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The novel *Shewanella putrefaciens*-infecting bacteriophage Spp001: genome sequence and lytic enzymes

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Abstract *Shewanella putrefaciens* has been identified as a specific spoilage organism commonly found in chilled fresh fish, which contributes to the spoilage of fish products. Limiting *S. putrefaciens* growth can extend the shelf-life of chilled fish. Endolysins, which are lytic enzymes produced by bacteriophages, have been considered an alternative to control bacterial growth, and have been useful in various applications, including food preservation. We report here, for the first time, the complete genome sequence of a novel phage Spp001, which lyses *S. putrefaciens* Sp225. The Spp001 genome comprises a 54,789-bp DNA molecule with 67 open reading frames and an average total G + C content of 49.42 %. In silico analysis revealed that the Spp001 open reading frames encode various putative functional proteins, including an endolysin (ORF 62); however, no sequence for genes encoding the holin polypeptides, which work in concert with endolysins, was identified. To examine further the lytic activity of Spp001, we analyzed the lytic enzyme-containing fraction from phages released at the end of the phage lytic cycle in *S. putrefaciens*, using diffusion and turbidimetric assays. The results show that the partially purified extract contained endolysin, as indicated by a high hydrolytic activity towards bacterial peptidoglycan decrease in the OD_{590} value by 0.160 in 15 min. The results will allow further investigation of the purification of natural Spp001 endolysin, the extension of Spp001 host range, and the applications of the phage-encoded enzymes.

Keywords *Shewanella putrefaciens* · Endolysin · Complete genome sequence · Bacteriophage

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Introduction

Bacterial contamination is the major cause of spoilage in most fish products. However, only some microbes—the specific spoilage organisms (SSOs)—participate in the spoilage process. A gram-negative bacterium, *Shewanella putrefaciens*, is an SSO found in seafood products, especially in ice-chilled marine fish [16]. *S. putrefaciens* alters the nutrient profile of fish and fish products by consuming trimethylamine oxide (TMAO), sulfate, nitrate, manganese, and iron [13]. The amount of *S. putrefaciens* in fish inversely correlates with the shelf-life of fish. For example, at the end of the recommended storage period, *S. putrefaciens* accounts for about 58.4 % of all bacteria in *Paralichthys olivaceus* stored at 4 °C [41]. Similarly, in *Pseudosciaena crocea*, *S. putrefaciens* was the dominating SSO, constituting 75.5 and 59.6 % of all SSOs at the end of the recommended storage period, with storage at 0 and 5 °C, respectively [32]. Furthermore, in some cases, *S. putrefaciens* infection was observed in humans [1, 4, 9, 21, 30], indicating that there is a need for an effective method to inhibit *S. putrefaciens* growth in fish.

Endolysins are lytic enzymes produced by bacteriophages that hydrolyze cell wall peptidoglycans and induce bacterial host cell lysis at the end of the lytic cycle, resulting in the dispersal of newly formed bacteriophages [12, 24]. The use of endolysins in the prevention of bacterial infection has advantages over antibiotics and chemical

bactericidal agents because of the high specificity of endolysins for host bacteria, minimal effects on beneficial microflora [8], and low chances of development of bacterial resistance [12, 20, 42]. Endolysins exhibit a wider antimicrobial spectrum compared to their bacteriophage hosts [23], and their high activity enables rapid bacterial cell lysis, within minutes or even seconds [34].

The use of endolysins as antibacterial agents has attracted interest in the past two decades [8, 15]. Most of the recent studies have revealed the ability of lysins to prevent pathogenic bacteria such as *Streptococci, Staphylococcus aureus* and MRSA, *Enterococci*, *Bacillus anthracis* in pre-clinical trials [10, 31, 39, 42]. The use of endolysins for the bio-control of pathogens in food and animal feeds involves the direct addition of the purified enzyme to food or to the raw product [5, 8, 25], for example, against *streptococci* and *staphylococci* in milk [6]; to control *C. perfringens* in raw poultry products [44]; or to inhibit *L. monocytogenes* contamination during the ripening of soft cheese [14]. Although the use of such applications is increasing, endolysins with gram-negative background have not been exploited [38]. The SSO-targeting endolysin from the *S. putrefaciens* phage has not been reported previously.

In our previous work, the *S. putrefaciens* strain Sp225 isolated from chilled fish was identified as the SSO for *Paralichthys olivaceus*. We used this strain as a host to isolate a bacteriophage, Spp001, which was strictly lytic and, therefore, invariably lethal to bacterial cells once infection was established, and could clarify a suspension of *S. putrefaciens* in 2–3 h [27]. Availability of the complete genome sequence of this phage would be considerably useful in advancing the practical use of its endolysin(s), but until now, the genome of the *S. putrefaciens* Spp001 phage has not been sequenced. In this study, we aimed to sequence the Spp001 genome fully and to provide a preliminary bioinformatics analysis, mostly focusing on the Spp001 endolysin gene(s). In addition, the enzymatic activity of the Spp001 endolysin in the crude lysates was assessed.

Materials and methods

Bacterial strain and bacteriophage used, and culture conditions

S. putrefaciens Sp225 (CCTCC AB2010225) was isolated and identified as the SSO for *Paralichthys olivaceus* stored at 4 °C. The strain stored at −80 °C was thawed and cultured in nutrient broth (NB) (Beijing Land Bridge Technology Co. Ltd. Beijing, China) at 25 °C overnight with continuous shaking (150 rpm).

S. putrefaciens bacteriophage 001 (Spp001; CCTCC M2011142) was isolated from sewage sampled from Nanshan market (Qingdao, China). Spp001 was propagated in inorganic salt broth (0.12 g NaCl, 3.56 g K_2HPO_4, 2.02 g KH_2PO_4, 0.075 g $MgSO_4{\cdot}7H_2O$, 0.006 g $CaCl_2$, 5 g $(NH_4)_2SO_4$, 2 g sodium citrate, pH 7.0–7.5, in 1 L) at 25 °C for 5 h, which added the culture of *S. putrefaciens*. The resultant lysate was filtered through 0.22-μm syringe filters and stored at 4 °C until use.

DNA extraction and sequencing, and genomic analysis

Spp001 was propagated as described above. A high-titer Spp001 lysate (about 1×10^{10} pfu mL^{-1}) was used for DNA extraction. The lysate was centrifuged at 20,000×*g* for 2 h to remove the residual debris, and 24.5 μL of 1 M $CH_3COONa{\cdot}3H_2O$, pH 5.0, and 12.3 μL of 100 mM $MgSO_4{\cdot}7H_2O$ were added to 200 μL of the supernatant. The mixture was incubated with 10 μL of 15 U/ml DNase at 37 °C for 30 min, followed by 5 μL of 25 mg/ml RNase at 37 °C for 15 min to remove bacterial genomic DNA and RNA, respectively. After that, 2 μL of 150 mM EDTA was added to the phage suspension, and the resultant solution was incubated at 65 °C for 15 min. The phage DNA was extracted directly from the crude lysate using a UNIQ-10 plant Genomic DNA Preps Kit (Sangon Biotech Co., Shanghai, China), and sequenced by the shotgun full-sequencing strategy using a 454 Genome Sequencer FLX titanium sequencer (Shanghai Personal Biotechnology Co., Ltd, Shanghai, China).

Sequence similarity searches were performed against a non-redundant database using alignment search tools (BLASTP, Psi-BLAST, BLASTX, BLASTN) available at the National Center for Biotechnology Information (NCBI) web site (http://www.ncbi.nlm.nih.gov/) [29, 40]. Screening of putative Spp001 proteins was performed based on the local alignment of each Spp001 ORF product to the amino acid sequences of the proteins in NCBI database. The InterProScan program (http://www.ebi.ac.uk/Tools/pfa/iprscan/) was used to predict putative conserved domains. Computed molecular weights and the isoelectric points (pIs) of putative proteins were predicted using proteomics tools from ExPASy Bioinformatics Resource Portal (http://www.expasy.org/proteomics) [18]. Signal peptides and transmembrane domains were identified using the SignalP 3.0 Server (http://www.cbs.dtu.dk/services/SignalP/) and the hidden Markov model (TMHMM2) (http://www.cbs.dtu.dk/services/TMHMM/), respectively [18]. Enzyme classes were predicted using the online ArchaeaFun 1.0 Server (http://www.cbs.dtu.dk/services/ArchaeaFun/). The complete genome sequence of virulent *S. putrefaciens* Spp001 phage was deposited in GenBank (Spp001 KJ002054).

Phage growth curve

The phage growth curve was constructed by using a modified one-step growth assay [26]. For this, 5 mL of the *S. putrefaciens* Sp225 culture in NB grown to an optical density of 1.0 at 590 nm (OD_{590}) was added to 300 mL of inorganic salt broth and incubated at 25 °C on a rotary shaker (150 rpm) for 3 h. The culture was inoculated with 10 mL of a Spp001 phage solution (1×10^9 pfu mL^{-1}). After a 10-min adsorption, the phage-bacterial suspension was incubated again at 25 °C on a rotary shaker (150 rpm). Aliquots were sampled at the indicated intervals until the complete lysis of bacterial cells (6 h), and Spp001 titers were immediately evaluated using the double-layer agar plate method [19].

Extraction and partial purification of lytic enzymes

The crude extraction of lytic enzymes from the *S. putrefaciens* bacteriophage was performed as described above, with some modifications. Twenty milliliters of *S. putrefaciens* culture grown to 1.0 OD_{590} in NB was added to 1,200 mL of inorganic salt broth and incubated at 25 °C on a rotary shaker (150 rpm) for 3 h. The culture was inoculated with 40 mL of *S*pp solution (1×10^9 pfu mL^{-1}), and after 10 min at room temperature, the phage-host suspension was cultured for an additional 15 min at the same conditions to allow Spp infection of the *S. putrefaciens* cells. The phage-infected *S. putrefaciens* cells were harvested by centrifugation at 3,300×*g* for 15 min at 4 °C, and re-suspended in 5 mL of inorganic salt broth. The suspension was incubated in the above conditions for 5 h until complete cell lysis and release of lytic enzymes. Cell debris and phages were removed by centrifugation at 6,010*g* for 30 min, and ammonium sulfate (up to 40 %) was added to the supernatant and gently mixed for 4 h at 4 °C. The unwanted precipitated proteins were removed by centrifugation at 8,000×*g* for 30 min, and ammonium sulfate was again added to the enzyme-containing supernatant at a final concentration of 50 %. After 4 h at 4 °C, the precipitate containing endolysin was pelleted by centrifugation at 8,000×*g* for 30 min, re-suspended in 1 mL of buffer A (20 mM Tris–HCl, pH 7.0), and dialyzed against 5 L of the same buffer at 4 °C for 24 h. The extract containing partially purified lytic enzymes was stored at 4 °C until further use [11, 28].

Detection of lytic activity

Qualitative diffusion assay

The diffusion method was used to assess qualitatively crude enzyme activity by zymogram analysis, with some modifications [17, 20, 35, 36]. The crude extract of *S. putrefaciens* cell wall peptidoglycan was prepared as described by Clarke [7]. The separating gels were prepared using a 30 % acrylamide:bis-acrylamide (29.2:0.8) stock (3 mL), 0.4 M Bis–Tris buffer pH 6.8, 0.1 % (w/v) SDS, 20 μM riboflavin (3 mL), 2 M Bis–Tris buffer containing 0.25 % (w/v) bacterial cell wall peptidoglycan (1 mL), 10 % (w/v) ammonium persulfate (40 μL), and N,N,N′,N′-Tetramethylethylenediamine (TEMED) (5 μL). Holes were made in the polymerized gel using a borer, and crude enzymes were added to the holes. Buffer A and phage inoculum (1×10^9 pfu mL^{-1}) were used as negative controls, and egg white-derived lysozyme (1 mg mL^{-1}) (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) served as the positive control. Substrate-containing gels were placed in a closed box and incubated at 25 °C for 16 h. The gels were then stained with 0.01 % (w/v) KOH in 0.1 % (w/v) methylene blue for 2 h, followed by destaining with deionized water until translucent circles were observed.

Quantitative turbidimetric assay

The lytic activity of the crude enzyme preparation against *S. putrefaciens* was assayed by monitoring the decrease in OD_{590} [23–28]. Briefly, *S. putrefaciens* Sp225 was cultured overnight until the OD_{590} reached 0.8. Cells were centrifuged at 3,300×*g* for 15 min, washed twice with SM buffer (5.8 g NaCl, 2 g $MgSO_4 \cdot 7H_2O$, and 50 mL of 1 M Tris–HCl, pH 7.5, in 1 L), and re-suspended in the same buffer to adjust the OD_{590} to 1.5. The reaction mixture consisted of 100 μL of the crude enzyme extract and 100 μL of the substrate cells. The lysis of host cells was monitored in 96-well plates by measuring a decrease in OD_{590}. The amount of crude lytic enzyme extract required for a reduction in the OD_{590} value by 0.01 in 15 min at 25 °C was considered as 1 lytic unit.

Protein concentration assay

The Coomassie Brilliant Blue G-250 assay developed by Bradford [2] was used to determine the protein concentration of the crude enzyme extracts.

Results and discussion

Genomic analysis of the Spp001 bacteriophage

The entire genomic sequence of the *S. putrefaciens* Spp001 phage was determined and deposited into the NCBI GenBank database under accession number Spp001 KJ002054. The Spp001 genome comprises a single 54,789-bp, double-stranded DNA molecule with a total G + C content

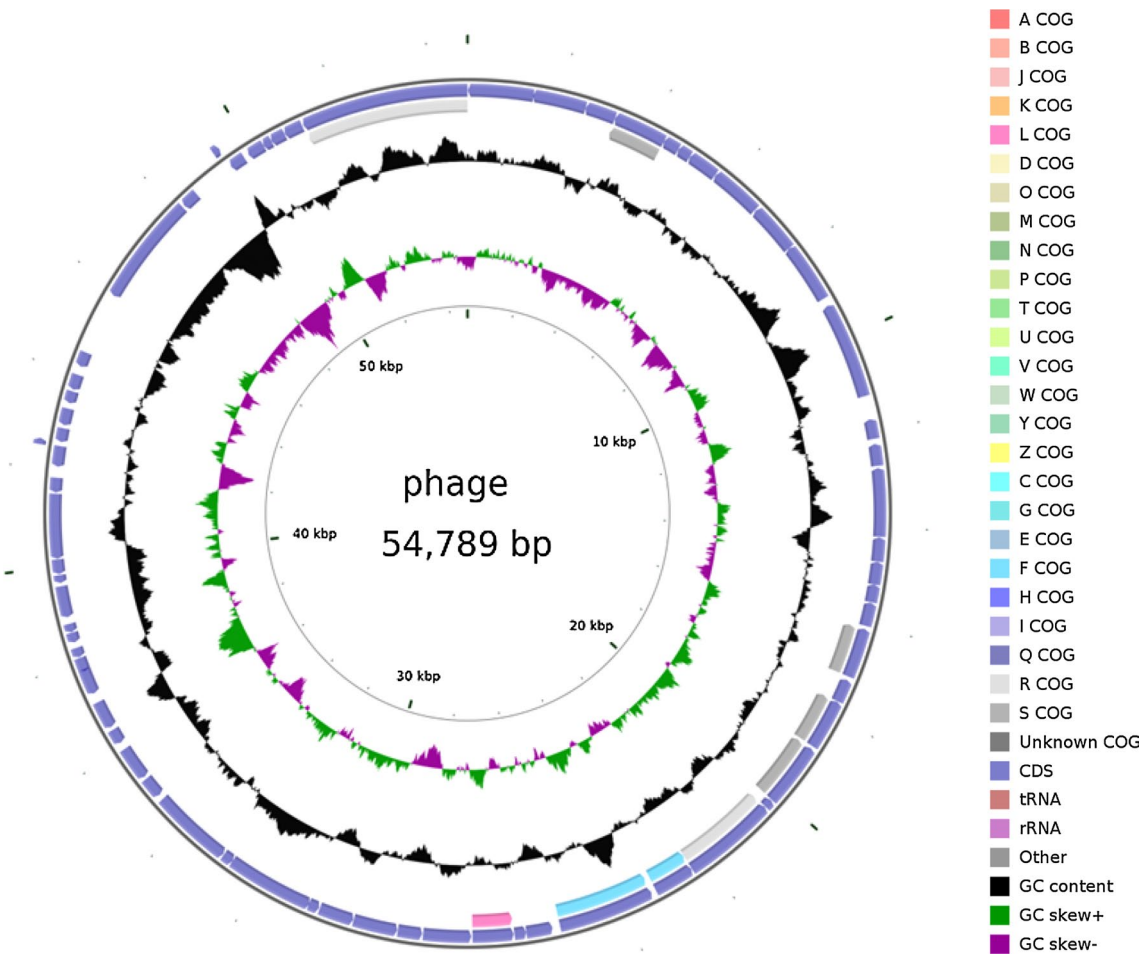

Fig. 1 A graphical representation of the phage Spp001 genome. *Circles* show (from the outside to the inside): (1) ORFs transcribed in the clockwise or counterclockwise direction. ORFs encoding different proteins are in different colors. *A* RNA processing and modification; *B* Chromatin structure and dynamics; *C* Energy production and conversion; *D* Cell cycle control, cell division, chromosome partitioning; *E* Amino acid transport and metabolism; *F* Nucleotide transport and metabolism; *G* Carbohydrate transport and metabolism; *H* Coenzyme transport and metabolism; *I* Lipid transport and metabolism; *J* Translation, ribosomal structure and biogenesis; *K* Transcription; *L* Replication, recombination and repair; *M* Cell wall/membrane/envelope biogenesis; *N* Cell motility; *O* Posttranslational modification, protein turnover, chaperones; *P* Inorganic ion transport and metabolism; *Q* Secondary metabolites biosynthesis, transport and catabolism; *R* General function prediction only; *S* Function unknown; *T* Signal transduction mechanisms; *U* Intracellular trafficking, secretion, and vesicular transport; *V* Defense mechanisms; *W* Extracellular structures; *Y* Nuclear structure; *Z* Cytoskeleton. (2) G + C % content (in a 1-kb window and a 0.1-kb incremental shift). Values greater than 49.42 % (average) are towards the outside, while values lower than 49.42 % are towards the inside. (3) GC skew (G − C/G + C, in a 1-kb window and a 0.1-kb incremental shift). Values greater than zero are in *green*, while those lower than zero are in *purple*. (4) Physical map scaled in kb

of 49.42 % (Fig. 1). Highly redundant sequences covered 3,573 bp, and a single sequence read had an average length of 739 bp. A total of 67 open reading frames (ORFs) predicted to encode proteins ranging from 5 kDa (ORF 61) to 125 kDa (ORF 67) were identified (Table 2). However, no rRNA- or tRNA-encoding genes were predicted by RNAmmer 1.2 or tRNA scan-SE (1.3.1) software.

Using a standard Psi-BLAST protocol, 38 of the 67 ORFs showed homology with previously characterized proteins against a non-redundant database, whereas the other ORFs represented new entries in the database. Putative proteins encoded by 25 ORFs had a conserved domain, while only 13 ORFs showed sequence similarity to the functionally characterized genes in the database (Table 1). Thus, this was a novel virulent phage, with no analogs in GenBank.

Early genes

ORFs identified in the Spp001 genome were predicted to code for essential enzymes associated with nucleotide metabolism, such as ribonucleoside-diphosphate reductase beta chain (ORF 25), ribonucleotide-diphosphate reductase subunit alpha (ORF 26), and 5′–3′ exonuclease (ORF 32). Most of the lytic phages encode their own

Table 1 Complete genome sequence for bacteriophage Spp001

ORF number	Start	End	Length	Molecular mass	pi	Gene name	Best match (identities %)	Best E value
01	7	1,401	464	50,068.42	8.73	Phage protein	Phage protein (33) [*Enterobacteria* phage phiEcoM-GJ1]	4E-67
02	1,389	2,531	380	41,010.52	9.08	Phage protein	Phage protein (25) [*Enterobacteria* phage phiEcoM-GJ1]	3E-11
03	2,531	3,187	218	24,393.15	6.43	pf11041 family protein	Protein of unknown function (DUF2612); pfam1104 (30) [*Erwinia* phage vB_EamM-Y2]	2E-23
04	3,187	4,329	380	41,583.26	5.41	Bacteriophage protein	Hypothetical protein (35) [*Erwinia* phage vB_EamM-Y2]	1.00E-63
05	4,326	4,673	115	12,839.98	7.76	Phage protein	gp81 (41) [*Erwinia* phage vB_EamM-Y2]	6.00E-19
06	4,673	4,960	95	9,452.63	5.83	Paar protein	Alanine racemase (33) [*Pantoea* sp. aB]	2.00E-08
07	4,962	5,615	217	23,674.99	8.32	Bacteriophage protein	Putative baseplate assembly protein (34) [*Enterobacteria* phage phiEcoM-GJ1]	9.00E-18
08	5,621	6,724	367	41,196.78	5.25	Phage protein	Hypothetical protein (36) [*Enterobacteria* phage phiEcoM-GJ1]	4.00E-10
09	6,741	7,793	350	38,768.87	5.26	Hypothetical phage protein	Hypothetical protein (24) [*Enterobacteria* phage phiEcoM-GJ1]	2.00E-08
10	7,793	9,064	423	45,266.44	5.05	Hypothetical phage protein		
11	9,229	11,253	674	74,041.78	5.73	Hypothetical Phage protein		
12	11,741	12,154	137	15,549.53	4.73	Hypothetical phage protein	Hypothetical protein (48) [*Aeromonas* phage PAS-1]	2.00E-39
13	12,260	12,715	151	16,373.70	5.3	Phage-related protein	Hypothetical protein (40) [*Erwinia* phage vB_EamM-Y2]	2.00E-30
14	12,761	14,179	472	50,118.50	5.21	Bacteriophage protein	Hypothetical protein (32) [*Erwinia* phage vB_EamM-Y2]	8.00E-59
15	14,191	14,691	166	19,337.16	6.31	Hypothetical phage protein	Hypothetical protein (33) [*Erwinia* phage vB_EamM-Y2]	1.00E-22
16	14,696	15,169	157	17,084.23	5.3	Hypothetical phage protein	Hypothetical protein (46) [*Enterobacteria* phage phiEcoM-GJ1]	1.00E-27
17	15,169	15,540	123	14,437.49	5.54	Phage protein	Phage protein (37) [*Enterobacteria* phage phiEcoM-GJ1]	2.00E-13
18	15,550	16,041	163	18,203.80	5.85	Hypothetical phage protein		
19	16,103	17,146	347	38,731.76	5.12	Major capsid protein	Major capsid protein (56) [*Enterobacteria* phage phiEcoM-GJ1]	3.00E-121
20	17,219	17,704	161	16,971.20	6.83	Hypothetical phage protein	Hypothetical protein (39) [*Enterobacteria* phage phiEcoM-GJ1]	9.00E-18
21	17,713	18,771	352	38,494.65	4.77	Phage-related protein	gp65 (36) [*Erwinia* phage vB_EamM-Y2]	1.00E-41
22	18,821	20,173	450	50,149.17	7.7	Phage-associated hi1409 family	Putative portal protein (57) [*Enterobacteria* phage phiEcoM-GJ1]	2.00E-167
23	20,185	20,367	60	6,704.67	8.18	Hypothetical phage protein		
24	20,377	22,383	668	75,994.45	5.57	Phage protein	Phage protein (34) [*Sphingomonas* sp.LH128]	6.00E-69
25	22,392	23,264	290	32,518.31	4.82	Ribonucleoside diphosphate reductase beta chain	Ribonucleotide-diphosphate reductase beta subunit (49) [*Pseudoalteromonas* phage pYD6-A]	2.00E-94
26	23,366	25,423	685	76,109.11	8.71	Ribonucleotide-diphosphate reductase subunit alpha	Ribonucleoside triphosphate reductase alpha chain (34) [*Cronobacter* phage VB_CsaM_GAP31]	3.00E-110

Table 1 continued

ORF number	Start	End	Length	Molecular mass	pi	Gene name	Best match (identities %)	Best E value
27	25,586	26,149	187	21,082.24	8.9	gp56	Hypothetical protein (31) [*Erwinia* phage vB_EamM-Y2]	1.00E-11
28	26,151	26,402	83	9,719.90	4.71	Hypothetical phage protein		
29	26,417	27,292	291	33,137.26	9.24	DNA ligase	Hypothetical protein (31) [*Burkholderia* thailandensis]	2.00E-21
30	27,316	28,338	340	37,444.81	6.17	Hypothetical protein EIO_2033	Hypothetical protein (34) [*Ketogulonicigenium vulgare* Y25]	4.00E-10
31	28,357	28,878	173	20,122.76	9.9	Phage protein	Hypothetical protein (43) [*Erwinia* phage vB_EamM-Y2]	2.00E-37
32	28,890	29,837	315	35,962.94	6.01	5–3 exonuclease	5′-3′ exonuclease (38) [*Erwinia* phage vB_EamM-Y2]	2.00E-68
33	29,848	30,591	247	27,029.26	4.6	Hypothetical phage protein	Hypothetical protein (36) [*Erwinia* phage vB_EamM-Y2]	4.00E-35
34	30,609	30,836	75	9,109.60	6.4	DNA polymerase	DNA polymerase (45) [*Erwinia* phage vB_EamM-Y2]	4.00E-16
35	30,827	32,683	618	70,439.02	6.19	DNA polymerase	DNA polymerase (46) [*Enterobacteria* phage phiEcoM-GJ1]	6.00E-167
36	32,680	32,910	76	8,445.01	9.03	Hypothetical phage protein		
37	32,925	34,640	571	64,413.07	6.37	Primase/helicase	DNA helicase/primase (47) [*Erwinia* phage VB_EaMM-Y2]	2.00E-177
38	34,746	35,183	145	15,045.74	4.42	Hypothetical phage protein		
39	35,235	35,948	237	27,725.17	8.57	Hypothetical phage protein	Hypothetical protein (29) [*Enterobacteria* phage phiEcoM-GJ1]	5.00E-16
40	36,101	36,415	104	11,777.07	10.42	Hypothetical phage protein		
41	36,481	37,065	194	20,321.49	5.43	Hypothetical phage protein		
42	37,263	37,808	181	19,926.95	6.41	appr-1-p processing domain-containing protein	Hypothetical protein (45) [*Salmonella* phage FSL SP-058]	3.00E-28
43	37,762	38,055	97	11,001.62	7.88	Hypothetical phage protein		
44	38,072	38,275	67	7,899.09	8.34	Hypothetical phage protein		
45	38,393	38,602	69	7,672.60	9.65	Hypothetical phage protein		
46	38,613	38,792	59	6,727.71	10.19	Hypothetical phage protein		
47	38,932	39,585	217	24,549.80	5.43	Hypothetical phage protein		
48	39,651	39,818	55	6,091.87	5.87	Hypothetical phage protein		
49	39,839	40,144	101	11,703.45	5.71	Hypothetical phage protein		
50	40,157	41,497	446	50,251.56	9.36	Hypothetical phage protein		
51	41,523	41,828	101	11,085.55	4.89	Hypothetical phage protein		
52	42,460	42,585	41	4,547.29	5.71	Hypothetical phage protein		
53	42,057	42,485	142	16,400.77	9.12	gtp-binding protein	Hypothetical protein (43) [*Synechococcus* sp. PCC 6312]	2.00E-26
54	42,569	42,859	96	10,972.60	4.67	Hypothetical phage protein		
55	42,923	43,276	117	13,156.00	6.73	Hypothetical Phage protein		
56	43,390	43,644	84	9,474.03	9.51	Hypothetical phage protein		
57	43,682	44,002	106	12,493.68	10.31	Hypothetical phage protein		
58	44,181	44,492	103	11,374.78	4.03	Hypothetical phage protein		
59	45,799	48,144	781	87,680.69	5.78	RNA polymerase	RNA polymerase, partial (67) [*Aeromonas* phage PAS-1]	1.00E-171
60	48,185	48,544	119	13,188.02	4.39	Hypothetical phage protein		
61	49,322	49,474	50	5,761.51	5.05	Hypothetical phage protein		
62	49,465	49,800	111	12,201.08	9.1	Cell wall hydrolase	Hypothetical protein (41) [*Vibrio* phage eugene 12A10]	3.00E-08

Table 1 continued

ORF number	Start	End	Length	Molecular mass	pi	Gene name	Best match (identities %)	Best E value
63	49,907	50,275	122	14,317.43	9.5	Peptidase m15a	Peptidase M15A (40) [*Vibrio* sp. EJY3]	2.00E-27
64	50,275	50,466	63	6,824.17	8.04	Hypothetical phage protein		
65	50,467	50,781	104	11,760.35	5	Hypothetical phage protein		
66	50,787	51,191	134	14,704.80	6.06	Hypothetical phage protein		
67	51,216	54,788	1,190	125,108.46	9.16	Phage tail fiber repeat family protein	Phage tail fiber repeat protein (32) [*Escherichia coli*]	4.00E-28

replication-related enzymes. The putative helicase gene (ORF 37) was homologous to that of the *Enterobacteria* phage phiEcoM-GJ1, with an amino acid (AA) identity of 47 %. ORF 37 was found to encode a conserved domain which related to helicase and primase, suggesting that this ORF partially encodes this enzyme. ORFs 34 and 35 were found to be related to DNA polymerase. ORF 35 encoded a putative protein that showed homology to that encoded by an *Enterobacteria* phage phiEcoM-GJ1 genome sequence, with up to 46 % AA identity. The Spp001 phage DNA ligase (ORF 29) was found to be similar to that of *Pseudomonas* phage Bf7 (27 % AA identity). ORF 53 was related to a GTP-binding protein, and ORF 59-encoded protein was found to be similar to the RNA polymerase of *Aeromonas* phage PAS-1 genome (67 % AA identity).

Late genes

As a conserved protease, terminase is the main component of bacteriophage packaging system [22]. The ORF 24-encoded polypeptide showed sequence similarity to the large subunit of *Erwinia* phage vB_EamM-Y2 terminase (57 % AA identity), whereas that encoded by ORF 22 was homologous to the portal protein of *Enterobacteria* phage phiEcoM-GJ1 [2, 14].

Putative structural proteins

ORF 19 was found to encode a putative major capsid protein similar to that of the *Enterobacteria* phage phiEcoM-GJ1 (56 % AA identity). Bacteriophages are known to recognize host receptors through other structural proteins such as the phage tail proteins. A putative tail protein gene encoded by ORF 67 showed 32 % AA identity to the phage tail fiber repeat protein of *Escherichia coli* genome.

Endolysins and holin

The group of endolysins includes lysozyme, endopeptidases, amidases, glucosidases, and transglycosylases [5]. Most bacteriophages disrupt the bacterial cell wall through the combinatorial effects of endolysins and phage-encoded polypeptides termed holins [7]. We analyzed all 67 proteins predicted to be encoded by the Spp001 genome for possible similarities to currently known endolysins and holins. The ORF most likely to belong to a gene encoding a putative endolysin was found to be ORF 62. This ORF was found to encode a hydrolase_2 (pfam07486) domain and a SleB (COG3773) domain, which encodes cell wall hydrolases (Fig. 2). Hydrolase_2 is the enzyme implicated in the hydrolysis of the cell wall in *Bacillus subtilis*, and SleB domain-containing cell wall hydrolyses have been shown to be involved in spore germination. The ORF 62-encoded protein was homologous to the cell wall hydrolase of *Bdellovibrio bacteriovorus* HD100 (31 % AA identity) and the SleB hydrolase of *Escherichia* phage bV_EcoS_AKFV33 (40 % AA identity). However, bioinformatics analysis of all 67 ORF-encoded putative proteins of the Spp001 phage revealed no similarity to any known bacteriophage holin. The apparent lack of holin genes has also been reported for other phages such as *Bacillus cereus* phages and *Listeria* phage A511 [23]. Moreover, the ORF 62-encoded protein had transmembrane region(s) and a signal peptide region. The enzyme class prediction analysis by the ArchaeaFun 1.0 Server indicated the possibility that ORF 62 may encode a transferase. Our results suggest that the lytic mechanism of the Spp001 phage is more likely to depend exclusively on endolysin, rather than a combined effect of holins and endolysins.

Extraction of partially purified enzymes

The majority of recent studies on endolysins examined the recombinant protein rather than the natural enzyme extracted from the bacteriophage-host system, because natural extracts contain multiple proteins, are very unstable, and have low extraction yields [9]. However, safety concerns hampered the production of endolysin by genetically engineered organisms [7]. In addition, parameters such as the nature of the expression vector, protein secretion, and folding affect the expression of the recombinant endolysin. Thus, we chose to get endolysin from the natural extracts of bacteriophage-host cultures.

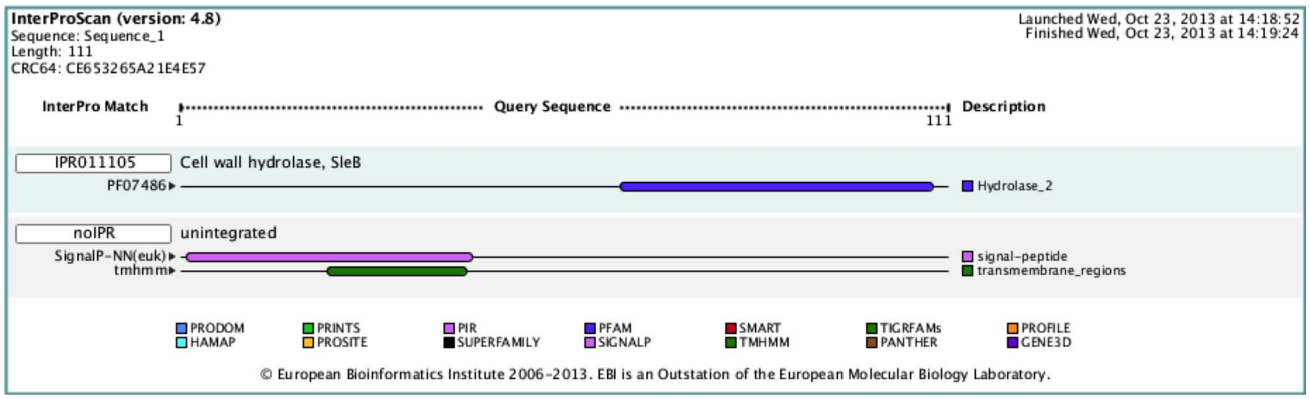

Fig. 2 Putative proteins encoded by ORF 62 (cell wall hydrolase/endolysin). The four lines display (from the top): (1) the length of the ORF 62-encoded protein; (2) The conserved domain of the ORF 62-encoded protein (shown in *blue*); (3) The signal peptide region of the ORF 62-encoded protein (shown in *purple*); (4) The transmembrane region of ORF 62-encoded protein (shown in *green*)

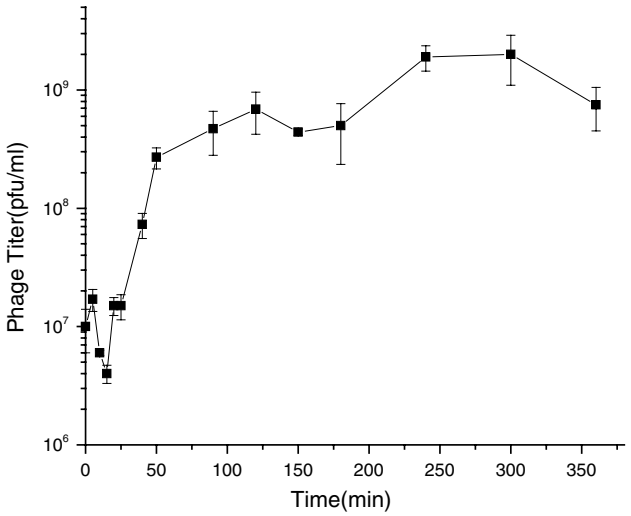

Fig. 3 The Spp001 phage growth curve. Each time point was assessed in triplicate, *Error bars* show standard error

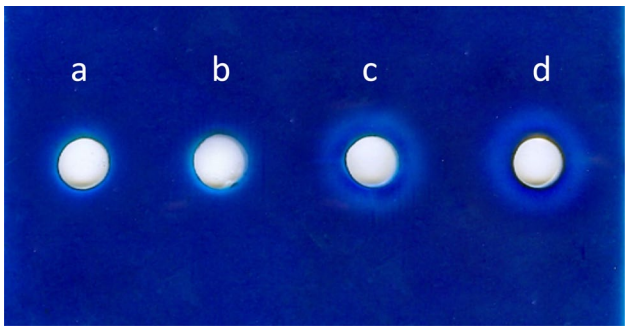

Fig. 4 The lytic activity as determined by the diffusion assay. **a** Phage inoculum (1×10^9 pfu mL^{-1}); **b** buffer A (20 mM Tris–HCl, pH 7.0); **c** partially purified lytic enzymes; **d** lysozyme (1 mg mL^{-1})

The phage growth curve was used to determine the best time for the extraction of crude lytic enzymes. The Spp001 phage growth curve revealed a latent period of about 40 min, a rise period of 200 min, and a plateau period of 60 min (Fig. 3). The time interval from the bacteriophage infection of the host bacteria to the end of the first centrifugation (excluding 10 min pre-incubation) was less than the latent period, when most phage particles were adsorbed by bacterial cells, but the newly formed bacteriophage particles had not yet been released. Phage-host co-precipitates obtained from the first centrifugation were re-suspended in a volume less than the original in order to increase the concentration of the released mature phage particles expressing endolysin. A relatively high concentration of lytic enzymes was detected in the supernatant, which contained the lysates, after centrifugation. Here, we describe a general method of obtaining endolysin-containing crude extracts. Compared with the assay described by Fischetti [12], our extraction procedure did not include high-speed centrifugation, and hence did not affect enzymatic activity in the natural extract. Thus, our method provides the advantages of easy operation and convenience for the scaling up production, and can be applied to the extraction of natural products from other bacteriophages.

Lytic activity of partially purified enzymes

A diffusion assay that used an SDS–polyacrylamide gel (pH 6.8) containing the bacterial cell wall peptidoglycan was utilized to analyze peptidoglycan hydrolysis by phage crude extracts. A water-soluble dye, methylene blue, was used to stain negatively charged anionic peptidoglycan in the gel, and clear zones indicating peptidoglycan hydrolysis could be visually detected [35]. As demonstrated in Fig. 4, lysozyme and the enzymes in the crude extract efficiently degraded the peptidoglycan substrate,

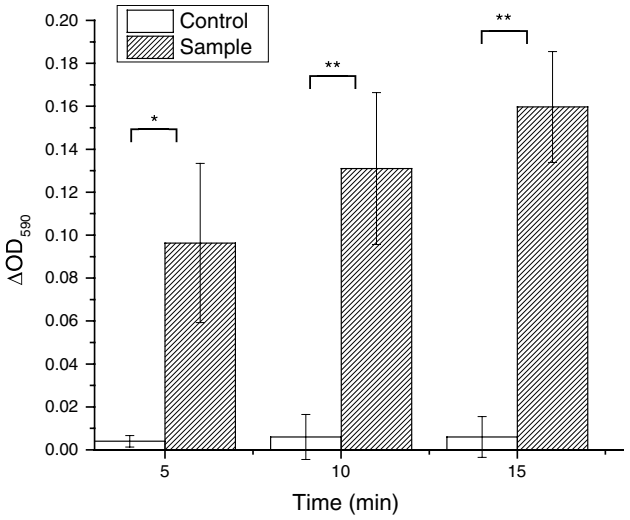

Fig. 5 The lytic activity as determined by the turbidimetric method. $\Delta OD_{590} = OD_{590}$ (× min) − OD_{590} (0 min). Each time point was assessed in triplicate, *Error bars* show standard error. *, ** indicate significant differences between the experiment group and the corresponding control (*$P < 0.05$, **$P < 0.01$)

Table 2 Detection of lytic activity by turbidimetry

Procedure	$\triangle OD_{590}$ (control)[a]	Vol. (mL)	Protein (mg/mL)	Concentration (U/mL)	Specific activity (U/mg)
1. Crude enzyme	0.04 (0.006)	20	0.196	40	204.08
2. Ammonium sulfate	0.16 (0.006)	1	0.287	160	557.49

[a] The decrease in the OD_{590} value for the experiment group. The decrease in the OD_{590} value for the control group is provided in parentheses

producing clear zones on a blue background. Phage inoculum and buffer A used as negative control showed no activity towards cell wall peptidoglycan. The translucent lytic zone generated by the partially purified Spp001 lytic enzymes was even clearer than that of lysozyme, suggesting that the partially purified Spp001 enzymes had high lytic activity.

The activity of the partially purified endolysin-containing extract was assessed by a decrease in OD_{590}, which corresponded to bacterial viability. The enzyme activity measured by the turbidity of the bacterial suspension is shown in Fig. 5. In the first 5 min, the maximum drop in sample turbidity was observed, and after 15 min, the decrease in turbidity was 0.16 (16 units), whereas in the control sample, it was only 0.006 (Table 2). Cell lysis by the Spp001 phage was not observed (data not shown).

Recent studies show that endolysins from gram-positive, bacteria-infecting phages could cause host cell rapid lysis, as no membrane is present to inhibit endolysins to the cell wall. Fischetti [12] and Raina [33] have reported that natural lysates contain a lytic enzyme that has a good ability to lyse cells of *streptococcal* species. However, studies on endolysin activity towards gram-negative bacteria have been limited, because their outer membrane prevents access to the cell wall peptidoglycan. Recent studies using EDTA, chloroform, chitooligosaccharides, or an EDTA-nisin combination together with lytic enzymes have demonstrated an effective lysis of host bacteria, indicating that treatment with specific compounds can render the outer membrane permeable to the external endolysin [22, 34, 43]. Many reports have already shown that endolysins by genetic engineering from gram-negative bacteria were highly lytic peptidoglycan hydrolases and active on the cells treated with an outer membrane permeabilizer was used [3, 37]. In this respect, it should be noted that our assay was performed with an *S. putrefaciens* suspension without any pre-treatment. The lytic enzymes from Spp001 can directly lytic host cells like endolysins with gram-positive background.

Conclusion

We demonstrate that Spp001 phage encodes putative enzymes with strong lytic activity. In future investigations, we aim to identify the endolysin-encoding gene of *S. putrefaciens* Spp001 phage by combining genetic and biochemical approaches. These studies may provide the basis for further investigations to explore the purification of natural lytic enzymes from bacteriophages.

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