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Genome-wide screens reveal *Escherichia coli* genes required for growth of T1-like phage LL5 and rV5-like phage LL12

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Abstract: Factors affecting the host-virus interaction must be understood for the effective application of bacteriophages to combat bacterial pathogens. Two novel *E. coli* phages, the T1-like siphophage LL5 and the rV5-like myophage LL12, were subjected to forward genetic screens against the Keio collection, a library of single non-essential gene deletions in *E.coli* str. BW25113. These genome-wide screens and subsequent experiments identified eight genes required for efficient propagation of phage LL5 and six genes required for propagation of LL12. The majority of the genes identified were involved in production of the phage receptors. *E. coli* mutants deficient in heptose II and the phosphoryl substituent of heptose I of the inner core lipopolysaccharide (LPS) were unable to propagate phage LL5, as were mutants deficient in the outer membrane protein TolC. Mutants lacking glucose I of the LPS outer core failed to propagate LL12. Two cytoplasmic chaperones, PpiB and SecB, were found to be required for efficient propagation of phage LL5 but not LL12. This approach may be useful for identifying phage receptors and required host factors in other phages, which would provide valuable information for their potential use as therapeutics and for phage engineering.

Keywords: bacteriophage; phage-host interactions; phage receptors; high-throughput screen; tail fibers

1. Introduction

Escherichia coli is a Gram-negative facultative anaerobic bacterium which is commonly found as a member of the commensal gut flora in mammals [1]. While commensal *E. coli* strains generally do not cause disease in humans, multiple strains of *E. coli* have acquired virulence factors and may cause disease with symptoms ranging from mild discomfort to life-threatening bacteremia. These strains have been categorized into several pathotypes, including enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC), and diffusely adherent *E. coli* (DAEC) [4].

Enterotoxigenic *E. coli* strains can be distinguished from other *E. coli* pathotypes by the presence of heat-labile enterotoxin (LT) and/or heat-stable enterotoxin (HT) [4]. These enterotoxins induce traveler's diarrhea (TD), characterized by mild to severe watery diarrhea [4], which may be accompanied by nausea, vomiting, abdominal pain, fever or blood in stool [5]. TD is one of the most common illnesses contracted by people from developed countries during international travel, with ETEC being the major causative agent of TD in Latin America, Africa, South Asia and the Middle East [6]. TD typically self-resolves or may be successfully treated with antibiotics, but the global increase in the emergence of antibiotic resistance warrants evaluation of alternative treatment approaches [6].

Bacteriophages (phages) are the natural viral predators of bacteria. Due to the ongoing emergence of multidrug-resistant bacteria and limited development of new antibiotics, there has been a renewed interest in the use of phages as antimicrobials [7-10]. However, knowledge on the basic biology of phages outside of a number of well-studied model organisms is limited. Phages are generally diverse at the genetic level and they are constantly co-evolving with their bacterial hosts [11,12]. In the use of phages as therapeutics, many novel phages need to be isolated and deployed [13]; for phage therapy to succeed, a thorough understanding of phage-host interactions on a broader scale is required [7].

With the availability of modern genetic resources, host-phage interactions can be studied efficiently on a large scale. A number of genome-wide screens have been conducted to study host factors required for viral replication in organisms such as HIV [14], Influenza virus [15], phage λ [16] and phage T7 [17]. We conducted forward-genetics screens of the novel *E. coli* phages LL5 and LL12 against the Keio collection, a library of single-gene deletions of all non-essential genes in *E. coli* K-12 strain BW25113 [18], in order to characterize host functions required for propagation of these two potentially therapeutic phages. Such host factors may include the identity of the phage receptors, chaperones, and other host accessory functions. Understanding host-virus interaction is also critical to facilitate the engineering of robust phages [17]. The results of this screen are discussed in terms of host factors required for infection and propagation of phages LL5 and LL12.

2. Materials and Methods

2.1 Bacterial strains and plasmids

The Keio collection was purchased from Thermo Scientific [18]. Keio strains deleted for *tolC* and *rfaC* were obtained directly from the Coli Genetic Stock Center at Yale University. Keio strain deleted for *rfaF* was purchased from the Dharmacon, Inc. Strains from the ASKA library used for complementation were purchased from National BioResource Project (NIG, Japan) [19]. To complement phenotypes associated with *tolC*, *rfaC* and *rfaF*, respective genes from *E. coli* str. MG1655 were cloned into the pBAD24 vector and expressed *in trans* as previously described [20]. The parental *E. coli* strain BW25113 was obtained from Ry Young (Texas A&M University, College Station, TX). *E. coli* strains from the Keio collection and their transductants were cultured in LB (Lennox) broth [10 g L^{-1} Bacto tryptone (BD), 5 g L^{-1} Bacto yeast extract (BD), 5 g L^{-1} NaCl (Avantor)] or LB agar [LB broth amended with 15 g L^{-1} Bacto agar (BD)] at 37 °C amended with 30 μg mL^{-1} kanamycin (LB kan) and strains containing plasmids from the ASKA library were maintained on LB amended with 10 μg mL^{-1} chloramphenicol (LB cm). Plasmid DNA from ASKA library strains was extracted using a QIAprep Spin Miniprep Kit (Qiagen). In complementation experiments with the ASKA plasmids, LB plates or top agar were supplemented with 0.05 - 0.1 mM IPTG to induce protein expression [19]. Strains containing pBAD24-based plasmids were maintained on LB amended with 100 μg mL^{-1} ampicillin (LB amp). In complementation experiments with pBAD24-based plasmids, LB plates were supplemented with 0.1 mM L-arabinose. L-arabinose was omitted during complementation of *tolC* because of the toxicity of TolC overexpression. Leaky expression of TolC from the uninduced complementing plasmid was sufficient for restoring the plating efficiency of phage LL5. All primers used in this study will be provided upon request.

2.2 Phage isolation and culture

The phages LL5 and LL12 were isolated against clinical isolates of enterotoxigenic *E. coli* (ETEC) obtained from John Deaton (Deerland Enzymes, Kennesaw, GA). Phages were isolated by the enrichment method [21] from filter-sterilized (0.22 μm) wastewater influent collected in College Station, TX in 2011. Both phages were subsequently cultured using *E. coli* strain DH5α as host. Phage lysates were prepared by the confluent plate lysis method [22] using LB (Miller) bottom plates (10 g L^{-1} Bacto tryptone, 5 g L^{-1} Bacto yeast extract, 10 g L^{-1} NaCl, 15 g L^{-1} Bacto agar) and top agar consisting of 10 g L^{-1} tryptone, 10 g L^{-1} NaCl, 5 g L^{-1} Bacto agar. Phages were harvested and stored as filter-sterilized (0.22

μm) lysates in lambda diluent (25 mM Tris-HCl pH 7.5, 100 mM NaCl, 8 mM $MgSO_4$, 0.01% w/v gelatin) at 4 °C.

Plaque assays were conducted using both spot titer and full-plate titration methods [22]. For spot titers, 10 μL of serially diluted phage was spotted on solidified lawns of 4 ml top agar inoculated with 100 μL of a fresh overnight host culture prepared as described above. For full-plate titers, 100 μL of serially diluted phage was mixed with 100 μL of host culture in 4 ml of molten top agar and poured over LB plates as described above. Plaques were enumerated after 16-18 h incubation at 37 °C. The efficiency of plating (EOP) was calculated as the ratio of the number of plaques appearing on the lawn of a test strain to the number of plaques on the reference strain.

2.3 Phage genome sequencing and annotation

Phage DNA was purified from high-titer lysates by a modified Wizard DNA purification kit (Promega) as previously described [23]. Phage LL5 was sequenced by 454 pyrosequencing at the Emory GRA Genome Center (Emory University, GA); trimmed FLX Titanium sequence reads were assembled into a single contig at 19.9-fold coverage using Newbler 2.5.3 (454 Life Sciences) at default settings. Phage LL12 was sequenced by Illumina TruSeq as unpaired 100-base reads; reads were quality-controlled by FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and assembled with Velvet 1.1 [24] into a single contig at 28.3-fold coverage. Assembled phage contigs were confirmed to be complete by PCR using primers facing off each end of the contig and sequencing of the resulting products. Structural annotation was conducted using Glimmer3 [25] and MetaGeneAnnotator [26] with tRNAs predicted by ARAGORN [27] or tRNAscan-SE [28] and gene functions predicted by InterProScan [29] or Conserved Domain Database [30], TMHMM (http://www.cbs.dtu.dk/services/TMHMM), BLASTp [31] and HHpred [32]. Phage genome annotation was conducted using the Phage Galaxy instance hosted by the Center for Phage Technology at Texas A&M University (cpt.tamu.edu). The annotated phage genomes were deposited in NCBI Genbank under accession no. MH491968 (LL5) and MH491969 (LL12).

2.4 Transmission electron microscopy

Phages were stained with 2% uranyl acetate and imaged in a JEOL 1200EX transmission electron microscope (TEM) under 100 kV accelerating voltage at the Texas A&M University Microscopy and Imaging Center, as previously described [20,33]. The size parameters of phages were measured electronically using ImageJ [34].

2.5 Screening and confirmation of phage-insensitive mutants

In order to optimize the input phage concentrations and incubation times, phages LL5 and LL12 stocks were serially diluted in fresh LB and 160 μL of each dilution was aliquoted into 96-well sterile transparent polystyrene flat-bottom plates (Greiner Bio-one). The plates were then inoculated with the Keio parental strain BW25115 using a 96-pin replicator (Phenix) and incubated at 37 °C for 6, 8, 10 and 18 hrs. The optical density (OD) at 550 nm was measured in a Tecan M200 plate reader at each time interval and the average OD was analyzed to determine the lowest phage concentration that inhibited bacterial growth.

The Keio collection consists of 90, 96-well plates containing two independently-generated sets of 3,985 single-gene knockouts in the *E. coli* BW25113 background [18]. The Keio strains were replicated into 96 well sterile polypropylene U-bottom microplates (Greiner Bio-one) containing LB kan + 8% glycerol using sterile plastic 96-pin replicators (Phenix). Plates were incubated at 37 °C overnight and stored frozen at -80 °C. These plates were used as the working stocks for the following screens. The odd- and even-numbered plates have identical gene deletion mutants created by independent experiments [18], and only the odd-numbered 45 plates were used for the initial screen. Initial screens were conducted in 96-well sterile transparent polystyrene flat-bottom plates (Greiner Bio-one). Phages LL5 and LL12 were diluted in fresh LB to obtain working stocks of 10^6 PFU mL^{-1} for LL5 and 10^3 PFU

mL^{-1} for LL12. 160 μL of the phage working stocks were aliquoted into all wells, Keio strains were inoculated into the phage lysates from the 96-well working stocks with 96-pin replicators, and the plates were incubated for 8 hours at 37°C. The OD_{550} was measured and the wells with OD_{550} higher than the predetermined cutoff values (0.2 for phage LL5 and 0.11 for phage LL12) were scored as positive for growth.

The positive mutants obtained from the first screen were verified by repeating the assay with the same strains and their corresponding mutant strains from the even-numbered Keio collection plates, side-by-side with eight replicates per assay. Mutants that returned mean OD_{550} above the designated cutoff in either the even- or odd-numbered set were retained for further characterization by measurement of phage efficiency of plating (EOP) by spot assays on soft agar lawns [22] as described above. EOP was calculated as the number of plaques observed on the mutant strain divided by the number of plaques observed on the parental *E. coli* strain BW25113. Mutants with EOP's of less than 10^{-2} were confirmed by enumerating plaques on full plates. When possible, mutant alleles were moved into the parental BW25113 background by P1 transduction using the kanamycin resistance cassette as the selectable marker [35]. All gene disruptions were confirmed by PCR using primers flanking the predicted insert followed by sequencing of the PCR product to confirm disruption of the gene. All mutants were complemented by transforming the original Keio mutant or its P1 transductant with a plasmid expressing the corresponding gene.

3. Results and Discussion

3.1 Isolation and characterization of phages LL5 and LL12

Phages LL5 and LL12 were isolated from municipal wastewater in College Station, TX by enrichment against enterotoxigenic *E. coli* (ETEC) clinical isolates. Shortly after isolation, both phages were determined to plate efficiently on *E. coli* K-12 strains including MG1655, DH5α and the Keio parental strain BW25113 and these phages were subsequently propagated on *E. coli* DH5α or BW25113 for the remainder of the study.

Phages LL5 and LL12 have distinct morphology as observed by transmission electron microscopy (Figure 1). LL5 is a siphophage with a head diameter of 61 nm (± 2 nm) and a flexible tail 156 nm (± 10 nm) in length. Phage LL12 is a large myophage with head diameter of 86 nm (± 2 nm) and a tail 112 nm (± 4 nm) in length with a pronounced baseplate.

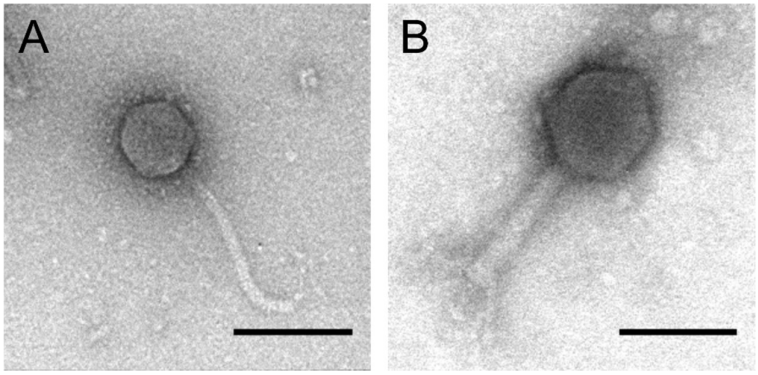

Figure 1. Transmission electron micrographs of phages LL5 (A) and LL12 (B). Phage LL5 has a capsid diameter of 61 nm (±2 nm) and a flexible, non-contractile tail 156 nm (±10 nm) in length. Phage LL12 has a capsid diameter of 86 nm (±2 nm) and a non-contractile tail 112 nm (±4 nm) in length. Dimensions are an average of ten measurements and the error represents standard deviation. The scale bar denotes 100 nm.

LL5 has a genome of 49,788 bp with 88 predicted protein-coding genes and no tRNA genes. The genome produced a circular assembly and was reopened to be syntenic with other T1-like phages in the NCBI database such as TLS (NC_009540) and T1 (NC_005833). Thirty-three LL5 encoded proteins could be assigned putative functions (Table S1, Figure 2A). Genes responsible for different stages of phage infection cycle have been identified in LL5, including a DNA primase/helicase (gp58), ATP-dependent helicase (gp60) and helicase (gp75). Structural proteins including the portal protein (gp31), major capsid protein (gp36), minor tail proteins (gp41, gp42, gp47, gp48), tail tube protein (gp43), tape measure protein (gp46), and tail fiber proteins (gp51, gp57) were identified. The small and large terminase subunits were identified as gp29 and gp30, respectively. Like its T1-like relatives, LL5 encodes a canonical lysis cassette composed of a holin (gp70), endolysin (gp71) and unimolecular spanin (gp72).

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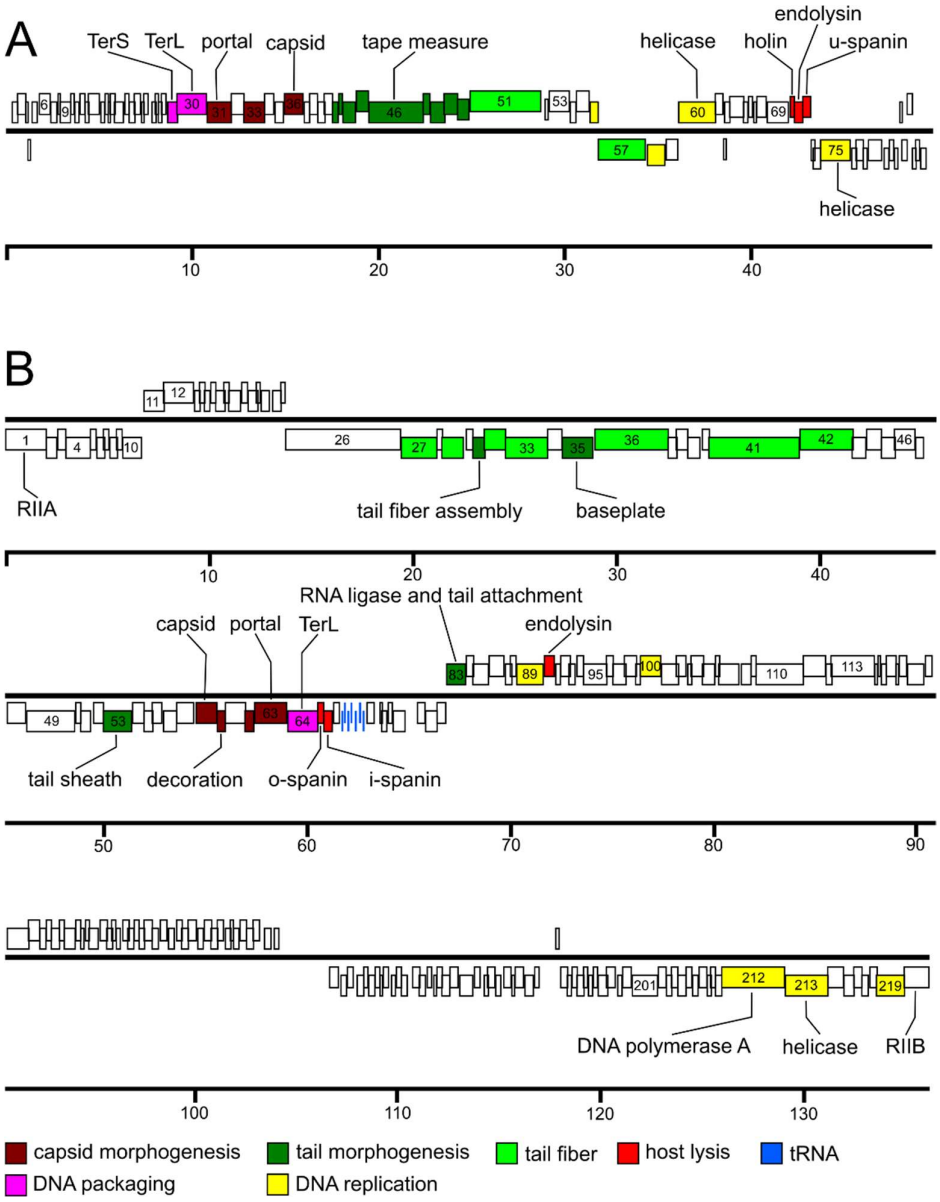

Figure 2. Genomic map of phages LL5 (A) and LL12 (B). The predicted genes of phages LL5 and LL12 are represented as boxes, which are numbered to match locus number. The boxes above the line denote genes on the positive strand, whereas those below the line denote genes on the negative strand. Predicted gene products playing important roles in phage infection cycle are indicated. The colored boxes represent genes predicted to perform similar functions. The scale bar represents the DNA position in kilobases.

Phage LL5 is closely related to the T1-like coliphage TLS (NC_009540) [36], with 96% sequence identity over 90% query coverage of the LL5 genome based on BLASTn analysis. As would be expected given this close similarity, the LL5 genome is syntenic with TLS, with 75 LL5 proteins having homologs in the TLS genome detectable by BLASTp with an E-value of less than 10^{-5}. Phage LL5 encodes two predicted tail fiber proteins, gp51 and gp57, in a genomic arrangement similar to that found in phage

TLS and T1. LL5 gp51 is closely related to predicted tail fibers in other phages including gp51 of TLS (also called TspJ, YP_001285540, 98% identity), gp33 of phage T1 (FibA, YP_003912, 67% identity) and the central tail fiber protein J of phage lambda (NP_040600, 23% identity). Approximately 3 kb downstream and on the opposite strand from gp51, gp57 is similar to other T1-like tail fibers only in its N-terminal domain, with 46% identity to T1 FibB (YP_003919) from residues 1-290. The C-terminal domain of gp57 is more closely related to tail fiber proteins found in T5-like phages such as DT57C and DT571/2 [37]. LL5 gp57 is 57% identical with a C-proximal region spanning residues 515 to 830 of the 1,076-residue DT57C LtfA protein (YP_009149889), which is within the host specificity region of this protein [38].

The LL12 genome was determined to be 136,026 bp in length and encodes 213 predicted protein-coding genes and 7 tRNAs. The genome produced a circular assembly and was reopened at a point between the genes encoding T4 RIIA and RIIB homologs, to retain its general synteny with other RIIAB-encoding myophages. Analysis of the raw Illumina reads by PhageTerm [39] suggests the presence of a non-permuted terminal redundancy of 459 bp spanning bases 104,966 - 105,424 in the genome as presented here. This predicted terminal repeat is located in a non-coding region of DNA between two convergent transcripts and corresponds to the location of non-permuted terminal repeats observed in phage phi92 [40]. Fifty LL12-encoded proteins could be assigned putative functions, as shown in Table S2 and Figure 2B. Major components for head morphogenesis including capsid protein (gp59), prohead protease (gp62), portal protein (gp63), and the large terminase subunit (TerL, gp64) were identified. The components for tail morphogenesis including baseplate (gp35), tail sheath protein (gp53), and multiple predicted tail fiber proteins (gp27, gp29, gp32, gp33, gp36, gp41, gp42) were identified. DNA replication proteins such as DNA polymerase (gp212), DNA replicative helicase/primase (gp213), and a helicase (gp219) were also identified. The genes encoding the large terminase subunit (gp64) and DNA polymerase (gp212) are disrupted by predicted intron sequences. These introns appear to be relatively short (~275-325 bp) and do not contain any significant protein-coding ORFs. The boundaries of these introns were determined based on protein sequence similarity to homologous proteins found in other phages that were not disrupted by introns (AKU44155 in the case of gp64, and AKU44295 for gp212). Like other large myophages, genes responsible for phage lysis are distributed across the LL12 genome rather than co-localized to a contiguous cassette; the phage endolysin (gp90), i-spanin (gp66) and o-spanin (gp65) were identifiable but the phage holin could not be positively identified. Based on analysis of predicted protein sequences by BLASTp with an E-value cutoff of 10^{-5}, LL12 is most closely related to other V5-like myophages, including rV5 (NC_011041) which shares 206 proteins with LL12, and ΦAPCEc02 (KR698074) which shares 204 proteins. LL12 is also more distantly related to the *E. coli* phage phi92 (NC_023693), with 48 common proteins detectable by BLASTp.

Like the related phages rV5, phi92 and ΦAPCEc02, phage LL12 encodes an extensive set of predicted tail fibers: gp27, gp29, gp32, gp33, gp36, gp41 and gp42 (Figure 2B, Table S2). All seven of these LL12 tail fibers are similar to tail fibers found in rV5 and ΦAPCEc02, with protein identities (by Dice coefficient) ranging from 4%-100% (Table S3). Six of these seven proteins are also detectable in the more distantly-related phage phi92, with three of these, gp29, gp33 and gp42 producing alignments to nearly the full-length phi92 proteins 147, 142 and 141, respectively (Table S3). CryoEM reconstructions of phi92 have indicated that this phage possesses multiple sets of tail fibers that are mounted to the baseplate in downward, sideward, and upward orientations [40]. These multiple tail fibers may contribute to a broadened host range in this phage and its relatives [40]. The electron density of the downward-facing tail fiber was assigned to gp143, which is not conserved in LL12 [40]. LL12 gp27 shows weak similarity to the N-terminus of phi92 gp150, which is predicted to form downward-facing tail spikes in cryoEM reconstructions [40]. LL12 gp41 also possesses similarity to rV5 gp41 (Table S3), however LL12 gp41 is missing the C-terminal chaperone of endosialidase domain (pfam13884) of rV5 gp41 spanning residues 1151-1200.

3.2 Host range determination for phages LL5 and LL12

Infection by STEC strains can result in watery or bloody diarrhea, hemolytic uremic syndrome, microangiopathic hemolytic anemia and thrombocytopenia [41]. *E. coli* strains belonging to several pathotypes tend to be clonal and are grouped as serotypes based on the O-antigens (lipopolysaccharide) and H-antigens (flagella) [4]. As phage LL12 bears similarity to phages rV5 and ΦAPCEc02, both of which infect STEC serotype O157:H7 [42,43], we sought to determine if phages LL5 and LL12 are also able to infect STEC representatives.

Phages LL5 and LL12 were spotted on soft agar overlays of STEC strains and EOP compared to the Keio parental strain BW25113. Phage LL5 was unable to form plaques on any of the tested STEC strains, and phage LL12 exhibited EOPs of close to 1 on STEC strains with serotypes O157:H7, O145:NM, O121:H19, O146 and O121:H19, demonstrating a relatively broad host range among STEC serotypes for this phage (Table 1).

Table 1. Host range of phages LL5 and LL12. Phages LL5 and LL12 were tested for their ability to infect Shiga toxin-producing *Escherichia coli* (STEC) by spotting serially diluted phages on the soft agar lawns of respective STEC isolates. The efficiency of plating (EOP) is relative to the number of plaques formed on the Keio collection parental *E. coli* strain BW25113. Cells marked with "-" indicate an EOP of less than 10^{-7} (insensitive to phage). The data is the average of two biological replicates.

STEC serotype	Isolate ID	Phage LL5 EOP	Phage LL12 EOP	LPS Core types[b]
Not STEC	BW25113	1.0	1.0	K-12
O157:H7[a]	USDA-FSIS 380-94	-	0.8	R3
O104:H21	ATCC BAA-178	-	-	
O145:NM[a]	83-75	-	0.7	R1, K-12
O26:H11[a]	H30	-	-	R3
O111:H-[a]	JBI-95	-	-	R3
O121:H19	ATCC BAA-2219	-	0.7	
O146	ATCC BAA-2217	-	1.0	
O103:H11	ATCC BAA-2215	-	-	
O145:Nonmotile	ATCC BAA-2192	-	-	R1, K-12
O26:H11	ATCC BAA-2196	-	-	R3
O45:H2	ATCC BAA-2193	-	-	
O103:H2[a]	CDC 90-3128	-	-	R3
O121:H19[a]	CDC 97-3068	-	0.6	
O45:H2[a]	CDC 96-3285	-	-	

[a] Sources of these isolates are described in [44]

[b] LPS core types information obtained from [45]

3.3 Development and optimization of screening assay

Multiplicity of Infection (MOI) is the ratio of the number of the phages to host cells in a culture. The purpose of the phage Keio screen was to identify host genes required for the phage to successfully infect, replicate within, and lyse their host cells. To determine this, it was imperative to optimize MOI

for each phage as excessively high MOI's could result in bacterial growth inhibition if the phage were able to infect the cells but still not produce progeny, while MOI's which were too low could result in false positive results [46]. Initially, the lowest input phage concentration required to control growth of parental BW25113 strain after 8 hr incubation at 37 °C was determined. A log higher phage concentration was applied in this screen so as to minimize false positives. The number of bacterial cells inoculated by the 96-pin replicator were determined by viable counts. Based upon the cells inoculated and PFU of phages used, the initial MOI of LL5 and LL12 in this screen was calculated to be 1.0 and 0.001, respectively.

3.4 Genes required for propagation of phage LL5

Phage LL5 was screened against the 3,985 single-gene knockouts of the Keio collection as described in the Materials and Methods and in the Supplementary Text. Following screening, confirmation and genetic complementation, eight genes were determined to be required for efficient propagation of phage LL5 (Table 2). Strains deleted for genes *lpcA*, *rfaE*, *rfaC*, *rfaP*, *rfaF* and *tolC* showed severe plating defects with the EOP of phage LL5 less than ~10^{-7}. This plating defect was also observed in the P1 transduced *rfaP* mutant. The plating efficiency of phage LL5 could be restored when the respective genes were provided *in trans* (Table 2). Two additional Keio mutants, *secB* and *ppiB*, exhibited milder defects in supporting phage LL5 growth, with EOP reductions of ~10- to 100-fold relative to the parental *E. coli* strain BW25113; these mutants could be transduced by P1 into the parental background and could also be complemented *in trans* (Table 2).

Table 2. *E. coli* genes required for phages LL5 and LL12 infection cycle. The genes required for phage infection cycle can be determined by testing the efficiency of plating. Eight genes were found to be required for phage LL5 plaque formation, whereas 6 genes were required for phage LL12. The kanamycin resistance cassette in the Keio strains were P1 transduced into parental BW25113 when possible in the initial screen, as denoted by "#". The plating phenotype was complemented in P1 transductants, when applicable. The data represents average and standard deviation of three biological replicates.

Phage	Gene	Detection method	EOP	Complemented EOP
LL5	*lpcA*	Targeted screen	$< 7.5 \times 10^{-8}$	0.8 ± 0.2
	rfaE	Targeted screen	$< 7.5 \times 10^{-8}$	1.0 ± 0.5
	rfaC	Targeted screen	$< 5.3 \times 10^{-7}$	0.7 ± 0.3
	rfaP#	Initial screen	$< 7.5 \times 10^{-8}$	2.2 ± 2.0
	rfaF	Targeted screen	$< 7.9 \times 10^{-7}$	0.7 ± 0.3
	tolC	Targeted screen	$< 5.3 \times 10^{-7}$	0.4 ± 0.3
	secB#	Initial screen	0.06 ± 0.02	0.2 ± 0.04
	ppiB#	Initial screen	0.09 ± 0.05	1.5 ± 0.3
LL12	*lpcA*	Initial screen	$< 4.4 \times 10^{-9}$	1.1 ± 0.4
	rfaE	Targeted screen	$< 4.4 \times 10^{-9}$	1.1 ± 0.5
	rfaC	Targeted screen	$< 5.1 \times 10^{-9}$	0.9 ± 0.4
	rfaP#	Initial screen	0.02 ± 0.01	1.0 ± 0.4
	rfaF	Targeted screen	$< 6.5 \times 10^{-9}$	1.5 ± 0.5
	rfaG	Initial screen	$5.1 \times 10^{-6} \pm 1.0 \times 10^{-6}$	1.1 ± 0.2

The genes *lpcA*, *rfaE*, *rfaC*, *rfaP* and *rfaF* are parts of the core LPS biosynthesis pathway. The severe plating defects associated with multiple genes in this pathway indicate that phage LL5 uses core LPS as its receptor. Heptose (Hep) I and Hep II are transferred to LPS from the nucleotide precursor molecule ADP-L-glycero-D-manno-heptose, which is synthesized in a separate pathway involving *lpcA*, *rfaE* and *rfaD* (Figure 3A) [2]. In the absence of either LpcA or RfaE, heptoseless LPS core is formed [47], whereas *rfaD* mutants can still incorporate the stereoisomer D-glycero-D-manno-heptose into the LPS [48]. Addition of Hep II by RfaF is the last step in the LPS pathway with a strong observed plating defect for LL5, indicating that the Hep II residue of the core LPS is required for host recognition by phage LL5. However, absence of RfaP also results in a strong plating defect. RfaP adds phosphate or 2-aminoethyl diphosphate (PPEtN) to Hep I of the inner core LPS (Figure 3B) [49]. Its absence still allows the addition of Hep II to the core LPS but blocks addition of Hep III by RfaQ and Hep II phosphorylation by RfaY [49]. Since the loss of RfaP still allows addition of Hep II, this suggests that the phosphate/PPEtN group itself is recognized by LL5 for infection. The plating efficiency on the *rfaY* mutant was normal and only a mild defect was observed on the *rfaQ* mutant (Table S4), indicating that these residues are not required for LL5 infection.

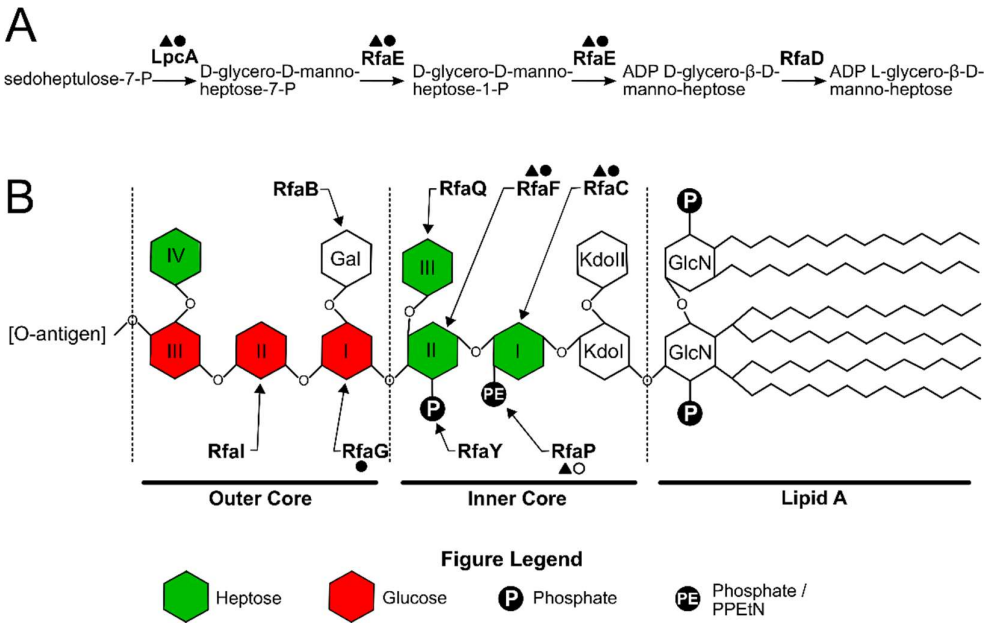

Figure 3. Genes and biosynthetic pathway of the *E. coli* core lipopolysaccharide (LPS) required for replication of phages LL5 and LL12. Proteins in the pathway are denoted in bold and label each step in biosynthesis. Black triangles indicate proteins that are required by LL5 for plaque formation, and black circles indicate proteins required by LL12. **Panel A:** The nucleotide sugar precursor ADP L-glycero-β-D-manno-heptose is used as a substrate for the transfer of heptose (green in panel B) to the *E. coli* core LPS. ADP L-glycero-β-D-manno-heptose is synthesized from sedoheptulose-7-P via a pathway comprised of *lpcA*, *rfaE* and *rfaD*. Both *lpcA* and *rfaE* are required for growth of phages LL5 and LL12. **Panel B:** LPS is composed of four distinct domains: Lipid A, inner core, outer core and O-antigen. The enzymes responsible for the addition of sugar residues and phosphoryl constituents relevant to this study are denoted. RfaC, RfaF, RfaQ, RfaG, RfaI and RfaB add hexo or hepto sugar residues to LPS, and RfaP and RfaY add phosphoryl substituents to heptose residues I and II, respectively. RfaC, RfaP and RfaF are required for plaque formation by phage LL5. RfaC, RfaF and RfaG are required by LL12, and absence of RfaP (open circle) results in a ~50-fold reduction in plating efficiency for phage LL12. Panel A is adapted from [2] and panel B from [3].

The predicted central tail fiber protein gp51 of LL5 bears strong similarity to the TspJ fiber of phage TLS as described above. Phage TLS utilizes the outer membrane protein TolC and the LPS core to recognize and infect its *E. coli* host [36]. Since the LPS receptor requirement of phage LL5 appears similar to that of phage TLS [36], the dependence of LL5 on TolC for infection was also tested. The plating efficiency of phage LL5 in a tolC deletion mutant was less than 5×10^{-7}, and this phenotype could be complemented *in trans* (Table 2). This indicates that, like TLS, LL5 also requires TolC for recognizing its *E. coli* host. While the data presented here indicates phage LL5 utilizes the Hep II and Hep I phosphorylation of the LPS inner core and the TolC OMP for infection, it is not clear from this data which of its two tail fibers, the central fiber gp51 and L-shaped side fibers gp57, recognize which of these features.

Apart from the genes encoding surface features, the plating efficiency of phage LL5 was also reduced in strains with deletions in two other genes, *secB* and *ppiB*. The EOP of phage LL5 was 0.06 and 0.09 in *secB* and *ppiB* strains, respectively. The plating defect in both strains could be restored to normal levels when the gene was supplied *in trans* (Table 2). SecB and PpiB are chaperones that contribute to protein translocation and proline peptide bond isomerization, respectively [50,51].

Proteins, once synthesized in cytoplasm, are sorted into compartments of the cell by different protein transport systems. SecB, a tetrameric cytoplasmic chaperone, is a component of the general secretory (Sec) system that transports proteins synthesized in the cytoplasm, post-translationally, to the extracytoplasmic compartments. Post-translational transport is primarily preferred for periplasmic and outer membrane proteins [52,53]. SecB binds to polypeptides and keeps them in an unfolded state until cytoplasmic ATPase SecA directs the bound polypeptide to the SecYEG transmembrane channel [53]. The translocation of polypeptides across the SecYEG channel is powered by the cytoplasmic ATPase SecA [52]. Eighteen *E. coli* proteins have been reported to be dependent on SecB-mediated translocation [50] [53]. We do not know if any of these SecB-dependent proteins play a role in the infection cycle of phage LL5, or if the potential accumulation of cytoplasmic protein aggregates in *secB* mutants hampers phage replication. In the absence of SecB, other cytoplasmic chaperones have been reported to be upregulated to stabilize secretory proteins during their delayed translocation and/or to rescue protein aggregates [54]. This compensatory mechanism by other chaperones may be the reason why the EOP defect of phage LL5 in the *secB* mutant is relatively mild (EOP = 0.06).

Another chaperone affecting the plating efficiency of phage LL5 is PpiB, which belongs to peptidyl-prolyl *cis*/*trans* isomerase (PPIase) superfamily of proteins, catalyzing protein folding at the peptide bonds preceding proline residues [51]. Although PPIases play a role in several biological processes, there is no evidence of any biological process depending solely on any PPIases [51]. The genome of *E. coli* K-12 encodes eight PPIases, belonging to three families: FKPBs, cyclophilins and parvulins [51]. The cyclophilins family consist of PpiA and PpiB, which are periplasmic and cytoplasmic proteins, respectively [51]. To our knowledge, there is only one reported instance of the requirement of a PPIase for a phage infection cycle: SlyD, belonging to FKBP family of PPIases, has been shown to be required for plaque formation by the ssDNA phage ΦX174 [55]. SlyD is required to stabilize ΦX174 lysis protein E, so that it can accumulate to optimum levels to lyse the host cell [56]. Since the infection cycle of phage LL5 has not been characterized, it is difficult to explain which aspect of phage replication is affected by the absence of PpiB.

3.5 Genes required for propagation of phage LL12

Phage LL12 was screened against the 3,985 single-gene knockouts of the Keio collection as described in the Materials and Methods and in the Supplementary Text. Following confirmation and genetic complementation, six genes were determined to be required for efficient propagation of phage LL12 (Table 2). All mutants in which phage LL12 showed plating defects were deleted for genes in the LPS biosynthesis pathway. Phage LL12 showed severe plating defects (EOP < 10^{-8}) in *lpcA*, *rfaE*, *rfaC* and *rfaF* deletions, and an EOP of ~10^{-6} in the *rfaG* deletion. Deletion of *rfaP* resulted in a milder plating

defect (EOP ~0.02). These defects could be restored when the respective genes were supplied *in trans*. (Table 2).

The functions of genes *lpcA*, *rfaE*, *rfaC*, *rfaP* and *rfaF* in LPS biosynthesis have been explained in context of phage LL5 above. RfaG links glucose (Glc) I to Hep II of the LPS inner core (Figure 3) [57,58], and marks the start of the outer core domain of the *E. coli* LPS. Sugar residues Glc II and galactose (Gal) are linked to Glc I by RfaI and RfaB respectively [59]. The plating efficiency of phage LL12 in the respective *rfaI* and *rfaB* mutants were close to wild type (~0.5) suggesting that Glc II and the Gal sidechain do not play significant roles in phage LL12 infection (Table S5). The strongly reduced EOP of phage LL12 on *rfaG* deletions suggests a crucial role of the outer core Glc I in the host recognition mechanism of phage LL12. A milder plating defect was observed in the *rfaP* mutant (Table 2), which lacks the phosphate/PPEtN modification to Hep I, the phosphate modification to Hep II by RfaY, and the addition of Hep III by RfaQ [49]. However, the *rfaQ* deletion did not appear in the initial screen, and deletion of *rfaY* shows only a minor reduction in plating efficiency (EOP ~0.1), suggesting that LL12 may also interact the phosphate/PPEtN modification to Hep I.

The Hep II - Glc I linkage is conserved in K-12, and R1 - R4 LPS core types in *E. coli* [45]. As shown in Table 1, phage LL12 is able to infect *E. coli* strains with K-12, R1 and R3 LPS core types, which is consistent with the finding that the Gal sidechain residue linked to Glc I in the K-12 core and the residues downstream of Glc I are not involved in phage receptor binding. To our knowledge, LL12 is the first candidate from the group of V5-like phages for which the host receptor has been characterized. Based upon the sequence similarities of their tail fibers (Table S3), other closely related V5-like phages such as rV5, ΦAPCEc02, and the O157:H7 typing phages 4, 5 and 14 are likely to use the same or similar receptors as phage LL12.

Genetic analysis has established phage LL12 recognizes the *E. coli* LPS outer core as its host receptor in a K-12 background. However, LL12 is also able to infect multiple different serotypes of *E. coli* with varying O-antigen (Table 1). In the case of phage P1, which also recognizes the LPS outer core [60], the extensive O-antigen expressed by hosts such as *E. coli* O157:H7 and Salmonella Typhimurium is able to obscure the LPS core. Loss of the O-antigen in these strains results in bacterial sensitivity to phage P1 [61,62]. This observation suggests that phage LL12 has developed a mechanism to deal with the presence of O-antigen that may mask its receptor in the LPS core. Several phages are known to have evolved mechanisms to penetrate bacterial O-antigen in order to reach their major receptors on the cell surface. The tail spike protein (TSP) of *Salmonella* phage P22 recognizes O-antigen as its receptor and also has endorhamnosidase activity and cleaves its glycosidic linkages resulting in the shortening of the O-antigen [63,64]. Coliphage G7C also expresses tail spikes with enzymatic activity against O-antigen that is involved in phage adsorption [65].

4. Conclusions

Phages LL5 and LL12 were initially isolated against pathogenic *E. coli* hosts and phage LL12 was shown to infect representatives of several prominent STEC serovars. Phage LL5 is a T1-like siphophage closely related to phage TLS and encodes a central tail fiber with almost identical to that of TLS but a side tail fiber which is more closely related to the L-shaped fibers of coliphage T5. LL12 is a large (~136 kb) myophage and a member of the V5-like phages, which are known to infect pathogenic *E. coli* strains. A total of eight and six *E. coli* genes were found to affect the propagation of phages LL5 and LL12, respectively (Table 2). Phage LL5 exhibited severe plating defects (< 10^{-7}) in *E. coli tolC* and other mutants defective in LPS inner core biosynthesis, which suggest that LL5 requires the outer membrane protein TolC, and the Hep II and phosphoryl modification of Hep I of the LPS inner core, to recognize its host. Phage LL5 also exhibited mild plating defects in *E. coli* mutants defective in *ppiB* and *secB*, which are cytoplasmic chaperones that may be involved in the production of phage components or proteins required for proper receptor expression. Phage LL12 showed severe plating defects in *E. coli* mutants defective for LPS inner and outer core synthesis, indicating LL12 requires the LPS outer core Glc I for infection.

The Keio collection is a library of single-gene deletion of non-essential genes in *E. coli* K-12 strain BW25113. The library consists of 7970 mutants, with each mutant generated independently and distributed in odd- and even-numbered plates [18]. This study was initiated with the screening of phages LL5 and LL12 against the Keio library to investigate the host factors required for successful phage propagation. Initial screens identified 37 *E. coli* genes necessary for phage LL5 and LL12 propagation (Tables S4, S5 and supplementary text), but on further analysis only five of these were found to be associated with significant defects in propagation, and an additional four genes were identified by targeted re-testing of specific gene knockouts. These observations highlight the generally noisy nature of high-throughput screens and the requirement for additional confirmatory experiments following screening.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Table S1: Gene products of phage LL5, Table S2: Gene products of phage LL12, Table S3: Homologs of putative LL12 tail fibers identified in related phage genomes, Table S4: Results of initial (untargeted) screening and targeted re-screening of phage LL5 against the Keio *E. coli* knockout collection. and Table S5: Results of initial (untargeted) screening and targeted re-screening of phage LL12 against the Keio *E. coli* knockout collection.

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