that ΦTIS1 has high stability under various temperature and pH conditions, making them potential candidates for phage-based biocontrol in different environmental settings (Jamal et al., 2015).

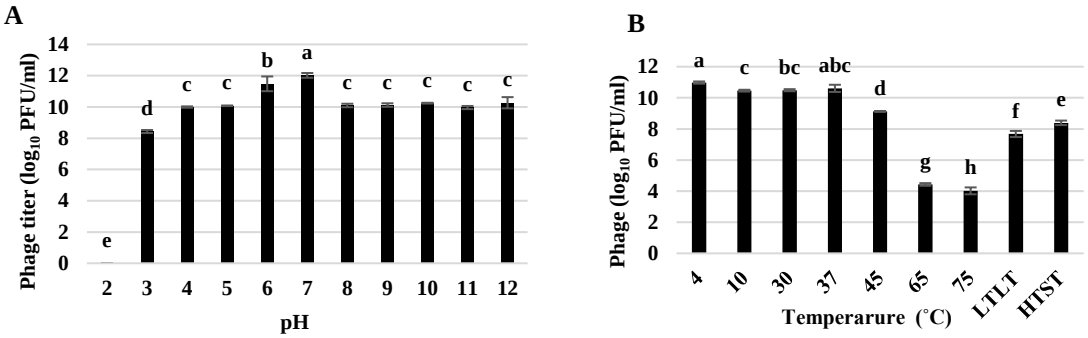

Figure 6 pH stability) A (and thermal stability (B) of phage ΦTIS1 .Values are means of 3 readings with ± SD .Nutrient broth without adjusted were used as a control. pH and thermal stability were analyzed by one-way ANOVA. The means of the triplicate for each incubation were compared by Tukey's test ($p \leq 0.05$). LTST represents low-temperature long-time pasteurization (62.5°C for 30 min), and HTST represents high-temperature short-time pasteurization (72°C for 30 s).

4.8 Whole-genome sequencing of phages and in silico analysis

The partial genome of *Pseudomonas* phage ΦTIS1 comprises a linear double-strand DNA. The genome assembly resulted in two long contigs with high coverage depth. The total genome size was 87,529 bp with a GC content of 54.71% (Figure 7). The BLASTn analysis with the NCBI database represented a sequence identity more than 98% with the query coverage higher than 98% to *Pseudomonas* phage vB_PaeM_G1 (Accession no. NC_041968.1) (https://blast.ncbi.nlm.nih.gov/Blast.cgi accessed on 5 December 2022). The predicted amino sequences were determined by BLASTp with protein available in NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi accessed on 5 January 2023) and tRNAscan-SE search. Out of 162 ORFs were predicted from 150 ATG start codon and 12 GTG start codon. Forty-four ORFs were identified as putative functional protein, which represented 19 genes on the positive strand and 25 genes on negative strand, while the 118 ORFs were classified as hypothetical protein. The putative functional gene of phage ΦTIS1 were separated into 3 groups including DNA replication and genome metabolism, host cell lysis, and structural proteins (Table 3). The three tRNAs were analyzed consist of tRNA-Asn (gtt), tRNA-Gln (ttg), and tRNA-Tyr (gta). The genome did not contain any gene coding antibiotic resistance, virulent factor, or toxin as well as no necessary gene for lysogenic cycle of phage.

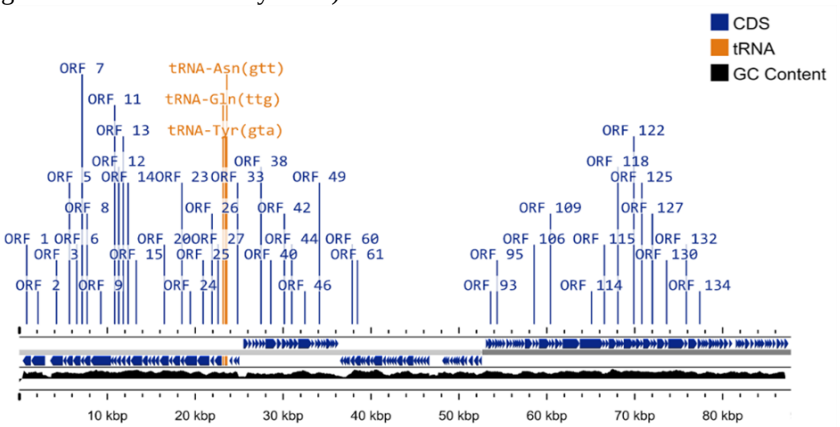

Figure 7 Genome structure of phage ΦTIS1 created using the CGView program. From upper to lower, the lines represent: 1 = forward strand, 2 = the nodes of genome, 3 = reverse strand, 4 = GC content. The element colors of each line are indicated.

4.9 *In vitro* lytic activity of the phage

The lytic activity of phage ΦTIS1 was tested against *P. fluorescens* TISTR 1887 in the exponential growth phase ($OD_{600} = 0.5$) using various MOIs. For the prevention experiment, the optimal MOI for ΦTIS1 resulting in the highest lytic activity was found to be 10 (Figure 8). All MOIs showed a significant effect on reducing bacterial growth, with a maximum reduction of approximately 5.7 log CFU/mL after 12 h of incubation. In the treatment experiment, the optimal MOI for ΦTIS1 resulting in the highest lytic activity was found to be 0.1 (Figure 9). When *P. fluorescens* TISTR 1887 was treated with ΦTIS1 at MOI 0.1, the maximum inactivation of was 5.4 log, achieved after 12 h of phage-based biocontrol. Several studies have suggested that phages have the potential to inhibit the growth of *Pseudomonas* in milk. For example, Tanaka et al., (2018) demonstrated that the HU1 phage reduced viable bacterial cell counts in rich medium, skim milk, and whole milk. Hu, Meng, & Liu (2016) reported that a phage cocktail led to a 2-log reduction in *Pseudomonas* cell number in UHT milk inoculated with *Pseudomonas* and a 1-log reduction in psychrotrophic bacteria and total bacteria counts in raw milk stored at 4°C for 5 days. In another study, do Nascimento et al., (2022) found that two lytic bacteriophages, UFJF_PfDIW6 and UFJF_PfSW6, were effective against *P. fluorescens*, reducing bacterial counts throughout the entire exponential growth phase in broth formulated with milk at both 4°C and 10°C. These results also found that phage-resistant mutants can emerge when using phages as a therapeutic treatment, and this can reduce the effectiveness of phage-based biocontrol. There are several strategies that can be employed to address this problem including the use of phage cocktail that target different receptors on the bacterial cell surface (Abedon, Danis-Wlodarczyk, & Wozniak, 2021; Oechslin, 2018) or the use phage-antibiotic combination therapy (Diallo, & Dublanchet, 2022).

Table 3 The ORFs of putative functional protein in phage ΦTIS1 genome and best similarity with databases

ORF	Position		Strand	Predicted function	Group of putative protein	E-value	Identity (%)	GenBank ID
	Start	Stop						
1	371	1,289	-	tail fiber assembly	Structural protein	1e-84	100.00%	YP_008858049.1
2	1,334	2,911	-	putative tail fiber protein	Structural protein	0	99.62%	UKM53946.1
3	3,479	4,945	-	baseplate wedge subunit	Structural protein	0	99.80%	YP_004306772.1
5	5,318	6,076	-	baseplate spike	Structural protein	0	99.60%	YP_008858044.1
6	6,073	6,954	-	baseplate hub	Structural protein	0	98.63%	YP_009124472.1
7	6,955	7,320	-	virion structural protein	Structural protein	4e-84	100.00%	YP_004306768.1
8	7,320	8,087	-	tail fiber protein	Structural protein	0	99.22%	YP_008858041.1
9	8,084	10,420	-	measure protein	Structural protein	0	99.87%	YP_008858040.1
11	10,652	11,038	-	tail assembly chaperone	Structural protein	3e-85	98.44%	YP_008857650.1
12	11,052	11,555	-	virion structural protein	Structural protein	6e-117	99.40%	YP_008856890.1
13	11,555	12,058	-	virion structural protein	Structural protein	2e-119	100.00%	YP_004306762.1
14	12,111	12,632	-	DUF3277 family protein	Structural protein	8e-124	100.00%	WP_016038315.1
15	12,643	13,965	-	tail sheath	Structural protein	0	99.77%	YP_004306760.1
20	15,943	16,998	-	major capsid protein	Structural protein	0	100.00%	WP_016038309.1
23	18,280	18,696	-	DNA methyltransferase	Genome metabolism	2e-96	99.28%	YP_008856879.1
24	18,698	20,185	-	portal protein	Structural protein	0	99.80%	YP_008857637.1
25	20,196	21,614	-	phage terminase large subunit	Genome metabolism	0	100.00%	WP_016038304.1
26	21,696	22,121	-	head fiber protein	Structural protein	8e-88	98.58%	YP_009604677.1
27	22,194	23,012	-	terminase large subunit	Genome metabolism	0	99.63%	YP_009206013.1
28	23,130	23,206	-	tRNA-Gln(ttg)	Genome metabolism			
29	23,408	23,495	-	tRNA-Tyr(gta)	Genome metabolism			
30	23,553	23,629	-	tRNA-Asn(gtt)	Genome metabolism			
33	24,652	25,008	-	DUF5681 domain-containing protein	Structural protein	1e-77	99.15%	WP_016038299.1
38	27,295	27,684	+	dCMP deaminase	Genome metabolism	2e-91	100.00%	YP_008857030.1
40	27,972	29,243	+	ATP-dependent DNA ligase	Genome metabolism	0	100.00%	YP_004306738.1
42	29,856	30,419	+	endolysin	Cell lysis	5e-137	99.47%	YP_008857786.1

Table 3 Cont.

ORF	Position		Strand	Predicted function	Group of putative protein	E-value	Identity (%)	GenBank ID
	Start	Stop						
44	30,674	31,276	+	DprA-like DNA recombination-mediator protein	Structural protein	2e-143	97.50%	YP_009206000.1
46	31,694	33,256	+	DNA helicase	Genome metabolism	0	100.00%	YP_004306732.1
49	33,921	34,349	+	peptidase HslV family	Genome metabolism	4e-96	100.00%	YP_004306729.1
60	37,612	38,112	-	DUF1643 domain-containing protein	Structural protein	8e-121	100.00%	YP_008857008.1
61	38,093	38,812	-	ead/Ea22-like family protein	Structural protein	4e-173	99.58%	WP_016038272.1
93	671	1,234	+	endolysin	Cell lysis	7e-137	99.47%	UVD32698.1
95	1,478	1,894	+	Rz-like spanin	Structural protein	4e-94	98.55%	YP_008856907.1
106	5,763	6,083	+	lipoprotein	Structural protein	0	100.00%	YP_004306787.1
109	7,679	7,864	+	RNA ligase	Genome metabolism	0	99.51%	YP_004306790.1
114	11,110	13,767	+	DNA primase/helicase	Genome metabolism	0	99.85%	YP_004306795.1
115	13,784	13,990	+	DNA polymerase	Genome metabolism	0	99.89%	YP_009604728.1
118	15,342	15,536	+	structural protein	Structural protein	0	99.66%	QIQ65134.1
122	17,166	17,327	+	putative exodeoxyribonuclease	Genome metabolism	0	99.45%	YP_008857692.1
125	18,057	18,272	+	HNH endonuclease signature motif containing protein	Genome metabolism	5e-135	100.00%	WP_223699727.1
127	19,253	19,477	+	DNA polymerase exonuclease subunit	Genome metabolism	0	99.67%	YP_008858086.1
130	20,794	21,168	+	putative thymidylate synthase	Genome metabolism	0	100.00%	YP_009604743.1
132	23,027	23,392	+	ribonucleotide-diphosphate reductase subunit alpha	Genome metabolism	0	100.00%	WP_223699730.1
134	24,514	24,996	+	puative ribonucleotide reductase	Genome metabolism	0	100.00%	YP_004306815.1

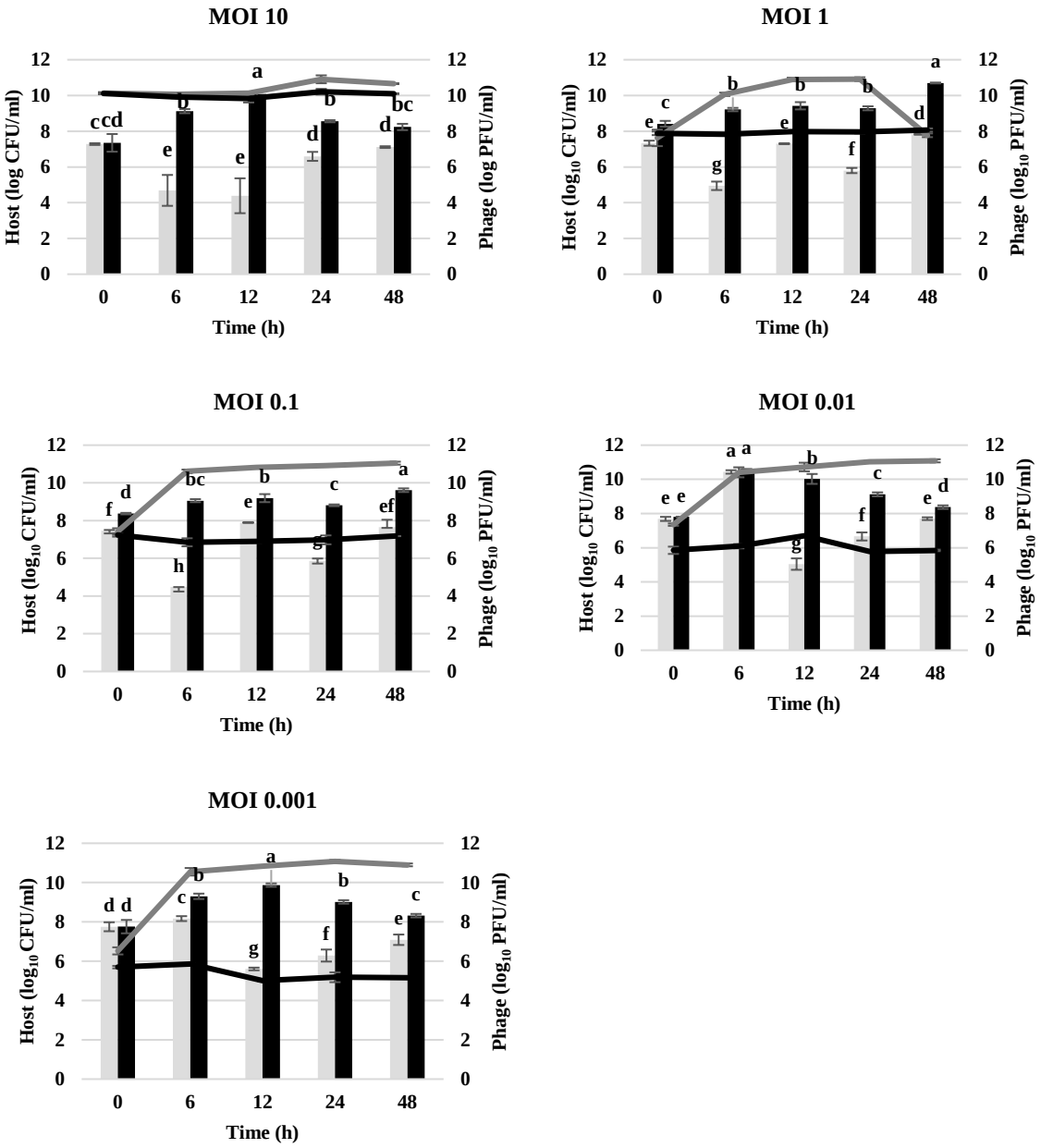

Figure 8 Inactivation of *P. fluorescens* TISTR 1887 by phage ΦTIS1 during 48 h at different MOIs in prevention experiment. Black bars represent bacteria titers in the bacteria control; grey bars represent bacteria titers with phage; black line represent the number of plaques counted in the phage control; grey lines represent the number of plaques counted in phage treatment. Values represent the mean of three experiments; error bars represent the standard deviation.

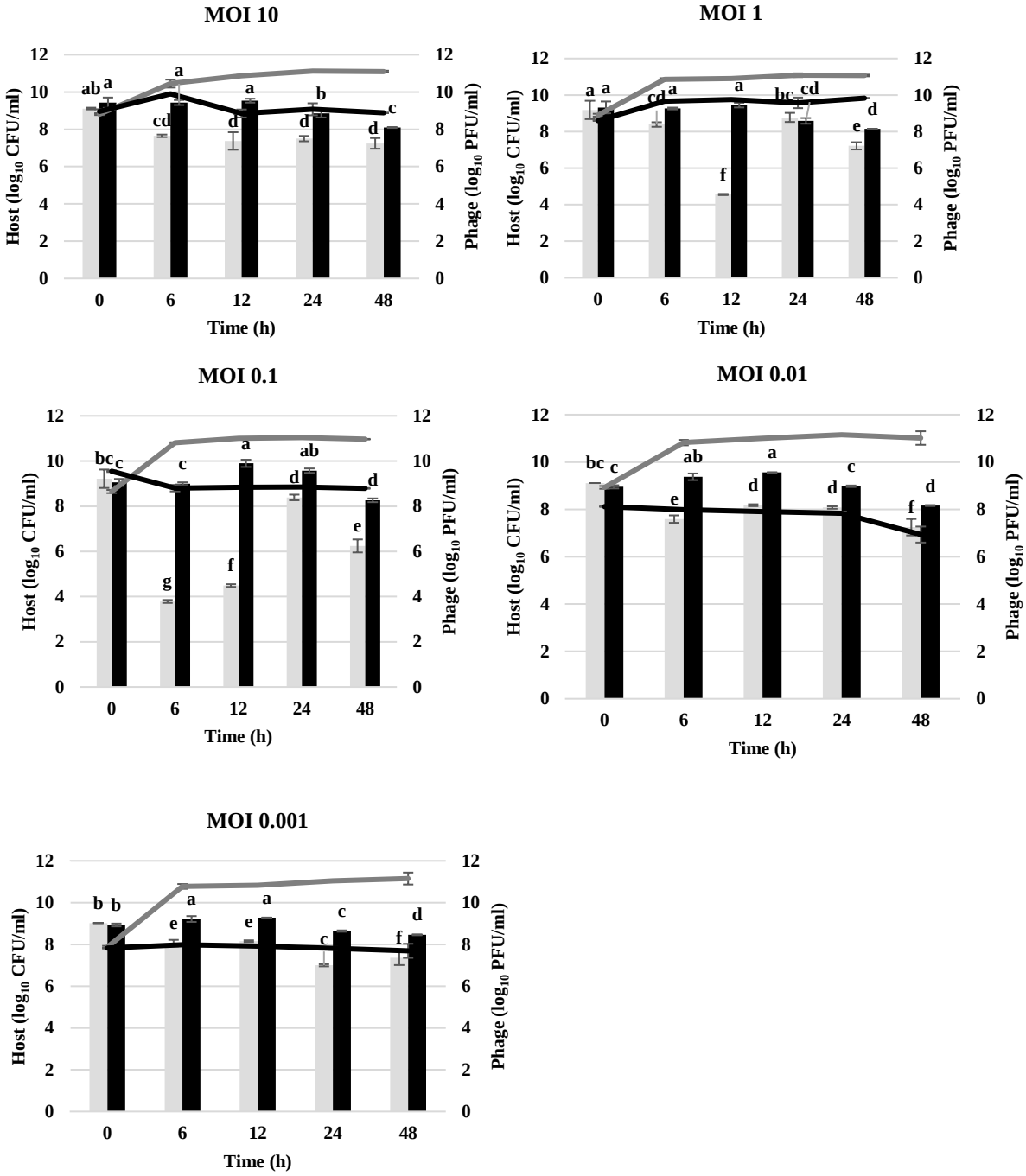

Figure 9 Inactivation of *P. fluorescens* TISTR 1887 by phage ΦTIS1 during 48 h at different MOIs in treatment experiment .Black bars represent bacteria titers in the bacteria control; grey bars represent bacteria titers with phage; black line represent the number of plaques counted in the control; grey lines represent the number of plaques counted in phage treatment. Values represent the mean of three experiments; error bars represent the standard deviation.

5. Conclusion

In this study, *P. fluorescens* phage ΦTIS1 was phenotypically and genotypically characterized. The phage exhibited large burst sizes and was found to be stable across a broad range of pH and temperatures. The partial ΦTIS1 genome was found to be a linear double-stranded DNA with a total length of 87,529 bp and a G + C content of 54.71%. The genome did not contain any gene coding antibiotic resistance, virulent factor or toxin as well as no necessary gene for lysogenic cycle of phage. These findings suggest that ΦTIS1 may be a useful tool for biocontrol of *P. fluorescens* in the dairy and other food-related industries.

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