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ORIGINAL PAPER

Identification of a lytic *Pseudomonas aeruginosa* phage depolymerase and its anti-biofilm effect and bactericidal contribution to serum

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Abstract

Pseudomonas aeruginosa (*P. aeruginosa*) infection has imposed a great threat to patients with cystic fibrosis. With the emergence of multidrug-resistant *P. aeruginosa*, developing an alternative anti-microbial strategy is indispensable and more urgent than ever. In this study, a lytic *P. aeruginosa* phage was isolated from the sewage of a hospital, and one protein was predicted as the depolymerase-like protein by genomic sequence analysis, it includes two catalytic regions, the Pectate lyase_3 super family and Glycosyl hydrolase_28 super family. Further analysis demonstrated that recombinant depolymerase-like protein degraded *P. aeruginosa* exopolysaccharide and enhanced bactericidal activity mediated by serum in vitro. Additionally, this protein disrupted host bacterial biofilms. All of these results showed that the phage-derived depolymerase-like protein has the potential to be developed into an anti-microbial agent that targets *P. aeruginosa*.

Keywords *Pseudomonas aeruginosa* · Bacteriophage · Depolymerase · Biofilm · Exopolysaccharide

Introduction

Pseudomonas aeruginosa is a Gram-negative, facultative anaerobic, rod-shaped bacterium that thrives in soil and aquatic habitats, and colonizes the surfaces of plants, animals, and humans [1]. *P. aeruginosa* is a nosocomial pathogen that commonly causes human infection in skin, ear, eye, urinary tract, heart, airway and lung tissues [2]. During the past decades, *P. aeruginosa* has become a leading cause of morbidity and mortality in intensive care units [3]. Chronic airway infection by *P. aeruginosa* is also responsible for morbidity of patients with cystic fibrosis and chronic obstructive pulmonary disease [4, 5]. Complement is a kind of serum protein includes a group of plasma proteins that promoting antibody kills pathogens [6]. It can mediate immune and inflammatory reaction in human, vertebrate serum and tissue fluids. Complement proteins in serum can be activated by antigen–antibody complex or microorganism to cause lysis of pathogenic microorganisms or be swallowed [7]. As a significant component of innate immunity, the complement system plays a crucial role especially against Gram-negative bacteria [8]. But barrier formed by *P. aeruginosa* versatile phenotypes, such as planktonic transition to biofilms and lipopolysaccharide, prevent lysis of complement and penetration of antibiotics [9–11]. These

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factors make clinical treatment of *P. aeruginosa* infection challenging.

Biofilms are defined as highly organized multicellular communities of bacteria enclosed in extracellular polymeric substances (EPS) that protect the encased microorganisms from attack by the host immune system [12]. Bacteria in biofilms also increase their resistance to antimicrobials [13]. Although the compositions of EPS are diverse according to the bacteria that initiate their formation, most consist of proteins, polysaccharides, and extracellular DNA [14]. Exopolysaccharides produced by *P. aeruginosa* include at least three distinct polymers (Psl, Pel, and alginate) [15]. Production of alginate confers *P. aeruginosa* with a mucoid phenotype, and Psl and Pel are commonly present on the surface of non-mucoid *P. aeruginosa* strains [16]. Although the mechanism of biofilms mediating host immune ineffectiveness and antibiotic resistance is not well defined [17], it is usually recognized that destructing biofilms or the exopolysaccharides secreted from bacteria is helpful to control bacterial mediated infection [14].

Phage-based therapeutic strategies have been reconsidered to prevent bacterial infection. Some studies have confirmed that some phages with depolymerase activity exhibit improved performance in terms of adsorption, invasion, decomposition, and biofilms destruction of target bacterial cells [18]. With the development of genetic engineering technologies, research on phage depolymerase has been increasing. Lin et al. cloned and expressed a capsule depolymerase from a *Klebsiella pneumoniae* phage, and found that this enzyme is specific for the capsule type of K1 [19]. Tait el al. reported that a cocktail of three phages with depolymerase activities eradicate *Enterobacter cloacae* biofilms completely [20]. These studies indicate that phage depolymerases might have potential application in controlling bacterial infection.

In this study, we expected to screen a *P. aeruginosa* phage capable of producing depolymerase and determined its antibiofilm effect and bactericidal contribution to serum.

Materials and methods

Bacteriophage isolation

The bacteria *P. aeruginosa* 1193 (*Pa.1193*) was collected and stored at the Beijing Institute of Microbiology and Epidemiology. *Pa.1193* was used to screen bacteriophages from raw sewage samples collected at a hospital. The sewage was filtered through a 0.22-μm filter. Then, 5 mL of the filtrate was mixed with 1 mL of *Pa.1193* in the exponential growth phase and 3 mL of 3 × Luria broth (LB). The mixture of bacteria and filtrate of sewage was incubated at 37 °C for 3 h to obtain more phages. when the mixture became clear, it was centrifuged and the supernatant was used for subsequent phage isolation. *Pa.1193* in the exponential growth phase was mixed with the above supernatant and then inoculated on double-layered agar plates. After overnight culture, a single clear plaque was selected and reamplified in the host bacterium *Pa.1193*. After three purifications by selecting a single clear plaque, the final selected plaque was inoculated into *Pa.1193* to amplify the bacteriophage, and the obtained phage was designated as IME180.

Whole genome sequencing and genome annotation

For genomic DNA preparation, 1×10^9 pfu of IME180 was used according to a previously published method [21] with some minor modifications. Briefly, the phage lysate solution was treated with DNase I and RNase A at 37 °C overnight and then inactivated at 80 °C for 15 min. The sample was mixed with lysis buffer (0.5% SDS, 20 mM EDTA, and 50 μg/mL proteinase K) and incubated at 56 °C for 1 h. An equal volume of a phenol and chloroform mixture was added to the above mixture for purification. The genomic DNA was precipitated with an equal volume of isopropanol and dissolved in nuclease-free water. The purified genomic DNA of IME180 was fragmented, ligated with adaptors, and then sequenced on the Ion Torrent Personal Genome Machine sequencer (Life Technologies, San Francisco, CA), according to the manufacturer's instructions. The complete genome sequence was assembled with Newbler software [22]. Potential coding sequences (CDSs) were predicted by RAST [23]. The putative functions of the CDSs were annotated by RAST and BLASTP against the National Center for Biotechnology Information (NCBI) database. The genomic sequence and annotated results were deposited in the NCBI database under accession number *MF788075*.

Cloning and confirmation of the phage-encoded depolymerase

The gene encoded depolymerase-like protein was annotated as depolymerase (DP) by BLASTP and the gene was amplified with forward primers containing BamH I site (5′-GGG GATCCATGAGTACG TTGAGAGTAGACACTCTAC-3′) and a reverse primer containing Not I site (5′-GGGCGG CCGCTTAACGGGTCATATAGGAGAACG-3′). After sequence confirmation by Sanger sequencing, the amplified PCR products were cloned into pET28a prokaryotic expression vectors and fused with an N-terminal His-tag. The recombinant plasmid (pET28-DP) was transformed into BL21 (DE3) competent cells. The cells containing recombinant plasmids were selected on LB agar plates in the presence of 30 μg/mL kanamycin and then cultured to the exponential growth phase, followed by induction at 37 °C for 4 h using 1 mM IPTG. Cells were collected by centrifugation

and then sonicated on ice. The bacterial lysate was centrifuged at 13,000 × *g* for 10 min at 4 °C, and the supernatant was filtrated through a 0.45-μm filter. The filtrate was applied to an Ni–NTA column and then washed with five column volumes of 100, 300, and 500 mM imidazole. The eluted products in various concentrations of imidazole were collected and electrophoresed by SDS–polyacrylamide gel electrophoresis (SDS-PAGE). The concentration of the expressed DP was determined by the Bradford assay, and then serial 10-fold dilutions of the depolymerase were prepared and transferred onto *Pa.1193* lawns to test the depolymerase activity.

Determination of the exopolysaccharide-degrading ability of IME180-encoded DP

To further determine the enzymatic activity of the expressed DP, the hot phenol method was used to extract bacterial exopolysaccharide, and a silver stain was used to observe the exopolysaccharide pattern [24]. Briefly, 1 mL of overnight-cultured *Pa.1193* was collected by centrifugation and dissolved in 270 μL phosphate-buffered saline (PBS), and then 30 μL of the purified DP (0.5 μg/μL), Dp4 (a depolymerase from a *Klebsella pneumoniae* phage; 0.5 μg/μL as a negative control), or PBS was added, followed by incubation at room temperature for 1 h. The treated bacteria were collected and resuspended in 150 μL deionized water. An equal volume of water-saturated phenol was added to the bacterial resuspension that was then vortexed vigorously. The mixture was incubated at 65 °C for 20 min and then mixed with an equal volume of chloroform. The mixture was centrifuged for 5 min at 10,000×*g*. The top immiscible phase was collected, mixed with protein loading buffer, and then boiled at 100 °C for 5 min. Twenty microliters of the mixture was separated on a 12% SDS-PAGE gel at 100 V for 1 h. The gel was then subjected to silver staining (PAGE rapid staining kit; Solarbio, Beijing, China).

Bactericidal contribution and biofilm inhibition mediated by the phage-encoded DP

For the bactericidal assay, 10 μL of overnight-cultured *Pa.1193* and 180 μL monkey serum (Bossbio, Beijing, China) were mixed in a 1.5 ml tube. and then combined with 10 μL purified DP (final concentration was 25 μg/mL) or an equal volume of PBS as the control. To ensure the accuracy of the results, we also mixed 10 μL of overnight-cultured *Pa.1193* and 190 μL PBS, and another experiment was that mixed 10 μL of overnight-cultured *Pa.1193* and 180 μL PBS and then combined with 10 μL purified DP (final concentration was 25 μg/mL). These mixtures were incubated at 37 °C for 3 h, serially diluted in PBS, and then plated on solid LB agar plates for bacterial enumeration. This bactericidal assay was independently performed three times, and the results were presented as the means of three experiments.

Pa.1193 biofilms were formed according to a previous report [25] with some minor modifications. An exponentially growing culture of *Pa.1193* ($OD_{600}=0.5$) was diluted to $OD_{600}=0.01$ using LB. For the biofilms inhibition assay, ninety microliters of the diluted culture was added to each well of a 96-well plate, 10 μL of purified DP was added to the corresponding wells at final concentrations of 6 or 30 μg/mL (PBS was used as the control), and the mixture were incubated at 37 °C for 18 h. Each group included six wells. The supernatant in each well was removed carefully and then washed twice with 150 μL saline. 150 μL of 0.1% crystal violet was added to each well and incubated at room temperature for 30 min. The wells were washed twice with 150 μL saline. The mixture of cold acetone and alcohol (1:4, 200 μL) was added to each well and incubated for 15 min to extract the crystal violet retained by the cells. The extract was used to determine the amount of biofilm by measuring its A_{590} with a Synergy HT Multi-Detection Microplate Reader (BioTek).

For the biofilms removal assay, 90 μL of the above diluted bacteria culture was added to each well of a 96-well plate, and the culture was incubated at 37 °C for 18 h. Subsequently, 10 μL of purified DP was added to the wells (PBS was used as the control), and the bacteria cultures were continued at 37 °C for additional 2 h. Each group included six wells. The residual biofilm in the wells were washed, stained, and extracted as described above. The A_{590} was measured to determine the amount of biofilm.

Statistical analysis

One-way ANOVA was conducted to analyze differences in bacterial counts. All analyses were performed using Prism 6 software (GraphPad, Inc., La Jolla, CA, USA). $p<0.01$ was considered as statistically significant.

Results

Identification of IME180-encoded depolymerase and complete sequence analysis

Commonly, a lytic bacteriophage forms a transparent plaque after infecting host bacteria. However, infection by phages that encode a depolymerase results in halo development around the plaques. Infection by the isolated IME180 phage was found to produce a clear plaque that was surrounded by a halo, and the scope of the halo was expanding as observed at 24, 48, and 72 h (Fig. 1). These results indicated that the isolated phage might encode a depolymerase. Results of annotation and BLASTP searching showed that the genome

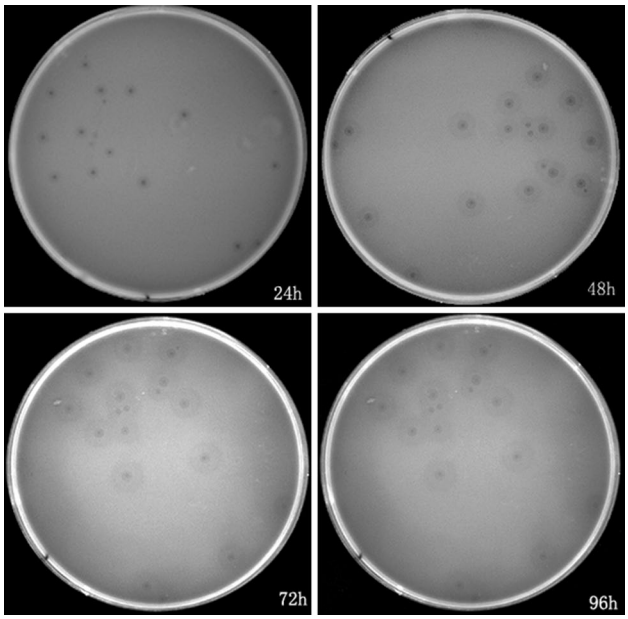

Fig. 1 Halo development on a lawn of *Pa.1193*. IME180 infection induced clear plaques on the lawn in an agar plate. A translucent halo appeared around the plaques at 24 h post-infection and kept increasing with incubation time, as observed at 48, 72, and 96 h

of IME180 contained 49,494 bp with 44.6% G + C content, 74 CDSs, and the phage genome had a high similarity with genomes of *P. aeruginosa* phage O4 (KU535860, 98%) and TC6 (MG676466, 96%). Phage O4 was isolated from seawater, and TC6 was isolated from hospital sewage. Both phages were classified into the family of *Podoviridae* under the order of *Caudovirales*. Based on International Committee on Taxonomy of Viruses guidelines and sequence similarity, IME180 was considered to be a member of *Podoviridae* and the genome of IME180 was a linear dsDNA. A genome map of the IME180 phage was drawn with DNA plotter and is presented in Fig. 2a.

A coding sequence with a length of 1395 bp in the genome of IME180 was found to be highly homologous to depolymerase that were previously proven to be exopolysaccharide-degrading enzymes [26]. As shown in Fig. 2b, the region of Lys^{71} to Met^{140} was predicted as Pectate_ lyase_3 super family and another region Leu^{29} to Leu^{108} was predicted as Glyco_hydro_28 super family by BLASTP. The coding sequence was designated as a depolymerase in the IME180 phage genome and used for the following assays. Other genes in IME180 are presented in Table 1.

Expression, purification, and activity determination of the IME180-encoded DP

The *E. coli* BL21 (DE3) cells harboring pET28-DP were induced, harvested, sonicated and centrifuged, then the supernatant was loaded on a gel to verify whether the protein was expressed (Fig. 3A). Most of the expressed DP was collected in 300 mM imidazole by protein purification (Fig. 3B). A specific protein band with a molecular weight of < 50 kDa appeared on the SDS-PAGE gel which was in agreement with the theoretical molecular weight of 48.65 kDa. After dialyzing against PBS, the purified DP was serially diluted to get four different concentrations, 500 μg/mL, 50 μg/mL, 5 μg/mL, and 0.5 μg/mL. We used 3 μL of the different concentrations DP to treat *Pa.1193* cells. Consequently, a halo appeared similarly to the phage infection on the *Pa.1193* lawn, even with 1.5×10^{-3} μg of the purified DP (Fig. 3c). These results indicated that the selected coding sequence encoded a depolymerase-like enzyme that was designated as DP in following experiments.

Determination of the ability to degrade exopolysaccharide by the phage-derived DP

As shown in Fig. 4, the expressed DP significantly degraded exopolysaccharides on the surface of *Pa.1193*, resulting in a ladder distribution pattern on the gel. In contrast, treatment with a confirmed *K. pneumoniae* phage depolymerase, Dp4, or PBS did not cause exopolysaccharide degradation on the surface of *Pa.1193*. Therefore, the depolymerase encoded by the IME180 phage had the ability to degrade host bacterial exopolysaccharides. These results further demonstrated that the isolated phage produced a halo during infection, which was due to the DP that was encoded by the phage genome.

Bactericidal contribution and biofilm inhibition mediated by the phage-encoded DP

In the bactericidal assay, we found no obvious decrease or increase in the number of *Pa.1193* when either serum or DP was applied to bacteria. However, when serum and DP were simultaneously applied to the bacteria, bacterial enumeration showed that the combined treatment reduced the number of *Pa.1193* on the agar plate by two orders of magnitude (Fig. 5a). This result suggested that the presence of DP decreases serum-invading bacteria to achieve bactericidal effects, and treatment with 25 μg/mL DP significantly enhanced serum-mediated bactericidal activity.

In the biofilm inhibition and disruption assay, 6 and 30 μg/mL DP was used to treat *Pa.1193*. The results showed that the DP derived from IME180 not only inhibited the formation of host bacterial biofilms, but also disrupted preformed host bacterial biofilms (Fig. 5b). These results also indicated that, although complete biofilm inhibition or removal was not observed, the phage-encoded DP exhibited dose-dependent inhibition or removal of *Pa.1193* biofilms.

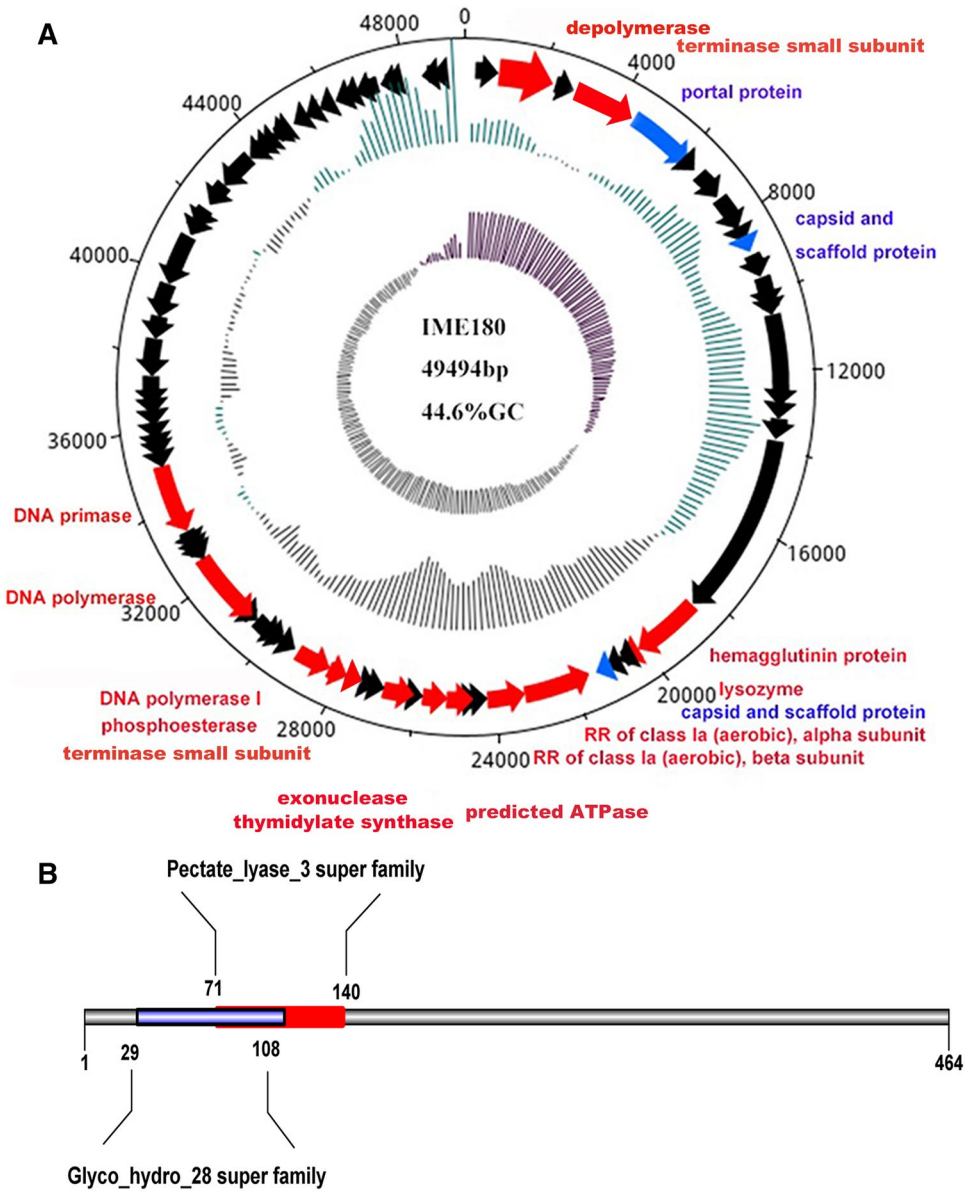

Fig. 2 Genome map of IME180 (**a**) and catalytic regions map of DP encoded by IME180 (**b**). **a** This map is divided into four circles. The first circle represents the full length genome and the second circle represents the sequences coding for amino acids in proteins. In the second circle, red arrows represent genes encoding functional proteins, blue arrows represent genes encoding structural genes, and black represent genes encoding hypothetical proteins. The third circle represents GC content, wherein gray and green represent less and more than the total genome average GC content, respectively. The fourth circle of purple and blue describes the GC skew of G−C/G+C, wherein purple is greater than 0, and blue is less than 0. **b** Conserved domains predicted by BLASTP. DP was a protein of 464 amino residues. The region of drawn red region Lys^{71} to Met^{140} was predicted as Pectate_ lyase_3 super family and blue region Leu^{29} to Leu^{108} was predicted as Glyco_hydro_28 super family by BLASTP

Discussion

In this study, a depolymerase from a lytic *P. aeruginosa* bacteriophage was identified and confirmed to actively degrade bacterial exopolysaccharides. In vitro application of the depolymerase inhibited biofilms formation and sensitized host *P. aeruginosa* to serum complement proteins.

Extracellular polymeric substances are found on bacterial surfaces, which are produced by bacteria when they live in biofilm communities [18]. EPS include polysaccharides, proteins, nucleic acids, and lipids [27]. Biofilms are frequently associated with healthcare contamination, anti-microbial resistance, and severe persistent infections because of their diffusion limitation [28, 29]. Recently, phage and phage-derived components have been reconsidered for use as therapeutic anti-microbial agents in the era of increasing emergence of multidrug-resistant and opportunistic bacterial pathogens [30, 31]. Phage-encoded depolymerases are commonly identified by a constantly increasing halo surrounding phage plaques and associated with degrading polymers on the bacterial surface or EPS involved in biofilms [32, 33]. Previous reports have shown that these phage-derived depolymerases are highly diverse and classified as sialidases, levanases, xylosidases, dextranases, hyaluronidases, peptidases,

Table 1 Features of bacteriophage IME180 gene products, and functional assignment

Gene	Start	End	Putative function	Length (aa)	BLASTP alignment	Organism	Percent identify	*E* value	GenBank
1	271	864	Hypothetical protein	197					
2	861	2255	Depolymerase	464	Pectin lyase fold	Vibrio phage	59%	1e−05	AUS02990.1
3	2308	2811	Hypothetical protein	167					
4	2815	4422	Terminase large subunit	535	Putative terminase large subunit	Citrobacter phage	64%	0.0	YP_009168386.1
5	4435	6189	Portal(connector) protein	584	Portal (connector) protein	Vibrio phage	56%	0.0	YP_009056231.1
6	6189	6446	Hypothetical protein	85					
7	6572	7330	Hypothetical protein	252					
8	7341	8282	Hypothetical protein	313					
9	8296	8652	Hypothetical protein	118					
10	8662	8928	Capsid and scaffold protein	88	Head fiber protein	Bacillus virus	57%	2e−04	AAA32281.1
11	8982	9602	Hypothetical protein	206					
12	9604	10,407	Hypothetical protein	267					
13	10,417	10,605	Hypothetical protein	62					
14	10,589	12,904	Hypothetical protein	771					
15	12,891	13,220	Hypothetical protein	109					
16	13,181	13,732	Hypothetical protein	183					
17	13,743	18,407	Hypothetical protein	1554					
18	18,407	20,185	Hemagglutinin protein	592	Hypothetical protein	Citrobacter phage	31%	9e−62	YP_009168398.1
19	20,172	20,609	Lysozyme	145	Putative lysozyme	Acinetobacter phage	63%	4e−48	AXF40585.1
20	20,611	20,757	Hypothetical protein	48					
21	20,759	21,118	Hypothetical protein	119					
22	21,118	21,396	Capsid and scaffold protein	92	Hypothetical protein	Variovorax paradoxus	99%	8e−56	PZQ78016.1
23	21,553	23,247	Ribonucleotide reductase of class Ia (aerobic), alpha subunit	564	Ribonucleoside-diphosph	Variovorax paradoxus	95%	0	PZQ78017.1
24	23,241	24,209	Ribonucleotide reductase of class Ia (aerobic), beta subunit	322	Ribonucleoside-diphosph	Vibrio phage	64%	1e−149	ASV43422.1
25	24,193	24,441	Hypothetical protein	82					

Table 1 (continued)

Gene	Start	End	Putative function	Length (aa)	BLASTP alignment	Organism	Percent identify	*E* value	GenBank
26	24,413	24,568	Hypothetical protein	51					
27	24,552	25,205	Phosphate starvation-inducible protein	217	Phosphate starvation-inducible protein	Vibrio phage	54%	4e−77	AUG88377.1
28	25198	25839	Thymidylate synthase	213	FAD-dependent thymidylate synthase	Vibrio diabolicus	65%	1e−104	WP_104972658.1
29	25820	26047	Hypothetical protein	75					
30	26049	26816	Exonuclease	255	3′–5′ Exonuclease	Salinivibrio phage			YP_007010541.1
31	26809	27060	Hypothetical protein	83					
32	27044	27412	Hypothetical protein	122					
33	27402	27824	Terminase small subunit	140	Terminase small subunit	Escherichia phage	35%	4e−18	YP_009208194.1
34	27799	28299	Phosphoesterase	166	Putative serine/threonine protein phosphatase	Acinetobacter phage	54%	1e−52	AXF40675.1
35	28278	29153	DNA polymerase I	291	5′–3′ Exonuclease	Vibrio phage	49%	1e−95	AUR96964.1
36	29282	29593	Hypothetical protein	103					
37	29586	29759	Hypothetical protein	57					
38	29753	30475	Hypothetical protein	240					
39	30475	30648	Hypothetical protein	57					
40	30657	32534	DNA polymerase	625	DNA polymerase	Salinivibrio phage	59%	0	YP_007010534.1
41	32527	32685	Hypothetical protein	52					
42	32678	32860	Hypothetical protein	60					
43	32847	33353	Hypothetical protein	168					
44	33353	35068	DNA primase/helicase	638	PutativeDNA primase/helicase	Citrobacter phage	52%	0	YP_009168430.1
45	35069	35341	Hypothetical protein	90					
46	35338	35514	Hypothetical protein	58					
47	35511	35807	Hypothetical protein	98					
48	35800	36099	Hypothetical protein	99					
49	36132	36416	Hypothetical protein	94					
50	36409	36639	Hypothetical protein	76					
51	36636	37385	Hypothetical protein	249					

Table 1 (continued)

Gene	Start	End	Putative function	Length (aa)	BLASTP alignment	Organism	Percent identify	*E* value	GenBank
52	37385	38335	Hypothetical protein	316					
53	38332	38880	Hypothetical protein	182					
54	38883	39743	Hypothetical protein	286					
55	39753	41024	Hypothetical protein	423					
56	41102	41293	Hypothetical protein	63					
57	41348	41875	Hypothetical protein	175					
58	42004	42624	Hypothetical protein	206					
59	42617	43549	Hypothetical protein	310					
60	43551	43781	Hypothetical protein	76					
61	43781	43951	Hypothetical protein	56					
62	43972	44292	Hypothetical protein	106					
63	44305	44433	Hypothetical protein	42					
64	44435	44677	Hypothetical protein	80					
65	44955	45239	Hypothetical protein	94					
66	45310	45708	Hypothetical protein	132					
67	45701	46063	Hypothetical protein	120					
68	46149	46373	Hypothetical protein	74					
69	46370	46561	Hypothetical protein	63					
70	46697	47290	Hypothetical protein	197					
71	47353	47502	Hypothetical protein	49					
72	47513	47779	Hypothetical protein	88					
73	48419	48568	Hypothetical protein	49					
74	48664	48879	Hypothetical protein	71					

BLASTP hits with an *E* value < 0.01 were included in the table

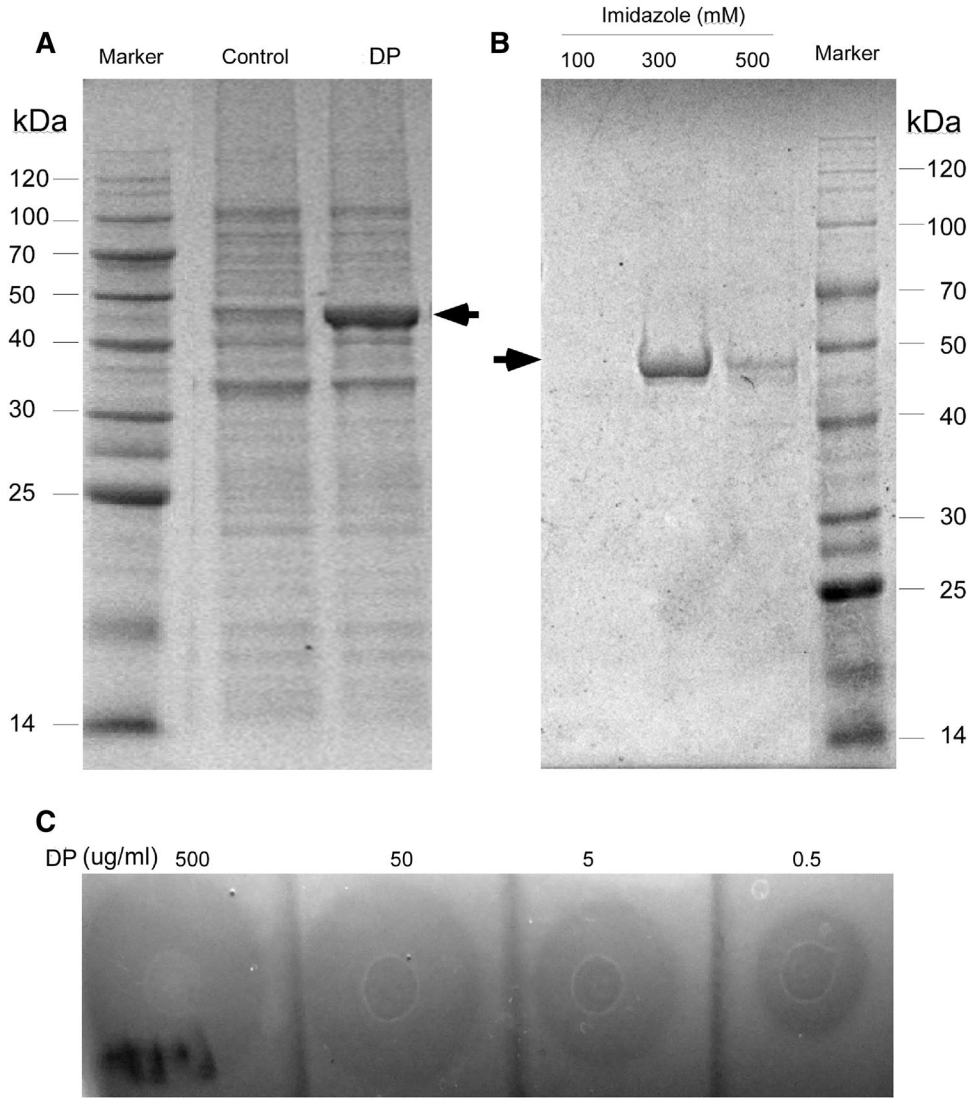

Fig. 3 Expression (**a**), purification (**b**) and activity confirmation of DP (**c**). A. Expression of IME180 DP was induced by IPTG, and a protein band with a molecular weight of < 50 kDa was confirmed by SDS-PAGE (12%), the lane marked as control was the supernatant of sonicated and centrifuged *E. coli* BL21 (DE3) cells harboring pET-28a (+) that induced using the same condition for depolymerase expression. B. His-tagged DP was eluted from an Ni–NTA column by 100, 300, and 500 mM imidazole and then detected on a 12% SDS-PAGE gel. C. The eluted DP was spotted on a lawn of *Pa.1193*, and the translucent halo began appearing at 30 min post-spotting. The results were obtained at 24 h post-spotting. The black arrow indicates the position of the expressed and purified DP on the gel

alginate lyases, and pectate lyases based on their targeted substrates [18]. As of 2015, 160 phage-derived depolymerases has been identified, but most of them were identified by prediction through genomic annotation. The diversity and biological roles of these phage-encoded depolymerases are not yet fully explored [18]. There are more than 200 genomes of *P. aeruginosa* phages in the NCBI database. However, few *P. aeruginosa* bacteriophage-encoded depolymerase genes have been confirmed and reported, although the first description of a *P. aeruginosa* bacteriophage-derived depolymerase was reported by Bartell et al. in 1966 [34]. A 37 kDa bacteriophage-derived alginate lyase was obtained by gel filtration chromatography and proven to increase *P. aeruginosa* susceptibility to phagocytosis [33]. With the exponential growth of fully sequenced phages, a substantial number of phage genomes have been deposited in public databases, and genomic annotation has predicted more phage-encoded depolymerases. However, considering that bacteriophages are highly diverse in their natural environment, it was thought that their depolymerases might have great diversity, and this characteristic often resulted in false prediction. Thus, identifying more phage-derived depolymerases through combined bioinformatics and molecular biology is still essential.

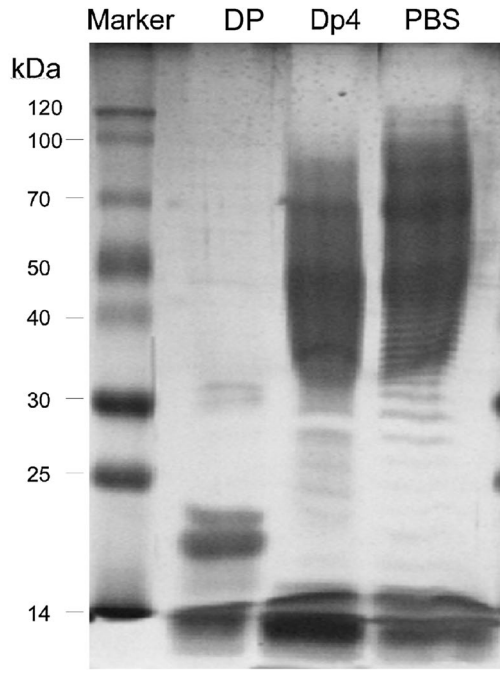

Fig. 4 Confirmation of phage depolymerase activity by degrading exopolysaccharides on the surface of bacteria. The hot phenol method was used to extract bacterial polysaccharide after treatment with 25 μg/mL DP. The degraded exopolysaccharides were separated on a 12% SDS-PAGE gel, and silver staining was performed on the gel. Dp4 is a depolymerase from a *K. pneumoniae* phage, which was used as the control

Commonly, most bacteriophage depolymerases are located in structural modules of phage genomes, including tail fibers, base plates, and the neck, or in close proximity to such genes, and some are distant from any structural genes [18]. As the most plentiful microorganisms on the planet, the amount of bacteriophages has been estimated to be ten-fold higher than that of their host bacteria. All of these factors, including the richness of bacteriophages, diversity of depolymerases, and their locations on phage genomes, often made identification of a phage-derived depolymerase difficult.

This study suggested that solely targeting exopolysaccharides might be difficult to eradicate biofilms because of their complex composition. A similar bactericidal pattern was also found in the serum-mediated anti-microbial assay. Despite a high dose of the depolymerase, 10% of the *P. aeruginosa* evaded serum-mediated disinfection and survived the combined treatment in this study. Therefore, the combination of phage-derived depolymerases and other anti-microbial agents such as antibiotics might be helpful to control antibiotic-resistant bacterial infections. In addition, identifying more depolymerases encoded by bacteriophages and exploring their biological functions may facilitate clarifying the mechanism of depolymerase-mediated bactericidal activity and enrich the library of phage-derived anti-microbial agents.

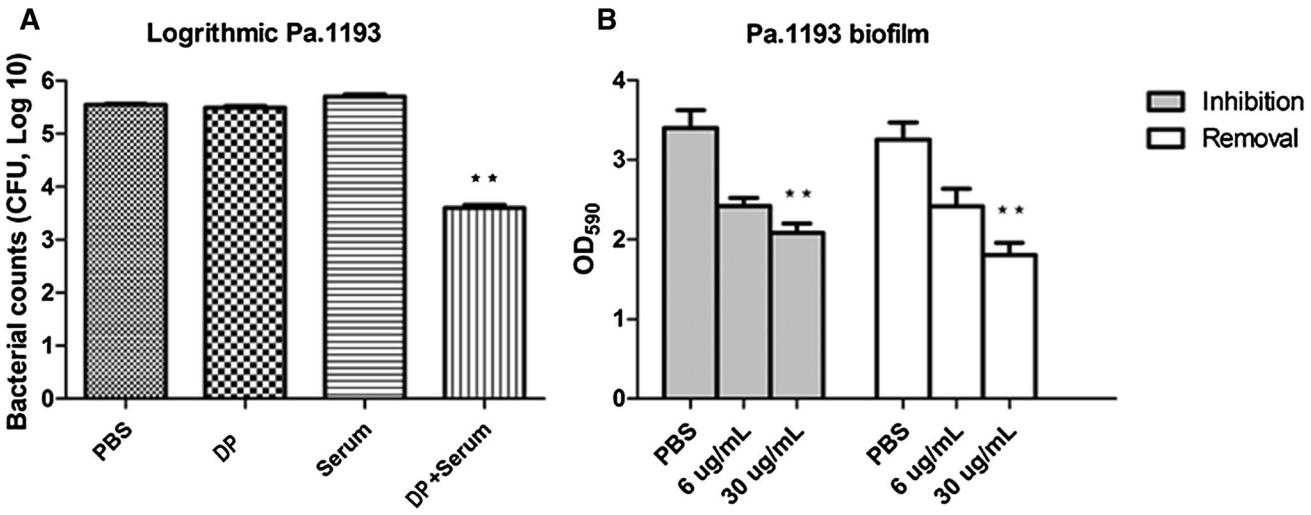

Fig. 5 DP increases host bacteria susceptibility to serum (**a**) and affects biofilm growth (**b**). **a** Treatment with 25 μg/mL DP significantly enhanced serum-mediated bactericidal activity. **b** Inhibition of *Pa.1193* biofilm growth and removal of the preformed biofilm were observed in the presence of 6 or 30 μg/mL DP. The double black star indicates a significant difference compared with the group without depolymerase treatment ($p < 0.01$)

Author contributions MLY conducted the identification, expression, and evaluation of the depolymerase and drafted the manuscript. LYN screened and identified the *Pa.1193* bacterium. WC performed the exopolysaccharide extraction and staining. GS performed the bacterial biofilm experiment. HTT isolated the IME180 phage. XSZ conducted the depolymerase purification. HY, FH, and ZXLL performed genomic analyses and annotation. TYG, YWG, and MZQ revised the manuscript. BCQ and HF conceived and designed the experiments. All authors read and approved the final manuscript.

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Compliance with ethical standards

Conflict of interest The authors have no conflicts of interest to declare.

Research involving human participants and/or animals All procedures involving animals were in accordance with ethical standards.

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