

RESEARCH ARTICLE

The Tail Associated Protein of *Acinetobacter baumannii* Phage Φ AB6 Is the Host Specificity Determinant Possessing Exopolysaccharide Depolymerase Activity

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Citation: Lai M-J, Chang K-C, Huang S-W, Luo C-H, Chiou P-Y, Wu C-C, et al. (2016) The Tail Associated Protein of *Acinetobacter baumannii* Phage Φ AB6 Is the Host Specificity Determinant Possessing Exopolysaccharide Depolymerase Activity. PLoS ONE 11(4): e0153361. doi:10.1371/journal.pone.0153361

Editor: Mark J van Raaij, Centro Nacional de Biotecnología - CSIC / CIF Q2818002D, SPAIN

Received: February 23, 2016

Accepted: March 29, 2016

Published: April 14, 2016

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Data Availability Statement: All relevant data are within the paper.

Funding: This work was supported by grants TCSP98-03-01 from Buddhist Tzu Chi General Hospital and TCIRP98003-02 from Tzu Chi University. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

Abstract

Acinetobacter baumannii is a non-fermenting, gram-negative bacterium. In recent years, the frequency of *A. baumannii* infections has continued to increase, and multidrug-resistant strains are emerging in hospitalized patients. Therefore, as therapeutic options become limited, the potential of phages as natural antimicrobial agents to control infections is worth reconsidering. In our previous study, we isolated ten virulent double-stranded DNA *A. baumannii* phages, ϕ AB1–9 and ϕ AB11, and found that each has a narrow host range. Many reports indicate that receptor-binding protein of phage mediates host recognition; however, understanding of the specific interactions between *A. baumannii* and phages remains very limited. In this study, host determinants of *A. baumannii* phages were investigated. Sequence comparison of ϕ AB6 and ϕ AB1 revealed high degrees of conservation among their genes except the tail fiber protein (ORF41 in ϕ AB1 and ORF40 in ϕ AB6). Furthermore, we found that ORF40 ^{ϕ AB6} has polysaccharide depolymerase activity capable of hydrolyzing the *A. baumannii* exopolysaccharide and is a component of the phage tail apparatus determining host specificity. Thus, the lytic phages and their associated depolymerase not only have potential as alternative therapeutic agents for treating *A. baumannii* infections but also provide useful and highly specific tools for studying host strain exopolysaccharides and producing glycoconjugate vaccines.

Introduction

Acinetobacter baumannii is a non-fermenting, gram-negative bacterium. According to the 2014 report by the Center for Disease Control, Taiwan, *A. baumannii* is an emerging pathogen

ranked first among gram-negative bacteria that can cause outbreaks of nosocomial infections such as bloodstream infections, endocarditis, ventilator-acquired pneumonia, meningitis, urinary tract infections, and wound infections with rising antibiotic resistance rates [1]. Therefore, as therapeutic options become limited, the search for novel agents becomes a priority. To fight this bacterium, one of the promising alternative approaches to conventional antibiotics treatment is phage therapy. Bacteriophages (phages) are non-cellular microorganisms that infect and lyse specific bacteria. Phages and their products are receiving renewed attention as bioagents for the treatment or prophylaxis of bacterial infectious diseases. However, the high host specificity of phages narrows the potential application of phage therapy. Therefore, an understanding of phage specificity is needed to predict the success and consequences of phage therapy.

The first step of phage infection is adsorption of the phage virion to a susceptible host cell, which is determined by the specific interaction between phage receptor-binding protein (RBP) and a specific receptor on the surface of the host cell [2–4]. To overcome the host specificity barriers, it is vital to understand the appendages of phages used to recognize the receptors on the host cell surface. The detailed recognition mechanisms vary among phages [5]. Most of the information on RBPs and phage-host interactions comes from studies carried out on coliphages and lactic acid phages. These RBPs have been identified as the central tail fibers or protruding baseplate proteins and have been well studied. For example, the major host range determinant in T-even phages is the distal end of their long tail fibers, which recognizes receptors on the surface of the *Escherichia coli* host [6]. Wang et al. [7] demonstrated that only the C-terminal 249 amino acids of the tail fiber protein in λ phage could bind with the LamB receptor and are thus involved in host specificity. In contrast, T5 and BF23 phages use the tail shaft, which is located above the straight tail fiber, as an RBP [8,9], and the RBPs from *Lactococcal* phages TP901-1 and Tuc2009 are lower baseplate proteins [10].

Unfortunately, phage-host interactions remain poorly understood in *A. baumannii*, even as increasing numbers of novel phages are reported. In our previous study, we isolated ten virulent double-stranded DNA bacteriophages, ϕ AB1–9 and ϕ AB11, and found that each has a narrow host range [11], indicating that the RBPs vary. In addition, we previously published the complete genome of the first *A. baumannii* lytic phage ϕ AB1 [12]. Phage ϕ AB1 possesses an isometric head of 60 nm, a short tail of 9×11 nm, and a linear double-stranded DNA genome containing 41,526 bp with a GC content of 39%. We identified 46 open reading frames (ORFs), including a putative tail fiber protein assigned as ORF41. Moreover, the 150 N-terminal residues of ORF41 were similar to the N-terminal regions of gp49 from LKA1 and gp38 from ϕ KMV, a conserved region found among tail fibers of the T7-like group [13] in a BLASTp analysis. Ceysen et al. [13] reported that the C-terminal regions of the tail fiber proteins in LKA1 are responsible for binding to the host-surface receptor. Thus, based on the bioinformatic analysis, we speculate that the gene product of *orf41* is important for ϕ AB1 recognition of *A. baumannii*: the 150 N-terminal residues are responsible for attaching the tail fibers to the phage virion, and the C-terminal region is responsible for specific binding to the host-surface receptor.

In this study, we sequenced the genome of phage ϕ AB6, which belongs to the same Autographivirinae subfamily as ϕ AB1 but exhibits a different host range. Analysis of the similarities and differences of these phages will provide a useful model for identifying and studying the phage genetic determinant (RBP) for host specificity.

Materials and Methods

Bacterial strains and phages

A. baumannii M68316 and 54149 were used as the propagation and the indicator host of ϕ AB1 and ϕ AB6, respectively. Phage sensitivity was tested by spot tests, which were performed by dropping 5 μ L phage lysate (ca. 1×10^9 PFU/mL) onto a lawn of *A. baumannii* cells in the top agar. Clearing zone formation indicated that the cells were phage sensitive.

Plaque and adsorption assay

Phage titers were determined by plaque assay on host strain M68316 or 54149 in an LB agar plate, and adsorption assays were performed as previously described [11]. Briefly, *A. baumannii* cells (0.6 U of OD₆₀₀) were infected with phages at an MOI of 0.0001, and samples (100 μ L) were collected after 5-min incubation at 37°C with shaking, diluted in 0.9 mL cold LB, and centrifuged (12,000 \times g, 5 min). The supernatants were titrated by plaque assay. Percent adsorption of the phage was calculated as [(initial titer – residual titer in the supernatant)/initial titer] \times 100%. Error bars represent the standard deviation of the three independent experiments.

Protein expression and purification

A fragment of *orf40* ^{ϕ AB6}, encoding a putative polysaccharide depolymerase, was amplified from purified phage ϕ AB6 by PCR using the primers AB6TFF1 (5' -GGATCCATGAATATACTACGCTCATTTA-3') and AB6TFR1 (5' -GTCGACTTAACTCGTTGCTGTAAATG-3'). Underlined nucleotides indicate recognition sequences for *Bam*HI and *Sal*I. The PCR fragment was excised by *Bam*HI and *Sal*I inserted into the pET-30a expression vector (Novagen, Madison, WI, USA). The resulting pET-*orf40* ^{ϕ AB6} plasmid was transformed into *E. coli* BL21 (DE3) cells. The recombinant His-tagged ORF40 protein was expressed under 0.1 mM IPTG induction at 20°C overnight then purified from the soluble fraction using a Ni-NTA column (Qiagen, Valencia, CA) according to the manufacturer's instructions and analyzed by SDS-PAGE.

Polyclonal antibody preparation

Polyclonal antibodies were raised in rabbits by Protech Technology Enterprise Co., Ltd. (Taipei, Taiwan) using its standard protocol. Initial immunizations of ORF40 ^{ϕ AB6} were complemented with Freund's complete adjuvant with five subsequent booster injections of protein. Protein concentrations in the initial immunization and booster injections were 150–200 μ g/mL. The final serum samples were acquired 11 weeks after the initial immunizations.

Determination of the polysaccharide depolymerase activity

The spot test was performed to observe polysaccharide depolymerase activity [14]. In brief, LB agar in a petri dish was overlaid with 0.7% top agar inoculated with 300 μ L fresh bacterial culture. Phage or purified recombinant polysaccharide depolymerase (1 μ L, 1 μ g/ μ L) was spotted onto the double-layer plate. After overnight incubation at 37°C, plates were observed for formation of lytic or semi-clear spots.

Immunogold detection of ORF40 ^{ϕ AB6} in virions

For gold immunolabeling, purified phage particles were dialyzed and incubated overnight at room temperature with primary antibody, anti-ORF40 ^{ϕ AB6}, diluted 1:1000 in buffer. Next, 6-nm gold-conjugated goat anti-rabbit immunoglobulin G secondary antibody (Electron

Microscopy Sciences, PA, USA) diluted 1:20 (wt/wt) with buffer was added for 2 h at room temperature. After fixation for 30 min at room temperature in phosphate-buffered saline with 0.25% glutaraldehyde, virions were negatively stained with 2% uranyl acetate and visualized by transmission electron microscopy.

φAB6 genome DNA sequencing

Phage φAB6 DNA was sequenced using pyrosequencing (454 technology). A total of 22,736 reads were assembled to a single contig at 122-fold coverage. The location of the terminal repeats was suggested using the φAB1 genome (GenBank accession number HQ186308) as the reference and confirmed with primer walking of the φAB6 DNA. Genes were predicted using GeneMark.hmm [15] and annotated using NCBI-BLAST [16]. The genomic sequence of φAB6 was deposited in GenBank (accession number KT339321).

Construction of chimeric phage φAB1tf6

To construct the chimeric phage φAB1tf6 carrying the tail fiber protein (ORF40) of φAB6 instead of φAB1 ORF41, overlapping PCR was used. A fragment containing upstream *orf41* with a unique *Ban*II restriction site was PCR amplified from φAB1 DNA with the primers AB1gp41F (5' -AGTACACCTAGTGGGGCTCC-3') and AB1gp42FR (5' -TAGCTTCTTGTCCTTGTTGCA-3'). The DNA fragment comprising the φAB6 tail fiber to the DNA maturase containing a unique *Bgl*II restriction site was amplified from φAB6 DNA with the primers AB1gp42FF (5' -TGCAACAAGCACAAGAAGCTA-3') and AB1gp45R (5' -GTTGTGCTTC CGATAGATCTTT-3'). The φAB1 *orf41* and φAB6 *orf40* fusion DNA fragment was amplified from the two amplicons described above using overlapping PCR with primers AB1gp41F and AB1gp45R, and the region of primers AB1gp42FR and AB1gp42FF was designed to link the φAB1 *orf41* and φAB6 *orf40*. Chimeric phage φAB1tf6 was generated by electroporation of the ligated left *Ban*II arm, the right *Bgl*II arm of φAB1, and *orf40* containing the *Ban*II-*Bgl*II DNA fragment of φAB6 into *A. baumannii* strain 54149 using a Bio-Rad Gene Pulser II system at 2.5 kV and 25 μF with the pulse controller adjusted to 200 Ω. A single plaque was subsequently isolated and amplified with *A. baumannii* strain 54149 to check the chimeric phage φAB1tf6. To confirm the recombinant φAB1tf6 phage carried *orf40* from φAB6, a set of primers (pf1: 5' -GCGTAATGGTAAGGAAC-3' and pr3: 5' -TCGCATCTACTTGAGTTT-3') annealing upstream and downstream of *orf40*, which is homologous in φAB1 and φAB6, was used.

Results

Phage characteristics

Phages φAB1 and φAB6 were isolated from sewage water in our laboratory, as reported previously [11]. Electron microscopy revealed that they are members of Podoviridae, both with a head diameter of approximately 60 nm and a short tail with 9-nm diameter and 11-nm length [11]. In addition, the host spectrum of each phage was different: φAB1 was shown to infect *A. baumannii* strain M68316 and φAB6 to infect strain 54149. In this study, adsorption assays were performed to test whether host specificity was determined by initial adsorption or some other resistance mechanisms. The results demonstrated that φAB1 was unable to adsorb to strain 54149, and φAB6 failed to bind to strain M68316 (see below in section "Construction and characterization of chimeric phage φAB1tf6 with altered host specificity"), suggesting that φAB1 and φAB6 may encode different RBPs that are responsible for their differential host specificity. In host range test, φAB1 lysed 23% (191/832) of the clinical *A. baumannii* isolates:

in contrast, ϕ AB6 was able to lyse 58% (483/832) of the isolates, indicating that ϕ AB6 has a much wider host range than ϕ AB1.

Genome sequencing and ORF identification for ϕ AB6

To carry out molecular characterization of the phage, we previously determined the sequence for the whole genome of ϕ AB1 [12]. In this study, we determined the sequence of the ϕ AB6 genome. The sequencing results revealed that the ϕ AB6 genome is 40,559 bp in size; includes direct terminal repeats (DTRs) of 410 bp, as determined by a combination of shotgun and primer walking approaches and confirmed by direct sequencing with outward-directed primers, leading to termination of the sequencing reaction at the ends of the genome; and has a GC content of 39%, which is similar to that of the host *A. baumannii*. Analysis showed that ϕ AB6 (accession number KT339321) shares 82.3% DNA sequence similarity with ϕ AB1 (accession number HQ186308). Potential ORFs of ϕ AB6 were identified based on gene prediction tools, tBLAST homology searches, and visual inspection of potential ORFs and their Shine-Dalgarno sequences, leading to prediction of 46 ORFs. The genome organization was largely conserved with that of ϕ AB1 (Table 1), although some notable differences were observed: 1) ORF6, 17, and 19 of ϕ AB1 are absent from ϕ AB6, and ORF9, 12, and 18 of ϕ AB6 are absent from ϕ AB1; 2) the DNA polymerase gene is separated into two domains (ORF18 and ORF20) by the putative HNH-AP2 endonuclease gene (ORF19) in ϕ AB1 but it remains a complete ORF encoding 766 aa in ϕ AB6 (ORF19); and, most interestingly, 3) there was a low degree of identity (34%) in amino acid sequence between the predicted tail fibers of ϕ AB1 (ORF41) and ϕ AB6 (ORF40), suggesting that the tail fiber protein might be the determinant for host specificity.

BLASTp analysis revealed that the 150-aa N-terminal domain of ORF40 ^{ϕ AB6} is similar to the N-terminal regions of LKA1 gp49 and ϕ KMV gp38, which are conserved among tail fibers of the T7-like group [13]. In addition, amino acid sequence alignment of ORF41 ^{ϕ AB1} and ORF40 ^{ϕ AB6} showed that their N-terminal 198 aa shared 95% identity; however, almost no similarity was observed in their C-terminal regions (Fig 1A). Further, the central domain of 194 residues (aa 226–419) of ORF40 ^{ϕ AB6}, which is not present in ORF41 ^{ϕ AB1}, has similarity to the pectate lyase 3 superfamily domain (Fig 1B), which is most closely related to glycosyl hydrolase. This finding suggests that this region may be able to hydrolyze the glucosidic bond of bacterial exopolysaccharides. These observations imply that the 198 N-terminal residues of ORF40 ^{ϕ AB6} are responsible for attaching to the phage virion and that the C-terminal residues are responsible for host specificity and exopolysaccharide depolymerization, thus destroying the integrity of the host cell wall for phage entry.

To determine the location of ORF40 ^{ϕ AB6}, we performed immunogold electron microscopy analysis. As shown in Fig 2, the electron-dense gold-labeled antibodies appeared as black spots attaching to the tail of ϕ AB6, demonstrating ORF40 ^{ϕ AB6} to be the tail fiber protein.

Analysis of the polysaccharide depolymerization activity of ORF40 ^{ϕ AB6}

The plaques manifested by ϕ AB6 were surrounded by translucent halos (Fig 3), indicating that host cell exopolysaccharides were depolymerized. This phenomenon suggested that the phage produced a depolymerase enzyme that could diffuse through the agar layer to degrade bacterial exopolysaccharides into oligosaccharide units during infection. It is known that the tail spike or tail fiber often exhibits depolymerase activity [17]. To test whether this was true for the ϕ AB6 tail fiber, we cloned *orf40* ^{ϕ AB6} into the pET30a expression vector and purified the His-tagged ORF40 ^{ϕ AB6} protein. As expected, recombinant protein with a size of 77 kDa was obtained (Fig 4A, lane 3). In spot tests, the purified recombinant ORF40 ^{ϕ AB6} protein caused depolymerization of the exopolysaccharide of host strain 54149, forming translucent halos, but

Table 1. Comparison of the putative open reading frames (ORFs) of ϕ AB6 with ϕ AB1.

ϕ AB6				Predicted function	ϕ AB1				% aa identity
ORF	Start	End	Length (aa)		ORF	Start	End	Length (aa)	
1	1582	2091	169	Hypothetical protein	1	1574	2083	169	95.8
2	2093	2494	133		2	2085	2486	133	96.2
3	2481	2714	77		3	2473	2706	77	100.0
4	2789	3385	198		4	2781	3377	198	99.5
5	3645	4043	132		5	3637	4035	132	99.2
					6	4025	4132	35	
6	4122	4601	159		7	4211	4690	159	100.0
7	4603	5037	144		8	4692	5126	144	99.3
8	5048	5215	55		9	5137	5304	55	100.0
9	5202	5393	63						
10	5390	5623	77		10	5478	5696	72	52.0
11	5613	5822	69		11	5686	5904	72	57.5
12	5844	6920	148	HNH endonuclease	17				45.2
					24				39.3
13	6620	7075	151	DNA primase	12	5894	6694	266	96.0
14	7075	7392	105		13	6694	7011	105	96.2
15	7392	7628	78		14	7011	7247	78	98.7
16	7642	8940	432	DNA helicase	15	7260	8558	432	100.0
17	8943	9923	326	ATP-dependent DNA ligase	16	8555	9541	328	99.4
18	9916	9999	27						
				HNH endonuclease	17	9801	10247	148	
19	10241	12541	766	DNA polymerase	18	10244	11104	286	96.2 ^a
				HNH endonuclease	19	11088	11525	145	
				DNA polymerase	20	11494	12978	494	91.3 ^b
20	12550	13029	159	HNH endonuclease	21	12987	13466	159	98.1
21	13047	13937	296	Hypothetical protein	22	13484	14374	296	100.0
22	14146	15120	318	DNA exonuclease	23	14583	15539	318	98.7
23	15083	15520	145	HNH endonuclease	24	15520	15957	145	95.9
24	15517	15957	146	DNA endonuclease VII	25	15954	16394	146	98.4
25	15961	16896	311	Hypothetical protein	26	16398	17333	311	100.0
26	16896	17546	216	dNMP kinase	27	17333	17983	216	96.3
27	17555	19972	805	RNA polymerase	28	17992	20409	805	98.0
28	20075	20272	65		29	20512	20709	65	98.5
29	20262	20520	83	Structural protein	30	20706	20957	83	97.6
30	20529	22805	518	Head-tail connector protein	31	20966	22522	518	99.4
31	22094	22954	286	Scaffolding protein	32	22531	23391	286	96.5
32	22970	24001	343	Capsid protein	33	23407	24438	343	98.8
33	24036	24241	61		34	24491	24676	61	95.1
34	24253	24546	97	Hypothetical protein	35	24688	24981	97	95.9
35	24737	25297	186	Tail tubular protein A	36	25106	25732	208	98.9
36	25306	27597	763	Tail tubular protein B	37	25741	28032	763	98.7
37	27597	28268	223	Internal virion protein B	38	28032	28706	224	96.3
38	28281	31166	961	Structural protein	39	28719	31604	961	98.2
39	31176	34274	1032	Internal virion (core) protein	40	31614	34712	1032	98.6
40	34281	36380	699	Tail fiber	41	34719	37367	882	95.5 ^c
41	36511	36846	111	Holin	42	37484	37813	109	100.0

(Continued)

Table 1. (Continued)

φAB6					φAB1				
ORF	Start	End	Length (aa)	Predicted function	ORF	Start	End	Length (aa)	% aa identity
42	36833	37390	185	Chitinase-like endolysin	43	37800	38357	185	96.8
43	37509	37817	102	DNA maturase A	44	38476	38784	102	99.0
44	37827	39764	645	DNA maturase B	45	38794	40731	645	99.5
45	39855	40058	67		46	40822	41025	67	95.5

^aIdentity is calculated based on the first 290 residues of ORF19 in φAB6 aligned to ORF18 in φAB1.

^bIdentity is calculated based on the C terminus (amino acids 290–766) of ORF19 in φAB6 aligned to ORF20 in φAB1.

^cIdentity is calculated based on the alignment between the first 200 residues of the two proteins.

doi:10.1371/journal.pone.0153361.t001

not that of non-host strain M68316 (Fig 4B). In parallel experiments, *orf41*^{φAB1} was cloned and expressed in *E. coli*. However, the recombinant protein ORF41^{φAB1} (97 kDa; Fig 4A, lane 6) did not exhibit depolymerase activity (Fig 4B).

Construction and characterization of chimeric phage φAB1tf6 with altered host specificity

Given that the phage tail fiber is often responsible for the adsorption specificity of phages [5,18], we hypothesized that replacement of ORF41 in phage φAB1 with ORF40 from phage φAB6 would lead to infection of strain 54149 instead of strain M68316. We therefore constructed the chimeric phage φAB1tf6. The resulting chimeric φAB1tf6 phage was confirmed by PCR using pf1 and pr3 primers, which were designed to amplify a 3.5-kb fragment from φAB1 and a 2.9-kb fragment from φAB6. When *orf41*^{φAB1} was successfully replaced by *orf40*^{φAB6}, i.e., φAB1tf6 was used as the template, pf1-pr3 primers amplified a PCR product of the same size as that from φAB6 (Fig 5). In addition, *SphI*-digested restriction fragment length polymorphism (RFLP) was used to verify that gene swapping had indeed occurred. As shown in Fig 6, the restriction pattern of φAB1tf6 (1, 2, 2.2, 9, and >12 kb) was almost identical to that of φAB1 (1, 2, 2.2, 3.6, 6, and >12 kb) except that the 3.6- and 6-kb fragments in φAB1 were missing and replaced by a 9-kb fragment, indicating that the *orf41* region was derived from φAB6. Both the PCR and RFLP results indicated that the obtained chimeric phage φAB1tf6 was recombinant and not a contamination of other unrelated phages. Spot assays showed that the chimeric φAB1tf6 was able to form clearing zones on strain 54149 but not on the original host strain M68316 for φAB1 (Fig 7). To test if the inability of φAB1tf6 to infect M68316 was due to a defect in phage adsorption, an adsorption assay was performed. The result (Fig 8) showed that φAB1tf6 displayed strong binding to 54149 but limited adsorption to M68316. The adsorption capability observed for the chimeric φAB1tf6 was similar to that of φAB6 but different from that of its parental phage φAB1. These data indicated that replacement of the tail fiber gene changed the host specificity of phage φAB1.

Discussion

Multidrug-resistant *A. baumannii* causes severe nosocomial infections among immunocompromised individuals [19]. Due to the paucity in development of novel antibiotics, phage therapy has been proposed as a potential alternative treatment for multidrug-resistant *A. baumannii* infection. However, knowledge about the mechanisms of phage infectivity and phage resistance in *A. baumannii* remains limited. In particular, little is known about the initial

(A)

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ORF41 $\phi$ AB1 MNILRSFTETVVTTPDTFPISFEYDEKYDAVHVFLNDVAVEDLGYTVSQVNAVTLKVEP 60
ORF40 $\phi$ AB6 MNILRSFTETVVTTPDIFPISFEYDEKYDAVHVFLNDVAVEDLGYTVSQVNAVTLKIEP 60

ORF41 $\phi$ AB1 AITEGTVRIERETDIDKMKYIFDAGALFIDQNVDADFRQIVHSQQEVRDGFIKLRGDVLP 120
ORF40 $\phi$ AB6 AIPDGTVRIERETDIDKMKYIFDAGALFIDQNVDADFRQIVHSQQEVRDGFIKLRGDVLP 120

ORF41 $\phi$ AB1 LVHGLQEALQQAQEASEAAQEANAEVAAAQTQYLKYFNPEIVPKNARIMLDNGDIV 180
ORF40 $\phi$ AB6 LVHGLQEALQQAQEASEAAQEANAEVAASQTQYLKYFNPEIVPKNARIMLDNGDIV 180

ORF41 $\phi$ AB1 RSIVANNIVNPNVDMTGWFNEGTNVGFVESIADMLAINNPHNGQVVYVKGYAATNSALA 240
ORF40 $\phi$ AB6 RSTVVNNTSNPNVDMTGWVKVSSSQIFDET---YNITQ----- 216

ORF41 $\phi$ AB1 QPSGGGHRIYVASRKTENDGFLCINGWVLQVENNTVTPEHAGAIGNNKDFDQSIPLNKV 300
ORF40 $\phi$ AB6 -----SVINGNLITVDNFGAK-----GDGVTDDSAA 242

ORF41 $\phi$ AB1 VNSGFDVSLSATTYYVSKPVRKWKRGGKITGTGELTTKIVKTTNNIEGFGKTTINGKVMN 360
ORF40 $\phi$ AB6 FQAYCDSALTGQNLYLGAKGRYILKN---QVDLKGKLVGNGCGKVSEFYNLGCIDVD- 298

ORF41 $\phi$ AB1 FDVDAVLILMPDVSQSDWYAQQLHLNGFSLSYDSLTNRGYGLYAPLICQSSLRYISISN 420
ORF40 $\phi$ AB6 -----GSSPDLQGKTAFINCGPTIQNLTARCSNGAGKQVSFIEIDG 339

ORF41 $\phi$ AB1 CANGVYSDSWMVTWERVEASADKPFIFGAPETNWLPNNTSQTFISCWAKNAKGSDSYAW 480
ORF40 $\phi$ AB6 YLANIDHITLINFYNQIVVKQALVGFNFTNAWLYSQNAGIYCEDPLNRVSTTGTFHNIY 399

ORF41 $\phi$ AB1 YLLNMQYSTFIS---CGNDFNGTDGSPINSLIYMRYSDVSFIDMGFEFAHAYNLLYCEDS 537
ORF40 $\phi$ AB6 FQLGDGHAMIFDRDVHGCDFDNIIFESM-----NGIKARTVAHCGFGKFW 445

ORF41 $\phi$ AB1 VANFVAPNIVGFENKYQREVTWNTYNAMFNCVSNSTVKISGRINTMYTSTNSTTVAAFS 597
ORF40 $\phi$ AB6 CENLKTATSKDWLEVTGANSCYGNSFTGYVKLLGGWTSKTSPTLDSLPTN---NYGGVS 501

ORF41 $\phi$ AB1 MCQGNSKLIVDLPIRFEGSNRLEEIAKTTSSTALLPAFGMYCDSTSKVDGYINDHIVLA 657
ORF40 $\phi$ AB6 VSAEG-----ISIVNAG---NKAKMLMLPSGFKTGNATIDETHIS 538

ORF41 $\phi$ AB1 PTNDRFLTTSQIRSSPINPQDVNLNDIFGFAGNYAQPSGALAQASRNYPESFVATDILSM 717
ORF40 $\phi$ AB6 SSTVTPLVKRRVIGADSSGAQYLASDTYTKLSRKWGTYNHGSNNAGAFYAPMMLTYDQSF 598

ORF41 $\phi$ AB1 SNTQIAGVSYDGGGNLYFRVKNWSFGINAWRQILSSGNTTVDANGFIKAASPIVKLFAD 777
ORF40 $\phi$ AB6 STPQNN-----NGWKIVKESTGVYRVERVSGNTSVITNGHIVVGSPLMGSRLG 646

ORF41 $\phi$ AB1 KIELNDEAEQNIIFEKLGVGDYLIKNSAGFSNDGWYIEAPKDANGNLFALVYKTLENG 837
ORF40 $\phi$ AB6 TGTGATHGIQM-----IETYAGSWTSY-----TEAAGFKVFWRDSSNA 684

ORF41 $\phi$ AB1 DISVKTYAKKFDIETASIVADISKPVDIKKDRWIDIRLNDINLKD 882
ORF40 $\phi$ AB6 LVDPHRFTVAFTATS----- 699
    
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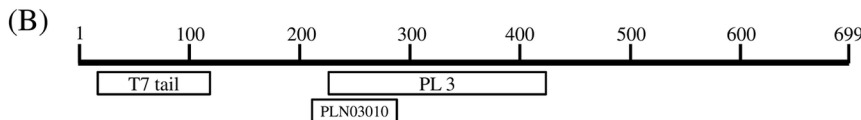


Fig 1. Sequence alignment of tail fibers of *A. baumannii* phages. (A) Amino acid sequence alignment of the phage tail fibers encoded by ϕ AB1 ORF41 and ϕ AB6 ORF40, respectively. (B) Predicted domain structure of ϕ AB6 ORF40. The numbers on the top indicate size in amino acid residues. The domains are identified as follow: T7 tail (aa 10–112), T7 phage tail domain; PLN03010 (aa 218–274), the PLN03010 domain of polygalacturonase; and PL 3 (aa 226–419) pectate lyase 3 superfamily.

doi:10.1371/journal.pone.0153361.g001

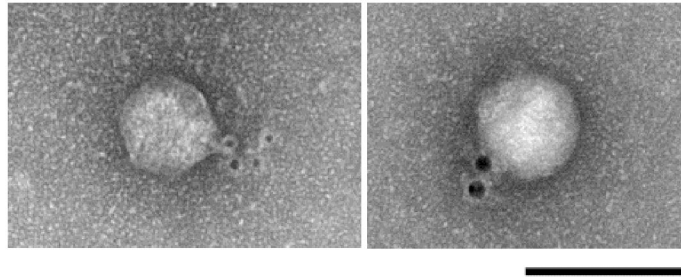


Fig 2. Immunogold-labeling electron microscopy of ϕ AB6 phage particles. The primary antibody targeting ϕ AB6 ORF40 is described in Materials and Methods. The secondary antibody was conjugated to 6-nm diameter gold particles (black dots). Scale bar, 100 nm.

doi:10.1371/journal.pone.0153361.g002

irreversible adsorption of *A. baumannii* phage to its host cells, a key step in the specific interaction between host receptor and phage RBP. Phage RBPs have been proposed as potential targets for genetic modification to broaden the host range, thus contributing to practical applications of phage therapy and the detection of bacteria by their specific phages [2,4,20]. In this study, we sequenced one of the *A. baumannii* podophages, ϕ AB6, and showed its high similarity to the previously sequenced phage ϕ AB1. Despite their high sequence similarity, ϕ AB1 and ϕ AB6 exhibited distinct host specificity. Detailed comparative sequence analysis revealed striking sequence diversity between the C-termini of the putative tail fibers of ϕ AB1 and ϕ AB6. This finding is similar to previous studies showing that the RBPs of *Lactococcus lactis* phages sk1, bIL170, and TP901-1 have a conserved N-terminus and a variable C-terminus that serves as the host specificity determinant [3,21]. Similarly, in λ phage, the C-terminal domain of the tail fiber protein is responsible for binding to the receptor protein LamB [7,22]. To test whether

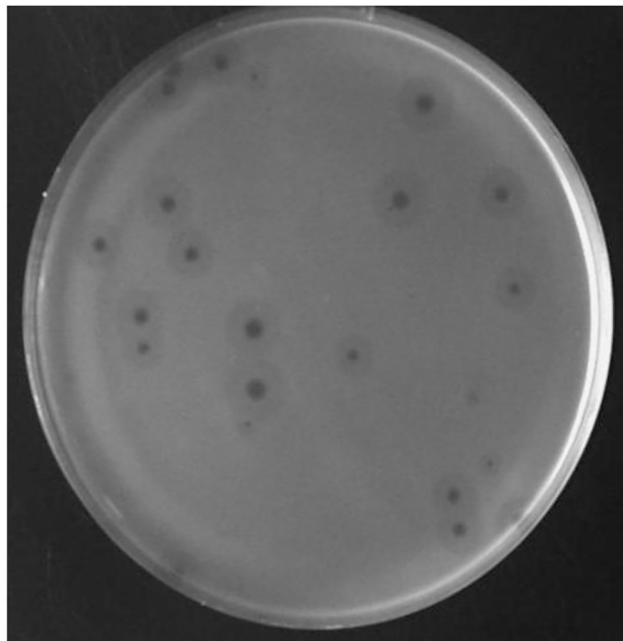


Fig 3. Translucent halo formation by phage ϕ AB6. Clear plaques surrounded by translucent halos were observed in the plaque assay of phage ϕ AB6 with *A. baumannii* strain 54149 as the indicator host.

doi:10.1371/journal.pone.0153361.g003

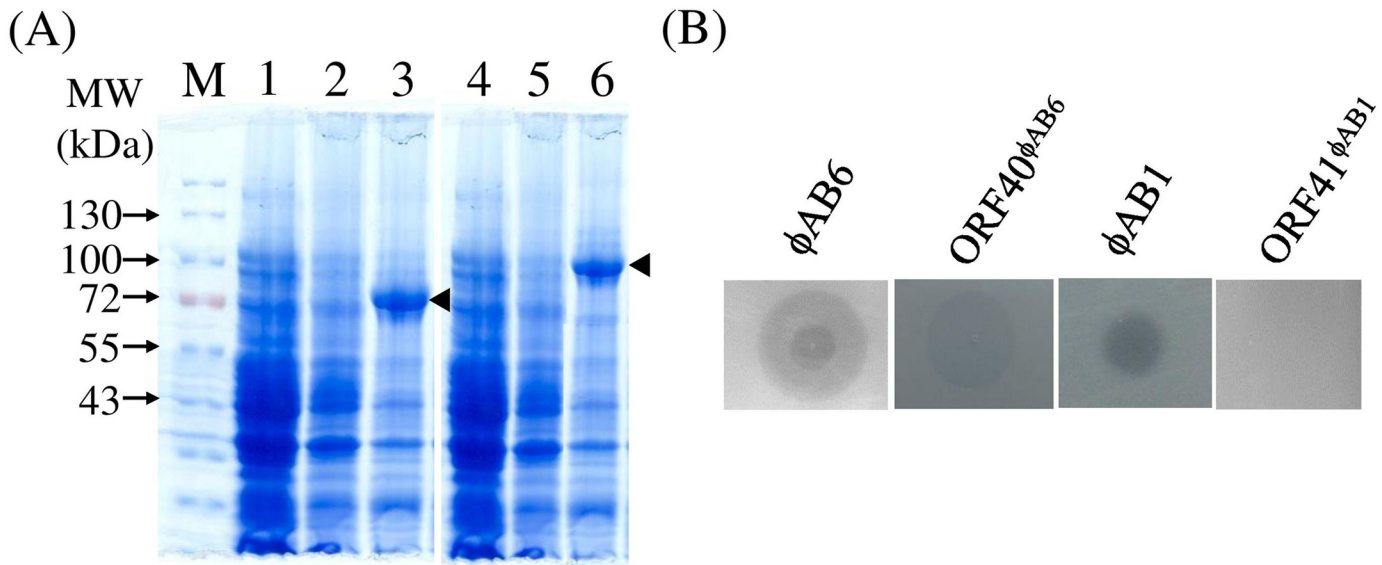


Fig 4. Overexpression and depolymerization activity test of ORF40^{φAB6} and ORF41^{φAB1} proteins. (A) SDS-PAGE of the recombinant proteins overexpressed. Lanes: M, protein markers; 1 and 4, BL21(DE3) harboring an empty plasmid; 2, uninduced BL21(DE3)/pET-orf40^{φAB6}; 3, BL21(DE3)/pET-orf40^{φAB6} induced with 0.1 mM IPTG; 5, uninduced BL21(DE3)/pET-orf41^{φAB1}; 6, BL21(DE3)/pET-orf41^{φAB1} induced with 0.1 mM IPTG. The arrowheads indicate the overexpressed recombinant proteins. (B) Enzyme activity of purified proteins was tested by spot test. Halo surrounding stands for positive activity.

doi:10.1371/journal.pone.0153361.g004

this domain was responsible for host specificity in *A. baumannii* podophages, we constructed a chimeric phage φAB1tf6 by replacing ORF41 in phage φAB1 with the corresponding gene (ORF40) from φAB6. The resultant chimeric phage φAB1tf6 acquired the host range of phage φAB6, demonstrating that the tail fiber protein is responsible for host specificity and that tail fiber substitution in related phages can lead to acquisition of different host ranges.

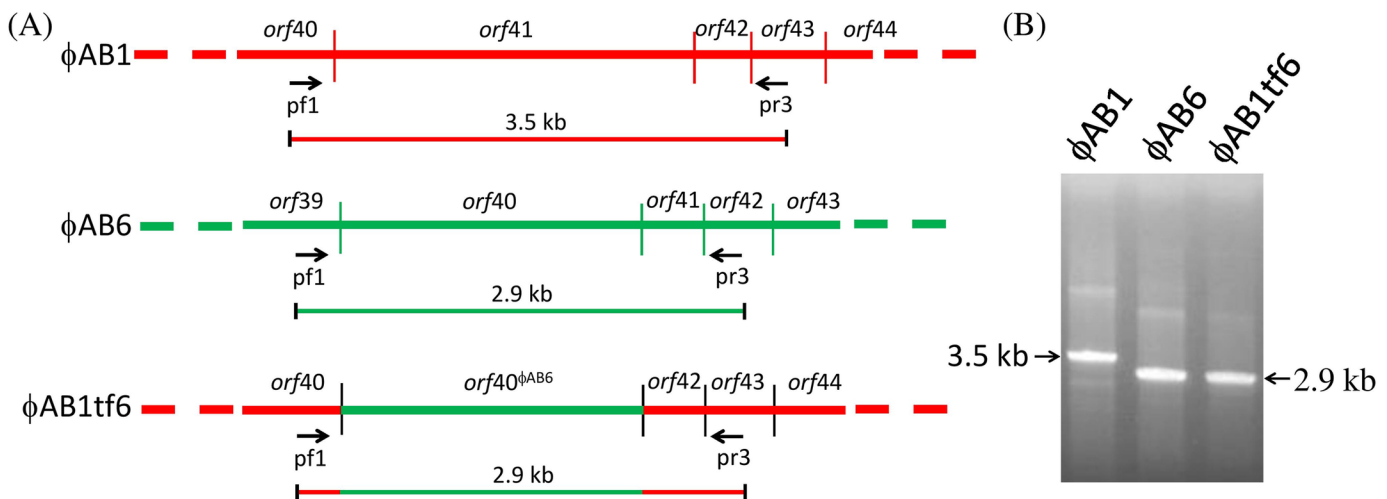


Fig 5. Confirmation of chimeric phage φAB1tf6 by PCR. (A) Maps of orf41^{φAB1}/orf40^{φAB6} and the surrounding genes. (B) The PCR reactions were performed on the genomes of φAB1, φAB6, and φAB1tf6 as the templates with pf1 and pr3 primers and the amplicons were subjected to separation in 0.8% agarose gel.

doi:10.1371/journal.pone.0153361.g005

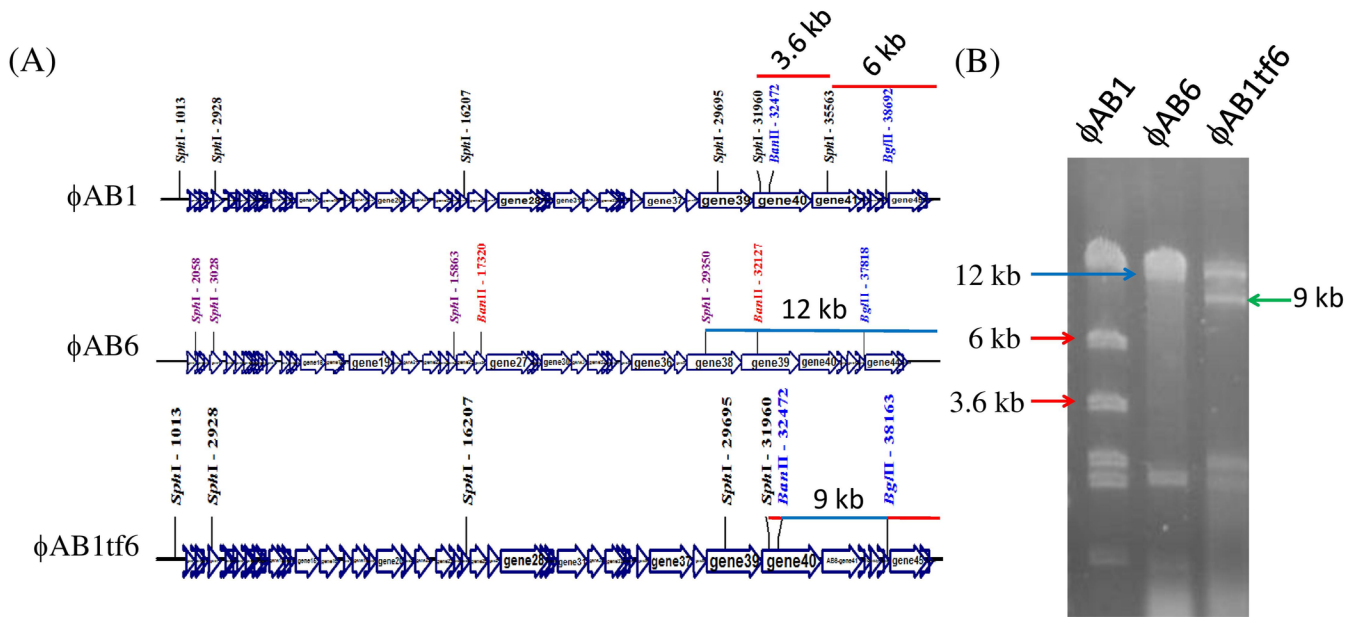


Fig 6. Confirmation of chimeric phage phiAB1tf6 by RFLP. (A) The *SphI*-restriction map shows *orf41*^{phiAB1}/*orf40*^{phiAB6} and surrounding genes. (B) RFLP was performed on genomic DNA of phiAB1, phiAB6, and phiAB1tf6 with *SphI* digestion. The digests were subjected to separation in 0.8% agarose gel.

doi:10.1371/journal.pone.0153361.g006

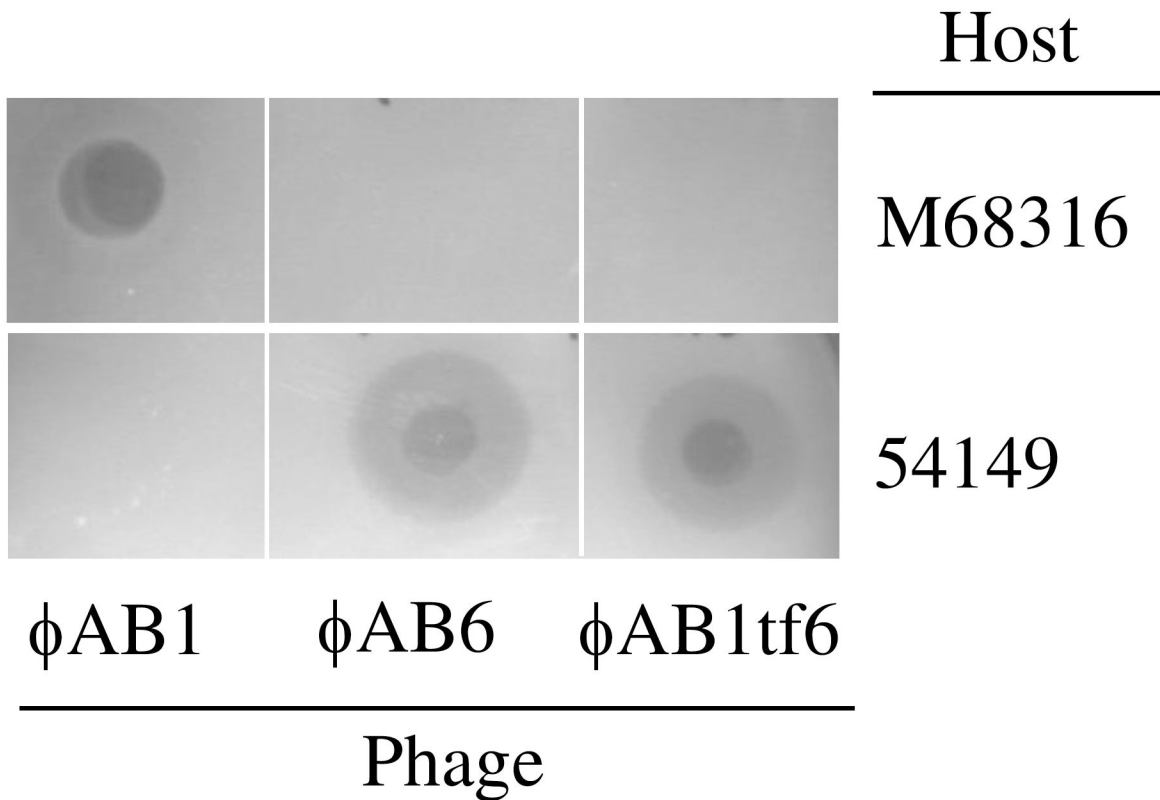


Fig 7. Spot assay for testing the infection ability of phiAB1, phiAB6, and phiAB1tf6 for *A. baumannii* strains 54149 and M68316.

doi:10.1371/journal.pone.0153361.g007

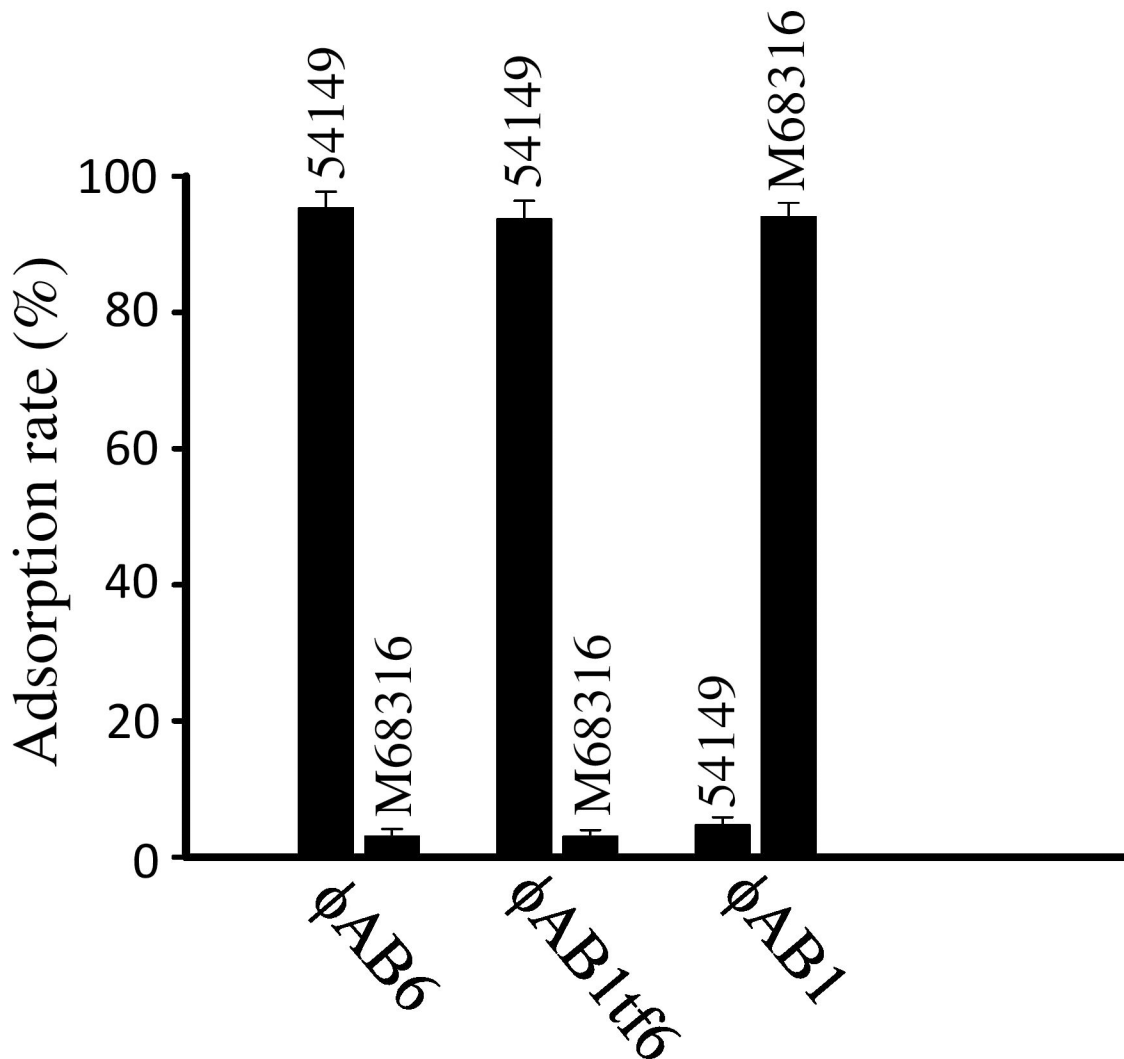


Fig 8. Adsorption assay for testing the specificity of ϕ AB1, ϕ AB6, and chimeric phage ϕ AB1tf6, as described in Materials and Methods, in binding to *A. baumannii* strains M68316 and 54149.

doi:10.1371/journal.pone.0153361.g008

Further, our spot tests revealed that ORF40 $^{\phi$ AB6 exhibited exopolysaccharide depolymerase activity toward *A. baumannii*, but no depolymerase activity was detected for ORF41 $^{\phi$ AB1. This result suggests that the pectate lyase 3 domain is responsible for the exopolysaccharide depolymerase activity. To our knowledge, no phage-derived enzymes that degrade the *Acinetobacter* exopolysaccharide matrix have been previously described, even though polysaccharide depolymerases are common constituents of tail spikes among phages [17] and include the activities of endorhamnosidases [23,24], alginate lyases [25], endosialidases [26], and hyaluronidases [27]. Furthermore, the pectate lyase 3 domain of ORF40 $^{\phi$ AB6 is unique among reported phage exopolysaccharide depolymerases, suggesting that it represents a novel phage-encoded exopolysaccharide depolymerase. Our results suggest that host exopolysaccharides are the specific receptor for ϕ AB6, since neither the phage nor the purified ORF40 $^{\phi$ AB6 causes lysis or translucent halo formation on the host strain of ϕ AB1. The specificity of ϕ AB6 could thus be due to the phage-encoded polysaccharide depolymerase, which can recognize the *A. baumannii* 54149 exopolysaccharide structure distinct from other exopolysaccharide types. Since

exopolysaccharide depolymerase is capable of destroying the outer layer of bacteria, which in turn reduces biofilm formation and antibiotic resistance by impeding drug penetration, ORF40^{φAB6} has the potential to be developed into a biofilm-degrading enzyme for controlling *A. baumannii* infections.

To our knowledge, this is the first report on the identification of a phage genetic determinant involved in *A. baumannii* host recognition, an RBP that possesses polysaccharide depolymerase activity. The well-characterized host specificity, the full genomic sequence of phage φAB6, and the polysaccharide depolymerase revealed in this study will be helpful for further elucidating the mechanistic details that determine the host range of this phage. The depolymerase activity of the ORF40^{φAB6} could be applied toward revealing exopolysaccharide structures. Finally, polysaccharides can be used as immunogens for glycoconjugated vaccine production [28]. Our data indicating that φAB6 is able to lyse 58% (483/832) of the clinical *A. baumannii* isolates suggest that vaccine prepared from the ORF40^{φAB6}-digested *A. baumannii* polysaccharide could possibly protect from over half of the infections caused by *A. baumannii* in Taiwan.

Acknowledgments

We thank the Electron Microscopy Laboratory of Department of Anatomy of Tzu Chi University for technical assistance.

Author Contributions

Conceived and designed the experiments: NTL. Performed the experiments: MJL SWH CHL PYC. Analyzed the data: MJL KCC NTL. Contributed reagents/materials/analysis tools: MJL KCC CCW NTL. Wrote the paper: NTL.

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