RESEARCH ARTICLE   **Open Access**

Genomic characterization of JG068, a novel virulent podovirus active against *Burkholderia cenocepacia*

Karlene H Lynch[1†], Ashraf H Abdu[1†], Max Schobert[2] and Jonathan J Dennis[1*]

Abstract

Background: As is true for many other antibiotic-resistant Gram-negative pathogens, members of the *Burkholderia cepacia* complex (BCC) are currently being assessed for their susceptibility to phage therapy as an antimicrobial treatment. The objective of this study was to perform genomic and limited functional characterization of the novel BCC phage JG068 (vB_BceP_JG068).

Results: JG068 is a podovirus that forms large, clear plaques on *Burkholderia cenocepacia* K56-2. Host range analysis indicates that this phage can infect environmental, clinical, and epidemic isolates of *Burkholderia multivorans*, *B. cenocepacia*, *Burkholderia stabilis*, and *Burkholderia dolosa*, likely through interaction with the host lipopolysaccharide as a receptor. The JG068 chromosome is 41,604 base pairs (bp) in length and is flanked by 216 bp short direct terminal repeats. Gene expression originates from both host and phage promoters and is in the forward direction for all 49 open reading frames. The genome sequence shows similarity to *Ralstonia* phage φRSB1, *Caulobacter* phage Cd1, and uncharacterized genetic loci of blood disease bacterium R229 and *Burkholderia pseudomallei* 1710b. CoreGenesUniqueGenes analysis indicates that JG068 belongs to the *Autographivirinae* subfamily and φKMV-like phages genus. Modules within the genome encode proteins involved in DNA-binding, morphogenesis, and lysis, but none associated with pathogenicity or lysogeny. Similar to the signal-arrest-release (SAR) endolysin of φKMV, inducible expression of the JG068 SAR endolysin causes lysis of *Escherichia coli* that is dependent on the presence of an N-terminal signal sequence. In an *in vivo* assay using the *Galleria mellonella* infection model, treatment of *B. cenocepacia* K56-2-infected larvae with JG068 results in a significant increase in larval survival.

Conclusions: As JG068 has a broad host range, does not encode virulence factors, is obligately lytic, and has activity against an epidemic *B. cenocepacia* strain *in vivo*, this phage is a highly promising candidate for BCC phage therapy development.

Keywords: *Burkholderia cepacia* complex, Phage therapy, *Autographivirinae*, φKMV-like phages, SAR endolysin, *Galleria mellonella*

Background

In recent years, the emergence of antibiotic-resistant Gram-negative pathogens – including *Acinetobacter*, *Klebsiella*, and *Pseudomonas* – has become a serious global concern [1]. As the treatment options for these bacteria become increasingly limited, scientists and clinicians

alike have turned to bacteriophage (or phage) therapy as a possible alternative to antibiotic delivery. By preventing and/or treating infections with phages – viruses that specifically infect bacteria – one can target pathogens that are resistant to conventional drug treatment while avoiding possible antibiotic side effects, such as disruption of the patient's normal flora [2]. Phages infecting the Gram-negative bacteria *Escherichia coli* and *Pseudomonas aeruginosa* have already been shown to be safe for human administration in multiple volunteer and phase I trials [3-6]. Although relatively little data are currently available for controlled human efficacy studies, a recent

* Correspondence: jon.dennis@ualberta.ca
†Equal contributors
[1]Department of Biological Sciences, 6–008 Centennial Centre for Interdisciplinary Science, University of Alberta, Edmonton, AB T6G 2E9 Canada
Full list of author information is available at the end of the article

phase I/II trial showed that *P. aeruginosa*-specific phages were clinically active [6].

The *Burkholderia cepacia* complex (BCC) is a group of antibiotic-resistant Gram-negative species that also appear to be a promising target for phage therapy. These bacteria cause transmissible and potentially fatal opportunistic infections in cystic fibrosis (CF) and immuno-compromised patients. Similar to *P. aeruginosa*, BCC bacteria are innately antibiotic-resistant owing to a variety of mechanisms (reviewed in [7]). Antibiotics such as meropenem, minocycline, and ceftazidime show partial efficacy against some clinical isolates, but the vast majority of strains are not susceptible to even the administration of multiple drugs [8]. Although phage therapy for *Burkholderia* species has not yet reached clinical trials, preliminary studies have shown it to be safe and effective in the protection of crop seedlings and in both invertebrate and mammalian infection models [9-12].

One of the most important aspects of phage therapy development is the selection of appropriate therapeutic phage candidates. To be clinically applicable, a phage should have a broad host range (that includes clinical isolates) and a sequenced and characterized genome that lacks genes encoding putative pathogenicity factors and lysogeny-related proteins (reviewed in [13]). Identifying BCC-specific phages that meet all of the above criteria has thus far proven challenging, particularly with respect to lysogeny genes. Although several phages have been isolated and characterized that have a broad host range (encompassing multiple strains and species within the BCC) and no virulence genes, almost all of these phages are temperate or encode proteins required for this lifestyle [13,14]. BCC-specific phages that are obligately lytic (or putatively so) are thus far suboptimal for phage therapy development because they either infect only environmental isolates (Bcep1, Bcep43, Bcep781, and BcepB1A) [15] or have genomes that are either not sequenced or not published (KS12, BcepF1, BcepNazgul, BcepGomr, BcepEtu, BcepFife, and BcepBrny) [13,16]. Although we have shown that BCC phages can be engineered to an obligately lytic form, such mutants can be difficult to construct and could potentially encounter additional hurdles with respect to regulatory approval [11,17]. As a result of these obstacles, a key objective of BCC phage isolation and characterization studies is to isolate naturally occurring obligately lytic phages that infect clinical strains and lack putative virulence or lysogeny genes. Here, we describe and characterize the complete genome sequence of podovirus JG068, a novel phage possessing each of these characteristics. Furthermore, using a well-characterized invertebrate infection model [10,18], we show that this phage is active against *Burkholderia cenocepacia in vivo*, providing further evidence that JG068 and other related phages are appropriate candidates for clinical development.

Results and discussion

Isolation, host range, and morphology

JG068 (vB_BceP_JG068) was isolated from a sewage processing plant using an uncharacterized strain of *Burkholderia dolosa*. Additional JG068 hosts were identified using spot tests and soft-agar overlays of BCC strains. A total of 32 strains were tested and seven were found to support lytic propagation of JG068 (with efficiency of plating [EOP] values in parentheses): *Burkholderia multivorans* ATCC 17616 (10^{-4}), *Burkholderia cenocepacia* K56-2 (10^0), J2315 (10^{-2}), and PC184 (10^0), *Burkholderia stabilis* LMG 14294 (10^{-1}), and *Burkholderia dolosa* AU0158 (10^0) and CEP021 (10^0). Excluding the soil isolate ATCC 17616, each of these strains was identified as a CF clinical isolate [19-21]. Furthermore, the three susceptible *B. cenocepacia* strains and AU0158 have all been linked to epidemic spread among CF patients [19,21]. This host range is relatively broad, clinically relevant, and distinct compared to the tropism of BCC phages that we have previously characterized [10,11,22-26]. On K56-2, JG068 forms large clear plaques, 1–3 mm in diameter.

Several BCC phages – including KS4, KS5, KS9, KS10, and KS12 – have been previously shown to use lipopolysaccharide (LPS) as a receptor [11], Abdu and Juárez Lara, unpublished data]. To assess if JG068 uses a similar receptor, a panel of both K56-2 [27,28] and PC184 [Abdu, unpublished data] LPS mutants were tested with the phage in spot tests. In K56-2 mutants, JG068 (propagated on wildtype K56-2 [EOP: 10^0]) less efficiently infected *wbxE* and *waaL* mutants (EOP: 10^{-4}) and did not infect *wabR*, *wabS*, *wabO*, or *waaC* mutants (EOP: <10^{-6}; Table 1). In PC184 (EOP: 10^0), this JG068 stock less efficiently infected a *wabP* mutant (EOP: 10^{-2}) and did not infect *wabO* or *waaC* mutants (EOP: <10^{-6}; Table 1). As JG068 does not infect mutants with significant deficits in the core LPS structure, the tail of this phage likely interacts with the LPS core of K56-2, PC184, and potentially other hosts. As noted in a previous BCC phage study, further experiments are required to validate this prediction as LPS truncation may also result in secondary changes to the cell surface structure of the mutants [11,27].

Electron microscopy of JG068 virions (Figure 1) shows that this phage has a short tail and belongs to the C1 morphotype of the order *Caudovirales* and family *Podoviridae* [29]. This morphology is relatively rare for a BCC phage as the only *Burkholderia* podoviruses identified to date are the BPP-1-like BcepC6B and the Bcep22-like DC1, Bcep22, BcepIL02, and BcepMigl [14]. The JG068 capsid is icosahedral and 60.93 ± 2.83 nm in diameter (Figure 1).

Genome sequencing and assembly

In order to determine if the genome sequence of JG068 was novel, several EcoRI genomic DNA fragments were

Table 1 JG068 LPS mutant host range

B. cenocepacia strain	Mutant strain	Mutation	Reference	Phenotype	EOP
K56-2	wildtype	none	[19]	wildtype	10^0
	RSF19	*wbxE*	[27]	truncated O-antigen	10^{-4}
	XOA7	*waaL*	[28]	no O-antigen	10^{-4}
	XOA15	*wabR*	[28]	truncated outer core	$<10^{-6}$
	XOA17	*wabS*	[28]	truncated outer core	$<10^{-6}$
	XOA8	*wabO*	[28]	truncated inner core	$<10^{-6}$
	CCB1	*waaC*	[28]	truncated inner core	$<10^{-6}$
PC184	wildtype	none	[19]	wildtype	10^0
	Δ*wabP*	*wabP*	[Abdu, unpublished data]	truncated inner core	10^{-2}
	Δ*wabO*	*wabO*	[Abdu, unpublished data]	truncated inner core	$<10^{-6}$
	Δ*waaC*	*waaC*	[Abdu, unpublished data]	truncated inner core	$<10^{-6}$

cloned into pUC19 and sequenced. Resulting reads were analyzed using BLASTN and found to be similar (but not identical) to a variety of sequences, including those from previously characterized podoviruses. The genome sequence was completed using ion semiconductor technology on the Ion Torrent platform. A total of 1.07×10^5 sequence reads were aligned into a single contig with over 400-fold coverage. Regions of ambiguity and genome ends were analyzed using Sanger sequencing.

The JG068 genome is 41,604 bp in length and has a 60.7% GC content. Based on the similarity of the sequence to characterized φKMV-like *Autographivirinae* genomes (discussed below), the chromosomal termini of JG068 are likely to be short direct terminal repeats (DTRs) as canonically found in T7 [30]. To identify these termini, JG068 DNA was directly sequenced using primers that bind the following four loci: within the left DTR,

Figure 1 Transmission electron micrograph of phosphotungstic acid-stained JG068 virions at 140,000-fold magnification. Scale bar represents 50 nm.

extending to the left terminus; immediately outside of the left DTR, extending to the left terminus; within the right DTR, extending to the right terminus; and immediately outside of the right DTR, extending to the right terminus. Primers that bind *within* the left DTR will amplify both the left and right ends of the genome simultaneously (as the primer binding site is in both DTRs). A reduction to one-half intensity in the sequence chromatogram represents the arrest of half of the reads (those originating from the left end of the genome) at the left chromosome terminus [31]. Primers that bind *immediately outside of* the left DTR will amplify only the left end of the genome (as the primer binding site is unique) and the sequence chromatogram will stop abruptly at the left chromosome terminus [30]. The same reasoning is true for primers binding within or near the right DTR. Using these methods, it was determined that the chromosomal termini of JG068 are 216 bp DTRs with an identical sequence on each end. The length of these repeats is shorter than that of other φKMV-like *Autographivirinae* such as LKD16 (428 bp), φKMV (414 bp), LKA1 (298 bp), and LIMElight (277 bp) [32-34].

In φKMV, genome replication is bidirectional with the origin and terminus in the DNA polymerase gene and an internal virion protein gene, respectively [33]. Using GenSkew analysis, a global co-minimum GC skew was identified in the JG068 DNA polymerase gene *18* and a global maximum skew was identified in the internal virion protein gene *38*, suggesting that the replication process of JG068 is similar to that of other φKMV-like *Autographivirinae*. In addition to the chromosomal termini and the putative replication origin, a third commonality with respect to the DNA of this genus of phages is that the JG068 genome lacks recognition sites for many common restriction enzymes, including BamHI, BglII, PstI, SacI, SmaI, and XhoI. As in other phages, including those that are φKMV-like, these sites tend to be lost as the phage evolves to avoid host restriction systems [32,33].
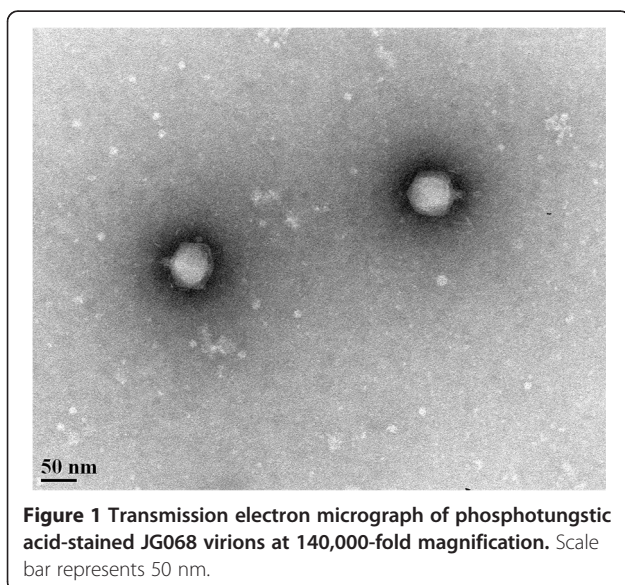
Relatedness

When the sequence of JG068 is analyzed using BLASTN, the most similar sequences (with E-values of $2e^{-87}$ or less) are those of *Ralstonia* phage φRSB1, a genetic locus of blood disease bacterium R229, a genetic locus of *Burkholderia pseudomallei* 1710b chromosome 1, and *Caulobacter* phage Cd1. In the BLASTN results, JG068 shows similarity to several members of the *Autographivirinae* subfamily and φKMV-like phages genus, including φRSB1, Cd1, and *Pseudomonas* phage Bf7. Members of this subfamily (with enterobacteria phage T7 being the best characterized) belong to one of several genera, including the T7-like phages, SP6-like phages, φKMV-like phages, and novel genera that have not yet been named [35,36]. These podoviruses all encode a single subunit RNA polymerase, as is found in JG068 [35]. To determine if JG068 belongs to this subfamily (and, if so, to what genus), we used CoreGenesUniqueGenes (CGUG) analysis to compare the genomes of JG068, T7 (NC_001604.1), SP6 (NC_004831.2), and φKMV (NC_005045.1) [37]. Using T7, SP6, or φKMV as the reference genome, the proteins of JG068 were 15.0%, 23.1%, or 44.9% similar, respectively. As ≥40% similarity indicates a genus-level relationship [35], we can conclude that JG068 is a novel member of the *Autographivirinae* subfamily and the φKMV-like phages genus. As noted above, the few BCC podoviruses that have been previously characterized are either Bcep22-like or BPP-1-like [14], thus JG068 is the first BCC *Autographivirinae* phage to be identified.

Genome annotation

The JG068 genome contains 49 putative open reading frames (ORFs) (Figure 2, Table 2). Similar to T7 and other *Autographivirinae*, all of the genes are transcribed in the forward direction [33,38]. Based on BLASTP analysis (with an E-value cutoff of 0.01), 19 JG068 proteins show no similarity to other proteins in the database (Table 2). The remaining 30 proteins have percent identities between 29% (Rz protein gp48, similar to hypothetical protein Bpse9_41836 of *B. pseudomallei* 91) and 64% (hypothetical protein gp8, similar to a hypothetical protein of *Ralstonia* phage RSB2) (Table 2), thus showing low-to-moderate similarity to other sequences at the protein level.

Similar to other phages, the JG068 genome has a modular organization. In both T7 and φKMV, class I early genes are clustered on the left end of the genome, class II genes for DNA-binding proteins are found centrally, and class III genes for virion morphogenesis and lysis are positioned on the right end [32,38]. The overall organization of JG068 is syntenic with both of these phages: left end genes encode hypothetical proteins, central genes encode DNA-binding proteins, and right end genes encode capsid morphogenesis and DNA packaging, tail morphogenesis, and lysis proteins (Figure 2). Using BTXpred analysis (to identify toxins) combined with BLASTP and HHpred comparisons (to identify other pathogenicity-associated proteins; Tables 2 and 3), no putative virulence modules were detected, indicating that JG068 is likely to be a safe candidate for clinical testing.

A module commonly identified in other BCC phages that is notably absent from the JG068 genome is that for lysogeny. Similar to other obligately lytic phages, JG068 forms clear plaques and does not encode either an integrase or a repressor. Most other characterized members of the *Autographivirinae* are also virulent (although putative prophage elements described as being T7-like have been identified in *Xanthomonas axonopodis*, *Pseudomonas putida*, and *B. pseudomallei*) [39,40]. In order to verify experimentally that JG068 was not capable of lysogenizing BCC strains, we isolated JG068-insensitive K56-2 from a JG068/K56-2 lysate. To determine if these cells were insensitive due to either receptor mutation or superinfection immunity, we attempted to lyse these isolates using a second putatively virulent BCC-specific phage, KS12. Previous assays have shown that KS12 uses LPS as a receptor [Juárez Lara, unpublished data]. If JG068-insensitive K56-2
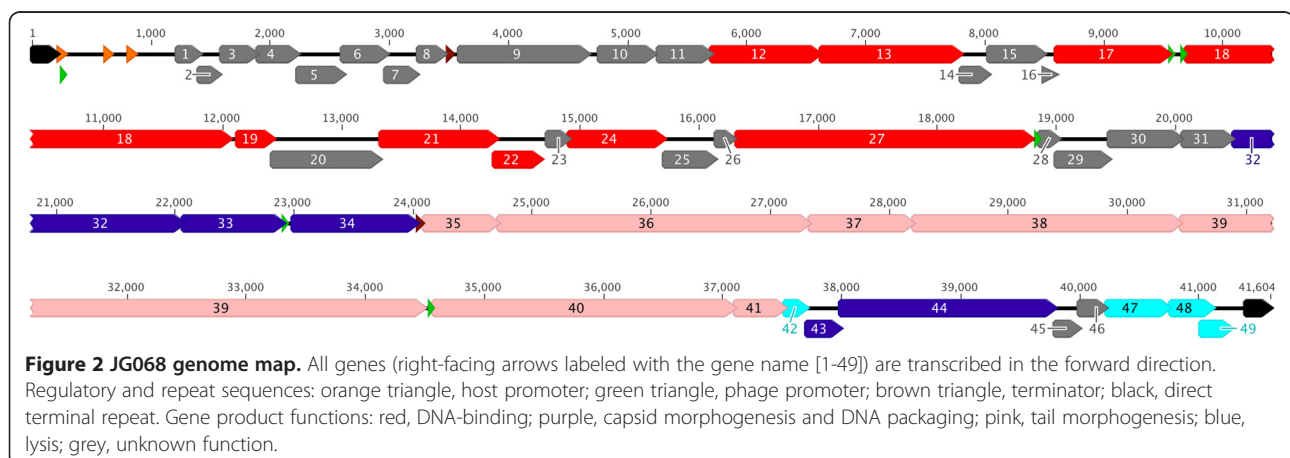

Figure 2 JG068 genome map. All genes (right-facing arrows labeled with the gene name [1-49]) are transcribed in the forward direction. Regulatory and repeat sequences: orange triangle, host promoter; green triangle, phage promoter; brown triangle, terminator; black, direct terminal repeat. Gene product functions: red, DNA-binding; purple, capsid morphogenesis and DNA packaging; pink, tail morphogenesis; blue, lysis; grey, unknown function.

Table 2 JG068 genome annotation

Gene	Start	End	Putative function	Strand	Putative ribosome binding site and start codon	Length (amino acids)	Closest relative	BLASTP alignment region (amino acids)	Percent identity	E-value	Organism	GenBank accession number
1	1211	1405	hypothetical	+	ACGGAGctagacgaATG	64	none					
2	1402	1566	hypothetical	+	GGAACGAtgctcgATG	54	none					
3	1590	1889	hypothetical	+	GGAGTAAcgatcATG	99	hypothetical protein φAB1_gp1	3-72/169	50	$1e^{-13}$	*Acinetobacter* phage φAB1	ADQ12705.1
4	1895	2227	hypothetical	+	GGGGTGAGttcgATG	110	none					
5	2224	2613	hypothetical	+	GGAGAAtcacGTG	129	hypothetical protein phAPEC8_0044	1-98/106	32	$6e^{-8}$	*Escherichia* phage phAPEC8	AFU62620.1
6	2603	2974	hypothetical	+	GAGGTGttgaATG	123	none					
7	2971	3231	hypothetical	+	AACGAGGccgcATG	86	none					
8	3242	3460	hypothetical	+	AAGGAGcaacacATG	72	hypothetical protein	27-54/109	64	$4e^{-6}$	*Ralstonia* phage RSB2	BAJ51800.1
9	3584	4672	hypothetical	+	AGGACAtcaATG	362	none					
10	4762	5223	hypothetical	+	AAGGAtttaacATG	153	none					
11	5252	5698	hypothetical	+	AAGGAActgacATG	148	none					
12	5702	6610	DNA primase	+	GGAGGctaaggcATG	302	putative DnaG-like primase	1-277/284	43	$1e^{-63}$	*Caulobacter* phage Cd1	ADD21651.1
13	6624	7802	DNA helicase	+	GAAGGTAAcacgcaaGTG	392	hypothetical protein BURPS1710b_1647	35-427/431	61	$2e^{-176}$	*Burkholderia pseudomallei* 1710b	YP_333051.1
14	7802	8032	hypothetical	+	GGGGGtgtgATG	76	none					
15	8032	8493	hypothetical	+	AAGGAGGGtgcgtgATG	153	none					
16	8493	8603	hypothetical	+	GTGGGGcgctgATG	36	none					
17	8600	9553	DNA ligase	+	GGAGGAAggtATG	317	PBCV-1 DNA ligase	4-309/309	37	$1e^{-41}$	*Burkholderia pseudomallei* 91	ZP_02453445.1
18	9688	12060	DNA polymerase	+	AAGGAAccATG	790	38L	1-806/808	60	0	*Burkholderia pseudomallei* 91	ZP_02453448.1
19	12122	12424	HNH endonuclease	+	AGGCAcggtcgcagcATG	100	endonuclease	30-112/249	47	$7e^{-18}$	*Bacteroides finegoldii* DSM 17565	ZP_05414969.1
20	12421	13314	hypothetical	+	AAGGAAcaagtATG	297	37L	8-260/276	50	$7e^{-71}$	*Burkholderia pseudomallei* 91	ZP_02453449.1
21	13327	14301	DNA exonuclease	+	AAGGAGGcaaggccATG	324	phosphodiesterase I	1-247/258	48	$5e^{-75}$	*Burkholderia pseudomallei* 1710a	ZP_04951398.1
22	14282	14677	DNA endonuclease	+	ATGAGAttgaccacaagcATG	131	DNA endonuclease VII	1-115/118	47	$2e^{-28}$	blood disease bacterium R229	CCA83273.1
23	14727	14909	hypothetical	+	GGCAGGttgattgcATG	60	none					
24	14910	15704	DNA exonuclease	+	GGAGAttaaATG	264	34L	1-263/263	60	$1e^{-115}$	*Burkholderia pseudomallei* 91	ZP_02453452.1

Table 2 JG068 genome annotation (Continued)

25	15704	16138	hypothetical	+	GGTGAGctaATG	144	hypothetical protein	16-128/138	33	$5e^{-6}$	Pantoea phage LIMElight	YP_007002888.1
26	16142	16297	hypothetical	+	GACGGAGtaacagATG	51	none					
27	16313	18799	RNA polymerase	+	GAGGAcactgATG	828	DNA-directed RNA polymerase	6-817/818	51	0	Burkholderia thailandensis MSMB43	ZP_02468154.1
28	18861	19025	hypothetical	+	AAGGAAccgGTG	54	none					
29	19003	19443	hypothetical	+	AAGGAActgcgcgATG	146	hypothetical protein BDB_mp60445	1-143/145	35	$5e^{-18}$	blood disease bacterium R229	CCA83279.1
30	19444	20058	hypothetical	+	AAGGAGAtttgaATG	204	none					
31	20068	20478	hypothetical	+	AAGGAGGGagcATG	136	none					
32	20488	22065	head-tail connector protein	+	AAGGAGAAgacATG	525	head portal-like protein from phage	9-494/512	50	$1e^{-165}$	blood disease bacterium R229	CCA83282.1
33	22066	22908	scaffolding protein	+	AAGGAGcgtaaATG	280	scaffolding-like protein from phage	59-249/255	36	$2e^{-18}$	blood disease bacterium R229	CCA83283.1
34	22991	24031	capsid protein	+	AGGAGGAAcctcaATG	346	major capsid-like protein	8-336/336	57	$6e^{-133}$	Ralstonia phage RSB1	YP_002213721.1
35	24099	24716	tail tubular protein A	+	AAGGAGActgctATG	205	tail tuber protein A from phage	1-200/203	40	$9e^{-42}$	blood disease bacterium R229	CCA83285.1
36	24726	27326	tail tubular protein B	+	AAGGAGGcattATG	866	tail tuber protein B from phage	2-858/858	48	0	blood disease bacterium R229	CCA83286.1
37	27339	28196	internal virion protein	+	AAGGGGGGtcagtcATG	285	hypothetical protein Bpse38_32600	5-199/301	37	$8e^{-22}$	Burkholderia thailandensis MSMB43	ZP_02468144.1
38	28206	30446	internal virion protein	+	AAGGAGGtagcATG	746	hypothetical protein RSB1_gp36	1-361/785	30	$2e^{-42}$	Ralstonia phage RSB1	YP_002213725.1
39	30458	34495	internal virion protein	+	AAAGGAGAAGtaaATG	1345	internal virion protein	9-1318/1333	30	$2e^{-131}$	Caulobacter phage Cd1	ADD21673.1
40	34572	37097	tail fiber protein	+	AGGAGGcaacATG	841	BcepGomrgp19	307-539, 321-459/669	44, 31	$3e^{-44}$, $2e^{-5}$	Burkholderia phage BcepGomr	YP_001210239.1
41	37107	37520	tail fiber assembly protein	+	AAGGAGGttttATG	137	hypothetical protein Bcep1808_1285	7-141/146	41	$2e^{-28}$	Burkholderia vietnamiensis G4	YP_001119130.1
42	37524	37706	holin	+	AGGAGtaagtaATG	60	none					
43	37703	37987	DNA maturase A	+	AGGAGcaagctATG	94	conserved hypothetical protein from phage	6-79/96	44	$2e^{-11}$	blood disease bacterium R229	CCA83292.1
44	37989	39788	DNA maturase B	+	GAGGCAttgatATG	599	TerL large terminase subunit-like protein	4-604/605	59	0	Ralstonia phage RSB1	YP_002213730.1
45	39788	39994	hypothetical	+	GGAGGAAgtaATG	68	none					
46	39998	40222	hypothetical	+	AAAGGAGtaattcATG	74	none					

Table 2 JG068 genome annotation *(Continued)*

47	40232	40753	SAR endolysin	+	AAGGAGGcagcATG	173	phage-type lysozyme	68-211/223	36	8e^{-27}	*Xanthomonas* phage Xp10	NP_858975.1
48	40763	41119	Rz	+	AGGAGAtaaccATG	118	hypothetical protein Bpse9_41836	5-105/106	29	6e^{-4}	*Burkholderia pseudomallei* 91	ZP_02453410.1
49	41016	41267	Rz1	+	AAGGGGAAGctgaATG	83	exported hypothetical protein	18-79/83	52	4e^{-11}	blood disease bacterium R229	CCA83297.1

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

Table 3 JG068 HHpred predictions

Protein	Motif of closest relative	Motif definition	Percent probability	E-value
gp12	2au3_A	DNA primase	100	$1.10e^{-42}$
gp13	3bgw_A	DNAB-like replicative helicase	100	$1.70e^{-48}$
gp15	1v58_A	Thiol:disulfide interchange protein DSBG	93.88	0.13
gp17	1fvi_A	Chlorella virus DNA ligase-adenylate	100	$3.90e^{-60}$
gp18	3pv8_A	DNA polymerase I	100	$1.60e^{-113}$
gp19	1u3e_M	HNH homing endonuclease	99.92	$2.90e^{-25}$
gp21	1exn_A	5'-exonuclease, 5'-nuclease	100	$3.90e^{-54}$
gp22	1e7l_A	GP49, recombination endonuclease VII	100	$8.10e^{-42}$
gp24	2gui_A	DNA polymerase III epsilon subunit	99.85	$7.30e^{-21}$
gp27	1msw_D	DNA-directed RNA polymerase, bacteriophage T7 RNA	100	$2.50e^{-210}$
gp29	4h89_A	GCN5-related N-acetyltransferase	96.86	0.002
gp32	3lj5_A	Portal protein, protein GP1	96.82	0.027
gp34	2xd8_A	GP10, T7-like capsid protein	100	$1.30e^{-48}$
gp39	1qsa_A	Protein (soluble lytic transglycosylase SLT70)	99.15	$6.20e^{-11}$
gp40	2ch7_A	Methyl-accepting chemotaxis protein	96.27	0.95
gp41	2kz6_A	Uncharacterized protein	99.67	$1.70e^{-16}$
gp44	3cpe_A	Terminase, DNA packaging protein GP17	99.97	$6.00e^{-30}$
gp47	3hde_A	Lysozyme	100	$3.80e^{-50}$

HHpred hits with a probability >90% were included in the table.

isolates are also insensitive to KS12 infection, they are likely LPS mutants, whereas if they are sensitive to KS12 infection, they are likely JG068 lysogens. Over 100 JG068-insensitive isolates were screened and all were found to be insensitive to both phages compared to wildtype K56-2. Twelve resistant colonies were PCR-screened using JG068-specific primers and each isolate was found to be amplification-negative. This evidence shows that resistance arises due to receptor mutation and not due to JG068 lysogeny.

In *Autographivirinae*, transcription is first initiated from host promoters found at the far left-hand side of the genome, followed by initiation from phage RNA polymerase-specific promoters found throughout the genome [33,38]. JG068 promoters putatively recognized by BCC RNA polymerase were identified using Neural Network Promoter Prediction. Using a cutoff of 0.95 and limiting the results to sequences found intergenically, three promoters were identified in the first 650 bp downstream of the left DTR (Figure 2 and Figure 3A). Phage promoters lack the conserved structure observed in bacterial promoters with −10 and −35 regions and are instead described as short consensus sequences that vary among different phages [41]. PHIRE was used to identify a strongly conserved 16 bp consensus sequence (Figure 3B) found six times at five intergenic loci in the JG068 chromosome: overlapping the first host promoter sequence, upstream of the DNA polymerase gene *18*

(2 promoters), upstream of the hypothetical protein gene *28* (downstream of the DNA-binding module), upstream of the capsid gene *34*, and upstream of the tail fiber gene *40* (Figure 2). Putative rho-independent terminators were identified using TransTermHP. Although multiple sequences were reported in the output, those shown in Figure 3C were chosen because they were intergenic (based on GeneMark predictions) and had a ΔG value of −10 kcal/mol or less. Similar to LIMEzero, the putative terminators are found upstream of the DNA-binding module and downstream of the capsid protein (Figure 2) [34].

Module analysis
DNA-binding proteins
One of the more notable aspects of the JG068 genome is its abundance of DNA-binding proteins. As discussed above, similar to T7 and φKMV, these genes are found centrally in the JG068 genome (Figure 2). In JG068 and other φKMV-like phages [32-34], the RNA polymerase gene is at the far right end of this module (upstream of the structural genes; Figure 2), whereas in T7 it is found close to the left end of the genome [38]. JG068 DNA-binding proteins include the primase gp12, helicase gp13, ligase gp17, DNA polymerase gp18, HNH endonuclease gp19, exonuclease gp21, endonuclease gp22, exonuclease gp24, and RNA polymerase gp27 (Table 2). The annotation of each of these proteins was based

Figure 3 Predicted promoter and terminator sequences in JG068. A, putative host promoter sequences identified using Neural Network Promoter Prediction [50]. Predicted −35/−10 boxes and transcription start sites are underlined. A canonical prokaryotic promoter is shown below. **B**, putative phage promoter consensus sequence identified using PHIRE [51] and plotted using WebLogo 3.3 [52]. **C**, putative rho-independent terminator sequences identified using TransTermHP [53]. The central loop region is separated by dashes and the 3′ stretch of thymidine residues is underlined.

on BLASTP analysis and subsequently confirmed using HHpred (Tables 2 and 3).

One of the unifying features of the *Autographivirinae* is the presence of a single-subunit phage RNA polymerase gene [35]. Based on CGUG analysis, the only JG068 DNA-binding protein that shows similarity to those of T7 is this polymerase. These two proteins have 31% identity based on BLASTP analysis. In contrast, almost all of the JG068 DNA-binding proteins are similar to proteins in φKMV with 29-48% identity. Furthermore, these genes are found in the same order in these two phages.

The three components within this module that are dissimilar between φKMV and JG068 are the genes encoding the ligase, HNH endonuclease, and DNA binding protein. In φKMV, a ligase gene is found between the helicase and DNA polymerase (as in JG068), but the sequences of these genes are dissimilar between the two phages [32]. Several HNH homing endonuclease genes have been identified in T7 [38], but only one is found in JG068 and none have been identified in φKMV. Although a DNA binding protein gene was identified upstream of the helicase gene in φKMV [32], such a gene was not identified in the JG068 annotation.

The position of the promoters driving the expression of genes in this module also differs between these phages. In φKMV, promoters upstream of these genes include four host promoters and two phage promoters (one between the DNA binding protein and primase genes and one between the DNA polymerase and endonuclease genes) [32]. In contrast, whereas the host promoters for JG068 are found at a similar locus as in φKMV, the phage promoters either overlap the host promoters or are found between the ligase (*17*) and DNA polymerase (*18*) genes (Figure 2). Based on this arrangement, all of the φKMV

genes in this module could be expressed from internal phage promoters (except for the gene encoding the DNA binding protein), whereas the primase (*12*), helicase (*13*), and ligase (*17*) genes of JG068 would be expressed from either a host promoter or the nested phage promoter on the far left end (Figure 2).

Morphogenesis proteins

The structural proteins of φKMV, LKA1, and LKD16 have been identified using mass spectrometry of proteins from purified virions [33,42]. Based on CGUG analysis, JG068 encodes proteins similar to the majority of these structural proteins. The putative structural genes *32–40* (excluding *33*, discussed below) are contained in a single 17 kbp module (Figure 2). In a CGUG comparison of φKMV and JG068, the head-tail connector (JG068 gp32), capsid protein (gp34), tail tubular protein A (gp35) and B (gp36), internal virion proteins (gp38 and gp39), and tail fiber protein (gp40) are all similar and each of these proteins was shown to be structural in φKMV virions [42]. In addition, when JG068 and LKA1 are compared using CGUG, a third internal virion protein (gp37) is identified as similar that is structural in LKA1 [33]. φKMV, LKA1, and LKD16 virions also contain some proteins not encoded similarly by JG068. The genes encoding these structural proteins are found either upstream of the head-tail connector gene or downstream of the tail fiber gene and have uncharacterized functions or are putatively involved in adsorption [33,42]. Based on our genome annotation, JG068 does not encode any additional unique structural proteins.

Additional head morphogenesis/DNA packaging and tail morphogenesis proteins are encoded by JG068 that are not predicted to be associated with the mature virion.

Although the scaffolding protein gp33 is encoded within the structural module discussed above, it is likely to function in capsid assembly but not to be part of the mature virion based on φKMV, LKA1, and LKD16 structural protein analysis [33,42]. Dissimilar to many other phages (but similar to others in the φKMV-like genus) [32-34], the DNA packaging genes 43 and 44 are found far downstream of the head morphogenesis genes (Figure 2). Gp33, gp43, and gp44 are all similar to proteins of φKMV based on CGUG analysis. The only putative morphogenesis protein that is unique to JG068 and not shared by φKMV or LKA1 is the putative tail fiber assembly protein gp41. Although HHpred analysis was uninformative (as the high probability matches to gp41 lack characterized functions; Table 3), this protein shows similarity in BLASTP analysis to tail fiber assembly proteins of *Cupriavidus necator* N-1, *Dickeya dadantii* 3937, and *Idiomarina xiamenensis* 10-D-4.

Lysis proteins

The lysis proteins of JG068, encoded on the far right end of the genome, include a putative pinholin, signal-arrest-release (SAR) endolysin, Rz, and Rz1. Although the lysis genes in most phages (including φKMV) tend to be arranged in a single block with the holin gene followed by the endolysin, Rz, and Rz1 genes [43], this order is not maintained in JG068. Here, the putative pinholin gene 42 is found upstream of the SAR endolysin (47), Rz (48), and Rz1 (49) genes, separated by genes encoding two DNA packaging proteins and two hypothetical proteins (Figure 2). Within the *Autographivirinae* subfamily, this gene arrangement is also observed in SP6 [44].

Although only two of the four JG068 lysis proteins are similar to those of φKMV based on CGUG analysis (the SAR endolysin gp47 and Rz gp48), these two phages are likely to use similar lysis mechanisms. The gp42 pinholin has analogous features to that of φKMV: ~60 amino acids in length (60 for JG068 and 66 for φKMV), two transmembrane domains (as predicted by TMHMM analysis), and positively-charged arginine and lysine residues at the C-terminus [43]. The gp48 Rz inner membrane protein has a single N-terminal transmembrane domain based on TMHMM analysis. Gene 49, encoding the Rz1 outer membrane lipoprotein, overlaps with gene 48 in the +1 reading frame and extends downstream of the 48 stop codon by 148 base pairs (Figure 2). LipoP analysis predicts a signal peptidase II cleavage site in gp49 between residues 18 (alanine) and 19 (cysteine).
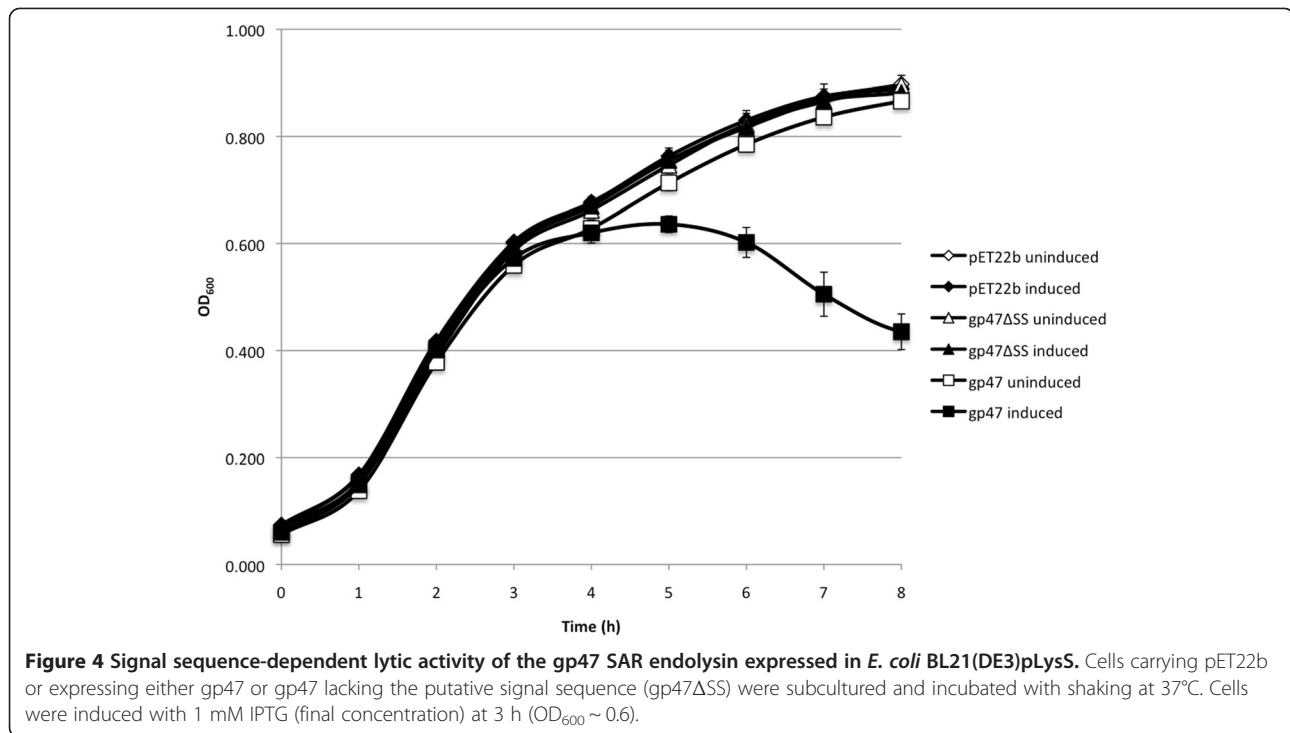
The SAR endolysin of JG068 has a very similar organization to that of φKMV. SignalP 3.0 predicts an N-terminal signal sequence probability of 0.97 for gp47 (later versions of SignalP do not make the same prediction, likely because the C-region is absent). The gp47 N-terminus contains two signal sequence regions followed by three putative catalytic residues of the lysozyme domain. Residues 1–7, including a positively-charged histidine, lysine, and arginine, make up the N-region. Residues 8–25 make up the hydrophobic glycine- and alanine-rich H-region, also predicted to be a transmembrane domain by OCTOPUS analysis. Using CD Search and BLASTP alignment with the φKMV SAR endolysin, the putative lysozyme catalytic residues were identified as 27E, 36D, and 45T, all found immediately downstream of the H-region. As in φKMV, because the C-region is absent, the protein will not be cleaved by a signal peptidase upon secretion and will instead remain associated with the inner membrane until release into the periplasm [43].

Using inducible expression of the φKMV SAR endolysin in *E. coli*, it was shown that this protein could decrease culture absorbance in the absence of a pinholin, but only if the N-terminal signal sequence of the endolysin was present [43]. We performed a similar experiment using the JG068 SAR endolysin to further characterize the export mechanism and biological activity of this protein. To allow for tightly controlled inducible expression, we cloned gene 47 into pET22b (with [gp47] or without [gp47ΔSS] the putative signal sequence in the first 25 residues) and transformed these plasmids into *E. coli* BL21 (DE3)pLysS. When these cells (or a pET22b blank control) are subcultured, their growth rates prior to IPTG induction are similar based on optical density measurements at 600 nm (OD_{600}; up to 3 h in Figure 4). However, following induction, expression of gp47 is lethal to the cells as the OD_{600} decreases from ~0.6 at 3 hours to ~0.45 at 8 hours (black squares, Figure 4). A very different trend is observed for the blank control, gp47ΔSS, and uninduced gp47 strains, where the OD_{600} increases from ~0.6 at 3 hours to ~0.9 at 8 hours, double that of the induced gp47 OD_{600} (Figure 4). As the lytic activity is dependent upon expression of not only the lysozyme domain but also the signal sequence, we can conclude that JG068 gp47 acts as a typical SAR endolysin in Gram-negative bacteria. While the classical endolysins of Bcep781/Bcep43 and BcepC6B have been functionally characterized [15,45], this is the first experimental evidence for SAR endolysin activity in a BCC phage. As these proteins have also been identified in the Bcep22-like viruses [25,46], similar experiments may be used to confirm the activity of SAR endolysins in these phages as well.

In vivo activity

The *Galleria mellonella* (greater wax moth) larvae model is commonly used to assess both strain virulence and phage therapy efficacy for members of the BCC, particularly *B. cenocepacia* [10,11,18]. Although this model is less complex than a mammalian system, it shows positive correlation with both mouse and rat models of infection [18]. To determine if JG068 possesses lytic activity against
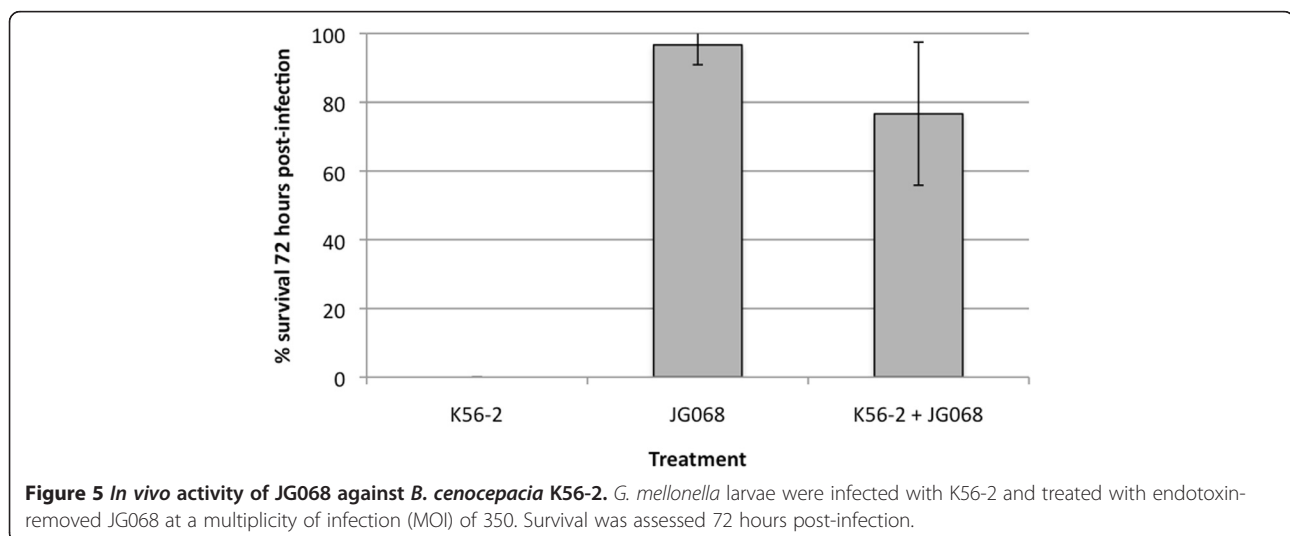
Figure 4 Signal sequence-dependent lytic activity of the gp47 SAR endolysin expressed in *E. coli* BL21(DE3)pLysS. Cells carrying pET22b or expressing either gp47 or gp47 lacking the putative signal sequence (gp47ΔSS) were subcultured and incubated with shaking at 37℃. Cells were induced with 1 mM IPTG (final concentration) at 3 h (OD$_{600}$ ~ 0.6).

B. cenocepacia in vivo, we infected *G. mellonella* larvae with 3.7×10^3 colony forming units (CFU) of K56-2 and treated with endotoxin-removed JG068 at a multiplicity of infection (MOI) of 350 (1.3×10^6 plaque forming units [PFU]). For infected, untreated controls, no larvae survived after 72 hours (Figure 5, left). Those larvae that received only JG068 remained healthy over the course of the experiment (Figure 5, centre), indicating that phage treatment produced no harmful effects. For infected, treated larvae, JG068 administration significantly increased survival to an average of 77% (from 0% in untreated controls) (Figure 5, right). In a previous study, the putatively virulent phage

KS12 was the most effective for immediate treatment of K56-2 infection in the *G. mellonella* model, resulting in 93 ± 12% survival (MOI = 5000) or 57 ± 6% survival (MOI = 500) [10]. Based on the *G. mellonella* data presented in Figure 5, JG068 is almost as effective as a significantly larger dose of KS12, indicating that JG068 is highly active *in vivo*.

Conclusions

One of the greatest challenges in BCC phage therapy development is the identification of phages with a broad, clinically relevant host range that are free of virulence

Figure 5 *In vivo* activity of JG068 against *B. cenocepacia* K56-2. *G. mellonella* larvae were infected with K56-2 and treated with endotoxin-removed JG068 at a multiplicity of infection (MOI) of 350. Survival was assessed 72 hours post-infection.

genes, obligately lytic, and active *in vivo*. Our characterization of the podovirus JG068 indicates that it is a rare example of a BCC phage that satisfies each of these requirements. JG068 infects strains of *B. multivorans*, *B. cenocepacia* (interacting with the LPS core as a receptor in K56-2 and PC184), *B. stabilis*, and *B. dolosa*. It is the first BCC virus to be identified as a member of the *Autographivirinae* subfamily and φKMV-like phages genus. The 41,604 bp genome sequence has a structure similar to that of other *Autographivirinae* chromosomes, with DTRs and genes encoding DNA-binding, morphogenesis, and lysis proteins expressed in the forward direction from phage and host promoters. JG068 lacks virulence genes, is obligately lytic, and encodes a functional SAR endolysin, the first to be experimentally verified for the BCC. Administration of JG068 to *B. cenocepacia* K56-2-infected *G. mellonella* larvae significantly decreases larval mortality, providing the first evidence that sequenced, obligately lytic BCC phages are active against *B. cenocepacia in vivo*. Given these characteristics, further study is warranted regarding the development of JG068 into an active antimicrobial for use in CF patients.

Methods

Bacterial strains and culture conditions

BCC strains were grown aerobically at 30°C overnight on half-strength Luria-Bertani (½ LB) solid agar or agarose plates or in ½ LB broth. Strains used for host range testing and phage propagation are members of the original and updated BCC experimental strain panels [19,20]. K56-2 LPS mutants [27,28] were grown at 30°C on ½ LB plates or in ½ LB broth containing 100 mg/l trimethoprim. *E. coli* DH5α transformed with pUC19 or pET22b was grown at 37°C on LB plates containing 100 mg/l ampicillin. *E. coli* BL21(DE3)pLysS was grown at 37°C in LB broth containing 25 mg/l chloramphenicol or, if transformed with pET22b, 25 mg/l chloramphenicol and 100 mg/l ampicillin.

Phage propagation, host range analysis, lysogeny screen, and electron microscopy

JG068 was isolated from a sewage plant in Steinhof near Braunschweig in Germany following an enrichment protocol previously described [47]. For propagation of JG068, 100 μl high-titre phage stock and 100 μl BCC liquid culture were combined and incubated 20 minutes at room temperature, mixed with 3 ml 0.7% ½ LB agar or 0.6% ½ LB agarose, overlaid on a ½ LB plate, and incubated at 30°C overnight. High-titre stocks were made in modified suspension medium (modified SM; 50 mM Tris–HCl [pH 7.5], 100 mM NaCl, 10 mM $MgSO_4$). Phage plates were overlaid with 3 ml modified SM and incubated >1 h at room temperature on a platform rocker. The supernatant was recovered, pelleted by centrifugation for 2 min

at 10,000 × g, filter-sterilized using a Millex-HA 0.45 μm syringe-driven filter unit (Millipore, Billerica, MA), and stored at 4°C.

Spot testing was used for preliminary host range analysis. Soft-agar overlays containing 100 μl BCC liquid culture were allowed to solidify for ≥10 minutes at room temperature, spotted with 10 μl drops of high-titer JG068 stock, and assayed for clearing and/or plaque formation after incubation at 30°C overnight. Strains that showed phage susceptibility in this assay were re-tested in soft-agar overlays containing both the strain and JG068. The host ranges for LPS mutants of K56-2 [27,28] and PC184 [Abdu, unpublished data] were tested similarly. Efficiency of plating (EOP) values were determined as previously described [46].

To assay for JG068-lysogenized K56-2, JG068 and K56-2 were plated in agar overlays at a multiplicity of infection of 10^0 and incubated at 30°C overnight. To recover surviving cells, plates were overlaid with 3 ml sterile water and incubated 1 h at room temperature on a platform rocker. Cells were pelleted by centrifugation for 2 min at 10,000 x g, washed and resuspended in sterile water, and plated to obtain isolated colonies. Colonies were struck out onto ½ LB plates and spotted with separate 10 μl drops of high-titer JG068 stock and high-titer KS12 stock. Isolates were scored as sensitive or insensitive compared to a sensitive wildtype K56-2 control that was lysed by both phage stocks. A subset of resistant isolates was replated and PCR-screened for lysogeny using JG068-specific primers (9F: GAACATCGGTAACGTCGTCAAGG; 9R: GGCGTGA CGAACAGCTTGGC). TopTaq DNA polymerase and buffers (Qiagen, Hilden, Germany) were used according to the manufacturer's instructions (template: 1 μl overnight culture incubated 5 min at 100°C).

For electron microscopy, phage stocks were prepared as described above with the following modifications: agarose plates and soft agarose were used for overlays, sterile water was used in place of modified SM, and a 0.22 μm filter was used for syringe-driven filtration. A carbon-coated copper grid was incubated with lysate for 5 min and stained with 2% phosphotungstic acid for 30 s. Transmission electron micrographs were captured using a Philips/FEI (Morgagni) transmission electron microscope with charge-coupled device camera at 80 kV (University of Alberta Department of Biological Sciences Advanced Microscopy Facility). The capsid diameter (mean ± standard deviation) was calculated using Microsoft Excel based on measurements from nine individual virions.

Phage DNA isolation, RFLP analysis, and sequencing

Phage DNA was isolated using a modified version of a Wizard DNA Clean-Up protocol [48]. Ten milliliters of filter-sterilized JG068 lysate (propagated using K56-2 on agarose medium) was treated with 10 μl DNase I (Thermo

Scientific, Waltham, MA), 100 μl 100x DNase I buffer (1 M Tris–HCl, 0.25 M $MgCl_2$, 10 mM $CaCl_2$), and 6 μl RNase (Thermo Scientific) and incubated 1 h at 37°C to degrade the bacterial nucleic acids. Four hundred microliters of 0.5 M EDTA and 25 μl of 20 mg/ml proteinase K (Applied Biosystems, Carlsbad, CA) were added and incubated 1 h at 55°C to inactivate DNase I. After cooling to room temperature, the lysate was added to 8.4 g of guanidine thiocyanate and briefly mixed. One milliliter of warmed, resuspended Wizard DNA Clean-Up Resin (Promega Corporation, Madison, WI) was added to the mixture and mixed by inverting for 15 minutes to allow for release of the phage DNA and binding to the resin. The mixture was pelleted by centrifugation at room temperature for 10 min at 5,000 x g and the supernatant was drawn off until ~5 ml remained. This mixture was resuspended by swirling, transferred into an empty syringe barrel attached to a Wizard Minicolumn (Promega Corporation), and pushed into the column. The column was then washed with 2 ml 80% isopropanol and dried by centrifugation for 2 min at 10,000 x g. JG068 DNA was eluted from the column following addition of 100 μl of 80°C nuclease-free water (Integrated DNA Technologies, Coralville, IA), incubation for 1 min, and centrifugation for ≥20 s at 10,000 x g.

Phage and plasmid DNA were quantified using a NanoDrop 2000c spectrophotometer (Thermo Scientific). For shotgun cloning, JG068 EcoRI fragments were purified using a GENECLEAN III kit (MP Biomedicals, Santa Ana, CA), ligated into pUC19, and transformed into *E. coli* DH5α (Invitrogen, Carlsbad, CA). Plasmids were purified using the GeneJET Plasmid Miniprep kit (Thermo Scientific) and sequenced using a 3730 DNA Analyzer (Applied Biosystems) by the University of Alberta Department of Biological Sciences Molecular Biology Service Unit (MBSU). Sequences were edited and assembled using Geneious [49].

The complete JG068 genome sequence was determined with the assistance of the MBSU using an Ion Torrent PGM (Applied Biosystems) and assembled using SeqMan NGen (DNASTAR, Madison, WI). Ambiguous regions in the assembly were resequenced using Sanger sequencing from EcoRI clones (for internal fragments) or PCR products (for terminal fragments). Chromosomal termini were identified using primers within the left direct terminal repeat (DTR), extending to the left end (IL: CAACCCTGTACAGCCGACCC), outside of the left DTR, extending to the left end (OL: CCTTGCTCTAT CTACCATGTTCCGC), within the right DTR, extending to the right end (IR: TGTGGATAGGGCGAAGTCT GAAGC), and outside of the right DTR, extending to the right end (OR: CTCCGACGAAGCATCCGC). Primers were used to directly sequence the JG068 DNA and sequence alignment was used to identify the loci where

sequence intensity dropped by 50% (for primers IL and IR) or 100% (for primers OL and OR). The JG068 sequence has been deposited in GenBank [GenBank: KC853746].

Bioinformatics analysis

Open reading frames (ORFs) were identified using GeneMark.hmm for prokaryotes [54] and annotated using BLASTP [55], HHpred [56], and CD-Search [57]. Gene *49*, which has a start codon within the last third of gene *48*, was identified using ORF Finder [58]. The replication origin and terminus were predicted using a GC-skew plot generated by GenSkew [59]. Genome restriction profiles were predicted using NEBcutter [60]. Whole genome comparisons were performed using CoreGenesUniqueGenes (CGUG) with a cutoff score of 75 [35,37]. Screening for bacterial toxins was performed using BTXpred [61]. Bacterial promoters, phage promoters, and rho-independent terminators were identified using prokaryotic Neural Network Promoter Prediction with a cutoff of 0.95 [50], PHIRE [51], and TransTermHP [53] on the PePPER server [62], respectively. Sequence logos were constructed using WebLogo [52]. Transmembrane domains were predicted using TMHMM [63] and OCTOPUS [64]. Lysis protein signal sequences were identified using SignalP 3.0 [65] and LipoP [66].

SAR endolysin expression

To assess the activity of the gp47 SAR endolysin, gene *47* including the signal sequence (bp 40,232 – 40,753; NdeI-SS-F: ATAATAACATATGCACCCCATCGTCAA GCGAG; HindIII-R: AAAAAGCTTCTATCGCCGGA TACCAGCAACG) or excluding the signal sequence (bp 40,307 – 40,753; NdeI-ΔSS-F: TAATAACATATGG ACGAGGGTATCCGGAACGTC; HindIII-R: as above) was PCR amplified using KAPA HiFi HotStart ReadyMix (Kapa Biosystems, Woburn, MA) and ligated in-frame into pET22b. These constructs were separately transformed into *E. coli* DH5α, sequenced to verify that the inserts were correct, and transformed into *E. coli* BL21 (DE3)pLysS using a standard protocol [67]. A pET22b blank control plasmid was isolated from *E. coli* MC4100 and transformed similarly into BL21(DE3)pLysS. To assay endolysin activity in *E. coli*, 5 ml liquid cultures of the three strains (in triplicate) were each subcultured into six wells of a 96 well plate by adding 10 μl culture to 190 μl LB broth containing 25 mg/l chloramphenicol and 100 mg/l ampicillin. The plate was then covered with plastic wrap and incubated with shaking at 37°C. Optical density measurements at 600 nm (OD_{600}) were measured at 1 h intervals with a Wallac 1420 VICTOR^2 multilabel counter (PerkinElmer, Waltham, MA). At 3 h (OD_{600} ~ 0.6), half of the samples were induced with IPTG (1 mM final concentration) and optical density measurements

continued at 1 h intervals up to 8 h. Averages and standard deviations were calculated using Microsoft Excel.

Galleria mellonella assays

G. mellonella infection and treatment assays were performed as described previously [10] with modifications. One milliliter of a 16 h K56-2 overnight culture was pelleted by centrifugation for 2 min at 10,000 × g, resuspended in 1 ml MgSO$_4$-ampicillin solution (10 mM MgSO$_4$, 1.2 mg/ml ampicillin), and diluted in this solution to 1:10^4. High-titre JG068 lysate was passaged through a Detoxi-Gel Endotoxin Removing Column (Thermo Scientific) and supplemented with ampicillin. Larvae (RECORP Inc., Georgetown, ON) were stored at 4°C and warmed to room temperature prior to injection with a 250 μl syringe with repeating dispenser (Hamilton Company, Reno, NV). In the left hindmost proleg, 5 μl of the diluted K56-2 suspension (3.7 × 10^3 CFU/5 μl) was injected into ten infected/treated larvae and ten infected/untreated larvae and 5 μl of MgSO$_4$-ampicillin solution was injected into ten uninfected/treated larvae. In the adjacent left proleg, 5 μl of the JG068 lysate (1.3 × 10^6 PFU/5 μl) was injected into ten infected/treated larvae and ten uninfected/treated larvae and 5 μl of MgSO$_4$-ampicillin solution was injected into ten infected/untreated larvae. Larvae were placed into petri dishes, incubated aerobically at 30°C for 72 h, and scored for survival. Experiments were repeated in triplicate. Averages and standard deviations were calculated using Microsoft Excel.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
KHL and AHA carried out the electron microscopy and sequenced and assembled the JG068 genome. KHL annotated the genome, performed bioinformatics analyses, and drafted the manuscript. AHA determined the BCC and LPS mutant host ranges, performed the SAR endolysin and *G. mellonella* experiments, and edited the manuscript. MS isolated JG068. JJD planned, supervised, and coordinated the study and edited the manuscript. All authors read and approved the final manuscript.

Author details
[1]Department of Biological Sciences, 6–008 Centennial Centre for Interdisciplinary Science, University of Alberta, Edmonton, AB T6G 2E9 Canada. [2]Institute of Microbiology, Technische Universität Braunschweig, Spielmannstr. 7, 38106 Braunschweig, Germany.

References

1. Falagas ME, Bliziotis IA, Kasiakou SK, Samonis G, Athanassopoulou P, Michalopoulos A: **Outcome of infections due to pandrug-resistant (PDR) Gram-negative bacteria.** *BMC Infect Dis* 2005, **5**:24.
2. Merril CR, Scholl D, Adhya SL: **The prospect for bacteriophage therapy in Western medicine.** *Nat Rev Drug Discov* 2003, **2**:489–497.
3. Bruttin A, Brüssow H: **Human volunteers receiving *Escherichia coli* phage T4 orally: a safety test of phage therapy.** *Antimicrob Agents Chemother* 2005, **49**:2874–2878.
4. Merabishvili M, Pirnay J-P, Verbeken G, Chanishvili N, Tediashvili M, Lashkhi N, Glonti T, Krylov V, Mast J, Van Parys L, Lavigne R, Volckaert G, Mattheus W, Verween G, De Corte P, Rose T, Jennes S, Zizi M, De Vos D, Vaneechoutte M: **Quality-controlled small-scale production of a well-defined bacteriophage cocktail for use in human clinical trials.** *PLoS ONE* 2009, **4**:e4944.
5. Rhoads DD, Wolcott RD, Kuskowski MA, Wolcott BM, Ward LS, Sulakvelidze A: **Bacteriophage therapy of venous leg ulcers in humans: results of a phase I safety trial.** *J Wound Care* 2009, **18**:237–243.
6. Wright A, Hawkins CH, Änggård EE, Harper DR: **A controlled clinical trial of a therapeutic bacteriophage preparation in chronic otitis due to antibiotic-resistant *Pseudomonas aeruginosa*; A preliminary report of efficacy.** *Clin Otolaryngol* 2009, **34**:349–357.
7. Mahenthiralingam E, Urban TA, Goldberg JB: **The multifarious, multireplicon *Burkholderia cepacia* complex.** *Nat Rev Microbiol* 2005, **3**:144–156.
8. Zhou J, Chen Y, Tabibi S, Alba L, Garber E, Saiman L: **Antimicrobial susceptibility and synergy studies of *Burkholderia cepacia* complex isolated from patients with cystic fibrosis.** *Antimicrob Agents Chemother* 2007, **51**:1085–1088.
9. Adachi N, Tsukamoto S, Inoue Y, Azegami K: **Control of bacterial seedling rot and seedling blight of rice by bacteriophage.** *Plant Dis* 2012, **96**:1033–1036.
10. Seed KD, Dennis JJ: **Experimental bacteriophage therapy increases survival of *Galleria mellonella* larvae infected with clinically relevant strains of the *Burkholderia cepacia* complex.** *Antimicrob Agents Chemother* 2009, **53**:2205–2208.
11. Lynch KH, Seed KD, Stothard P, Dennis JJ: **Inactivation of *Burkholderia cepacia* complex phage KS9 gp41 identifies the phage repressor and generates lytic virions.** *J Virol* 2010, **84**:1276–1288.
12. Carmody LA, Gill JJ, Summer EJ, Sajjan US, Gonzalez CF, Young RF, LiPuma JJ: **Efficacy of bacteriophage therapy in a model of *Burkholderia cenocepacia* pulmonary infection.** *J Infect Dis* 2010, **201**:264–271.
13. Lynch KH, Dennis JJ: **Cangene gold medal award lecture - Genomic analysis and modification of *Burkholderia cepacia* complex bacteriophages.** *Can J Microbiol* 2012, **58**:221–235.
14. Lynch KH, Dennis JJ: **Genomics of *Burkholderia* phages.** In *Burkholderia: From Genomes to Function.* Edited by Coenye T, Mahenthiralingam E. Hethersett: Horizon Scientific Press. In press.
15. Summer EJ, Gonzalez CF, Bomer M, Carlile T, Embry A, Kucherka AM, Lee J, Mebane L, Morrison WC, Mark L, King MD, LiPuma JJ, Vidaver AK, Young R: **Divergence and mosaicism among virulent soil phages of the *Burkholderia cepacia* complex.** *J Bacteriol* 2006, **188**:255–268.
16. Robinson SM, Hall J, Das A, Rosenbloom A, Gonzalez C, Young R, Summer EJ: *M-015. Genomic analysis of virulent soil phages of Burkholderia.* http://ieg.ou.edu/asm2006/data/papers/M_015.htm.
17. Pirnay J-P, Verbeken G, Rose T, Jennes S, Zizi M, Huys I, Lavigne R, Merabishvili M, Vaneechoutte M, Buckling A, De Vos D: **Introducing yesterday's phage therapy in today's medicine.** *Future Virol* 2012, **7**:379–390.
18. Seed KD, Dennis JJ: **Development of *Galleria mellonella* as an alternative infection model for the *Burkholderia cepacia* complex.** *Infect Immun* 2008, **76**:1267–1275.
19. Mahenthiralingam E, Coenye T, Chung JW, Speert DP, Govan JRW, Taylor P, Vandamme P: **Diagnostically and experimentally useful panel of strains from the *Burkholderia cepacia* complex.** *J Clin Microbiol* 2000, **38**:910–913.
20. Coenye T, Vandamme P, LiPuma JJ, Govan JRW, Mahenthiralingam E: **Updated version of the *Burkholderia cepacia* complex experimental strain panel.** *J Clin Microbiol* 2003, **41**:2797–2798.
21. Broad Institute: *Burkholderia dolosa database.* http://www.broadinstitute.org/annotation/genome/burkholderia_dolosa.
22. Seed KD, Dennis JJ: **Isolation and characterization of bacteriophages of the *Burkholderia cepacia* complex.** *FEMS Microbiol Lett* 2005, **251**:273–280.

23. Goudie AD, Lynch KH, Seed KD, Stothard P, Shrivastava S, Wishart DS, Dennis JJ: **Genomic sequence and activity of KS10, a transposable phage of the *Burkholderia cepacia* complex.** *BMC Genomics* 2008, **9**:615.
24. Lynch KH, Stothard P, Dennis JJ: **Genomic analysis and relatedness of P2-like phages of the *Burkholderia cepacia* complex.** *BMC Genomics* 2010, **11**:599.
25. Lynch KH, Stothard P, Dennis JJ: **Characterization of DC1, a broad-host-range Bcep22-like podovirus.** *Appl Environ Microbiol* 2012, **78**:889–891.
26. Lynch KH, Stothard P, Dennis JJ: **Comparative analysis of two phenotypically-similar but genomically-distinct *Burkholderia cenocepacia*-specific bacteriophages.** *BMC Genomics* 2012, **13**:223.
27. Loutet SA, Flannagan RS, Kooi C, Sokol PA, Valvano MA: **A complete lipopolysaccharide inner core oligosaccharide is required for resistance of *Burkholderia cenocepacia* to antimicrobial peptides and bacterial survival in vivo.** *J Bacteriol* 2006, **188**:2073–2080.
28. Ortega X, Silipo A, Saldfas MS, Bates CC, Molinaro A, Valvano MA: **Biosynthesis and structure of the *Burkholderia cenocepacia* K56-2 lipopolysaccharide core oligosaccharide: Truncation of the core oligosaccharide leads to increased binding and sensitvity to polymyxin B.** *J Biol Chem* 2009, **284**:21738–21751.
29. Ackermann H-W: **Frequency of morphological phage descriptions in the year 2000.** *Arch Virol* 2001, **146**:843–857.
30. Casjens SR, Gilcrease EB: **Determining DNA packaging strategy by analysis of the termini of the chromosomes in tailed-bacteriophage virions.** *Methods Mol Biol* 2009, **502**:91–111.
31. Summer EJ: **Preparation of a phage DNA fragment library for whole genome shotgun sequencing.** *Methods Mol Biol* 2009, **502**:27–46.
32. Lavigne R, Burkal'tseva MV, Robben J, Sykilinda NN, Kurochkina LP, Grymonprez B, Jonckx B, Krylov VN, Mesyanzhinov VV, Volckaert G: **The genome of bacteriophage φKMV, a T7-like virus infecting *Pseudomonas aeruginosa*.** *Virology* 2003, **312**:49–59.
33. Ceyssens P-J, Lavigne R, Mattheus W, Chibeu A, Hertveldt K, Mast J, Robben J, Volckaert G: **Genomic analysis of *Pseudomonas aeruginosa* phages LKD16 and LKA1: establishment of the φKMV subgroup within the T7 supergroup.** *J Bacteriol* 2006, **188**:6924–6931.
34. Adriaenssens EM, Ceyssens P-J, Dunon V, Ackermann H-W, Van Vaerenbergh J, Maes M, De Proft M, Lavigne R: **Bacteriophages LIMElight and LIMEzero of *Pantoea agglomerans*, belonging to the "φKMV-like viruses".** *Appl Environ Microbiol* 2011, **77**:3443–3450.
35. Lavigne R, Seto D, Mahadevan P, Ackermann H-W, Kropinski AM: **Unifying classical and molecular taxonomic classification: analysis of the *Podoviridae* using BLASTP-based tools.** *Res Microbiol* 2008, **159**:406–414.
36. Abbasifar R, Kropinski AM, Sabour PM, Ackermann H-W, Alanis Villa A, Abbasifar A, Griffiths MW: **The genome of *Cronobacter sakazakii* bacteriophage vB_CsaP_GAP227 suggests a new genus within the *Autographivirinae*.** *Genome Announc* 2013, **1**:e00122-12.
37. Mahadevan P, King JF, Seto D: **CGUG: in silico proteome and genome parsing tool for the determination of "core" and unique genes in the analysis of genomes up to ca. 1.9 Mb.** *BMC Res Notes* 2009, **2**:168.
38. Molineux IJ: **The T7 Group.** In *The Bacteriophages*. 2nd edition. Edited by Calendar R. New York: Oxford University Press; 2006:277–301.
39. Casjens S: **Prophages and bacterial genomics: What have we learned so far?** *Mol Microbiol* 2003, **49**:277–300.
40. Kawasaki T, Shimizu M, Satsuma H, Fujiwara A, Fujie M, Usami S, Yamada T: **Genomic characterization of *Ralstonia solanacearum* phage φRSB1, a T7-like wide-host-range phage.** *J Bacteriol* 2009, **191**:422–427.
41. Lavigne R, Villegas A, Kropinksi AM: ***In silico* characterization of DNA motifs with particular reference to promoters and terminators.** *Methods Mol Biol* 2009, **502**:113–129.
42. Lavigne R, Noben JP, Hertveldt K, Ceyssens P-J, Briers Y, Dumont D, Roucourt B, Krylov VN, Mesyanzhinov VV, Robben J, Volckaert G: **The structural proteome of *Pseudomonas aeruginosa* bacteriophage φKMV.** *Microbiology* 2006, **152**:529–534.
43. Briers Y, Peeters LM, Volckaert G, Lavigne R: **The lysis cassette of bacteriophage φKMV encodes a signal-arrest-release endolysin and a pinholin.** *Bacteriophage* 2011, **1**:25–30.
44. Dobbins AT, George M Jr, Basham DA, Ford ME, Houtz JM, Pedulla ML, Lawrence JG, Hatfull GF, Hendrix RW: **Complete genomic sequence of the virulent *Salmonella* bacteriophage SP6.** *J Bacteriol* 2004, **186**:1933–1944.
45. Walmagh M, Boczkowska B, Grymonprez B, Briers Y, Drulis-Kawa Z, Lavigne R: **Characterization of five novel endolysins from Gram-negative infecting bacteriophages.** *Appl Microbiol Biotechnol* 2012: 10.1007/s00253-012-4294-7.
46. Gill JJ, Summer EJ, Russell WK, Cologna SM, Carlile TM, Fuller AC, Kitsopoulos K, Mebane LM, Parkinson BN, Sullivan D, Carmody LA, Gonzalez CF, LiPuma JJ, Young R: **Genomes and characterization of phages Bcep22 and BcepIL02, founders of a novel phage type in *Burkholderia cenocepacia*.** *J Bacteriol* 2011, **193**:5300–5313.
47. Garbe J, Bunk B, Rohde M, Schobert M: **Sequencing and characterization of *Pseudomonas aeruginosa* phage JG004.** *BMC Microbiol* 2011, **11**:102.
48. Promega Corporation: *DNA Isolation From Lambda Lysates Using the Wizard® DNA Clean-Up System.* http://www.promega.ca/resources/articles/pubhub/enotes/dna-isolation-from-lambda-lysates-using-the-wizard-dna-cleanup-system.
49. Drummond AJ, Ashton B, Buxton S, Cheung M, Cooper A, Duran C, Heled J, Kearse M, Markowitz S, Moir R, Stones-Havas S, Sturrock S, Swidan F, Thierer T, Wilson A: *Geneious v5.6.* http://www.geneious.com.
50. Reese MG: **Application of a time-delay neural network to promoter annotation in the *Drosophila melanogaster* genome.** *Comput Chem* 2001, **26**:51–56.
51. Lavigne R, Sun WD, Volckaert G: **PHIRE, a deterministic approach to reveal regulatory elements in bacteriophage genomes.** *Bioinformatics* 2004, **20**:629–635.
52. Crooks GE, Hon G, Chandonia JM, Brenner SE: **WebLogo: a sequence logo generator.** *Genome Res* 2004, **14**:1188–1190.
53. Kingsford CL, Ayanbule K, Salzberg SL: **Rapid, accurate, computational discovery of Rho-independent transcription terminators illuminates their relationship to DNA uptake.** *Genome Biol* 2007, **8**:R22.
54. Lukashin AV, Borodovsky M: **GeneMark.hmm: New solutions for gene finding.** *Nucleic Acids Res* 1998, **26**:1107–1115.
55. Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ: **Gapped BLAST and PSI-BLAST: A new generation of protein database search programs.** *Nucleic Acids Res* 1997, **25**:3389–3402.
56. Söding J, Biegert A, Lupas AN: **The HHpred interactive server for protein homology detection and structure prediction.** *Nucleic Acids Res* 2005, **33**:W244–W248.
57. Marchler-Bauer A, Bryant SH: **CD-Search: Protein domain annotations on the fly.** *Nucleic Acids Res* 2004, **32**:W327–W331.
58. NCBI: *ORF Finder.* http://www.ncbi.nlm.nih.gov/projects/gorf.
59. *GenSkew – visualization of nucleotide skew in genome sequences.* http://genskew.csb.univie.ac.at.
60. Vincze T, Posfai J, Roberts RJ: **NEBcutter: A program to cleave DNA with restriction enzymes.** *Nucleic Acids Res* 2003, **31**:3688–3691.
61. Saha S, Raghava GP: **BTXpred: prediction of bacterial toxins.** *In Silico Biol* 2007, **7**:405–412.
62. *Transcription Terminator Prediction.* http://pepper.molgenrug.nl/index.php/pepper-tools/terminator-predictie.
63. Krogh A, Larsson B, von Heijne G, Sonnhammer ELL: **Predicting transmembrane protein topology with a hidden Markov model: Application to complete genomes.** *J Mol Biol* 2001, **305**:567–580.
64. Viklund H, Elofsson A: **OCTOPUS: Improving topology prediction by two-track ANN-based preference scores and an extended topological grammar.** *Bioinformatics* 2008, **24**:1662–1668.
65. Bendtsen JD, Nielsen H, von Heijne G, Brunak S: **Improved prediction of signal peptides: SignalP 3.0.** *J Mol Biol* 2004, **340**:783–795.
66. Juncker AS, Willenbrock H, von Heijne G, Brunak S, Nielsen H, Krogh A: **Prediction of lipoprotein signal peptides in Gram-negative bacteria.** *Protein Sci* 2003, **12**:1652–1662.
67. Silhavy TJ, Berman ML, Enquist LW: *Experiments with Gene Fusions.* Cold Spring Harbor Laboratory: Cold Spring Harbor; 1984.