RESEARCH ARTICLE

DNA Packaging Specificity of Bacteriophage N15 with an Excursion into the Genetics of a Cohesive End Mismatch

Michael Feiss, Jea Young Min†, Sawsan Sultana‡, Priyal Patel§, Jean Sippy *

Department of Microbiology, Carver College of Medicine, University of Iowa, Iowa City, Iowa, 52242, United States of America

† Current address: Division of Epidemiology, Department of Medicine, Vanderbilt University, 2525 West End Ave Suite 809–5, Nashville, Tennessee, 37205, United States of America
‡ Current address: Presence St. Joseph Medical Center, 333 N. Madison St. Joliet, Illinois, 60435, United States of America
§ Current address: University Hospitals and Clinics (UIHC), Department of Pathology, 200 Hawkins Dr. 6240 RCP, Iowa City, Iowa, 52242, United States of America
* jean-sippy@uiowa.edu

Abstract

During DNA replication by the λ-like bacteriophages, immature concatemeric DNA is produced by rolling circle replication. The concatamers are processed into mature chromosomes with cohesive ends, and packaged into prohead shells, during virion assembly. Cohesive ends are generated by the viral enzyme terminase, which introduces staggered nicks at cos, an approx. 200 bp-long sequence containing subsites cosQ, cosN and cosB. Interactions of cos subsites of immature concatemeric DNA with terminase orchestrate DNA processing and packaging. To initiate DNA packaging, terminase interacts with cosB and nicks cosN. The cohesive ends of N15 DNA differ from those of λ at 2/12 positions. Genetic experiments show that phages with chromosomes containing mismatched cohesive ends are functional. In at least some infections, the cohesive end mismatch persists through cyclization and replication, so that progeny phages of both allelic types are produced in the infected cell. N15 possesses an asymmetric packaging specificity: N15 DNA is not packaged by phages λ or 21, but surprisingly, N15-specific terminase packages λ DNA. Implications for genetic interactions among λ-like bacteriophages are discussed.

Introduction

Large DNA viruses, such as tailed bacteriophages and herpes viruses, use an ATP-powered motor to translocate viral DNA into the preformed empty shell, called the prohead or procapsid [1–3]. Recent structural and bioinformatic studies demonstrate that the DNA packaging machinery of these viruses is descended from that of an ancient common ancestor [4]. For example, the prohead shell is an icosahedral lattice principally constructed of many copies of the major capsid protein whose fold is conserved [5]. Similarly, one of the prohead’s 5-fold
vertexes, the unique portal vertex, contains the radially disposed dodecameric portal protein. The portal protein contains a channel through which DNA enters and exits the shell interior [6–9]. Terminase, also conserved in the herpes viruses and tailed bacteriophages, is usually a hetero-oligomer of small (TerS) and large (TerL) subunits [2,10–12]. TerS carries out viral DNA recognition. TerL is a motor protein whose N-terminal ATPase domain powers translocation of the DNA into the prohead. TerL also contains the C-terminal endonuclease domain that cuts concatemeric DNA into unit-length virion chromosomes.

TerS molecules contain three domains, as follows. An α-helical central domain oligomerizes TerS into cylindrical, 8- to 12-mer oligomers, depending on the virus. At the C-terminus is a β-barrel structure. The C-terminus contains a functional TerL binding domain, as shown for λ and 21 [13,14], P22 [15,16], and T4 [17,18]. In the case of P22’s TerL, short α-helical extensions at the extreme C-terminus may tether TerS to TerL [19]. At the TerS N-terminus is a DNA binding motif. In the cases of λ [20], Sf6 [21], T4 [18,22], and SF6 [23,24], the DNA binding motif is likely a classic helix-turn-helix. For SF6 TerS, the DNA binding domains are tethered to the central cylinder by linkers hence are highly mobile. It is proposed in a number of these cases that the DNA is wrapped around the TerS oligomer into a nucleosome-like structure by interactions with multiple TerS DNA binding domains [15,17,25,26]. Phage λ TerS is unusual in that in addition to oligomerizing, the DNA binding domain forms a tight dimer [20], as discussed further below.

Among the tailed dsDNA bacteriophages, pac phages use a headful mechanism to package virion DNA from concatemers, i.e., immature end-to-end multimers of virus DNA produced by rolling circle replication. An initial cut is made near a pac site, the TerS recognition site, and packaging is terminated by a non-specific cut when the prohead is full. The first termination cut is also the start of packaging of the next chromosome along the concatemer, as packaging is processive. The resulting viral chromosomes have a terminal redundancy, and individual chromosomes are circular permutations of the unique viral sequence. In contrast, virion DNAs of cos phages have a unique DNA sequence with short complementary cohesive ends. cos phages produce virion DNAs through TerL’s introduction of precisely staggered nicks in concatemeric DNA. The interaction of TerS with pac or cos recognition sites varies from phage to phage. pac phage P22’s TerS recognition site is a simple, 22 bp, asymmetric sequence located in the TerS gene [27]. In contrast, for pac phage SPP1, there are multiple TerS binding sites flanking the site where the initial cut is made [25]. For T4, the headful mechanism is used, but initiation is complex and not well understood [28].

For cos phage λ and its close relatives, virion chromosomes have cohesive ends, i.e., complementary, 5’-ended, 12 base-long, single-strand extensions that anneal, circularizing the DNA, upon entry into a host cell. The DNA segment containing the DNA packaging signals and the annealed cohesive ends is called cos. At late times during the lytic cycle, recombination and rolling circle replication produce concatemeric DNA [29]. During packaging, concatemeric DNA is recognized and processed by terminase into monomeric virion chromosomes. TerSλ and TerLλ are gene products of the Nu1 and A genes, respectively. TerSλ’s winged helix-turn-helix (wHTH) domain, which specifically binds cos, forms a tight dimer [20]. The simplest form of λ terminase is the protomer, a TerS2:TerL1 heterotrimer. Protomers further assemble into [TerS3:TerL1]4 tetramers [30,31]. TerSλ recognizes λDNA by specific binding to cosB, a cos subsite adjacent to cosN, the nicking site at which TerLλ introduces staggered nicks to generate the cohesive ends of mature virion chromosomes. Assembly of TerSλ on cosBλ positions TerLλ’s endonuclease to introduce the staggered nicks at cosN [32–34]. When cosB is deleted or re-positioned, cosN nicking is inefficient and inaccurate, indicating that anchoring by TerS is critical for nick introduction by TerL [33–36].
Packaging is initiated when terminase binds and nicks a cos on a concatemer. Following cosN nicking and cohesive end separation, terminase forms a tight complex, Complex I, on the cosB-containing chromosomal end [37,38]. Complex I then docks on the portal protein, gpB, of the prohead [39,40]. Following docking of Complex I on the portal, TerLλ’s ATPase is activated and ATP hydrolysis-powered translocation of the DNA into the shell ensues [41,42]. Terminase remains docked on the portal during translocation. When the next cos along the concatemer is encountered, terminase (1) nicks cosN, (2) dissociates from the portal, and (3) remains bound to the cosB-containing end of the next chromosome along the concatemer [43]. By remaining bound to the next chromosome along the concatemer as a Complex I, terminase processively packages downstream chromosomes. A third sub-site, cosQ, is essential for recognition and nicking of the downstream cos during termination of packaging [44,45]. In sum, cosN and cosB are required to initiate λDNA packaging, cosQ and cosN are required for termination, and all three subsites are required for processivity [46].

The 120 bp-long cosBλ is complex, containing three TerS binding sites, R3, R2, and R1 (Fig 1) [38]. Between R3 and R2 is a binding site [47], I1, for IHF, the E. coli site-specific DNA bending protein [48–51]. IHF bends DNA into an approx. 180° hairpin [52]. At cos, the IHF-induced bend at I1 positions R3 and R2 such that the wHTH motifs of dimeric TerS can be docked into the major grooves [20]. Complex I likely includes this nucleoprotein structure [53].

In this paper, we refer to the λ-like phages as those with λ-like cohesive ends, i.e., 12 base-long, 5’ extensions with significant identity to λ’s cohesive ends. This group includes λ, 434, φ80, 21, N15, Monarch, and gifsy-1 [54]. In the cosNs of this group, bp differences in the right half of the cohesive end sequences, i.e., bp 7–12, are common. For example N15’s cohesive

Fig 1. Comparison of the coses and terminase small subunit genes of λ, 21 and N15. A. Complex cos structure of phages λ, 21, Monarch. R3, R2 and R1 sequences are TerS binding sites, and I1 is an IHF binding site. B. Simple cos of N15 and relatives [58]. rR2 is a proposed accessory TerS binding site in the cos of N15 and relatives.
ends differ from λ’s at bp 9 and 12, and gify-1’s differ at positions 8, 9, and 11. In contrast, the 7 bp cosQ site is highly conserved. The cosBs of the λ-like phages differ both in structural complexity and in packaging specificity, as follows.

With respect to cosB structure, DNA sequence analysis suggest that many λ-like phages have a complex cosB similar to cosB\(^\lambda\), with three R sequences with R2 oriented opposite to R3 and R1, and with a likely IHF binding site between R3 and R2. Recently, N15 was shown to have a different, simpler cosB consisting of a single critical TerS binding site located approximately at the position of the R3 in complex cosBs. A second, inverted sequence that closely matches R3\(^{N15}\), called rR2, is located within the TerS gene, about 200 bp distal to R3\(^{N15}\), and though not critical for DNA packaging, is proposed to play a non-essential, accessory role in DNA packaging. The R1 sequence of cosB\(^{21}\) plays such a role [55]. Examination of prophages in Genebank identified an additional five prophages that have the R3 and rR2 sequences found in N15’s cosB [56]. In sum, two strikingly different cosB structures, complex and simple, are found in the λ-like phages.

In addition to structural differences, packaging specificity differences are found in the λ-like phages, as exemplified by the well-characterized specificities of λ and 21. λ and 21 have identical cosQ and cosN sequences, yet each package self-DNA >10\(^3\)-fold more efficiently than that of the other phage. Examination of the recognition helix and cosB R site sequences shows significant differences that account for the specificity difference (Fig 2) [20,57]. Genetic analysis identified recognition helix:R site contacts that differ between λ and 21, plus clashes involved in discrimination by TerS\(^\lambda\) and TerS\(^{21}\) against the heterologous DNA [56]. Looking at TerS\(^{N15}\) and R3\(^{N15}\) suggests that N15, and its relatives with simple cosBs might have a novel packaging specificity distinct from the λ and 21 specificities (Fig 2)[58]. Interestingly, a second group of N15-like prophages, exemplified by Monarch, have complex, tripartite cosBs. TerSMonarch and TerSN15 have identical recognition α-helixes and strong R site identity, suggesting that both subgroups share the same packaging specificity. Here we present a study of N15’s packaging specificity. Because of the strategy used, a preliminary analysis of the genetics of phage chromosomes with mismatched cohesive ends, λ versus N15, was carried out.

Results
Genetics of a cohesive end mismatch: Phages with mismatched cohesive ends are viable; the mismatches can persist to post-DNA replication

Strategy for studying DNA packaging specificity. To ask about the effects of cosN and cosB differences on DNA packaging, we used a helper phage/passive prophage approach (Fig 3). In these experiments, the DNA packaging substrate, a passive prophage, is provided to the DNA packaging machinery of a lytically growing “helper” phage, as follows. Two prophages integrated in tandem at attB contain between them a chromosome that mimics a chromosome in a concatemer. Terminase from a helper phage can initiate packaging at the cos of the upstream prophage, translocate DNA and terminate packaging at the cos of the downstream prophage. In our experiments, the helper phage was a heat-inducible (cI857) derivative of a λ strain that forms a plasmid prophage. Helpers with λ, 21 or N15 DNA packaging specificity were used. The 21- and N15-specific helpers were viable hybrid derivatives of λ in which a left chromosome end, the DNA segment including cosB\(^\lambda\) and much of the TerS\(^\lambda\)-encoding Nu1 gene, was replaced with the functionally analogous segment from phages 21 or N15, respectively. The 21- and N15-specific helpers are called λ21\(^{hy51}\) [59] and λN15\(^{hy4}\) [58], respectively. These helper phages are viable hybrid phages in which the left λ DNA end is replaced with the corresponding left end of the 21 and N15 chromosome, respectively. The left end segments,
each about 600 bp in length, include 21’s or N15’s cosB and the 5’ end of the TerS-encoding gene which contains the helix-turn-helix DNA binding specificity motif. The corresponding chimeric TerS proteins are called TerShy51 and TerShy4, respectively. When a helper prophage is heat-induced to carry out lytic growth, the tandem prophages remain repressed because the prophages have thermo-stable imm21 and imm434 immunity repressors that differ in specificity from the immλ helper. The maximum number of packageable passive prophages equals the number of bacterial chromosomes in the cell, so efficient packaging of a prophage gives a yield of 1–4 virions/infected cell; the helper yield was also low, at ~10–20/induced lysogen.

**Genetics of mismatched cohesive ends.** We did preliminary helper packaging experiments asking if the helper packaging approach was appropriate for N15 specificity studies. First, using in vivo phage crossing techniques, we made λ att+ N15hy4, phage ϕ1182 (Table 1),
for dilysogen construction. φ1182 was used to lysogenize MF532, which contains a partial λ imm434 ind prophage which includes the prophage segment from attL through cosλ. MF532 (φ1182) contains the passive prophage structure: imm21 cosQN15NN15B^N15 \rightarrow imm434 ind cosQN4B^4, where the arrow indicates the direction of DNA packaging. Packaging of the passive prophage by the λN15hy4 helper was expected to package a passive chromosome with mismatched cohesive ends. That is, initiation at the cosN15 of the upstream prophage and termination at the cosλ of the downstream prophage generates a helper-packaged chromosome with N15 left cohesive end (designated as ceLN15; Fig 4). It was uncertain whether phages carrying the mismatched passive chromosome would be viable. Would the chromosome with a 2-bp cohesive end mismatch be able to circularize? Would the λN15hy4 terminase, after initiating packaging at the cosN15 of the upstream prophage, be able to terminate packaging at the cosλ of the downstream prophage? Although it was unknown whether N15-specific terminase would terminate packaging at cosλ, previous work showed that λ and 21 terminases could terminate packaging at either cosλ or cos21. In fact, previous work indicated that termination appears to not depend on the presence of cosB [46]. Another termination issue was whether the helper’s N15-specific terminase would show preference for the downstream cosN sequence. Previous work demonstrated that TerL^λ efficiently nicks a cosN with bp changes in the right half of the cohesive end sequence [60], indicating cosN specificity was also not a likely issue.
Packaging of MF532 (φ1182)’s passive prophage by the λN15hy4 helper produced a modest yield (0.1 phages/induced cell) bearing passive prophages with mismatched cohesive ends. This yield is about 10% of the yield one might expect. The low yield of this cellN15 cellR λ chromosome might be due to inefficiency at any of the stages of DNA packaging, i.e., initiation, termination, or reduced viability of the virions carrying chromosomes with mismatched cohesive ends, or the infirm recA- uvrB- host cells. Plaque-formation by cellLN15/cellR λ mismatch phages indicates that the cohesive end mismatch is not lethal, i.e., the mismatch does not block cyclization of the injected DNA. Finding that phages with mismatched cohesive ends are viable indicated that none of the confounding possibilities prevented packaging of mismatched chromosomes into virions that were competent to carry out successful infections. Below we report experiments directly testing both cosN specificity and termination efficiency of λ- and N15-specific terminases at heterologous coses.

The cyclized DNA of the mismatch phages from MF532 (φ1182) contains a cosN with mismatches at positions 9 and 12 (Fig 4A). The mismatched bp might or might not be subject to repair prior to DNA replication. If the mismatches are repaired, then all the progeny of an infection by a mismatch phage will either be cosN<sup>λ</sup> or cosNN15, depending on the direction of the repair process. If the cosN mismatch is not repaired, then a mixed burst of both cosN<sup>λ</sup> and cosNN15 progeny phages are expected from an infected cell. To ask if the mismatched bp were or were not repaired, four well-isolated plaques produced by mismatch phages were suspended in buffer, replated, and individual plaques were subjected to DNA sequence analysis to

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**Table 1. Bacteria and Phages.**

| Strain/Plasmid | Genotype/Comments | Source/Reference |
|----------------|-------------------|-----------------|
| **A. Bacteria** |                   |                 |
| MF713          | λ b511 Δ<sub>Gal609</sub>[gal-attL-S], recA1 | [71] |
| MF532          | λ imm<sup>434</sup> ind<sup>Δ</sup>att<sub>A</sub>1[Nu1-attR-bio-uvrB-chlA] recA1 | [71] |
| MF611          | W3101 recA1       | [61] |
| MF3510         | MF611 galK103     | Feiss collection |
| C600           | thi leu thr supE / host for plaque assays | Our collection |
| C600 (λ Bam1)  | thi leu thr supE / Kn transduction recipient | Feiss collection |
| MF1427         | C1a galK          |                 |
| **B. Phages**  |                   |                 |
| λ-P1:5R Kn³ cl857 nin5 = φ1080 | Helper phage with λ packaging specificity | [55] |
| λ-P1:5R Kn³ 21<sup>hy5</sup> cl857 nin5 = φ1081 | Helper phage with 21 packaging specificity | [64] |
| λ-P1:5R Kn³ N15<sup>hy4</sup> cl857 nin5 = φ1187 | Helper phage with N15 packaging specificity | [58] |
| λ-P1:5R Kn³ cl857 nin5 Nu1ms1 = φ1082 | Helper phage | [55,62] |
| λ-P1:5R Kn³ cl857 nin5 cos2 | cosN deletion phage | [55] |
| λ imm<sup>21</sup> = φ72 | cos<sup>λ</sup> / dilysogen constructions | Feiss collection |
| λ N15<sup>hy4</sup> cosQ<sup>N15</sup>cosN<sup>N15</sup>cosD<sup>N15</sup> imm<sup>21</sup> rec3 = φ1182 | cosN<sup>N15</sup> / dilysogen constructions | This work |
| λ N15<sup>hy4</sup> cosQ<sup>N15</sup>N<sup>B</sup> imm<sup>434</sup> ind<sup>Δ</sup> = φ1197 | Dilyysogen constructions | This work |
| λ N15<sup>hy4</sup> cosQ<sup>N15</sup>N<sup>B</sup>imm<sup>434</sup> imm<sup>34</sup> ind<sup>Δ</sup> = φ1199 | Dilyysogen constructions | This work |
| λ cosQ<sup>N15</sup>N<sup>B</sup>imm<sup>434</sup> ind<sup>Δ</sup> b511 = φ1201 | Dilyysogen constructions | This work |
| λ cosQ<sup>N15</sup>N<sup>B</sup>imm<sup>434</sup> ind<sup>Δ</sup> b511 = φ1203 | Dilyysogen constructions | This work |
| λ 21<sup>hy3</sup> gal⁺ att⁺ imm<sup>21</sup> φ1221 | cos<sup>21</sup> / dilysogen constructions | This work |
| λ gal⁺ att⁺ imm<sup>21</sup> = φ1225 | cos<sup>λ</sup> / dilysogen constructions | This work |
| λ N15<sup>hy4</sup> cosN<sup>imm</sup>21 gal⁺ att⁺ = φ1227 | Passive prophage with N15 packaging specificity | This work |
| λ cl857 Δ(stf-tfa)::cat = φ1208 | cat gene provides resistance to chloramphenicol | [85] |
| λ imm<sup>434</sup> ind<sup>Δ</sup> stf-tfa::cat = φ1220 | Passive cos<sup>3</sup> prophage | This work |

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Packaging of MF532 (φ1182)’s passive prophage by the λN15<sup>hy4</sup> helper produced a modest yield (0.1 phages/induced cell) bearing passive prophages with mismatched cohesive ends. This yield is about 10% of the yield one might expect. The low yield of this cellN15 cellR λ chromosome might be due to inefficiency at any of the stages of DNA packaging, i.e., initiation, termination, or reduced viability of the virions carrying chromosomes with mismatched cohesive ends, or the infirm recA uvrB host cells. Plaque-formation by cellN15 cellR λ mismatch phages indicates that the cohesive end mismatch is not lethal, i.e., the mismatch does not block cyclization of the injected DNA. Finding that phages with mismatched cohesive ends are viable indicated that none of the confounding possibilities prevented packaging of mismatched chromosomes into virions that were competent to carry out successful infections. Below we report experiments directly testing both cosN specificity and termination efficiency of λ- and N15-specific terminases at heterologous coses.

The cyclized DNA of the mismatch phages from MF532 (φ1182) contains a cosN with mismatches at positions 9 and 12 (Fig 4A). The mismatched bp might or might not be subject to repair prior to DNA replication. If the mismatches are repaired, then all the progeny of an infection by a mismatch phage will either be cosN<sup>λ</sup> or cosNN15, depending on the direction of the repair process. If the cosN mismatch is not repaired, then a mixed burst of both cosN<sup>λ</sup> and cosNN15 progeny phages are expected from an infected cell. To ask if the mismatched bp were or were not repaired, four well-isolated plaques produced by mismatch phages were suspended in buffer, replated, and individual plaques were subjected to DNA sequence analysis to
determine the cohesive end sequence. We found that in the progeny of two of the four infections by mismatch phages, both cosN^λ and cosNN_15^λ phages were present (Table 2). We conclude that in at least some infections, the mismatch is not repaired and DNA replication produces a progeny phage burst in which some are cosN^λ and some are cosNN_15^λ. In practical terms, the MF532 (φ1182) helper packaging produced phages that were imm434 ind- cosQ^λ cosBN_15^λ and either cosN^λ (φ1197) or cosNN_15^λ (φ1199).

A second dilysogen, MF713 (φ1199), was constructed. MF711 contains a partial λ b511 prophage containing the prophage segment extending rightward from cos^λ. MF713 (φ1199) has the prophage structure: cosQ^λN_15^B → cosQ^λN_15^B N_15. Packaging MF713 (φ1199)'s passive prophage by the λ helper is expected to produce phages bearing chromosomes with the reciprocal mismatch, i.e., a ceL^λ/ceR^N_15 mismatch (Fig 4B). Using the λ-specific helper, the yield was 0.67 phages bearing passive prophage chromosomes/induced cell, indicating that the

Fig 4. Annealed cohesive ends of phage chromosomes with cohesive end mismatches. A. The mismatch of a phage DNA with a ceL^N_15/ceR^λ mismatch. B. The mismatch of a phage DNA with a ceL^λ/ceR^N_15 mismatch. Mismatched bp are underlined and in bold. Highly conserved bp flanking the nick sites are highlighted in grey.

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Table 2. Both cohesive end alleles are found in the progeny of phages with mismatched cohesive ends.

| Plaque of phage with mismatched cohesive ends | Cohesive end alleles of progeny phages |
|---------------------------------------------|---------------------------------------|
|                                             | λ        | N15     | λ        | N15     |
| 1                                           | 10       | 0       | 10       | 0       |
| 2                                           | 10       | 0       | 9        | 0       |
| 3                                           | 6        | 4       | 11       | 0       |
| 4                                           | 9        | 1       | 11       | 0       |
| 5                                           | —        | —       | 1        | 10      |
| Heteroallelic bursts total bursts            | 2/4      | 1/5     |

The ceL^{N15}-ceR^{N} phages resulted from packaging of the cosQ^{N15} N^{N15} B^{N15} —cosQ^{N}N^{N}B^{N} passive prophage of MF532 (φ1182) by the N15-specific helper, φ1187. The ceL^{λ}-ceR^{N15} phages resulted from packaging of the cosQ^{N}N^{N}B^{N} —cosQ^{N}N^{N15}B^{N15} passive prophage of MF713 (φ1199) by the λ-specific helper, φ1080. Progeny of phages with mismatched cohesive ends were sampled from well-separated plaques on lawns of MF1427.

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Quantitating effects of a cohesive end mismatch on infectious phage production. To ask about the quantitative effects of a cohesive end mismatch on infectious phage production,

Table 3. Effect of a cohesive end mismatch on viable phages production.

| Line | Helper Specification | Passive Prophage cos Structure^a | Prophage Yield [sem]b |
|------|----------------------|---------------------------------|----------------------|
| 1    | λ                    | Q^N15B^N15 → Q^N15B^N15         | 2.41 [0.61]          |
| 2    | N15                  | Q^N15B^N15 → Q^N15B^N15         | 1.24 [0.09]          |
| 3    | λ                    | Q^N15B^N15 → Q^N15B^N15         | 0.61 [0.10]          |
| 4    | N15                  | Q^N15B^N15 → Q^N15B^N15         | 0.27 [0.038]         |

^a The host bacteria were MF4943 for lines 1 and 2, and MF4942 for lines 3 and 4.

^b sem is the standard error of the mean, calculated from 3 to 5 independent observations.

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MF4942, with the prophage structure \(\text{imm}^{21}\cos Q^N\text{N}^{15}\text{B}^\text{N}^{15}\), and MF4943, with the prophage structure \(\lambda^{\text{imm}^{21}}\cos Q^N\text{B}^\text{N}^{15}\), were constructed and used in helper packaging experiments (see S1 Table for details). With the \(\lambda\)-specific helper, packaging the \(\text{ce}^{\text{N}^{15}/\text{ceR}\text{N}^{15}}\) mismatched chromosome, the yield was about 25% that of a chromosome with matched \(\text{ceL}^{\text{N}^{15}/\text{ceR}\text{N}^{15}}\) cohesive ends (Table 3, compare lines 1 and 3), indicating that the mismatch reduced the level of viable virion production by four-fold. Remarkably, the \(\lambda^{\text{N}^{15}/\text{hy}^{4}}\) helper phage was able to package \(\lambda\) DNA rather efficiently, at about 50% the efficiency of the \(\lambda\)-specific helper (Table 3, compare lines 1 and 2 and lines 3 and 4). As with the \(\lambda\)-specific helper, the yield of viable \(\text{ce}^{\text{N}^{15}/\text{ceR}\text{N}^{15}}\) mismatched chromosome-carrying phages was about 25% that of phages with matched cohesive ends (Table 3, compare lines 2 and 4).

To summarize, the \(\lambda^{\text{N}^{15}/\text{N}^{15}}\) cohesive end mismatch reduced the recovery of viable packaged chromosomes roughly fourfold, and the \(\text{N}^{15}\)-specific helper packaged \(\lambda\)-specific DNA at about 50% efficiency.

**N15’s Packaging Specificity**

**cosN specificity.** One explanation for the reduction in yield for mismatched phages is that packaging is inefficient due to reduced efficiency of \(\cos\) cleavage. For example, since both the \(\lambda\) and \(\text{N}^{15}\) terminases have TerL\(^\lambda\), both terminases might cleave \(\cos N^4\) more efficiently than \(\cos N^{N15}\). Alternatively, the Ter\(^{N15/hy4}\):cos\(^B\) interaction might affect TerL\(^\lambda\) positioning and the efficiency of \(\cos N\) cleavage. In either case, \(\lambda\) terminate might act more efficiently in phages with \(\cos B^\lambda\) than \(\text{N}^{15/hy4}\) terminate. To investigate this point, the yields of phage pairs expressing \(\lambda\) or \(\text{N}^{15/hy4}\) terminate, with \(\cos B^\lambda\) and either \(\cos N^4\) or \(\cos N^{N15}\), were determined. The yields were found not to depend on \(\cos N\): for both \(\lambda\) and \(\text{N}^{15/hy4}\) phages, the yield did not significantly differ between \(\cos N^4\) and \(\cos N^{N15}\) (Table 4). These results do not support the idea that \(\lambda\)- and \(\text{N}^{15}\)-specific terminases are \(\cos N^4\)-specific. An alternative explanation for the yield reduction caused by a cohesive end mismatch is that DNA packaging is fully efficient, but the infectivity of the virions with mismatched cohesive ends is reduced. The reduction in yield might be due to less efficient cyclization or a lethal event caused by attempted mismatch repair. Finding a lack of \(\cos N\) specificity is not surprising, as follows. First, the \(\lambda\) and \(\text{N}^{15}\) TerLs are quite similar, with 64.9% identity and 78.5% similarity. Second, the base pair differences in the cohesive end sequences are located in the right half of the cohesive end sequence, where sequence changes do not have measurable effects on \(\cos\) cleavage [60].

**Termination specificity.** Previous work showed that \(\lambda\)- and 21-specific terminases efficiently terminate DNA packaging at the downstream \(\cos\) regardless of \(\cos B\) specificity [61]. Later work showed that \(\cos Q\), \(\cos N\) and I2, the latter a segment between \(\cos N\) and \(\cos B\), were required for efficient packaging terminase [46]. To ask if \(\lambda\) and \(\text{N}^{15/hy4}\) terminases were also \(\cos B\)-non-specific for packaging termination, dilysogens were constructed in which the initial

| Line | Phage | Yield a pfu/infected cell [sem] b |
|------|-------|----------------------------------|
| 1    | \(\lambda\)–\(\text{N}^{15/hy4}\) cosQ\(^N\)N\(^{15}\)B\(^{N15}\) [\(\phi^{1199}\)] | 103 [13] |
| 2    | \(\lambda\)–\(\text{N}^{15/hy4}\) cosQ\(^N\)N\(^{15}\)B\(^{N15}\) [\(\phi^{1197}\)] | 111 [12.5] |
| 3    | \(\lambda\) cosQ\(^N\)N\(^{15}\)B\(^{N15}\) [\(\phi^{1203}\)] | 120 [8] |
| 4    | \(\lambda\) cosQ\(^N\)N\(^{15}\)B\(^{N15}\) [\(\phi^{1201}\)] | 132 [17] |

a Host and plating bacterium was MF1427.

b [sem] is the standard error of the mean for 3 or more independent observations.

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MF4942, with the prophage structure \(\text{imm}^{21}\cos Q^N\text{N}^{15}\text{B}^\text{N}^{15}\), and MF4943, with the prophage structure \(\lambda\) \(\text{imm}^{21}\cos Q^N\text{N}^{15}\text{B}^\text{N}^{15}\), were constructed and used in helper packaging experiments (see S1 Table for details). With the \(\lambda\)-specific helper, packaging the \(\text{ce}^{\text{N}^{15}/\text{ceR}\text{N}^{15}}\) mismatched chromosome, the yield was about 25% that of a chromosome with matched \(\text{ceL}^{\text{N}^{15}/\text{ceR}\text{N}^{15}}\) cohesive ends (Table 3, compare lines 1 and 3), indicating that the mismatch reduced the level of viable virion production by four-fold. Remarkably, the \(\lambda\)–\(\text{N}^{15/hy4}\) helper phage was able to package \(\lambda\) DNA rather efficiently, at about 50% the efficiency of the \(\lambda\)-specific helper (Table 3, compare lines 1 and 2 and lines 3 and 4). As with the \(\lambda\)-specific helper, the yield of viable \(\text{ce}^{\text{N}^{15}/\text{ceR}\text{N}^{15}}\) mismatched chromosome-carrying phages was about 25% that of phages with matched cohesive ends (Table 3, compare lines 2 and 4).

To summarize, the \(\lambda\)–\(\text{N}^{15}\) cohesive end mismatch reduced the recovery of viable packaged chromosomes roughly fourfold, and the \(\text{N}^{15}\)-specific helper packaged \(\lambda\)-specific DNA at about 50% efficiency.
cos was cosBN15 and the downstream cos was cosBN15 (MF4944) or cosB4 (MF4945). In a second pair of dilysogens, the initial cos was cosB4, and the downstream cos was either cosB4 (MF4946) or cosBN15 (MF4947). These dilysogens were then further lysogenized with the λ- and N15-specific helpers and used in helper packaging experiments. Neither helper displayed any cosB specificity for termination at the downstream cos (Table 5). These results extend the earlier observations that packaging termination is not dependent on cosB specificity [46]. These results are not unexpected given the strong sequence conservation for cosQ, cosN, and I2 in λ, 21 and N15.

Packaging specificities of λ, 21 and N15. The packaging specificities of λ and 21 are due to the TerS-cosB interaction at the start of DNA packaging. To study N15’s packaging initiation specificity, a set of three dilysogens was constructed in which the passive prophage’s upstream initiation cos was cosQNλB (MF4948), cosQNλB21 (MF4949), or cosQNλBN15 (MF4950). In each strain the downstream terminator cos was NQBN15. These dilysogens were then lysogenized with the λ-, 21-, or N15-specific helper phages. As in previous helper packaging experiments, the λ- and 21-specific terminases failed to package each other’s chromosomes (Table 6). Further, neither the λ-specific or 21-specific helpers packaged the N15-specific chromosome, suggesting that N15 has a unique packaging specificity. The inability of λ and 21 to package N15-specific chromosomes is not unexpected, given the apparent simplicity of cosN15 compared to the elaborate structures of cos3 and cos21. The possibility remained that N15 and λ might have the same packaging specificity, but that λ terminase was unable to utilize cosBN15 because of the lack of I1, R2 and R1. In previous work, it was shown that several Nu1 missense mutations affecting TerS (gpNu1) suppressed a variety of cosB3 defects. The Nu1mis1 mutation

| Table 5. Packaging termination does not depend on cosB specificitya. |
|---|
| Line | Prophage Structure | Helper Phage Specificity | HelperYield (ϕ/cell)[sem] | Prophage Yield (ϕ/cell) [sem]b |
| 1 | cosQN'B N15 → cosQN'B N15 | N15 | 11.1 [3.8] | 1.88 [0.37] |
| 2 | cosQN'B N15 → cosQN'B N15 | N15 | 13.5 [8.3] | 1.62 [0.76] |
| 3 | cosQN'B N15 → cosQN'B N15 | λ | 17.7 [4.7] | 2.20 [0.44] |
| 4 | cosQN'B N15 → cosQN'B N15 | λ | 22.4 [3.9] | 2.34 [0.35] |

a The host bacteria were MF4944 (line 1), MF4945 (line 2), MF4946 (line 3), and MF4947 (line 4)  
b [sem] is the standard error of the mean for 3 or more independent observations.

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| Table 6. Packaging specificities of phages λ, 21 and N15a. |
|---|
| Passive Prophage’s Specificity | Helper’s Specificity | λ | 21 | N15 |
| --- | --- | --- | --- | --- |
| λ | 1.75 | 8.1 x 10^-4 | 0.45 |
| [0.55]b | [1.7 x 10^-4] | [0.19] |
| 21 | 1 x 10^-4 | 1.24 | 3.5 x 10^-4 |
| [5 x 10^-6] | [0.11] | [6.5 x 10^-5] |
| N15 | 5 x 10^-4 | 2 x 10^-4 | 2.5 |
| [1.5 x 10^-4] | [6.5 x 10^-5] | [0.36] |

a The dilysogen strains were MF4948 (cos3→cos4), MF4949 (cos21→cos3) and MF4950 (cosN15→cos3).  
b [sem] is the standard error of the mean for 3 or more independent observations.

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which causes the TerS change L40F, for example, suppresses a number of cosB mutations, including the Δ[R2,1] mutation. The Δ[R2,1] deletion takes out cosB Δ bp 92–164, a segment containing R1, R2 and part of I1 [62]. Because Nu1ms1 terminase sponsors packaging of DNA containing only R3 of cosBΔ, we wondered if Nu1ms1 terminase would sponsor packaging of N15-specific DNA. We used the N15-specific, λ-specific, and Nu1ms1 helper phages to ask if Nu1ms1 terminase could sponsor packaging of N15-specific chromosomes in MF4950. While the N15-specific helper gave a yield of 3.5 prophages/induced lysogen, the prophage yields were 2.9 x 10⁻⁴ and 1.35 x 10⁻⁴ for the λ wild type and λ Nu1ms1 phages, respectively. As observed above with other dilsygens, the N15-specific helper packaged λ-specific chromosomes with reasonable efficiency, i.e., about 50% the efficiency of packaging self-specific N15 chromosomes. In sum, N15 has an asymmetrically unique packaging specificity, with N15 DNA’s specificity being distinct from those of λ and 21, but surprisingly, N15-specific terminase is able to package λ DNA reasonably efficiently.

Discussion

The goal of this work was to use helper packaging experiments to study N15’s packaging specificity, using λ N15hy4 [58]. For helper packaging experiments, the λ-versus-N15 cohesive end difference was a complication. That is, the 12 base-long cohesive ends of λ and N15 differ at bp 9 and 12, and initial helper packaging experiments generated chromosomes with a λ/N15 cohesive end mismatch. We found that chromosomes with mismatched cohesive ends are able to cyclize, and the mismatch persists past replication of the chromosome, in at least some infections. The cohesive end mismatch is correlated with a roughly fourfold reduction in viable phage yield. Burst size studies suggest that TerLλ acts with equal efficiency at cosNλ and cosNN15, and that cosN15 is efficiently utilized for packaging termination. The preliminary helper packaging experiments produced progeny phages, enabling helper packaging experiments on N15’s packaging specificity which did not generate mismatched chromosomes. N15’s DNA packaging specificity was found to be distinct from those of λ and 21. That is, neither λ nor 21 packages N15 chromosomes and N15-specific terminase does not sponsor 21 DNA packaging. Surprisingly, N15-specific terminase packages λ DNA at about 50% efficiency.

Functional asymmetry of the cohesive ends of λ-like phages

cosNλ, while showing significant two-fold rotational symmetry, is remarkably asymmetric functionally, as follows. An early study of cosN mutations found that many mutations affecting bp 7–12 in the right half of cosN had minimal phenotypic effects, whereas mutations affecting symmetric bp in the left half of cosN have severe phenotypic effects [60]. For example, the G2C mutation reduced virus yield about 10-fold, but the symmetrical C11G change had no phenotypic effect. Subsequent biochemical cos cleavage experiments confirmed that cosN is functionally asymmetric, and further showed that the asymmetry reflected the proximity of cosN’s right half to cosB [34]. Studies on the importance of the λ cosN-to-cosB spacing also indicate that the TerS-R3 interaction precisely positions Terl with respect to the cosN nicking sites for proper nicking by the TerL endonuclease [33,36]. These results suggest that anchoring of TerLλ to cosB makes bottom strand nicking relatively insensitive to base pair changes. The functional asymmetry of cosN correlates well with natural cosN sequence variation, as follows. The cohesive end sequence of φD326, determined long ago [63], differs from cosNΔ at positions 9 and 12, as does N15. Similarly, Salmonella phage Gifsy-1 differs from λ at cosN bps 8, 9, and 11. In contrast, the two base pairs flanking the nick positions, 5’-CG-3’ and 5’-CG-3’, are highly conserved, indicating that there is selection against changes of these bps. Similarly, natural variation in cosQ, the packaging termination signal, is not apparent. In sum, the results indicate that
sequence maintenance for cosQ and the left half of the cohesive end sequence is under selection but maintenance of bp positions 7–12 is not.

**Genetic consequences of the λ versus N15 cohesive end mismatch**

Our experiments show phages carrying λ chromosomes with mismatched cohesive ends, at bp positions 9 and 12, are able to circularize and replicate. For such phages, there is a modest, four-fold decrease in yield. The reduced yield is not due to effects of cosN bp changes on terminase efficiency, as both λ and N15hn4 terminases sponsor robust virus yields with either cosNλ or cosNN15. We speculate that the reduced yield of viable phages is due to either inefficient cyclization or lethal attempts at mismatch repair. One candidate for repair of the mismatch is the 5′-to-3′ exonuclease of DNA polymerase I, which could carry out nick translation through the mismatched bp. In at least some of the successful infections by phages with mismatched cohesive ends, the mismatch persists through replication of the parental chromosome, since some singly infected cells yield both cosNλ and cosNN15 progeny phages (Table 2). These results are surprising, since bp position 12 is adjacent to the bottom strand nick which presumably must be ligated prior to replication fork passage. The λ and N15hn4 terminases, with TerSλ and chimeric TerSN15hn4 subunits, respectively, do not show cosN specificity. For both, the large subunit is TerLλ which must be appropriately positioned to nick cosNλ. Given the functional asymmetry of cosNλ, efficient cleavage of both cosNλ and cosNN15 is as expected.

**Packaging specificity in the N15-like phages**

N15 is an unusual λ-like phage in having a simple cosB containing a single critical TerS binding site, R3N15. R3N15 extends from N15 bp 48–59, and is positioned similarly to R3λ. In cosBN15, there is a proposed additional remote site, “rR2”, in opposite orientation to the critical R3N15 sequence and located in the 1 gene at bp 249–260 [58]. The rR2 sequence strongly matches that of R3N15 and is found in all N15-like prophages with the simple cosB [58]. The rR2 sequence is not critical for N15 growth. Though not essential for plaque formation, rR2 is speculated to contribute to viral fitness for N15 and its close relatives [58]. Another group of prophages, typified by Monarch, has a strong match to R3N15 and TerSλ’s recognition helix, suggesting a shared packaging specificity (Fig 2). The Monarch group, however, does not have the simple cosBN15. Rather cosBMonarch is analogous to cosBλ, with three R sequences and an IHF binding site between R3 and R2, and the rR2 sequence is not present.

Comparing the R3 sequences of 21, N15, Monarch, and λ leads to some conclusions about TerS-R3 interactions (Fig 2). The strong identity of R3N15 and R3Monarch is apparent, as is the lack of similarity of R3N15 and R3Monarch to R3λ and R321. In R3λ and R321, bp 56 and bp 59 are critical for TerS recognition [56]. R3λ bp 58 is also important for TerSλ recognition [55]. R3N15 was identified by the packaging effects of two 6 bp-long block mutations, m1 and m2. In m1 and m2, bp 48–53 and 54–59, respectively, were scrambled, as follows. In m1, all six bp were changed—GAGGTT→TGTAGG, and in m2, four were changed—GTGTTT→GTGTGT [58]. The cosBN15m1 and m2 mutations reduced λ N15hn4’s yield by about 7-fold and 100-fold, respectively, indicating bp 54–59 are most important for recognition. That bp 54 to 59 include bp important for TerS interactions agrees with the locations of cosBλ and cosB21 bp 56 and 59, which are important bp for recognition by TerS [56].

Why can N15-specific terminase package λ DNA, but not 21 DNA? Looking at the R3 alignment gives hints, but first it is important to consider the validity of the alignment in Fig 2A. In the alignment, each sequence is equidistant from bp 1, the first base of the left cohesive end. It is possible to shift the N15 and Monarch sequences to increase the sequence identity to R3λ and R321. However in λ, the cosN-to-R3 distance is critical, because shifting the distance by
inserting or deleting more than one or two bp lowers virus yield and results in improper nick introduction in cosN [33,36]. These results indicate that when N15-specific terminase sponsors packaging of λ DNA, TerS\textsuperscript{N15hy4} anchors TerL\textsuperscript{λ} in a manner that closely matches TerS\textsuperscript{λ} anchoring of TerL\textsuperscript{λ}. In the cosB alignment, R3\textsuperscript{N15} and R3\textsuperscript{Monarch} have a match to the R3\textsuperscript{λ} TA bp 56, an important TerS\textsuperscript{λ} contact, R3\textsuperscript{λ} bp 56 also plays a role in λ/21 phage specificity, as follows. Genetic analysis indicates that Glu24 of the TerS\textsuperscript{λ} recognition helix clashes with GC bp 56 of R3\textsuperscript{21}, accounting in part for the inability of λ terminase to recognize 21-specific DNA (Fig 2B). The genetics also indicated that Glu24 of TerS\textsuperscript{λ} makes a favorable contact with TA bp 56 of R3\textsuperscript{λ}. We speculate that, in the case of N15’s recognition helix, perhaps the Val22 residue, at the position analogous the TerS\textsuperscript{λ}’s Glu24, makes a favorable contact with the bp 56 TA bp of cosB\textsuperscript{λ}, but is unable to make a favorable contact with the GC bp 56 of R3\textsuperscript{21}. It is also not clear whether the inability of λ terminase to package N15 DNA is due to a defect in cosN cutting, or in a post-cleavage defect such as formation of complex I. More research is required to understand these relationships.

\textbf{cosB architecture and function}

The likely shared packaging specificity of phages N15 and Monarch, and the finding that N15 packages λ DNA raise further interesting questions about cos structure and terminase recognition. The interaction of TerS\textsuperscript{λ} with cosB\textsuperscript{λ} includes a proposed IHF-assisted DNA hairpin that juxtaposes the major grooves of R3 and R2 for docking by dimeric gpNu1 [20]. The strong similarity of cosB\textsuperscript{λ} and cosB\textsuperscript{Monarch} suggests a hairpin-containing nucleoprotein structure also forms at cosB\textsuperscript{Monarch}. It is interesting that the TerS\textsuperscript{N15} DNA binding domain forms a tight dimer [58], even though cosB\textsuperscript{N15} is much simpler than cosB\textsuperscript{Monarch}. Does TerS\textsuperscript{N15} form a similar hairpin structure at cosB\textsuperscript{N15}, even though there is neither an IHF site nor an R2-equivalent? Elsewhere we propose that cosB\textsuperscript{N15} is derived from cosB\textsuperscript{Monarch} by unequal crossing over between R3 and R1. If so, the TerS\textsuperscript{N15} would be derived from TerS\textsuperscript{Monarch}, and might retain the ability to interact with cosB\textsuperscript{Monarch} in a manner similar to TerS\textsuperscript{λ} docking at cosB\textsuperscript{λ}. A related issue is packaging of λ DNA by N15-specific terminase. Does TerS\textsuperscript{N15hy4} utilize all three R sequences of cosB\textsuperscript{λ} when packaging λ DNA? These interesting questions await further studies.

What evolutionary steps account for the packaging systems of the λ-like phages? It seems clear that λ and 21 are descended from a common ancestor phage, as the basic cos structure is preserved. We earlier presented one possible path, in an early ancestor virus where a single R sequence and a terminase with low sequence specificity acquired greater packaging specificity by acquisition of three R sequences [64]. A scenario for N15 is that N15 has undergone a simplification process, by which a Monarch-like ancestor phage with three R sequences has lost two, and the IHF site, by unequal crossing over between R3 and R1. One would think that such an event would result in a major fitness reduction. It is possible however that the simplification and subsequent refinement, such as the acquisition of rR2, might take place in the prophage state, where fitness for lytic growth is moot [58,65].

\textbf{Genetic consequences of the asymmetric, shared packaging specificities of λ and N15}

The chromosomes of many bacteriophages, including the λ-like phages, are mosaics of DNA segments, indicating that extensive horizontal exchange is part of a phage’s evolutionary history [66]. Enteric bacteria frequently harbor λ-like prophages, so that an infecting λ-like phage has a strong likelihood of replicating in the presence of another λ-like phage chromosome. Of course several significant consequences may result from such an interaction. If a prophage shares the packaging specificity of the infecting phage that enters the lytic cycle, then the
packaging machinery of the infecting phage will initiate packaging at the prophage \( \cos \). For the \( \lambda \)-like prophages, \( \cos \) is located roughly in the center of the prophage, so that the packaging initiation encapsidates a DNA segment that will include adjacent bacterial DNA, stalling the motor when the head shell is full, which in turn produces a tailless, non-infectious particle. Production of such inactive phage structures reduces the production efficiency of viable progeny phages. One might think therefore, that it would be advantageous for \( \lambda \)-like phages to have diverse packaging specificities. So far, only modest diversity is observed, though not many examples have been studied. Among characterized \( \lambda \)-like phages with related \( \cos \) structures, the three packaging specificities of \( \lambda \), 21 and N15 have been documented. Other natural isolates, such as phages 434 and \( \phi80 \), share \( \lambda \)'s packaging specificity. N15 is an interesting case, as N15's packaging machinery retains the ability to package \( \lambda \) DNA, even though N15 has a significantly divergent DNA recognition system.

A second interaction between \( \lambda \)-like phages is recombination. If a packaging event initiated at a prophage \( \cos \), is accompanied by a downstream crossover between prophage and lytic phage DNAs, the recombinant is a novel progeny phage that contributes to genetic diversity. Some such genetic exchanges generate recombinants that are more robust in a changing environment. In the case of a cohesive end mismatch, such as in the present \( \lambda \) versus N15 situation, recombination may produce less fit progeny phages. Because the cohesive end mismatch does not persist, the reduced viability of a recombinant phage is transient.

DNA recognition by TerS

TerS molecules interact with their recognition sites through their N-terminal DNA binding domains, and with TerL through their C-terminal domains. These two specificity domains are separated by the long \( \alpha \)-helical core domain. In principal, phage diversity might be generated by swapping of the TerS DNA recognition domain for that of any other DNA binding protein with an asymmetric recognition site. In the present case, we note that both the \( \cos \) and TerS of N15 are recognizably evolutionarily related to those of phage \( \lambda \) [58]. Phage 21’s packaging specificity determinants also share a common ancestor with \( \lambda \) [56]. N15 and 21 were chosen for study as interesting variants of the \( \lambda \) paradigm, and as such these phages do not shed light on extreme possibilities for specificity swapping. We note that in a more systematic study in P22-like phages, considerable horizontal transfer of TerS gene segments has occurred, not only between highly diverged members of the P22 family, but also has occurred between P22-like phages and phages outside the P22-like group [16]. Acquisition of a TerS segment from a non-phage source such as a transcriptional regulatory system has not been observed. Surely further sequence acquisition and study will add to what we know about this issue.

Materials and Methods

Media

Luria broth (LB), LB agar, tryptone broth (TB), tryptone broth agar (TA), and tryptone broth soft agar (TBSA) were prepared as described [67], except TB, TA, and TBSA were supplemented with 0.01 M MgSO\(_4\). For phage infections, TB was supplemented with 0.2% maltose. When required, ampicillin, chloramphenicol, and kanamycin were added at 100 \( \mu \)g/mL, 10 \( \mu \)g/ml, and 50 \( \mu \)g/mL, respectively.

Bacteria, phages and plasmids

These are listed in Table 1. A list of dilysogens constructed for this work is in S1 Table.
Microbiological methods
Phage crosses and infections were done using standard protocols [67].

Helper phages
The helper phages used were in the thermo-inducible λ-P1:5R KnR nin5 genetic background. In λ-P1:5R cI857 KnR nin5, the DNA segment containing the λ site-specific recombination system is replaced by the phage P1 plasmid replication and partitioning segment, so the helper phage forms a plasmid prophage [68]. The KnR marker in λ-P1:5R cI857 KnR nin5 is a substitution for λ DNA between the two λ SalI sites [69]. The SalI sites are located in (1) the bet gene and (2) distal to gam, so that the helper phages are red-gam-. When growing in a recA host, red-gam- phages are unable to generate concatemers through rolling circle replication or recombination [29]. Consequently, the helper phage yield is low, about 10–20 phages/cell in the experiments reported here. For simplicity, λ-P1:5R cI857 KnR nin5 will simply be designated λ; this phage was used as the helper with λ packaging specificity. λ21hy51 [59] and λN15hy4 [58] were the 21- and N15-specific helpers, respectively.

Dilysogens
Dilysogens were constructed with two recA− strains with deleted prophages, MF713 and MF532 (Table 2). The λ b511 prophage of MF713 is deleted for a DNA segment extending from the bacterial gal operon thru the prophage early genes, including cl, while the segment containing cos, the head and tail genes, the b511 marker and attΔ●B are present. The genetic structure of MF713 is:

\[ \Delta[\text{gal} - \text{attL} - \text{cl} - S] - \text{cos}^\lambda - / / - \text{b511 attA}●B - \text{bio} - \]

where λ genes are underlined, slashes indicate the long DNA stretch of the λ head and tail genes, dashed lines indicate E. coli. Lysogenization of MF713 by λ imm434 ind cos generates the following genetic structure:

\[ \Delta[\text{gal} - \text{attL} - \text{cl} - S] - \text{cos}^\lambda - / / - \text{attP} - \text{imm}^{434} - \text{cos}^\lambda - / / - \text{attR} - \text{bio} - , \]

where cos indicates that the downstream cos, at which termination occurs, may have λ, 21 or N15 sequences. The passive packageable prophage is double-underlined. Care was taken to exclude isolates with two copies of the added prophage. For MF713 (λ imm434 cos), we confirmed that the chromosomes produced by helper packaging of the passive prophage carried the b511 marker. Generally, 10 isolates were tested by a PCR assay or a test for a b511 phage’s inability to lysogenize.

The λ imm434 prophage of MF532 is deleted rightwards from Nu1 through the head and tail genes, attR, and bacterial DNA including bio, uvrB and chlA. The genetic structure of MF532 is:

\[ \text{gal} - \text{attL} - \text{imm}^{434} - \text{cos}^\lambda - \Delta[\text{Nu1} - / / - \text{attR} - \text{bio} - \text{uvrB} - \text{chlA}] \]

Lysogenization of MF532 by λ imm21 cos gives the genetic structure:

\[ \text{gal} - \text{attL} - \text{imm}^{21} - \text{cos}^\lambda - / / - \text{attP} - \text{imm}^{434} - \text{cos}^\lambda - \Delta[\text{Nu1} - / / - \text{attR} - \text{bio} - \text{uvrB} - \text{chlA}] \]

where the passive packageable prophage is double-underlined. The cos at which packaging is initiated, cos, was either cos^λ, cos^{21} or cos^{N15}. The cos^{21} is from 21hy33, which also has gene l
and a chimeric 2/A gene. The \( N15^{hy4} \) provided \( \cos^{N15} \). Care was taken to ensure that dilysogen candidates with more than two prophages were eliminated.

For MF532 dilysogens, the upstream prophage carried the immunity of phage 21 (\( \text{imm}^{21} \)) and \( \cos \). The downstream prophage of MF532 carries \( \text{imm}^{434} \) and \( \cos^{\lambda} \). Helper packaging of the passive prophage of an MF532 dilysogen generates virions carrying chromosomes that are \( \text{imm}^{434} \). The immunity of 10 isolates was tested to confirm this expectation.

**MF611 (Tables 3 and 6).** To generate this dilysogen set, the \( \text{recA1 E. coli} \) strain MF611 was sequentially lysogenized by the two phages under study. Then helper phages were added. The genetic structure of dilysogens was determined by examining the genetic content of helper-packaged passive prophages. In all cases care was taken to show that isolates with more than two prophages were excluded from experiments.

**MF3510 (Table 5).** MF3510 is a \( \text{galK}^{-} \) derivative of MF611. Phages used carried \( \text{gal} \) or \( \text{cat} \) markers, and examination of packaged passive prophages was used to eliminate isolates with more than two prophages. In most cases, for all three dilysogen sets, multiple independent dilysogen isolates were used in experiments.

**Helper phages.** The helper phage genetic background was \( \lambda^{-} \)-\( \text{P1:5R} \) \( \text{KnR} \) \( cl857 \) \( nin5 \), and this phage was the \( \lambda^{-} \)-specific helper phage. The 21-specific helper phage was a recombinant containing the left chromosome end 21\(^{hy51} \), which includes \( \cos^{B21} \) and a chimeric small subunit gene in which the 5'-half was from the 21 \( I \) gene and the 3' half was from \( \text{Nu}I \). The N15-specific helper, \( \lambda^{-} \)-\( P1 N15^{hy4} \), is described in Results. For simplicity, these three helper phages will simply be designated as the \( \lambda^{-} \)-, 21-, and N15-specific helpers, resp.

**Helper packaging protocol.** Strains were grown overnight in LB+kanamycin, in standing culture, at 31°C, and 0.2 ml was used to inoculate 5 ml of LB. Cultures were shaken at 31°C for 2 hrs. An aliquot was removed and plated to determine the cell titer. Helper phages were induced by shifting to 42°C for 15 min, and the cultures were shaken at 37°C for an additional 70 min. Lysis was completed by addition of CHCl₃, debris removed by centrifugation, and the lysates titered at 37°C. Helper phages form clear plaques and phages bearing passive prophages form turbid plaques. For lysates with low levels of passive prophage-containing virions, dilutions were plated on C600(\( \lambda \)). If the progeny of phages with passive prophages were to be subjected to further analysis, the indicator strain was MF1427, an \( \text{E. coli C} \) strain. The level of defective \( \lambda^{-} \)-like prophage material in \( \text{E. coli C} \) is very low, and no \( \cos^{N14} \) is present, so that concern about recombination with defective prophage DNA was eliminated.

### Supporting Information

**S1 Table.** Dilysogens used in Helper Packaging Experiments. (DOCX)

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Author Contributions
Conceived and designed the experiments: MF. Performed the experiments: MF JYM SS PP JS. Analyzed the data: MF. Contributed reagents/materials/analysis tools: MF. Wrote the paper: MF JS.

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