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Isolation and characterization of virulent phages infecting *Shewanella baltica* and *Shewanella putrefaciens*, and their application for biopreservation of chilled channel catfish (*Ictalurus punctatus*)

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ABSTRACT

The growth of *Shewanella* spp., mainly *S. baltica* and *S. putrefaciens*, is responsible for the spoilage of chilled fresh fish. Phages are an alternative tool to control bacterial growth. In this study, virulent phages infecting 4 *S. baltica* and 6 *S. putrefaciens* strains were isolated and characterized. Transmission electron microscopy revealed that 6 out of 10 phages (3 phages infecting *S. baltica* and 3 phages infecting *S. putrefaciens*) belonged to Myoviridae, while the other 4 phages (1 phage infecting *S. baltica* and 3 phages infecting *S. putrefaciens*) belonged to Siphoviridae. Phage SppYZU01 and SppYZU05 showed the broadest host range, being lytic towards all the 4 *S. baltica* strains and 5 out of the 6 *S. putrefaciens* strains, respectively. The genome sequence of SppYZU01 had no similarity with known genome sequences, while that of SppYZU05 was 88.5% similar to the genome of a virulent *S. putrefaciens*-infecting phage (Spp001). According to the host range and lytic activity, 3 phages, including SppYZU01, SppYZU05, and SppYZU06, were combined into a cocktail (designated as SPMIX3-156). SPMIX3-156 showed potential as an antimicrobial agent to control *S. baltica* and *S. putrefaciens* strain growth in catfish matrices. Bacterial growth in the catfish muscle juice inoculated with 10^4 colony-forming units (CFU)/mL of *Shewanella* strains was partially inhibited by 10^5 plaque-forming units (PFU)/mL of SPMIX3-156 at both 25 °C and 4 °C. The catfish fillets inoculated with *Shewanella* strains were used as a model to evaluate the biopreservative effects of SPMIX3-156. Total viable counts of fillet samples treated with 10^7 PFU/mL of SPMIX3-156 were reduced by 3.21 and 2.75 log units after 1 day at 25 °C and 10 day at 4 °C, respectively, compared to those of untreated samples. Fillet quality indices, including pH, total volatile basic nitrogen, and sensory value of the SPMIX3-156-treated samples, considerably improved compared to those of the control samples at both 4 °C and 25 °C. Our results suggest that SPMIX3-156 is a promising biological agent against *S. baltica* and *S. putrefaciens*, and may have a potential use in chilled fish fillet biopreservation.

1. Introduction

Fish are highly prone to spoilage because of the high water activity, nutritive composition, and neutral pH, which make them a suitable substrate for bacterial growth during post-mortem storage (Gram and Huss, 1996). The iced or refrigerated storages are widely used by consumers and processors to reduce the deterioration rate and maintain the freshness of fish. These methods facilitate in temperature control and avoid the damage caused by ice crystals to the fish texture compared to the frozen storage (Love and Haraldsson, 2010). However, some psychrotrophic flora can grow and produce metabolites that result in off-odors during iced or refrigerated storages (Gram and Huss, 1996; Yu et al., 2017), leading to loss of quality and subsequent spoilage. Therefore, taking measures to control growth of psychrotrophic bacteria is necessary for delaying the decline in fish quality and extending the preservation life of fish during low temperature storage.

Shewanella spp. comprise the predominant psychrotrophic bacteria responsible for the spoilage of both fresh and packed fish, producing

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off-odors even at low cell numbers (Dalgaard, 1995; Gram and Dalgaard, 2002). *S. putrefaciens* and *S. baltica* are well-known specific spoilage organisms (SSOs) of chilled marine fish (Gram and Dalgaard, 2002; Gu et al., 2013; Vogel et al., 2005). Their spoilage action includes production of trimethylamine (TMA), hydrogen sulphide (H_2S), methyl mercaptan (CH_3SH), dimethyl sulphide ($(CH_3)_2S$), and other characteristic compounds leading to intensive off-odors (Gram and Huss, 1996; Herbert and Shewan, 1975; Lopez-Caballero et al., 2001). These *Shewanella* species are also the major spoilage flora in the chilled freshwater fish such as grass carp (*Ctenopharyngodon idella*), common carp (*Cyprinus carpio*), bighead carp (*Aristichthys nobilis*), and channel catfish (*Ictalurus punctatus*) (Liu et al., 2017; Tao et al., 2016; Wang et al., 2014). *S. putrefaciens* grows from 3.76 log colony-forming units (CFU)/g to 9.47 log CFU/g in grass carp fillets after 14 days at 4 °C, producing high levels of 1-octen-3-ol, nonanal, and 2-ethylcyclohexanol, which are volatile compounds causing strong off-odors (Wang et al., 2017). In our previous study, the relative abundance of *Shewanalla* spp. increased from 3.21% to 45.96% in channel catfish fillets during 12 days at 4 °C (Tao et al., 2016). Therefore, limiting growth of these *Shewanella* species is necessary for extending the shelf-life of chilled fish.

Phages have attracted great attention for application in food biopreservation during the last years, because they are self-replicating, self-limiting, ubiquitously distributed in nature, and considered a part of the natural food microflora (Sillankorva et al., 2012). Phages can even reduce the host bacteria in biofilms, which are not easy to remove or sanitize (Lee and Park, 2015). The virulent phages have been successfully isolated and used as food biocontrol agents against spoilage organisms such as *Lactobacillus* spp. in beer (Deasy et al., 2011), *Pseudomonas* spp. in milk (Hu et al., 2016), *Erwinia amylovora* in fruits (Schnabel et al., 1998), and *Brochothrix thermosphacta* in pork (Greer and Dilts, 2002). Virulent phages have also been used for the inactivation of main food-borne pathogens, including *Listeria monocytogenes*, *Staphylococcus aureus*, *Escherichia coli* O157:H7, *Salmonella enterica*, *Shigella* spp., *Campylobacter jejuni*, and *Cronobacter sakazakii* in different food systems (Greer, 2005; Perez et al., 2015). However, the isolation and application of *Shewanella* phages for the preservation of fish are very limited. Currently, only one virulent phage (designated as Spp001) infecting *S. putrefaciens* strain Sp225 has been isolated and sequenced (Han et al., 2014). This phage provided effective biocontrol of *S. putrefaciens* under chilled conditions and prevented spoilage of *Paralichthys olivaceus* fillets (Li et al., 2014). Although *S. baltica* is a main spoilage organism of fish and other food products (Gu et al., 2013), and it is considered as the most represented species among H_2S producers in ice-stored fish (Tryfinopoulou et al., 2007; Vogel et al., 2005), phages infecting *S. baltica* have not been reported to date. Despite the limited library of virulent phages infecting *Shewanella* available to date, there are no currently available genomic data on phages infecting *S. baltica*. In addition, since phages are usually highly specific, it is more valuable to isolate broad host range phages and create phage cocktails as broader spectrum antibacterial agents for biopreservation of chilled fish carrying both *S. baltica* and *S. putrefaciens*.

In this study, waste effluents from fish markets were screened to isolate a collection of broad spectrum virulent phages infecting *S. baltica* and *S. putrefaciens*. The selected potent virulent phages were identified morphologically, characterized by dynamics of infective characteristics and host range, and sequenced. Based on the results, virulent phages were cocktailed and evaluated for their potential and efficacy in biocontrol of *S. baltica* and *S. putrefaciens* strains in channel catfish (*Ictalurus punctatus*) muscle juice and fillets at both 25 °C and 4 °C storage conditions.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Four *S. baltica* strains (SYZU01, SYZU03, SYZU08, and SYZU09) and 6 *S. putrefaciens* strains (SYZU02, SYZU04, SYZU05, SYZU06, SYZU07, and SYZU10) were obtained from the Key Laboratory of Prevention and Control of Biological Hazard Factors (Animal Origin) for Agrifood Safety and Quality, Ministry of Agriculture of China, Yangzhou University (Jiangsu, China). Strain SYZU01 and SYZU02 were isolated from the spoiled fillets of olive flounder and rainbow trout, respectively. The other 8 strains (SYZU03 to SYZU10) were isolated from the spoiled fillets of freshwater fish (grass carp and channel catfish) collected from different locations in Jiangsu. All strains stored at −80 °C were thawed and cultured in Luria-Bertani (LB) medium (Huankai Microbial Sci & Tec. Co. Ltd. Guangzhou, China) overnight with shaking (150 rpm) at 25 °C.

2.2. Enrichment, isolation, and purification of phages

For enrichment, isolation, and purification of phages, methods were performed as previously described (Sambrook et al., 1989; Twest and Kropinski, 2009), with some modifications. Fifteen samples of wastewater (100 mL) were collected from three local freshwater and marine product marketplaces in Yangzhou. Thirty milliliters of each sample was centrifuged at 8000 ×*g* for 10 min to remove solid particles and filtered using 0.45-μm-pore syringe filters (Millex, Merck Millipore Ltd., Tullagreen, Carrigtwohill, Co. Cork, Ireland). For enrichment purposes, the filtrates (5 mL) were added to 5 mL of 2X LB broth with 100 μL of the exponential phase cultures of each individual host strain and incubated overnight at 25 °C with gentle shaking. After incubation, the cultures were centrifuged at 8000 ×*g* for 10 min, and supernatants were filtered with a 0.22-μm-pore size sterile filter (Millex, Merck Millipore Ltd., Tullagreen, Carrigtwohill, Co. Cork, Ireland).

To screen for the presence of virulent phages, the filtrates (phage enrichments) were used for plaque testing. Briefly, the overnight culture (100 μL) of the host strain was mixed with 5 mL LB soft agar (0.5% agar), overlaid onto LB agar (1.5% agar) plates, and solidified at 20 °C for 15 min. Ten microliters of each filtrate was spotted on the top of soft agar and incubated at 25 °C for 16 h. After incubation, the plates were examined for clear plaques in the bacterial lawns. Clear plaques of lysed agar were cut, dissolved into SM buffer (5.8 g/L NaCl, 2 g/L $MgSO_4{\cdot}7H_2O$, and 50 mmol/L Tris-HCl, pH 7.5), and centrifuged at 8000 ×*g* for 10 min. The supernatants were filtered through 0.22-μm-pore size sterile filters and used as phage crude extracts.

For isolating and purifying individual phages from phage crude extracts, 100 μL of 10-fold serial dilutions of phage crude extracts were mixed with 100 mL of exponential phase host strain cultures and incubated at 25 °C for 10 min. The suspensions were mixed with 5 mL of LB soft agar, poured onto LB bottom agar plates, and incubated at 25 °C for 16 h for plaque formation. The individual plaques were picked from agar plates based on their sizes and morphologies, and suspended in 1 mL of SM buffer at 4 °C for 24 h. The suspensions were filtered using 0.22-μm-pore size sterile filters to prepare the pure bacteriophage stocks. The purification process was repeated at least 3 successive times with each individual phage stock to ensure phage purity. The diameters of clear plaques were determined for preliminary evaluating lytic capability of phage.

2.3. Propagation and titration of phages

The phage stocks were propagated on host strains. Two hundred microliters of pure phage stocks (approx. 10^8 PFU/mL) were mixed with 200 μL of exponential phase host strain culture and incubated at 25 °C for 10 min. The suspensions were inoculated in 10 mL of LB broth and cultured at 25 °C for 16 h with shaking at 150 rpm. After incubation, the phage lysates were centrifuged at 8000 ×*g* for 10 min at 4 °C, and supernatants were filtered through 0.22-μm-pore size sterile filters and stored at 4 °C. Titers of the phage lysates were determined by plaque counting using the above described soft agar overlay technique and expressed as plaque-forming unit (PFU) per mL.

2.4. Determination of host range

For host range determination, the bacterial lawns were prepared with overnight cultures on LB agar plates as described above. Ten microliters of each phage dilution (10^8 PFU/ml) was spotted onto each bacterial lawn, dried at 25 °C for 10 min on a clean bench, and incubated at 25 °C for 24 h. The phages showing visible lytic zones at the point of application were marked as positive for lytic activities. Based on the spot test, phages forming a clear lysis zone on the lawn of the target strains were selected for further determination of efficiency of plaquing (EOP) using the double-layer agar method (Akhtar et al., 2014). The host strains were set as the reference strains. The EOP was defined as the ratio between the average titer of the target strain (PFU/mL) and that of the reference strain (PFU/mL). For each phage, three independent experiments were done.

2.5. Transmission electron microscopy (TEM)

The morphological characteristics of phages were examined using TEM. Stock cultures of purified phages were concentrated by polyethylene glycol (PEG) 8000 precipitation method as previously described (Jaomanjaka et al., 2016) and resuspended in a 50 μL of SM buffer. Ten microliters of suspension (approximately 10^{10} particles/mL) was placed on carbon-coated copper grids for 30 s, negatively stained with 0.5% phosphotungstic acid for 2 min, and dried at 25 °C for 5 min. The stained phages were examined by transmission electron microscopy (Tecnai 12; Tecnai, Eindhoven, the Netherlands) at the Physical and Chemical Testing Center, Yangzhou University.

2.6. One-step growth curve

The one-step growth curve was determined for each phage on its host strain. One milliliter of mid-log phase bacterial culture was harvested by centrifugation at 8000 ×*g* for 10 min at 4 °C and resuspended in 10 mL of fresh LB medium. Phages were added at a multiplicity of infection (MOI) of 0.1 and incubated at 25 °C for 10 min to allow phage adsorption. After the adsorption, the suspensions were centrifuged at 8000 ×*g* for 10 min, and the pellet containing infected cells were suspended in LB broth, and then incubated at 25 °C with shaking at 150 rpm. Two hundred microliters of samples were taken at 10-min intervals for 120 min, and the titers were determined using the classical double-layer plating technique. The one-step growth curves were plotted using logarithmic titer values (PFU/infected cell) vs. the time (min) from which the phage growth parameters such as latent period, burst time, and burst size were estimated as previously described (Pujato et al., 2015).

2.7. DNA extraction, sequencing, and genomic analysis

One milliliter of pure phage stocks was mixed with 1 mL of exponential phase host strain culture, and incubated at 25 °C for 10 min. The suspension was then inoculated into 100 mL of LB broth, and incubated at 25 °C for 8 h with shaking at 150 rpm. After incubation, the phage lysates were centrifuged at 8000 ×*g* for 10 min at 4 °C, and supernatants were filtered through 0.22-μm-pore size sterile filters. The filtrates were concentrated and purified by PEG 8000 precipitation and CsCl gradient centrifugation as described by Sambrook et al. (1989). The phage genomic DNA was extracted and purified using the ABigen Lambda Phage DNA Purification kit (AB1141, ABigen Corporation, Beijing, China). Total DNA was subjected to quality control by agarose gel electrophoresis and quantified by Qubit ® dsDNA HS Assay Kit in a Qubit 2.0 Flurometer (Life Technologies, Carlsbad, CA, USA).

The genome sequencing was performed with massively parallel sequencing (MPS) Illumina technology. A paired-end library with an insert size of 500 bp was constructed and sequenced using the Illumina Hiseq 2500 PE150 platform (Illumina, San Diego, CA, USA). Library construction and sequencing were performed at the Total Genomics Solution (TGS) Institute, Shenzhen, Guangdong, China. Quality control of both paired-end and mate-pair reads was performed using an in-house program. After this step, Illumina adapters and low quality reads were filtered. The filtered reads were assembled by SOAPdenovo (http://soap.genomics.org.cn/soapdenovo.html) to generate scaffolds. All reads were used for gap closure.

Gene prediction was performed by GeneMarkS (http://topaz.gatech.edu/) with an integrated model which combined the GeneMarkS generated (native) and heuristic model parameters. Gene annotation was achieved by the algorithms of the basic local alignment search tool (BLAST) search in the National Center for Biotechnology Information (NCBI) database against the non-redundancy (nr) nucleotide database with *E*-value ≤1e-5 and minimal alignment length percentage ≥ 40%. Screening for putative proteins was performed based on the local alignment of each open reading frame (ORF) product to the amino acid sequences of the proteins in the Non-Redundant Protein Database databases (NR), Kyoto Encyclopedia of Genes and Genomes (KEGG), Gene Ontology (GO), Virulence Factors of Pathogenic Bacteria (VFDB), and Antibiotic Resistance Genes Database (ARDB). The complete genome sequences of virulent phages (SppYZU01 and SppYZU05) were deposited in GenBank (https://www.ncbi.nlm.nih.gov/genbank) under the accession number KY622015 and KY709296. Comparisons of phage genome sequences in the present study with their homologous sequences in GenBank were performed using MUMmer (Kurtz et al., 2004).

2.8. Control of *S. baltica* and *S. putrefaciens* by phage in fish matrices

2.8.1. Experiments with fish muscle extract juice (FMEJ)

FMEJ was used as a model to determine the effectiveness of phages in biocontrol of *S. baltica* and *S. putrefaciens* in the fish matrix. Fresh channel catfish was purchased from a local market, and fish muscles on both sides of the spine were collected. For FMEJ preparation, 500 g of fish muscle was chopped, mixed with 1 L of water, and boiled for 10 min. Juice was separated from solids by centrifugation at 4000 ×*g* for 20 min and adjusted to pH 7.0 with NaOH (1.0 mol/L). The juice was supplemented with cysteine (Sangon Biotechnology Co., Ltd., Shanghai, China) (40 mg/L) and methionine (Sangon Biotechnology Co., Ltd., Shanghai, China) (40 mg/L), and autoclaved for the experiment. Overnight cultures of 4 *S. baltica* and 6 *S. putrefaciens* strains were adjusted to a final concentration of 10^6 CFU/mL with 0.85% NaCl solution and combined into a cocktail of 10 *Shewanella* strains. The bacterial cocktail (0.5 mL, the total bacterial cell concentration is 10^6 CFU/mL) was inoculated into 49 mL of FMEJ and mixed with SM buffer (0.5 mL) (control group), or individual phage dilutions (0.5 mL, at a titer of 10^7 PFU/mL), or cocktailed phage dilutions (0.5 mL, at a titer of 10^7 PFU/mL) (treated group). The mixtures were incubated at constant temperature of 25 °C for 2 d or 4 °C for 7 d, and the growth of the host was monitored periodically by measuring the optical density (OD) at 600 nm using a spectrophotometer UV-7504C (Xinmao Instrument Co., Ltd., Shanghai, China) in triplicate until stationary phase was reached. The change of phage titers were monitored using the double-layer plating technique as described in Section 2.3. Incubations were carried out at 25 °C for 16 h for plaque formation. Phage titers were expressed as plaque-forming units per mL (PFU/mL).

2.8.2. Experiments with catfish fillet artificially contaminated with *S. baltica* and *S. putrefaciens*

The phage cocktail that showed the most effectiveness in the FMEJ model was evaluated using the artificially contaminated fish fillet. Fish muscles of fresh catfish were cut into approximately 2 cm × 2 cm × 0.5 cm slices (length × width × thickness) under sterile conditions. The catfish fillets were soaked in sterile water containing 100 parts-per-million (ppm) of sodium hypochlorite at room temperature, gently shaken for 5 min, washed with sterile water for 10 times, and drained for 30 min on a clean

Table 1
Characterization of phages by plaque morphology and transmission electron microscopy.

Phage	Host strain		Plaque diameter (mm)	Morphological analysis by TEM[a]		Presumptive family
	Strain	Source		Head(diameter)	Tail (length × width)	
SppYZU01	*Shewanella baltica* SYZU01	Olive flounder	2.7	Isometric (55 nm)	160 nm × 13 nm, with contractile tail sheath	Myoviridae
SppYZU02	*Shewanella putrefaciens* SYZU02	Rainbow trout	3.0	Isometric (68 nm)	240 nm × 16 nm, with noncontractile tail	Siphoviridae
SppYZU03	*Shewanella baltica* SYZU03	Grass carp	0.8	Isometric (65 nm)	184 nm × 15 nm, with contractile tail sheath	Myoviridae
SppYZU04	*Shewanella putrefaciens* SYZU04	Channel catfish	0.7	Isometric (85 nm)	129 nm × 14 nm, with noncontractile tail	Siphoviridae
SppYZU05	*Shewanella putrefaciens* SYZU05	Channel catfish	2.5	Isometric (61 nm)	166 nm × 15 nm, with contractile tail sheath	Siphoviridae
SppYZU06	*Shewanella putrefaciens* SYZU06	Channel catfish	1.2	Isometric (63 nm)	233 nm × 16 nm, with noncontractile tail	Myoviridae
SppYZU07	*Shewanella putrefaciens* SYZU07	Grass carp	0.8	Isometric (95 nm)	163 nm × 16 nm with contractile tail sheath	Myoviridae
SppYZU08	*Shewanella baltica* SYZU08	Channel catfish	0.7	Isometric (71 nm)	169 nm × 14 nm, with contractile tail sheath	Myoviridae
SppYZU09	*Shewanella baltica* SYZU09	Channel catfish	0.8	Isometric (85 nm)	130 nm × 14 nm, with noncontractile tail	Siphoviridae
SppYZU10	*Shewanella putrefaciens* SYZU10	Grass carp	1.2	Isometric (65 nm)	175 nm × 15 nm, with contractile tail sheath	Myoviridae

[a] Transmission electron microscopy.

bench. The sterilized fish pieces (200 g) were immersed in 1 L of bacterial suspension of the 10-*Shewanella* strain cocktail (containing 10^6 CFU/mL of total bacterial cell, prepared as described in 2.8.1) for 2 min and drained for 30 min. The artificially contaminated fish pieces were randomly divided into two groups and soaked in 500 mL of SM buffer (control group) and 500 mL of phage cocktails (at a titer of 10^7 PFU/mL) (treated group) for 5 min at 4 °C, separately. The treated fish samples were removed, drained for 30 min, individually sealed in air-proof sterile polyethylene bags, and stored at 4 °C for 12 days and 25 °C for 48 h. Sampling was carried out at 2-day intervals for storage at 4 °C and 12-h intervals for storage at 25 °C. Samples from each treatment were collected in triplicate for the determination of bacterial total viable counts (TVCs), phage titer, pH, total volatile basic nitrogen (TVB-N), and sensory scores.

Samples (5 g) were mixed (1/10, w/v) with distilled water and homogenized in blenders. The homogenates were centrifuged at 8000 ×*g* for 5 min, and the supernatants were used for measuring pH with a digital pH meter (pHS-3C, Shanghai Lanke Apparatus Co., Shanghai, China) and determining TVB-N concentration according to Method for Analysis of Hygienic Standard of Meat Products, China (GB/T 5009.44-2003) (http://www.gov.cn/fuwu/bzxxcx/bzh.htm). For TVC determination, the homogenates were decimally diluted in sterile saline (0.85%), spread onto LB agar plates in duplicate, and incubated at 25 °C for 48 h. CFUs were counted manually to calculate the concentration of viable cells in the sample (CFU/g). Phage titer determination were carried out as described in Section 2.3, and expressed as plaque-forming units per mL (PFU/g). Sensory evaluation was performed according to the method described by Zhu et al. (2013). The attributes of fish fillets (appearance, odor, morphology, and texture) were evaluated by a panel of 13 trained members (six women and seven men), and each panel member was asked to rate the fish muscle using a scale of 1–5 scores for each attribute and record comprehensive preference scores, in which 20 was equivalent to the best quality, and 4 was indicative of complete spoilage.

2.9. Determination of the mutation frequencies of bacteriophage insensitive mutants

The bacteriophage insensitive mutants (BIMs) frequencies of *S. baltica* and *S. putrefaciens* strains were determined according to the method described by Gutierrez et al. (2015). Aliquots of 100 μL of overnight culture of each bacterial host (approx. 10^8 CFU/mL) were mixed with 100 μL of the phage cocktail SPMIX3-156 (10^{10} PFU/mL) and incubated at 25 °C for 10 min. The exact bacterial concentration of overnight culture was determined by plating serial dilutions onto LB agar plates. Five milliliters of LB soft agar (0.5%, w/v) were added to the mixture, poured onto a LB agar plate and incubated at 25 °C for 16 h. After incubation, each of surviving colonies were picked and suspended in fresh LB broth and cultured at 25 °C for 16 h. Each of these cultures (100 μL) was mixed with 5 mL LB soft agar, overlaid onto LB agar plates, and solidified at 20 °C for 15 min. Ten microliters of phage cocktail SPMIX3-156 (10^9 PFU/mL) was spotted on the top of soft agar and incubated at 25 °C for 24 h. The cultures without forming clear plaques in the bacterial lawns were confirmed as real BIMs. The BIM frequency was calculated as the ratio between the number of real BIM colonies and the initial number of bacteria incubated in the presence of phage.

2.10. Statistical analysis

All analyses were performed in triplicate. Data were expressed as mean values ± standard deviation of means. The least significant difference procedure was used to test for difference between means using the SPSS software 19.0 (SPSS Inc., Chicago, IL, USA) and Excel program. The significance level was set at $p < 0.05$.

3. Results and discussion

3.1. Collection and isolation of phages infecting Shewanella spp.

A total of 10 phages (designated as SppYZU01 to SppYZU10) infecting 4 strains of *S. baltica* and 6 strains of *S. putrefaciens* were isolated from wastewater samples (Table 1). All the phages formed clear plaques on their host lawn, with plaque diameter varying from 0.7 to 3.0 mm. Morphological analysis by TEM (Fig. 1) showed that all the phages have the isometric head (diameter 55 nm to 95 nm) and tail (length: 129 nm–240 nm; width: 13 nm–16 nm). Six phages (SppYZU01, SppYZU03, SppYZU05, SppYZU07, SppYZU08, and SppYZU10) have contractile tail sheath, while 4 phages (SppYZU02, SppYZU04, SppYZU06, and SppYZU09) had a noncontractile tail. The morphological characteristics of the group of six phages (3 infecting *S. baltica* and 3 infecting *S. putrefaciens*) and four phages (1 infecting *S. baltica* and 3 infecting *S. putrefaciens*) are typical of the Myoviridae and Siphoviridae families, respectively (Caudovirales order) (Fauquet et al., 2005).

Our successful isolation of phages supports that sewage from fish processing industry as a suitable environment for isolation purposes due to the presence of their host species *S. baltica* and *S. putrefaciens* in this habitat (Clokie et al., 2011). Of note, the virulent phage Spp001 infecting *S. putrefaciens*, previously described, belongs to Siphoviridae family (Kong et al., 2012), but not virulent phages infecting *S. baltica* have been reported to date. In the present study, the phages infecting different *S. baltica* and *S. putrefaciens* strains displayed diversity in plaque and structural morphology, and 6 phages (3 infecting *S. baltica* and 3 infecting *S. putrefaciens*) belong to Myoviridae and 4 phages (1 infecting *S. baltica* and 3 infecting *S. putrefaciens*) belong to Siphoviridae. These results showed morphological diversity of *S. putrefaciens* and *S. baltica*-infecting phages in the environment.

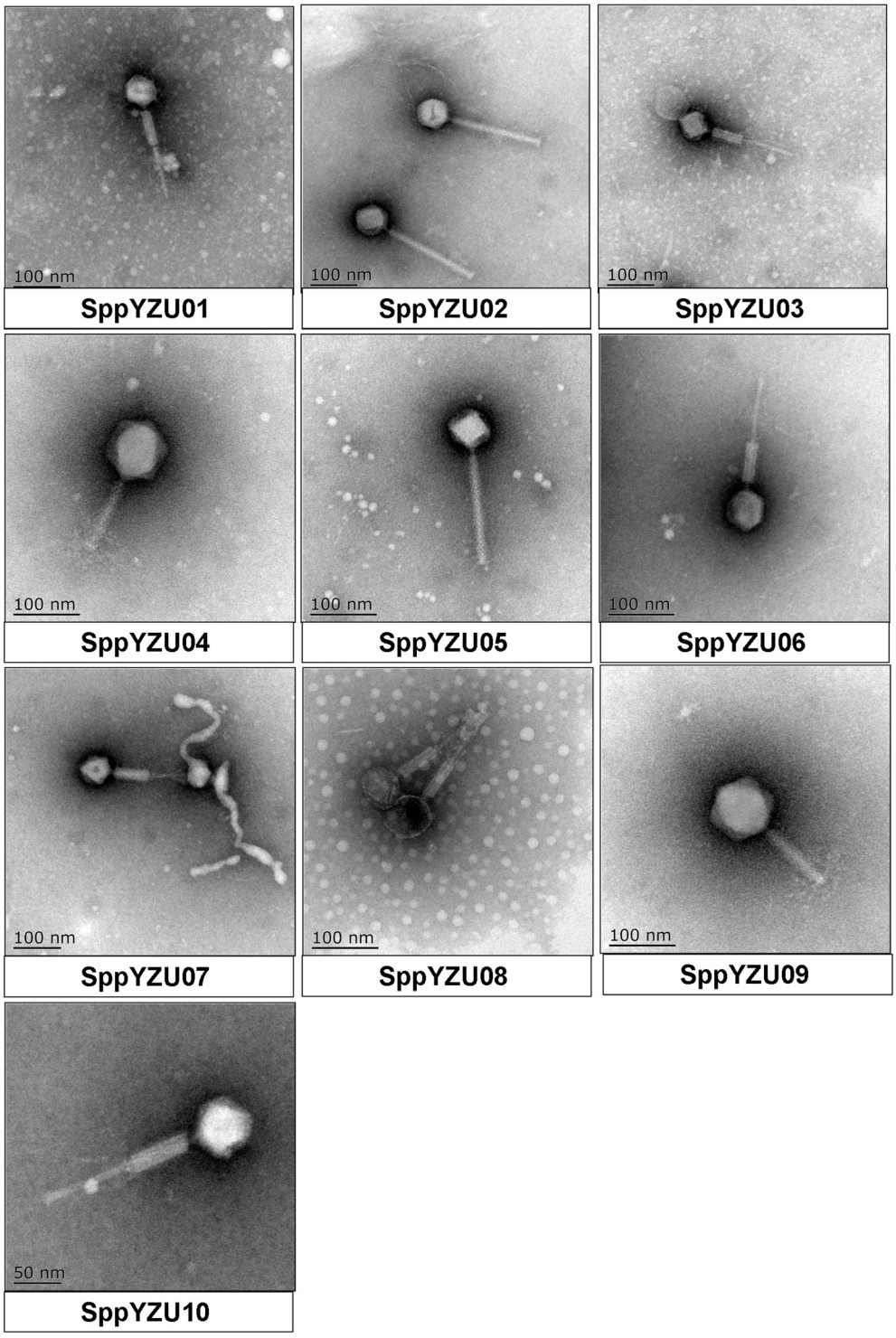


Fig. 1. Electron micrographs of *Shewanella baltica* and *S. putrefaciens* phages.

3.2. Host range of phages

Ten phages were screened by plaque testing on 10 strains of *Shewanella* strains (Table 2). Two of them (SppYZU03 and SppYZU06) were highly specific and only formed clear plaques on their own host strains, while the other 8 phages were capable to lyse multiple strains. SppYZU01 and SppYZU05 had the broadest spectrum of lytic ability. SppYZU01 was capable to lyse all the *S. baltica* strains. SppYZU05 lysed 5 out of 6 *S. putrefaciens* strains and one *S. baltica* strain (SYZU03).

The narrow host specificity of phages limits their application in food industry, because a single phage cannot be effective against all the strains of a particular species (Pereira et al., 2016a). Therefore, screening the broad host spectrum virulent phages is valuable for using phages as antibacterial agents in food biopreservation. In the present study, all host *Shewanella* strains came from different sources such as seawater fish and freshwater fish of different geographical locations, but the lytic activities of phages against the host strains have not been restricted by their sources and isolation locations, as already described by Wolf et al. (2003). SppYZU05 was found to be virulent for both *S. putrefaciens* and *S. baltica*. Cross-species-infecting phages were also

Table 2
Lytic spectra of *Shewanella* phages determined in 10 host strains.

Species	Strain	Lysis by phage [a]									
		SppYZU01	SppYZU03	SppYZU08	SppYZU09	SppYZU02	SppYZU04	SppYZU05	SppYZU06	SppYZU07	SppYZU10
S. baltica	SYZU01	Host	−	+ +	−	−	−	−	−	−	−
S. baltica	SYZU03	+ + +	Host	−	−	−	−	+ +	−	−	−
S. baltica	SYZU08	+ + +	−	Host	+	−	−	−	−	−	−
S. baltica	SYZU09	+ + +	−	−	Host	−	−	−	−	−	−
S. putrefaciens	SYZU02	−	−	−	−	Host	−	+ + +	−	−	−
S. putrefaciens	SYZU04	−	−	−	−	−	Host	+ + +	−	+ +	−
S. putrefaciens	SYZU05	−	−	−	−	+ +	+	Host	−	+ +	+
S. putrefaciens	SYZU06	−	−	−	−	−	−	−	Host	−	−
S. putrefaciens	SYZU07	−	−	−	−	−	−	+ + +	−	Host	+
S. putrefaciens	SYZU10	−	−	−	−	+ +	−	+ + +	−	−	Host
Number of host		4	1	2	2	2	2	6	1	3	3

+ + +, EOP 0.5 to 1.0; + + EOP 0.2 to 0.5; +, EOP 0.001 to 0.2; −, (< 0.001) bacterial strain was not susceptible to phage attack.

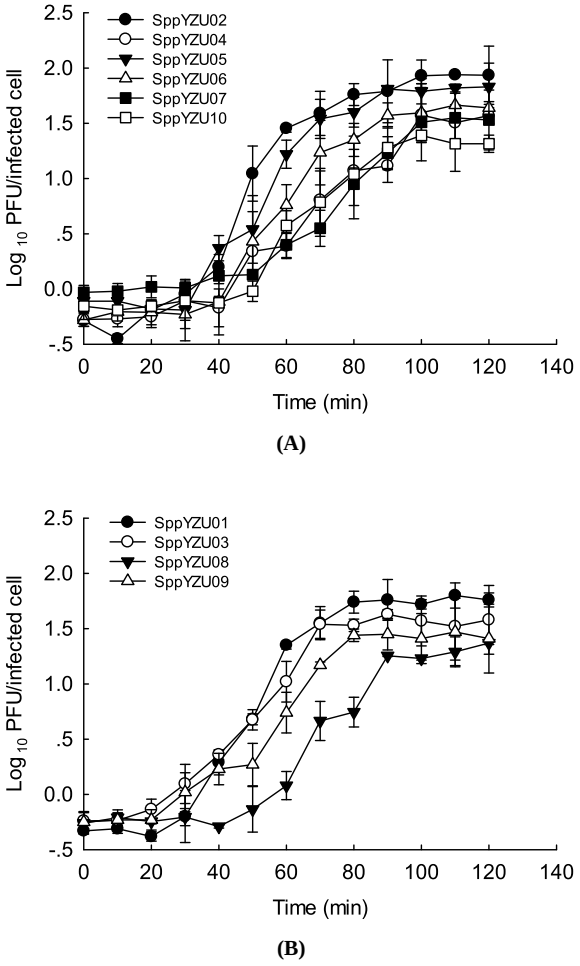


Fig. 2. One step growth curves of *Shewanella putrefaciens* phages (A) and *Shewanella baltica* phages (B). Curves were determined in LB broth at 25 °C at multiplicity of infection (MOI) ratio of 0.1. Values represent one of three independent experiments.

observed in previous studies (Jensen et al., 1998), suggesting that the phage host range was not always species-restricted. Wide host range phages exist in common sources (wastewater and soil) and are readily isolated from aquatic environments (Jensen et al., 1998). None of the phages isolated in the present study lysed all the strains, but the combination of SppYZU01, SppYZU05, and SppYZU06 had the capacity to form cleared zones on all the *Shewanella* strains (data not shown).

Table 3
Characterization of *Shewanella* phages by their lysis parameters estimated from the one step growth curve.[a]

Phages	Latent time (min)	Rise period (min)	Burst size (phages per infective cell)
SppYZU01	22.3 ± 2.1	47.8 ± 2.1	55 ± 3
SppYZU02	12.7 ± 2.1	54.0 ± 2.9	81 ± 16
SppYZU03	27.1 ± 2.4	53.0 ± 2.9	39 ± 5
SppYZU04	42.7 ± 2.5	56.0 ± 0.8	37 ± 4
SppYZU05	33.7 ± 2.6	41.0 ± 2.9	68 ± 5
SppYZU06	35.3 ± 4.7	52.7 ± 2.5	45 ± 6
SppYZU07	53.7 ± 2.4	54.7 ± 4.1	33 ± 9
SppYZU08	45.0 ± 4.1	55.7 ± 3.3	23 ± 3
SppYZU09	28.0 ± 1.6	57.7 ± 2.1	25 ± 5
SppYZU10	42.3 ± 2.1	49.7 ± 2.1	20 ± 1

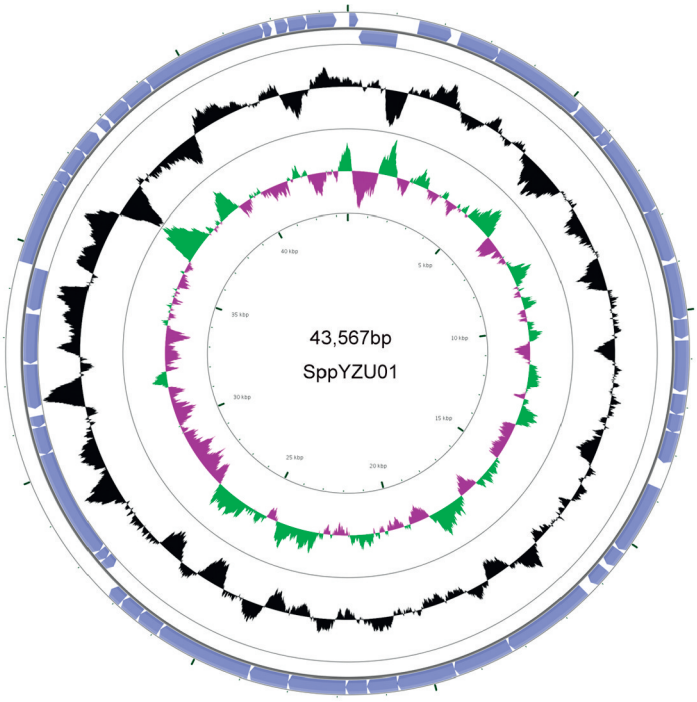
[a] One step growth curve of *Shewanella* phages in their host strain was determined in Luria-Bertani (LB) broth at 25 °C at multiplicity of infection (MOI) ratio of 0.1. Values represent one of three independent experiments.

3.3. One-step growth curve of phages

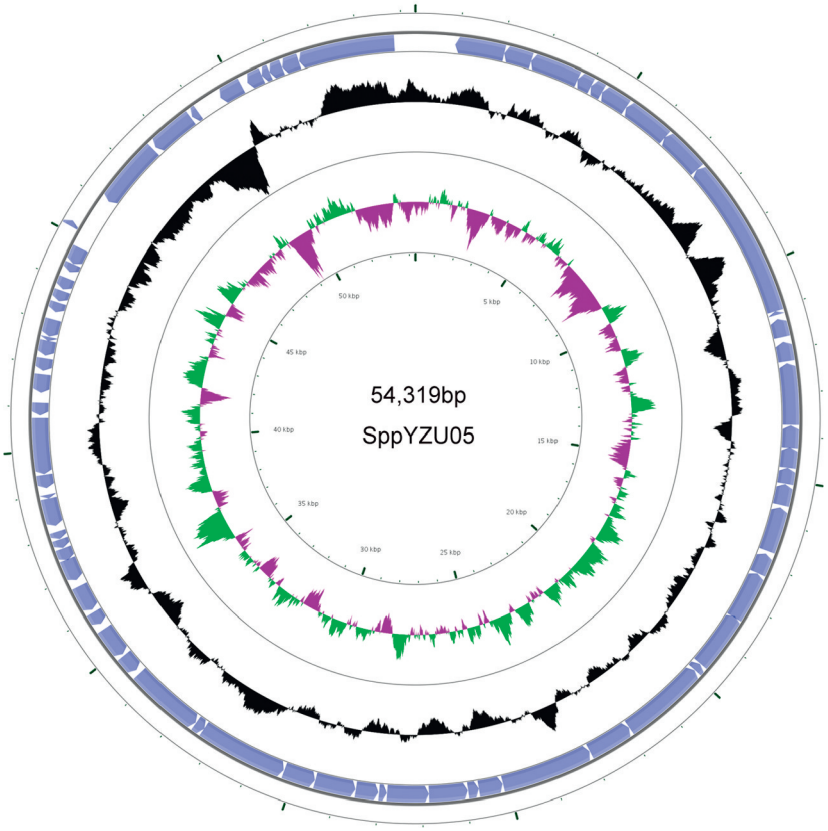
The one-step growth curves of the 6 *S. putrefaciens* phages and 4 *S. baltica* phages are shown in Fig. 2(A and B), and the latent period, the rise period and the burst size are shown in Table 3. These parameters for the 10 *Shewanella* phages ranged from 12.7 to 53.7 min (average 34.3 ± 12.2 min), 41.0 to 57.7 min (average 51.2 ± 5.5 min), and 20 to 81 phages per host cell (average 42.6 ± 20.1 phages per host cell), respectively. Phage burst size and latent periods are important factors to consider when phages are selected. Phages with high burst sizes and short latent periods are more effective in inactivating bacteria (Abedon and Culler, 2007). In the present study, phages SppYZU01, SppYZU02, SppYZU05, and SppYZU06 showed shorter latent time and higher burst size compared to other phages.

3.4. Genomic analysis of the phages

The complete genomes of two wide host range phages (SppYZU01 and SppYZU05) were sequenced, and the general features of the phage genomes are shown in Fig. 3(A and B). The *S. baltica*-infecting phage SppYZU01 genome consisted of 43,567 bp of double-stranded DNA, with the average GC content of 55.72%. The genome annotation analysis predicted 49 ORFs (average size of 834 bp), numbered consecutively from ORF1 to ORF49 (Fig. 3A, and Supplementary data, Table S1), representing 93.84% of the total genome. The genome of SppYZU01 had no similarity with other genomes in GenBank, suggesting that this was a novel virulent phage. Eleven out of the 49 predicted ORFs in SppYZU01 were homologous to genes encoding putative large terminase subunit, portal protein, putative protease, major capsid



(A)



(B)

Fig. 3. The genome of (A) *Shewanella baltica* phage SppYZU01 and (B) *S. putrefaciens* phage SppYZU05. Circles display (from the outside): (1) open reading frames (ORFs) transcribed in the clockwise or the counterclockwise direction. (2) G + C % content. Values > 55.72% (average) in (A) and 50.63% (average) in (B) are represented by outward peaks, and smaller values are represented by inward peaks. (3) GC skew (G − C/G + C in a 1-kb window and 0.1-kb incremental shift). Values greater than zero are in magenta, and smaller values are in green. (4) Physical map scaled in Kbp. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

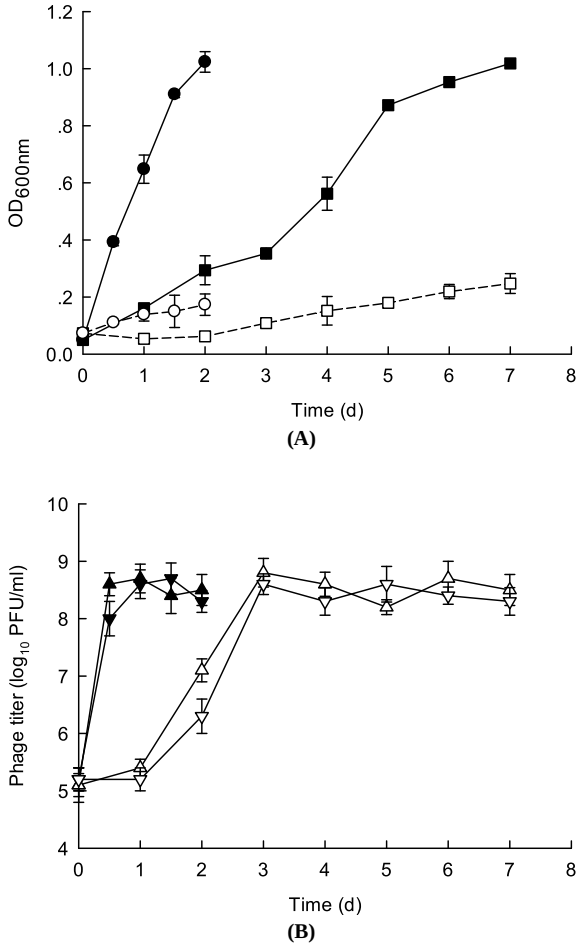


Fig. 4. Effect of phage cocktail SPMIX3-156 on the growth of *Shewanella* strain cocktail in fish juice medium incubated at 25 °C for 2 days and at 4 °C for 7 days. (A) Samples were spiked with 10^4 colony forming unit (CFU)/mL of *Shewanella* cell mixture including 4 *S. baltica* strains and 6 *S. putrefaciens* strains, and inoculated without phages (●, 25 °C; ■, 4 °C) or with 10^5 plaque forming unit (PFU)/mL of SPMIX3-156 (○, 25 °C; □, 4 °C). (B) The change of phage titers during incubation at 25 °C (▲, against *S. baltica*; ▼, against *S. putrefaciens*) and 4 °C (△, against *S. baltica*; ▽, against *S. putrefaciens*). Assays were performed in triplicate. Data reported are the mean ± standard deviation.

protein, tail protein of *Vibrio* phage H188 (ORF05, ORF08, ORF10, ORF12, and ORF24, respectively), putative DNA polymerase and tail proteins of *Vibrio* phage VP16T and VP16C (ORF32, ORF16, and ORF21, respectively), putative helicase of *Edwardsiella* phage MSW-3 (ORF38), and putative TetR transcriptional regulator of *Klebsiella* phage JD001 (ORF43). However, the majority of the predicted ORFs (38 ORFs out of 49) had no homology to known coding sequences, probably because of the insufficient information about the functional genes of phage genomes in GenBank.

The *S. putrefaciens*-infecting phage SppYZU05 genome contained 54,319 bp, with an average GC content of 50.63%, in which 65 ORFs were predicted (average size of 707 bp) (Fig. 3B, and Supplementary data, Table S2). The BLAST search revealed that the genome of SppYZU05 was homologous (88.5% of sequence identity) to the genome of Spp001 (GenBank accession no. KJ002054), a virulent *S. putrefaciens*-infecting phage isolated from Qinggdao city (China). In the SppYZU05 genome, 53 out of 65 ORFs showed homology with ORFs in Spp001, with sequence identity varying from 45.29% to 98.73%, but 38 out of 53 homologous ORFs encoded hypothetical proteins, and only 15 ORFs encoded proteins with known functions. Twelve ORFs in SppYZU05 had no homology with sequences in GenBank.

Analyses of the genome sequences of the phages enabled us to explore their relationships with each other and with their hosts (Zhao et al., 2009). However, to date, little is known about the genome sequences of *S. baltica* phages. The genomic information on *S. putrefaciens* phages is also very limited. Of note, the only *S. putrefaciens* phage genome sequence available in GenBank was reported by Han et al. (2014). To our knowledge, SppYZU01 is the only phage that infects *S.baltica.* As most of the predicted ORFs (38 out of 49) in the SppYZU01 genome had not homology with available sequences in GenBank, this phage can be defined as a novel virulent phage. The genome information on this *S. baltica* phage will provide insights into the molecular characteristics and taxon of *Shewanella* phages.

SppYZU05 is closely related to phage Spp001, which showed effective biocontrol on *S. putrefaciens* growth in fish fillet under chilled conditions in previous studies (Han et al., 2014; Li et al., 2014). Although the genome of SppYZU05 had 88.5% sequence identity with that of Spp001, the ORF sequence homology varied from 45.29% to 98.73%. The collinearity analysis showed that heterologous ORFs are located in the downstream of genomes (Fig. S1). The difference in genome content may reflect the phage evolution in different spoilage ecosystems (Culley et al., 2003; Desplats and Krisch, 2003; Papke and Doolittle, 2013), because the host of SppYZU05 (*S. putrefaciens* strain SYZU05) used in the present study was isolated from freshwater fish (*Ictalurus punctatus*), while the host of Spp001 (*S. putrefaciens* strain Sp225) was isolated from seawater fish (*Paralichthys olivaceus*) (Han et al., 2014). Further analyses and characterization of functional genes in both *Shewanella* phages would help in developing phage preservative agents with high lytic activity and wide host range.

3.5. Inhibition activity of Shewanella phage cocktail in FMEJ

FMEJ was used as a model to determine the effectiveness of phages in the biocontrol of *S. baltica* and *S. putrefaciens.* Fig. 4A shows that the OD_{600} values of FMEJ inoculated with 10^4 CFU/mL of the 10-*Shewanella* strain cocktail increased from 0.05 to 1.02 in 2 days at 25 °C and to 1.01 in 7 days at 4 °C (control group). A three-phage cocktail (SppYZU01 + SppYZU05 + SppYZU06), named SPMIX3-156, was screened as an effective anti-*Shewanella* agent. SPMIX3-156 was able to considerably reduce the OD_{600} of *Shewanella* hosts at MOI of 10 at both 25 °C and 4 °C, and kept the OD values at low level (OD_{600} < 0.2) throughout the storage period. The phage titers against *S. baltica* and *S. putrefaciens* increased approximately 3.5 $\log_{10}$ PFU/mL during the storage experiments at both 25 °C and 4 °C (Fig. 4B). No significant reduction in bacterial OD was observed when the inoculated FMEJ was treated with individual phage (data not shown). Phage cocktails are an effective strategy to broaden the spectrum of phage-based antibacterial agents, if the target population contains multiple strains (Cairns et al., 2009; Pereira et al., 2016b). SPMIX3-156 showed broad lytic activities and effectiveness in inhibiting growth of *S. baltica* and *S. putrefaciens* strain mixture in FMEJ. The other two phage combinations lacking SppYZU01 or SppYZU05, namely, a four-phage cocktail SPMIX-1267 (SppYZU01 + SppYZU02 + SppYZU06 + SppYZU07) and a five-phage cocktail SPMIX-38956 (SppYZU03 + SppYZU08 + SppYZU09 + SppYZU05 + SppYZU06), did not show considerable effectiveness in spite of their lytic spectra covering 10 *Shewanella* strains (data not shown).

3.6. The biopreservative potential of phage cocktail in fish fillets

SPMIX3-156 was tested for its biopreservative potential in sterile fish muscle fillets (FMF) artificially contaminated with 10-*Shewanella* strain cocktail. Fig. 5A shows that SPMIX3-156 inhibits the growth of *Shewanella* strains in FMF samples. In control groups (without treatment), TVCs increased from 3.48 log CFU/g to 9.72 log CFU/g after 2 days of storage at 25 °C and to 9.85 log CFU/g after 16 days of storage at 4 °C. Similar trend of growth was shown by TVC in control and experimental groups although a notorious reduction of 3.21 and 2.75 log units was respectively detected in the latter ones at day 1 (25 °C) and day 10 (4 °C). Fig. 5B shows that the phage concentrations in the

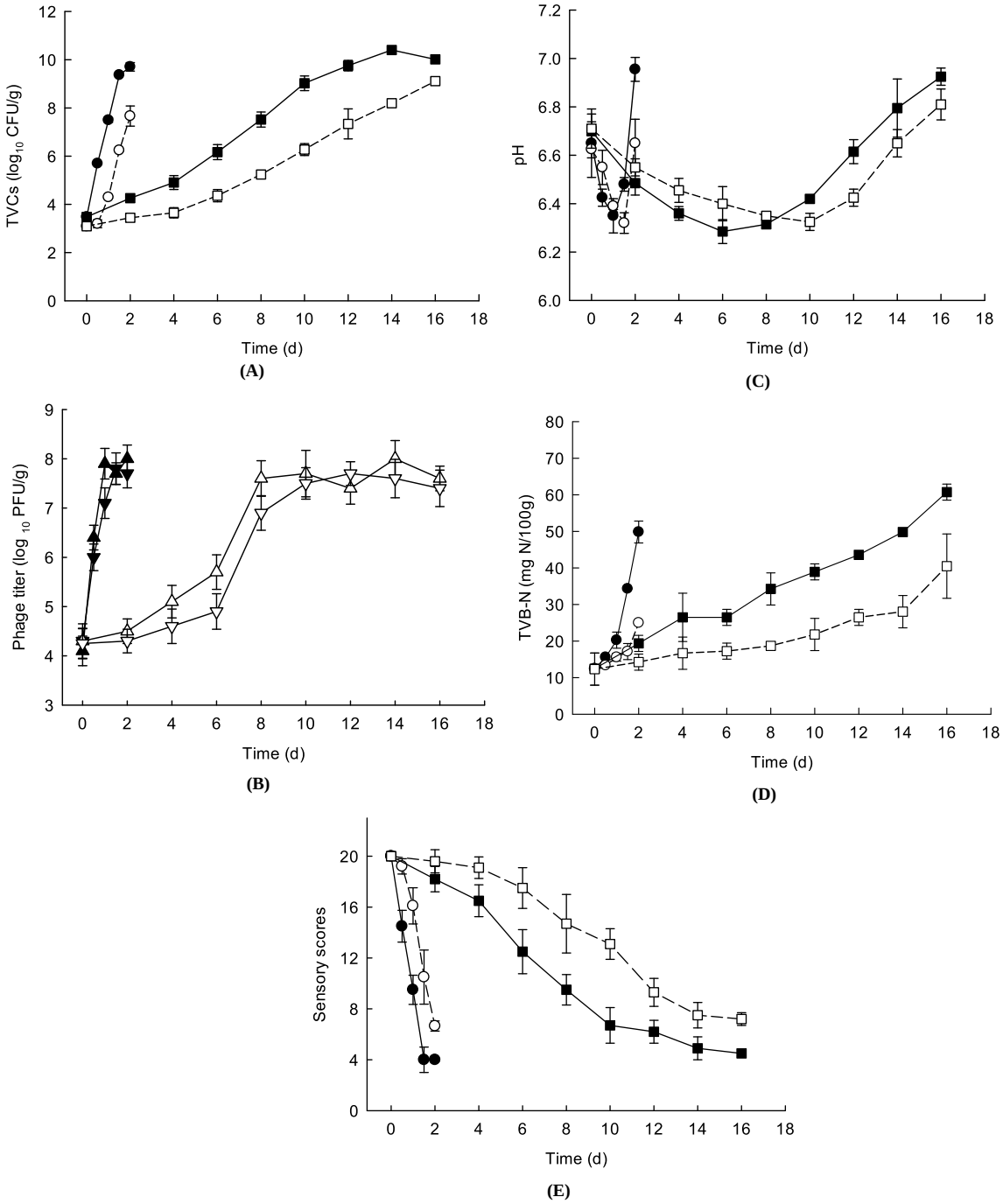


Fig. 5. Changes in total viable count (TVC) (A), phage titers (B), pH (C), total volatile basic nitrogen (TVB-N) (D), and sensory scores (E) of the artificially contaminated fish fillets during storage at 25 °C for 2 days (circles) and at 4 °C for 16 days (squares) in the absence (closed) and presence (open) of phage cocktail SPMIX3-156. The fish fillets were artificially contaminated with *Shewanella* strain cocktail suspension (cell counts of each strain was 10^6 colony forming unit (CFU)/mL). The control groups (●, ■) and experimental groups (○, □) were treated, respectively, with SM buffer and SPMIX3-156. The phage titer changes during storage at 25 °C (▲, against *S. baltica*; ▼, against *S. putrefaciens*) and 4 °C (△, against *S. baltica*; ▽, against *S. putrefaciens*) were determined. Assays were performed in triplicate. Data are expressed as the mean ± standard deviation.

experimental groups increased significantly during the storage at both 25 °C and 4 °C. The phage titers against *S. baltica* and *S. putrefaciens* increased 3.8 $\log_{10}$ PFU/g and 3.0 $\log_{10}$ PFU/g after 1 day of storage at 25 °C, and 3.4 $\log_{10}$ PFU/g and 3.3 $\log_{10}$ PFU/g after 10 days of storage at 4 °C, respectively.

S. putrefaciens has been reported to grow from 3.76 log CFU/g to 9.47 log CFU/g in sterile grass carp fillets during 16 days of storage at 4 °C (Wang et al., 2017). Even though our experiments were performed in channel catfish fillets contaminating with *S. baltica* and *S. putrefaciens* strain cocktail, similar observations were noted at 4 °C, despite the fact that the bacterial population increase during the first 10 days was quicker than that observed in the previous study. This may be due to the differences in inoculated strains, fish matrix, or both. SPMIX3–156 showed some effectiveness in inhibiting growth of *S. putrefaciens* and *S. baltica* in FMF at both 4 °C and 25 °C conditions. It was noted that the biocontrol effectiveness in the FMF is lower than those in FMEJ. The reasons may be that some bacterial cells on the FMF tissue have not been infected by phages during the phage soaking treatment, and these

Table 4
Frequencies of *S. baltica* and *S. putrefaciens* strains spontaneous mutations to phage cocktail SPMIX3-156 resistance.

Strain	Initial number of bacteria (CFU)	Number of BIM colonies (CFU)	Mutation frequency
S. baltica SYZU01	$(2.1 \pm 0.3) \times 10^7$	–	–
S. baltica SYZU03	$(3.9 \pm 0.2) \times 10^7$	–	–
S. baltica SYZU08	$(1.6 \pm 0.3) \times 10^7$	8.7 ± 1.5	$(5.4 \pm 1.0) \times 10^{-7}$
S. baltica SYZU09	$(7.5 \pm 0.2) \times 10^6$	4.0 ± 3.6	$(5.3 \pm 5.0) \times 10^{-7}$
S. putrefaciens SYZU02	$(3.7 \pm 0.4) \times 10^7$	7.3 ± 2.9	$(2.0 \pm 0.8) \times 10^{-7}$
S. putrefaciens SYZU04	$(2.5 \pm 0.3) \times 10^7$	11.3 ± 4.9	$(4.5 \pm 2.0) \times 10^{-7}$
S. putrefaciens SYZU05	$(3.7 \pm 0.4) \times 10^7$	–	–
S. putrefaciens SYZU06	$(4.6 \pm 0.2) \times 10^7$	6.7 ± 2.5	$(1.4 \pm 0.5) \times 10^{-7}$
S. putrefaciens SYZU07	$(2.1 \pm 0.3) \times 10^7$	18.3 ± 3.1	$(8.7 \pm 1.5) \times 10^{-7}$
S. putrefaciens SYZU10	$(7.5 \pm 1.1) \times 10^6$	21.3 ± 5.7	$(2.8 \pm 0.8) \times 10^{-6}$

Values represent mean value of three experiments; –, no BIM colonies detected.

bacterial cells grow in the subsequent storage. To increase the phage concentration or optimize soaking conditions (such as soaking time, frequency, temperature or composition of solution) for phages uniformly adsorbing to bacterial cells on the FMF will result in a higher effectiveness in the further application.

On the other hand, the quality indices (pH, sensory value, and TVB-N) for the SPMIX3–156-treated samples considerably improved compared to those in the control samples at both temperatures. Fig. 5C shows the pH changes of FMF during storage at 25 °C and 4 °C. During the storage, the initial pH (6.6–6.8) of FMF gradually decreased to 6.3–6.4 in both control and treated groups, and increased until the final storage time. The pH decrease during the initial storage time may be related to the accumulation of lactic acid, while the increase during the later storage stage may be due to the decomposition of amino compounds caused by the growth of spoilage bacteria (Gui et al., 2013). For the control groups (untreated), the pH increase occurred after 24 h at 25 °C and 6 days at 4 °C, while the pH increase in the experimental groups (treated with 10^7 PFU/mL of SPMIX3-156) occurred after 36 h at 25 °C and 10 days at 4 °C. At the final storage time at 25 °C and 4 °C, the FMF pH in the experimental groups were significantly lower than those in the control group ($p < 0.01$).

TVB-N results from degradation of proteins and non-protein nitrogenous compounds caused by microbial activity (Ruiz-Capillas and Moral, 2005). Fig. 5D shows the change of TVB-N contents in FMF during the storage at 25 °C and 4 °C. In the control group, TVB-N increased from an initial value of 12 mg/100 g to 51 mg/100 g at 25 °C and 63 mg/100 g at 4 °C at the final storage time. In contrast, after being treated with 10^6 PFU/mL of SPMIX3–156, TVB-N values in FMF gradually increased to 22 mg/100 g and 38 mg/100 g at the final storage time at 25 °C and 4 °C, respectively, and the TVB-N increase was significantly lower than that in control group ($p < 0.01$). This may be attributed to the fact that the growth of *Shewanella* strains in FMF samples was inhibited by SPMIX3-156.

Sensory evaluation results (Fig. 5E) showed that the sensory scores declined differently among groups. For control samples at 25 °C, an increasing off-odor was detected at 12 h (score of 14.5), and the fillets were totally spoiled after 36 h of storage (score of 4). For control samples at 4 °C, the fillets deteriorated more slowly, and the deterioration degree was unacceptable after 6 days of storage (score of 12.5). SPMIX3-156-treated fillets obtained higher scores than those of the control samples and were considered unacceptable after 36 h at 25 °C (score of 10.5) and after 10 days of storage at 4 °C (score of 13.1). Organoleptic quality deterioration is closely associated with microbial spoilage caused by the release of metabolites and changes of surface color of samples (Hui et al., 2016). Overall, our results support that the SPMIX3-156 treatments delayed the fillet organoleptic deterioration associated with *Shewanella* spoilage at both 25 °C and 4 °C.

3.7. Mutation frequencies of BIM to SPMIX3-156

The 4 *S. baltica* and 6 *S. putrefaciens* strains showed different mutation frequencies (varing from no detection to 2.8×10^{-6}) to the phage cocktail SPMIX3-156 (Table 4). No BIM colonies were detected for two strains of *S. baltica* (SYZU01 and SYZU03) and a strain of *S. putrefaciens* (SYZU05). BIMs of the other strains after treatment with SPMIX3-156 emerged at frequencies of 1.4×10^{-7} to 2.8×10^{-6}. *S. putrefaciens* strain SYZU10 showed the higher mutation frequency (2.8×10^{-6}), which indicated that this strain is more likely to format BIM. All BIM colonies showed smaller colony size and slower growth rate than those of the non-phage infecting bacteria (data not shown).

4. Conclusion

Shewanella spp., mainly *S. baltica* and *S. putrefaciens*, are the predominant psychrotrophic bacteria responsible for the spoilage of both fresh and packed fish. Phages may be used as an alternative to chemical antimicrobials against these spoilage bacteria. In this study, the isolation, characterization, genomic analysis, and biopreservative effect testing of virulent phages with high lytic activities against *S. baltica* and *S. putrefaciens* strains were performed. The phage cocktail SPMIX3–156 showed potential as antimicrobial agent to control the growth of *S. baltica* and *S. putrefaciens* strains in fish matrices at different temperatures. SPMIX3-156 is a promising biocontrol agent to reduce the *Shewanella*-associated fish deterioration at ambient or refrigerated conditions. The effectiveness of phages to reduce *Shewanella* load in fish processing equipment and environment needs to be further explored.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2018.12.020.

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