Isolation of a lytic bacteriophage vB_EfaS_PHB08 and its endolysin lys08 against *Enterococcus faecalis* biofilm

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Research

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Abstract

Background: *Enterococcus faecalis* is an opportunistic pathogen can cause a variety of diseases, such as urinary tract infections and wound infections in human and animals. Recently, bacteriophages and their derivatives represent a good effect on fighting against bacterial infections.

Methods: We isolated virulent bacteriophages of *E. faecalis* using the double-layer plate method. The bio-activities of the phage isolated were determined via one-step growth curve testing and bacterial killing assays. Illumina HiSeq sequencing was performed to determine the genetic characteristics and the lysins of the phage. Protein expression and antibiofilm assays were also performed to highlight the bio-activities of the phage lysins.

Results: We isolated a virulent bacteriophage vB_EfaS_PHB08 (thereafter PHB08) from the sewage nearby hospital. PHB08 possessed a linear double-stranded DNA genome with 55,244 bp in length, which encoded 91 putative coding sequences (CDS). We found that PHB08 could inhibit the growth of host bacteria for 12 h. In vegetable models, PHB08 can reduce $1´10^5$ Colony Forming Units (CFU) of *E. faecalis* per square centimeter at room temperature (25 °C) for 24 h. In addition, PHB08 and its endolysin can remove the biofilm formed by *E. faecalis*.

Conclusions: A virulent phage and endolysin displayed a good effect on reducing and/or eradicating *E. faecalis* infection and biofilm.

Background

The Gram-positive pathogenic bacteria *Enterococcus faecalis* is the causative agent of endocarditis, sepsis and meningitis in both animals and humans [1–3]. It has been reported that *E. faecalis* is the third most common pathogen that is associated with hospital-acquired infections and it accounts for 15% of catheter-associated urinary tract infections (CAUTI) and 5–15% of infective endocarditis (IE) cases [4–6]. *E. faecalis* strains are also closely associated with many hard-to-treat persistent interradicular infections, suggesting that *E. faecalis* plays a role in the pathogenesis of dental disease [7, 8]. In most cases, treatment of *E. faecalis* infections relies on the use of antimicrobials. However, due to the frequency of use of antibacterial drugs, the resistance of *E. faecalis* clinical isolates is increasing, suggesting a worrisome condition of using antimicrobials continuously [9–11]. Moreover, different genotypes of *E. faecalis* play a role in spreading drug resistance genes, which further promotes the clinically difficult treatment of *E. faecalis* infections [12].

During the infection, *E. faecalis* strains always form single and mixed-species biofilms on both tissue and medical devices in the host, often under exposure to fluid flow, giving rise to infections that are recalcitrant to treatment [13]. Research showed that produce biofilm of *E. faecalis* is related with clinical disease [14]. Formation of biofilms confers the bacteria capacity to escape the killing of antibiotics and the elimination of the host immune system [15]. Therefore, eradication of biofilms formed by *E. faecalis* during the infection is beneficial for clinical treatment.

Bacteriophages (thereafter phages) are the natural predator of bacteria and they are probably the most abundant biological entities in nature [16]. Regarding their ability to kill pathogens with high specificity, phages have been proposed as promising therapeutic tools since their discovery in 1915 [17]. Recently, *E. faecalis* phages have been found to have the potential to specifically inhibit (or kill) the reproduction and survival of *E. faecalis* as a biological control agent [18–20]. Phages and their derivates such as the lysins have also displayed a good effect on reducing and/or eradicating bacterial biofilms in addition to their effective bactericidal activity [21–23]. In this study, we isolated a virulent vB_EfaS_PHB08 (thereafter PHB08) specific infect for *E. faecalis* and constructed a recombinant plasmid expressing endolysin lys08. Further studies revealed that PHB08 as a potential biological agent in lettuce model. In addition, PHB08 and its derivative exhibited effectively for removing *E. faecalis* biofilms.

Methods

Bacterial strains and cultural conditions

E. faecalis strain EF3964 was recovered from a patient with urinary tract infections. It grows well on tryptic soy agars (TSA; Becton, Dickinson and company, MD, USA) and/or in tryptic soy broth (TSB; Becton, Dickinson and company, MD, USA) at 37° C for 16 h.

Phage isolation and purification

Phages against *E. faecalis* were isolated from sewages using *E. faecalis* strain EF3964 as an indicator by a double-layer plate methodology, as described previously [24]. In briefly, sewage samples from the hospital sewage were centrifuged at 4,000 × *g* for 10 min. The supernatants were harvested and were filtered using a 0.22 μm membrane to remove bacteria. After that, 300 μl of the filtrates were mixed with 300 μl of the bacterial culture of EF3964 at mid-log phase, and the mixture incubated using a double-layer TSA plate 37 °C for 12 h to form the phage plaques. Presumptive single plaque was picked and was resuspended in 6 ml of sterile SM buffer (5.8 g of NaCl, 2.0 g of $MgSO_4{\cdot}7H2O$, 50 ml of Tris-HCl [pH7.4], 5.0 ml of 2% gelatin). The phage-containing SM buffer was then centrifuged at 12,000 × *g* for 30 s and the supernatant was filtered through a 0.22 μm pore size membrane. In the next, the phage preparations were given serial 10-fold dilutions with sterile SM buffer. Finally, the phage preparations were inoculated into the indicator bacteria at mid-log phase, which was then incubated using a double-layer TSA plate 37 °C to the next cycle. Phage isolation by the double-layer agar method was repeated four more times. The phages were purified by CsCl gradient ultra-centrifugation and then stored at 4 °C [25]. The morphology of the phages was observed under transmission electron microscope (HITACHI H-7650, Japan).

Physical parameter of phage PHB08

For temperature sensitivity assay, 100 μl (10^9 Plaque Forming Units, PFU) of the purified phages were treated at different temperature (4, 20, 40, 50, 60, 70, and 80 °C) for 1 h. For acid-base sensitivity assay, 100 μl of phage (10^9 PFU) with 900 μl SM buffer of pH (3.0, 5.0, 7.0, 9.0, and 11.0), were treated at 37 °C

for 1 h. Phage titer was determined using the double-layer plate method. This experiment was repeated three times.

One-step growth curve

The one-step growth curve of PHB08 is determined as described previously [24]. In briefly, PHB08 with multiplicity of infection (MOI) of 0.01 was inoculated into the indicator bacteria at mid-log phase and the mixture was incubated at 37 °C for 5 min. After incubation, the mixture was centrifuged at 12,000 rotation per minute (rpm) for 30 s. The supernatant was discarded with equal volume TSB. The titer of phage was determined by double-layer plate method. This experiment was repeated three times.

Lytic Activity of PHB08

The lytic activity of phage PHB08 was analyzed in a 96-well microtiter plate by examining the optical density measurement method [26, 27]. Briefly, 100 µl of the mid-log phase *E. faecalis* EF3964 ($6.6 \cdot 10^7$ Colony Forming Units, CFU) mixed with 100 µl of phage PHB08 of different MOI (0.001, 0.01, 0.1, 1.0, 10, 100, and 1000) was incubated at 37 °C (160 rpm). Wells with equal volume of TSB medium or Phosphate buffer saline (PBS) buffer added were used as controls. The absorbance value of resulting supernatant was measured at 590 nm using a multimode microplate reader (Tecan Spark 10M). This experiment was performed in triplicate.

Killing assay in vegetable module

The effect of phage PHB08 on host strain EF3964 in vegetable module was evaluate as previously described [28]. Briefly, the vegetable was sterilized with sodium hypochlorite (100 µg/ml) for 5 min. After washing with sterile water, the vegetable sample was covered evenly host strain EF3964 (10^5 CFU/cm^2) until sample dried naturally. Subsequently, phage PHB08 with different MOI values (1000, 100, 10, and 1.0) was sprayed on the vegetable leaves at 25 °C for 6, 12, and 24 h, respectively. The control group was added equal volume phosphate buffered saline (PBS; pH = 7.4). The survival of EF3964 was counted by 10-fold dilutions method. This experiment was repeated three times.

DNA extraction and analysis of genome sequence

The phages' genomic DNA extracted using the phenol-chloroform protocol was dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH = 8.0]) and was sequenced on an Illumina HiSeq 2,500 sequencer with 2 × 100 bp read length. The short reads were assembled into the genome by means of SOAPdenovo [29]. Open reading frames (ORFs) were predicted using Glimmer [30, 31]. The final assembled sequence was searched against the current protein and nucleotide databases (http://www.ncbi.nlm.nih.gov/) by means of the basic local alignment search tool (BLAST). Protein BLAST (BLASTP) was used to identify putative homologies and proteins sharing similarities with the predicted phage proteins. The genomes were scanned for tRNAs using tRNA scan-SE [32] and ARAGORN [33]. Phylogenetic tree was analyzed using the ClustalW program in MEGA 6.0 [34]. The complete genome sequence of PHB08 was deposited in GenBank under the accession number MK570225.

Cloning, expression and activity identification of lys08

The putative lysin gene lys08 of phage PHB08 was amplified by polymerase chain reaction (PCR) with specific primers (5'-CGTGTGTCACATACCTGAATTG-3'; 5'-GCAGTAACAGCCATTCATCTATG-3'), and then was cloned into the expression vector pET-28a, generating pET-lys08, which was finally transformed into the expressed strain BL21 (DE3). Single colonies of BL21 were picked and inoculated into TSB medium containing 50 μg/ml of kanamycin, incubated overnight at 37 °C. After that, bacterial culture was transferred into fresh TSB containing 50 μg/ml of kanamycin and the mixture was cultured at 37 °C until Optical Density (OD_{600}) value reaches 0.6 ~ 0.8. The expression of protein Lys08 was induced by the addition of 0.6 mM/L IPTG in the bacterial culture and further incubation at 25 °C for 16 h. Lys08 was purified by Ni-nitrilotriacetic acid column as previously described [35]. The purified protein lys08 was dialyzed in a protein preservation solution (50 mmoL/l Tris, 0.3 mol/l NaCl, pH 8.0) in 0.45 μm membrane concentrated. Purified protein was quantified by the Bradford Protein Assay Kit (Thermo Fisher Scientific) and stored at -80 °C until uses.

Antibiofilm by phage PHB08 and its endolysins lys08

Biofilm formation was detected using a 96-well microtiter plate as previously described [36]. In briefly, 100 μl of the overnight cultured bacterial strain EF3964 was added to each well and was incubated at 37 °C for 24 h, 48 h, or 72 h, respectively. Equal volumes of PBS (pH = 7.4) was included as controls. Each well was washed three times with sterile PBS buffer. Subsequently, 100 μl phage PHB08 ($2.0{\cdot}10^9$ PFU/ml, $2.0{\cdot}10^8$ PFU/ml, $2.0{\cdot}10^7$ PFU/ml, and $2.0{\cdot}10^6$ PFU/ml) with TSB medium or 100 μl lys08 (50 μg and 100 μg) was added to every well at 37 °C for 4 h. After incubation, 1% crystal violet solution was added 100 μl to each well at 37 °C for 15 min, then washed three times with sterile PBS buffer until the liquid has no more crystal violet color. Finally, 150 μl of 33% acetic acid added to each well. The absorbance value of resulting supernatant was measured at 600 nm as mentioned above. This experiment was repeated three times.

Statistical Analysis

Student's unpaired t-test was used for statistical significance when comparing results for two groups; while ordinary one-way analysis of variance (ANOVA) with post-hoc analysis by Dunnett's test was used when comparing the results of more than two groups. Data are present as "Mean ± SD". Differences were considered statistically significant if $P < 0.05$ (∗). All statistical analyses were performed using GraphPad Prism software.

Results

Microbiological characteristics of phage PHB08

The isolated phage PHB08 had a clear, translucent, uniform size plaque on a double-layer agar plate (Fig. 1a). Under the observation of electron microscope, phage PHB08 had a rectangular head (length 124 mm ± 5, width 61 mm ± 5) and a long tail (158 mm ± 5) (Fig. 1b). Based on these morphological

characteristics and according to the latest International Committee on Taxonomy of Viruses (ICTV) classification, PHB08 was determined as a member of the family *Siphoviridae*.

Phenotypic parameters of phage PHB08

Phage PHB08 was treated in different pH (3.0–11.0) at 37 °C for 1 h and treated in different temperature (4–80 °C for 1 h. The Acid-base tolerance results exhibited that the activity of PHB08 is relatively stable between 5.0–11.0 (Fig. 2a). Temperature tolerance results showed that the titer of phage PHB08 is quite stable between 4 °C and 60 °C (Fig. 2b). PHB08 was treated at 70 °C for 40 min or at 80 °C for 20 min, no titer was observed (Fig. 2b).The one-step growth curve of PHB08 showed that PHB08 has a latency of 20 min and a high-speed growth period of 40 min with an average burst size of 64 phage particles per infected (Fig. 2c). The host range assays indicated that phage PHB08 specifically infected 15 out of 19 *E. faecalis* clinical isolated, but not infecting other species including *Enterococcus faecium* (Table 1).

Table 1
The host range of PHB08.

Strains	Isolated locations	Plaque formation
Enterococcus faecalis EF3964 (Host Strain)	Hubei, China	+
Enterococcus faecalis EF1833	Hubei, China	+
Enterococcus faecalis EFHB01	Hubei, China	+
Enterococcus faecalis EFHB02	Hubei, China	+
Enterococcus faecalis EFHB03	Hubei, China	+
Enterococcus faecalis EFHB04	Hubei, China	-
Enterococcus faecalis EF1067	Anhui, China	+
Enterococcus faecalis EF7011	Anhui, China	+
Enterococcus faecalis EFhn15	Hunan, China	+
Enterococcus faecalis EFhn20	Hunan, China	-
Enterococcus faecalis FJ1801	Fujian, China	+
Enterococcus faecalis Hu6	Unknow	+
Enterococcus faecalis Hu7	Unknow	+
Enterococcus faecalis Hu8	Unknow	-
Enterococcus faecalis XN01	Shanxi, China	+
Enterococcus faecalis XN02	Shanxi, China	+
Enterococcus faecalis 20170714-1	Unknow	+
Enterococcus faecalis 20170714-2	Unknow	+
Enterococcus faecalis 20170714-3	Unknow	-
Enterococcus faecium HBM101	Hubei, China	-
Enterococcus faecium HBM1-2	Hubei, China	-
Enterococcus faecium 14409	Beijing, China	-
*Enterococcus faecium*STT-03	Hainan, China	-
Enterococcus faecium STT-04	Hainan, China	-
Enterococcus faecium 21905	Hubei, China	-

NOTE:(+) indicates that plaques were observed ;(-) indicates no plaques were observed.

Strains	Isolated locations	Plaque formation
Enterococcus faecium 180724	Unknow	-
Enterococcus faecium 180801	Unknow	-
Enterococcus faecium SX11	Shanxi, China	-
*Enterococcus faecium*HN12	Hainan, China	-
Escherichia coli 18701	Hubei, China	-
Escherichia coli 14997	Hubei, China	-
Escherichia coli O157:H7	Hubei, China	-
Escherichia coli DH5α	Hubei, China	-
Salmonella 268	Hubei, China	-
Salmonella 140411	Hubei, China	-
Salmonella 1003	Hubei, China	-
NOTE:(+) indicates that plaques were observed ;(-) indicates no plaques were observed.		

Features of phage PHB08 genome

The complete genome of phage PHB08 was linear double-stranded DNA genome of 55,244 bp, with a G + C content of 40% (Fig. 3). The genome of PHB08 was predicted 91 putative CDS (gene annotation for each CDS of PHB08 shown in Table 2), and one tRNA (Trp-CCA) prediction. A BLASTn search revealed that the PHB08 was closely related to phage vB_EfaS_IME198 (GenBank accession no. KT932699.1; 96.18% identity, 89% coverage) and vB_EfaS_HEf13 (GenBank accession no. MH618488.1; 95.95% identity, 84% coverage). The phage PHB08 genome contains genes that encode the structure and assembly protein of phage PHB08, including tail fibers (CDS61), tail length tape-measure protein (CDS62), major capsid protein (CDS71), portal protein (CDS74), and terminase large subunit (CDS75). Encoding DNA replication and regulation modules such as DNA binding protein (CDS6), DNA polymerase I (CDS22), adenylate kinase and related kinases (CDS35), HNH homing endonuclease (CDS39, CDS58, and CDS87), replicative DNA helicase (CDS41), DNA replication protein (CDS42), and DNA primase (CDS44). Genes encoding endolysin protein ware found in the PHB08, such as phage lysin (CDS59), with 96% amino acid sequence identity to the CHAP domain protein of *Enterococcus* phage Entf1. No lysogeny associated gene and encoding known antibiotic resistance were predicted [37]. Phylogenetic tree analysis the amino acid sequence of the major capsid protein (CDS71) and terminase large subunit (CDS75) indicated that PHB08 belongs to Saphexavirus, *Siphoviridae* family (Fig. 4).

Table 2

Gene annotation for each CDS of PHB08.

CDS	Start	Stop	Length (bp)	Size (aa)	Function	Accession numbers	% identity	*E* value
1	151	17	135	44	hypothetical protein	–	–	–
2	603	229	375	124	hypothetical protein	–	–	–
3	982	596	387	128	hypothetical protein	–	–	–
4	1406	1083	324	107	hypothetical protein	–	–	–
5	2683	2171	513	170	hypothetical protein	–	–	–
6	2991	2686	306	101	Phage DNA binding protein	YP_009603915.1	99.01%	2e-71
7	3259	2993	267	88	hypothetical protein	–	–	–
8	3479	3252	228	75	hypothetical protein	–	–	–
9	3722	3513	210	69	hypothetical protein	–	–	–
10	3947	3735	213	70	hypothetical protein	–	–	–
11	4207	3947	261	86	hypothetical protein	–	–	–
12	4398	4204	195	64	hypothetical protein	–	–	–
13	4895	4476	420	139	hypothetical protein	–	–	–
14	5109	4909	201	66	hypothetical protein	–	–	–
15	5707	5120	588	195	hypothetical protein	–	–	–
16	6053	5730	324	107	hypothetical protein	–	–	–

NOTE:(–) indicates that no analysis or no results.

CDS	Start	Stop	Length (bp)	Size (aa)	Function	Accession numbers	% identity	*E* value
17	6259	6023	237	78	hypothetical protein	–	–	–
18	6521	6243	279	92	hypothetical protein	–	–	–
19	7275	6580	696	231	hypothetical protein	–	–	–
20	7663	7268	396	131	hypothetical protein	–	–	–
21	8120	7665	456	151	hypothetical protein	–	–	–
22	10726	8195	2532	843	DNA polymerase I (EC 2.7.7.7)	YP_009218931.1	98.34%	0.0
23	10991	10806	186	61	hypothetical protein	–	–	–
24	11232	11005	228	75	hypothetical protein	–	–	–
25	11585	11232	354	117	hypothetical protein	–	–	–
26	11798	11586	213	70	hypothetical protein	–	–	–
27	12010	11801	210	69	hypothetical protein	–	–	–
28	12231	12007	225	74	hypothetical protein	–	–	–
29	12439	12245	195	64	hypothetical protein	–	–	–
30	12620	12441	180	59	hypothetical protein	–	–	–
31	13026	12820	207	68	hypothetical protein	–	–	–
32	13154	13038	117	38	hypothetical protein	–	–	–
33	13720	13157	564	187	hypothetical protein	–	–	–

NOTE:(–) indicates that no analysis or no results.

CDS	Start	Stop	Length (bp)	Size (aa)	Function	Accession numbers	% identity	*E* value
34	14444	13812	633	210	hypothetical protein	–	–	–
35	15006	14437	570	189	adenylate kinase and related kinases	–	–	–
36	15437	15003	435	144	hypothetical protein	–	–	–
37	15903	15574	330	109	hypothetical protein	–	–	–
38	16931	15903	1029	342	hypothetical protein	–	–	–
39	17364	16924	441	146	HNH homing endonuclease	NP_389885.1	28.46%	1e-05
40	17661	17437	225	74	hypothetical protein			
41	19040	17676	1365	454	Replicative DNA helicase (DnaB)	NP_719448.1	24.37%	8e-04
42	19828	19052	777	258	DNA replication protein	YP_009036407.1	99.61%	0.0
43	20230	19877	354	117	hypothetical protein	–	–	–
44	21252	20305	948	315	DNA primase	–	–	–
45	21452	21264	189	62	hypothetical protein	–	–	–
46	21610	21452	159	52	hypothetical protein	–	–	–
47	21861	21607	255	84	hypothetical protein	–	–	–
48	22253	21861	393	130	hypothetical protein	–	–	–
49	22401	22255	147	48	hypothetical protein	–	–	–

NOTE:(–) indicates that no analysis or no results.

CDS	Start	Stop	Length (bp)	Size (aa)	Function	Accession numbers	% identity	*E* value
50	22585	22373	213	70	hypothetical protein	–	–	–
51	23471	23346	126	41	hypothetical protein	–	–	–
52	23856	23656	201	66	hypothetical protein	–	–	–
53	24242	23856	387	128	hypothetical protein	–	–	–
54	24679	24245	435	144	hypothetical protein	–	–	–
55	25556	24732	825	274	hypothetical protein	–	–	–
56	26060	26284	225	74	hypothetical protein	–	–	–
57	26286	26714	429	142	hypothetical protein	–	–	–
58	26732	27292	561	186	HNH homing endonuclease	QDB70581.1	97.85%	2e-138
59	28035	27319	717	238	Phage lysin	–	–	–
60	31337	28110	3228	1075	Phage minor structural protein	QBZ69423.1	76.49%	0.0
61	35341	31349	3993	1330	Phage tail fibers	YP_009604020.1	87.17%	0.0
62	38240	35355	2886	961	Phage tail length tape-measure protein	YP_009218894.1	98.96%	0.0
63	38477	38253	225	74	hypothetical protein	–	–	–
64	38928	38488	441	146	hypothetical protein	–	–	–
65	39761	39072	690	229	hypothetical protein	–	–	–
66	40216	39782	435	144	hypothetical protein	–	–	–

NOTE:(–) indicates that no analysis or no results.

CDS	Start	Stop	Length (bp)	Size (aa)	Function	Accession numbers	% identity	*E* value
67	40609	40229	381	126	hypothetical protein	–	–	–
68	40971	40594	378	125	hypothetical protein	–	–	–
69	41391	40987	405	134	hypothetical protein	–	–	–
70	41891	41451	441	146	Chitinase (EC 3.2.1.14)	AYH92720.1	97.95%	2e-100
71	42852	42046	807	268	Phage major capsid protein	YP_006488741.1	99.63%	0.0
72	43575	42901	675	224	hypothetical protein	–	–	–
73	44441	43686	756	251	hypothetical protein	–	–	–
74	45988	44453	1536	511	Phage portal protein	AYH92724.1	99.80%	0.0
75	47316	46045	1272	423	Phage terminase, large subunit	YP_009603889.1	99.53%	0.0
76	47627	47379	249	82	hypothetical protein	–	–	–
77	47990	47646	345	114	hypothetical protein	–	–	–
78	48603	48004	600	199	hypothetical protein	–	–	–
79	48884	49198	315	104	hypothetical protein	–	–	–
80	49198	49455	258	85	hypothetical protein	–	–	–
81	49455	49847	393	131	hypothetical protein	–	–	–
82	49849	50235	387	128	hypothetical protein	–	–	–
83	50232	50492	261	86	hypothetical protein	–	–	–

NOTE:(–) indicates that no analysis or no results.

CDS	Start	Stop	Length (bp)	Size (aa)	Function	Accession numbers	% identity	*E* value
84	50494	50700	207	68	hypothetical protein	–	–	–
85	50690	50992	303	100	hypothetical protein	–	–	–
86	51012	51380	369	121	hypothetical protein	–	–	–
87	51824	52582	759	252	HNH homing endonuclease	APU00279.1	98.81%	0.0
88	52657	53511	855	284	hypothetical protein	–	–	–
89	53512	53730	219	72	hypothetical protein	–	–	–
90	53732	53944	213	70	hypothetical protein	–	–	–
91	54122	54262	141	46	hypothetical protein	–	–	–

NOTE:(–) indicates that no analysis or no results.

Killing of PHB08 in vitro

The effect of the PHB08 on host strain EF3964 was evaluated in liquid medium at 37 °C for 12 h. As shown in Fig. 5a, phage PHB08 showed a strong antibacterial ability at different MOI value (from 0.0001 to 100) for 12 h. After two hours, the curve of the OD_{600nm} value showed a downward trend, implying that the host bacteria were killed by phage PHB08. The effect of phage PHB08 on host strain was evaluated in lettuce as a vegetable model at room temperature (25 °C). With higher (MOI = 1000) phage-treated, the number of living bacteria EF3964 have a significantly reduction ($P < 0.001$) compared to control group. With phage-treated at MOI = 1, no significantly reduction of living bacteria EF3964 was observed within 24 h (Fig. 5b).

Antibiofilm activity

The recombinant protein lys08 was predicted to be 26.4 kDa, which was consistent with Polyacrylamide Gel Electrophoresis (PAGE-Gel) test results (Fig. 6a). The purified lys08 was detected by the spot method. The results showed 5 μl lys08 (5 μg) can form translucent halo on the plate, suggesting bactericidal activity of lys08 (Fig. 6b). The effect of the lys08 and phage on the biofilm was evaluated using 96-well microtiter plate method. In phage challenge groups, PHB08 was able to remove the formation of biofilm in co-culture 24 h at 37 °C ($P < 0.001$) (Fig. 6c). In lys08-treated groups, obviously reduction was observed ($P < 0.001$) (Fig. 6d).

Discussion

In recent years, the use of bacteriophages and their derivates fighting against bacterial infections and antimicrobial resistance have received more attentions [17]. In the present study, we isolated a virulent phage PHB08 from sewages using *E. faecalis* EF3964 as the indicator bacterium. The morphology and phylogenetic tree analysis further revealed that phage PHB08 belonged to the *Siphoviridae* family. The phenotypic parameters assays showed that PHB08 had relatively good stability at temperatures between 4 °C and 60 °C, and pH between 5.0 and 9.0, the average survival rate of phages during incubation is maintained at 40–50%. Our results were consistent with those of phages including *Enterococcus* phage vB_EfaS_HEf13 [38] and *Enterococcus* phage EF-P10 [39] when they were exposed to the same temperature (4–60 °C) and acid-base (5.0–9.0). It is worthy note that PHB08 can lyse 15 of the 19 *E. faecalis* strains, with a host range of 78.9%, which has a wider lytic range than the other *E. faecalis* bacteriophages (7%-70.5%) [38, 40–42]. The average burst of PHB08 was 64 phage particles per infected, in line with the generally reported estimate of about 30–122 phage particles per infected [43, 44] .The genome sequence of phage PHB08 has the highest similarity (89–98%) with those phages (IME198, HEf13, Ef7.1, EF-P29, EF-P10, VD13, IME-EF1, SAP6, EF1c55, BC-611, Entf1, and Ef2.2) with ~ 40% G + C **(**Table 3**)**. Phylogenetic analysis of the complete genome sequences of PHB08 and other representative the complete *Saphexavirus* genome. Our data showed that PHB08 was closely related to phage SAP6 **(Figure S1)**. The results indicated those phages, which were isolated from different countries (China, USA, Japan, South Korea, Poland, Russia) may have the complex evolutionary relationship.

Table 3

Sequence information for the *E.faecalis* phages belonging to Saphexavirus subfamily used in this study.

Name	GenBank	Length	Similarity	G + C%	ORF	tRNA	Countries
Enterococcus phage vB_EfaS_PHB08	MK570225.1	55,244 bp	100%	40.00%	91	1	China
Enterococcus phage vB_EfaS_IME198	KT932699.1	58,000 bp	96.18%	40.02%	95	0	China
Enterococcus phage vB_EfaS_HEf13	MH618488.1	57,811 bp	95.95%	40.03%	95	1	South Korea
Enterococcus phage vB_EfaS_Ef7.1	MK721194.1	58,018 bp	94.09%	40.03%	102	3	The United States
Enterococcus phage EF-P29	KY303907.1	58,984 bp	96.07%	39.77%	101	0	China
Enterococcus phage EF-P10	KY472224.1	57,408 bp	96.05%	39.82%	127	0	China
Enterococcus phage VD13	KJ127303.1	55,726 bp	94.89%	40.01%	88	1	The United States
Enterococcus phage IME-EF1	NC_041959	57,081 bp	96.40%	40.05%	98	0	The United States
Enterococcus phage SAP6	NC_041960	58,619 bp	97.53%	40.00%	44	0	South Korea
Enterococcus phage vB_EfaS_EF1c55	MN103542.1	55,876 bp	89.20%	39.79%	94	0	Poland
Enterococcus phage BC-611	AB712291.1	53,996 bp	95.40%	40.45%	88	1	Japan
Enterococcus phage Entf1	MK800154.1	58,938 bp	95.24%	39.93%	105	1	Russia
Enterococcus phage vB_EfaS_Ef2.2	MK721189.1	58,400 bp	89.52%	39.92%	103	2	The United States

The harm caused by foodborne pathogens has become a serious challenge to humans [45]. Researchers began to use phages as natural antimicrobials in food to kill or inhibit foodborne pathogens, thus ensuring food safety [46, 47]. A series of studies have shown that phages can be used in food safety. For example, application phages on *Salmonella* in cantaloupes resulted *Salmonella* significantly reduced [48];

With phage treated, 95% reduction the number of *Campylobacter jejuni* was observed, and also founded that *Salmonella* can be killed or inhibited by phage [49]. In our study, killing assay exhibited the host strain EF3964 can be inhibited or killed at different MOI for 12 h in medium. Potential bactericidal ability at low MOI was similar to those of the single phage 13076 and phage 14028 [26]. In vegetable model, PHB08 can kill $1{\cdot}10^5$ CFU/cm^2 *E. faecalis* at 25 °C for 24 h. It can be inferred that phage-based biological control methods have great potential in improving the safety of food microorganisms. *E.faecalis* with biofilm formation can provide resistance to antibiotics [50], explore the new methods of against cell biofilms is currently one of the major problems in medicine [51]. Although some research have reported of phages fighting bacterial biofilms [52, 53], only a few studies on the effects of *E. faecalis* phage endolysin on host bacteria biofilms. In our study, endolysin lys08 and phage PHB08 can effectively remove the host strain biofilm at 37 °C for 72 h ($P < 0.001$), suggesting that phage and endolysin have the development potential to against the biofilm formation of *E. faecalis*.

Conclusion

A virulent phage of *E. faecalis* was isolated and characterized in this study. This phage displayed a high survival stability and capacity to lyse cells. *In vitro* tests revealed that PHB08 and its endolysin lys08 could remove the biofilm formed by *E. faecalis*. Overall, our study offers an option for treating *E. faecalis* infections through phage and its derivates.

Abbreviations

CDS
Coding Sequences
CFU
Colony Forming Units
CAUTI
Catheter-associated Urinary Tract Infections
TSA
tryptic soy agars
TSB
tryptic soy broth
PFU
Plaque Forming Units
MOI
Multiplicity of infection
rpm
Rotation per minute
PBS
Phosphate buffer saline

ORFs
Open Reading Frames
BLAST
Basic Local Alignment Search Tool
BLASTP
Protein BLAST
PCR
Polymerase chain reaction
OD
Optical Density
PAGE-Gel
Polyacrylamide Gel Electrophoresis

Declarations

Ethics approval and consent to participate: Not applicable

Consent for publication: Not applicable

Availability of data and materials

The datasets during and/or analysed during the current study available from the corresponding author on reasonable request

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Author Contributions

DY and YC performed the data and drafted the main manuscript; DY, YC, ES, and LH planned and performed experiments; BW, HC, and ZP were responsible for experimental design, project management, and manuscript revision. All authors reviewed and agreed the publication of this manuscript.

Conflicts of interest

The authors declare that they have no competing interests.

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Accession number(s). The complete genome sequence of phage was deposited in GenBank under the accession number MK570225.

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Figures

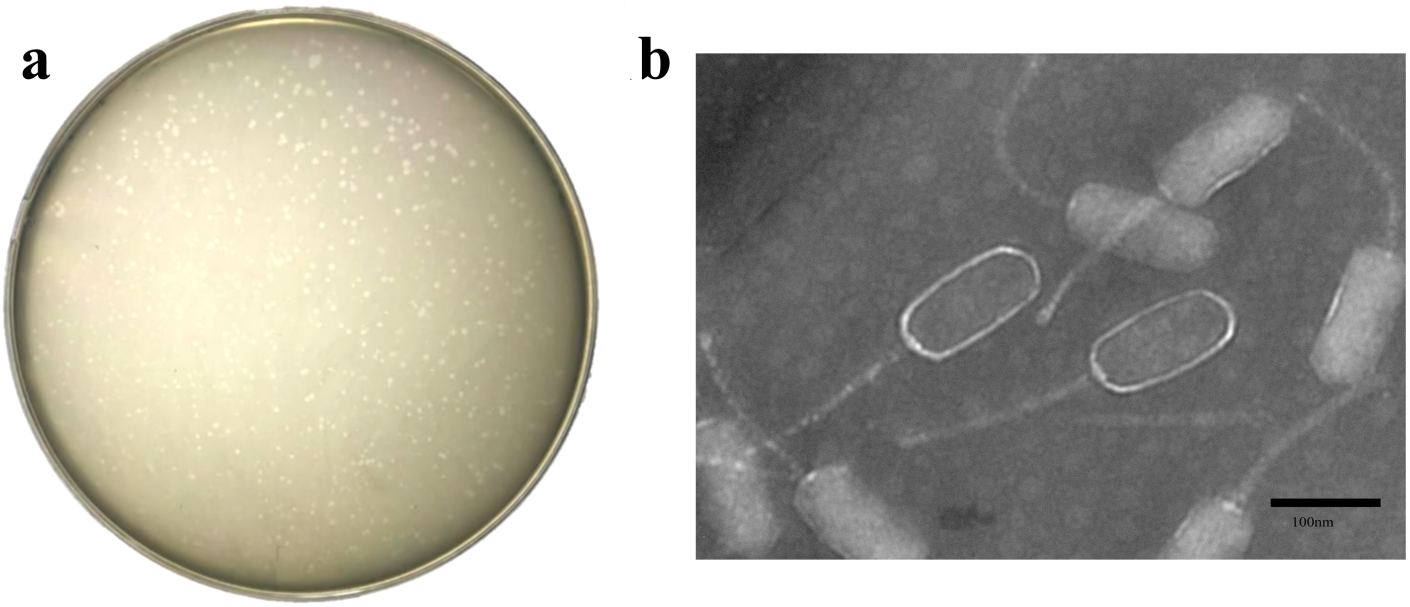

Figure 1

Morphological characteristics of phage PHB08. a. Appearance of phage PHB08 plaque on host strain EF3964. b. Electron microscopy of phage PHB08. Phage PHB08 has a rectangular head (length 124 mm ± 5, width 61 mm ± 5) and a long tail (158 mm ± 5). The scale in the right corner is 200 nm.

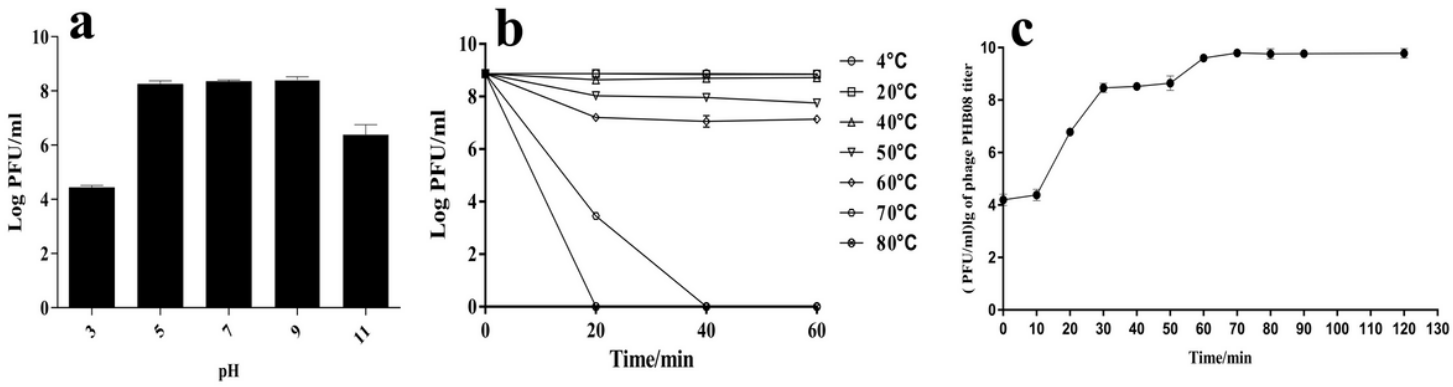

Figure 2

Biological characteristics of phage PHB08. a. Stability of phage PHB08 at different temperatures. b. Stability of phage PHB08 at different pH values. c. Curves for one-step growth of phage PHB08.

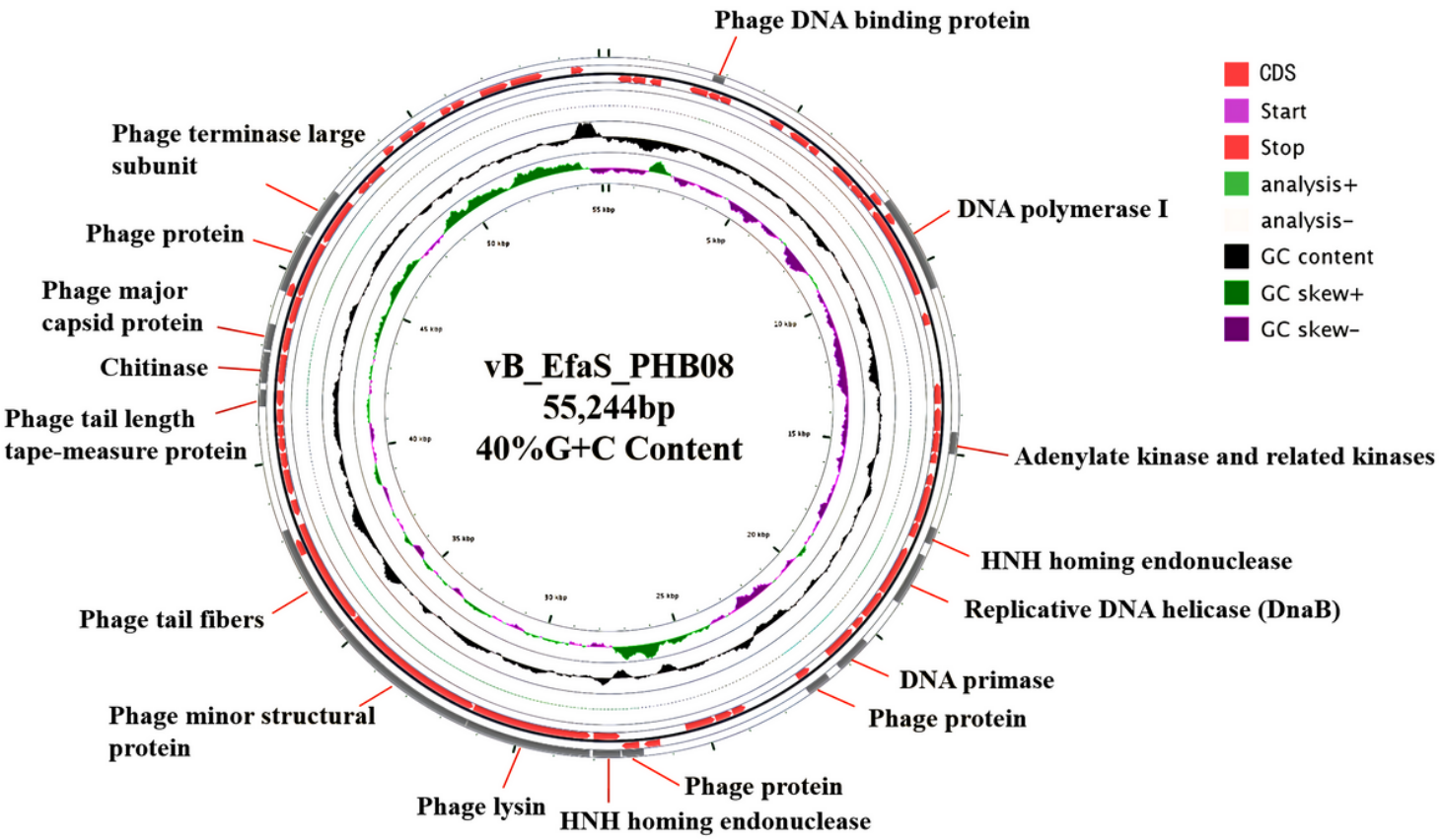

Figure 3

Circular genetic map of PHB08. The red part represents the distribution of the CDS region. The black part represents the total content of GC (40%). The green part represents the GC skew +, which means the GC shift on the leading chain is positive and the purple is the GC skew -.

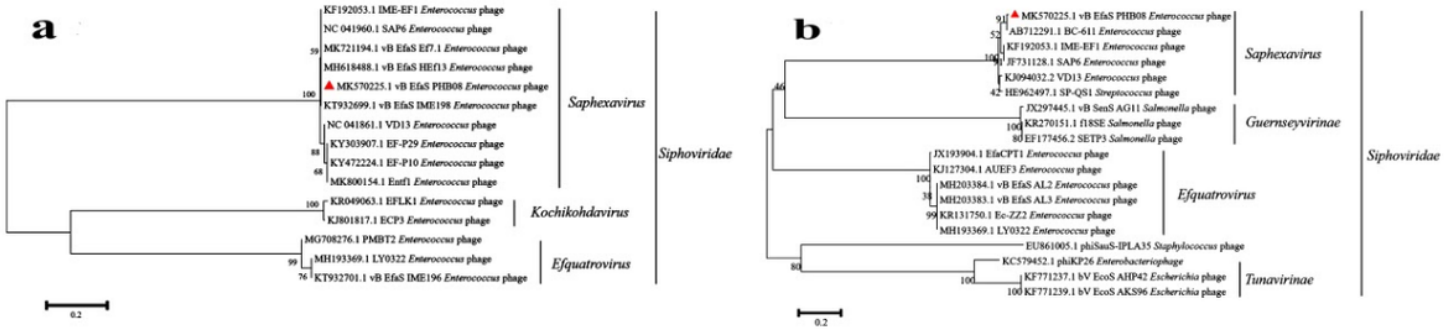

Figure 4

Phylogenetic tree analysis of phage PHB08. a. The amino acid of terminase large subunit large terminal subunit and b. the major capsid proteins of phage PHB08 was analyzed by MEGA6.0. The neighbor-joining method was used to construct phylogenetic with a bootstrap re-sampling analysis of 1,000 replications. The numbers next to the branches are bootstrap values.

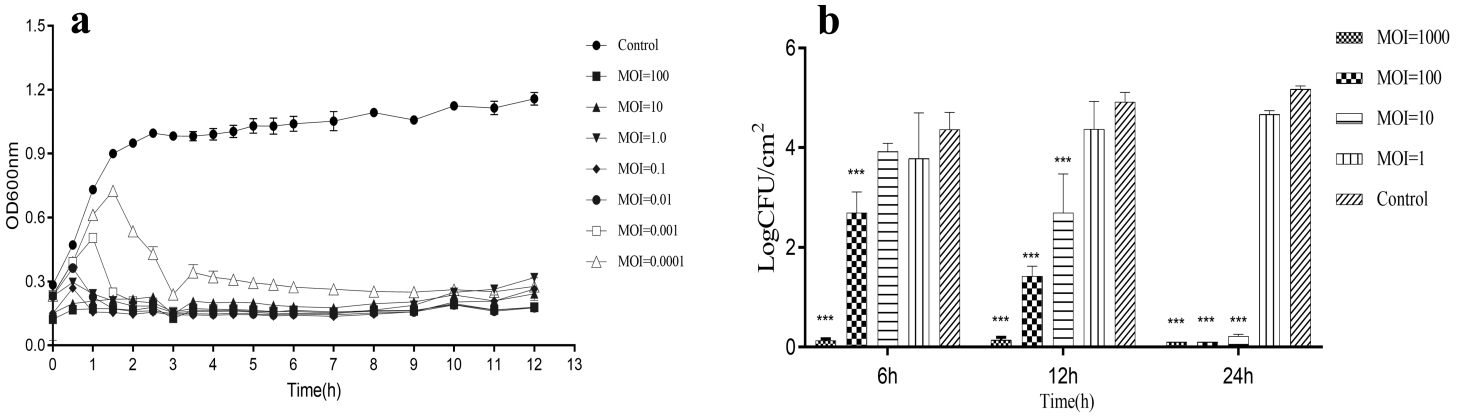

Figure 5

Lytic Activity of PHB08 in vitro. a. Killing assay in TSB medium. b. Killing assay in vegetable module. Data are expressed as the mean ± SD. Validation of the killing by CFU count of host bacteria EF3964 after 6 h, 12 h, and 24 h with or without treatment by PHB08 at MOIs of 101-104, respectively. Data are expressed as the mean ± SD. Significance was determined by ANOVA (***P < 0.001).

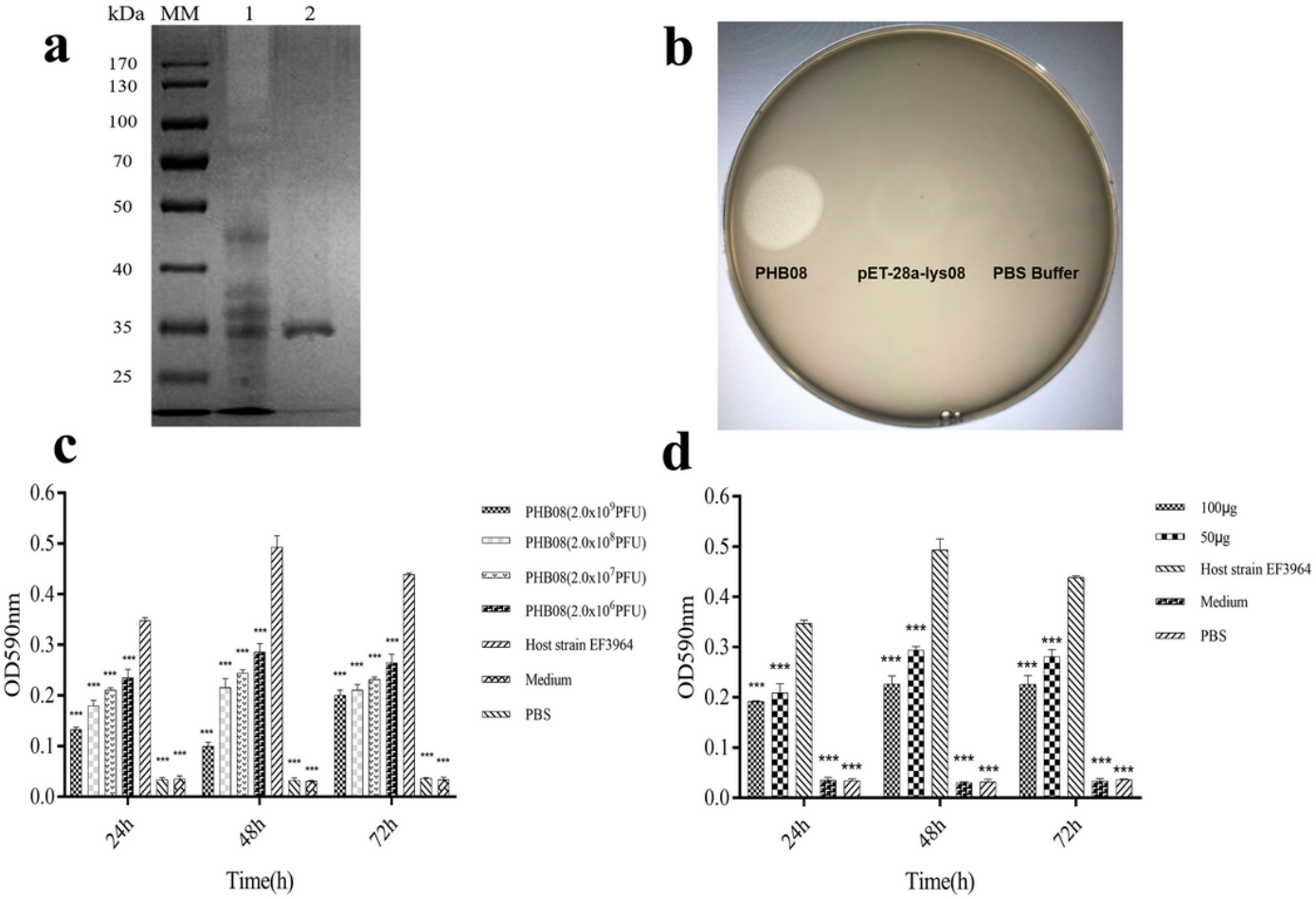

Figure 6

Antibiofilm activity. a. SDS polyacrylamide gel electrophoresis analysis of purified protein lys08. Lane 1: Unpurified protein, lane 2: Purified protein. b. The activity of PHB08 and purified lys08 was determined by spot tests. 5 µl endolysin lys08 (5 µg) spotted on the plated containing host strain EF3964 at 37°C for 12 h. c. The effect of the phage PHB08 on the biofilm and (d.) lys08 on the biofilm was evaluated using 96-well microtiter plate method. Three independent experiments were performed and data are expressed as means ± SD (n = 3). Significance was determined by ANOVA (***, P < 0.001)

Supplementary Files

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