37

The *Listeria* Bacteriophages

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B acterial viruses specific for the genus *Listeria* were discovered almost 60 years ago (57), and were early reported for their usefulness in phage typing (61) of isolates of the pathogen *Listeria monocytogenes* (65). In the following years, phage typing of *Listeria* isolates has proven to be a very useful method, and led to the isolation of more than 400 phages for *L. monocytogenes*, *L. ivanovii*, and the nonpathogenic species *L. innocua*, *L. seeligeri*, and *L. welshimeri* (5, 9, 16, 19, 22, 25, 26, 31–33, 48–51, 53, 55, 63). To date, no phages infecting organisms of the species *L. grayi* have been found. This chapter briefly summarizes our present knowledge on *Listeria* phages, and gives an overview on their general and particular properties, with respect to both the basic science and the various practical applications.

Ultrastructure, Composition, and Taxonomy of *Listeria* Phages

Electron microscopical examinations of more than 120 *Listeria* phages (2, 5, 7, 10, 33, 49, 51, 61, 71) revealed a relatively limited diversity (figure 37-1). Most phages belong to the morphotype group B1 of the *Siphoviridae* family (isometric capsid and long, noncontractile tail), in the order *Caudovirales* (1) (see chapter 2 for an overview of phage classification). These Siphoviruses are divided into five recognized species and one proposed species (table 37-1) based upon differences in tail length (3). The remaining phages were classified as *Myoviridae* of the morphotype A1 (isometric capsid and long, contractile tail). Two species were established, based on different particle dimensions and mode of sheath contraction. While members of the species A511 resemble a more commonly found phage morphotype, phages of species 4211 (such as 01761 depicted in figure 37-1) are unique and feature a rather unusual mode of sheath contraction in which the tail seems to contract toward the baseplate, thereby exposing the inner tail tube starting from the capsid.

Caudovirales generally have double-stranded DNA as genetic material. Restriction endonuclease analysis allowed discrimination of individual *Listeria* phages and calculation of genome sizes, which range from 36 to more than 100 kb (table 37-1). The G-C content lies between 35 and 41 mol % (36, 38, 54, 70; unpublished data). A significant correlation between ultrastructure and overall DNA homology was found, which supports the existence of at least five DNA homology groups among the phages investigated (38, 54). Structural proteins of more than 40 phages were analyzed by electrophoretic methods (33, 38, 70, 71), and, very recently, mass spectrometry (70). Several studies indicate that the major capsid proteins are proteolytically processed during maturation of the head, while from the tail sheath proteins only the N-terminal methionine is absent (36, 41, 70). The PSA virion employs a particularly interesting mechanism for synthesis of essential components, involving a translational +1 frame-shifting at the 5′ ends of the major structural protein genes for the capsid (*cps*) and tail (*tsh*) (70). Comparison of protein profiles permits differentiation of phages and establishment of similarity clusters, and generally corresponds well with ultrastructure and DNA hybridization patterns.

Host Ranges and Phage Receptors

All *Listeria* phages are strictly genus-specific. The temperate ones only recognize host bacteria of individual but specific serovar groups, while the virulent A511-like phages can attack strains of all species and serovars (4, 5, 31, 32, 50, 52). The *Listeria* O-antigens are largely determined by the variable structure and sugar substitution of poly-ribitolphosphate cell wall teichoic acids (17). It has been demonstrated by biochemical and genetical approaches that the teichoic acid substituents, N-acetylglucosamine and rhamnose, are major determinants of phage adsorption in serovar 1/2 strains, while N-acetylglucosamine and
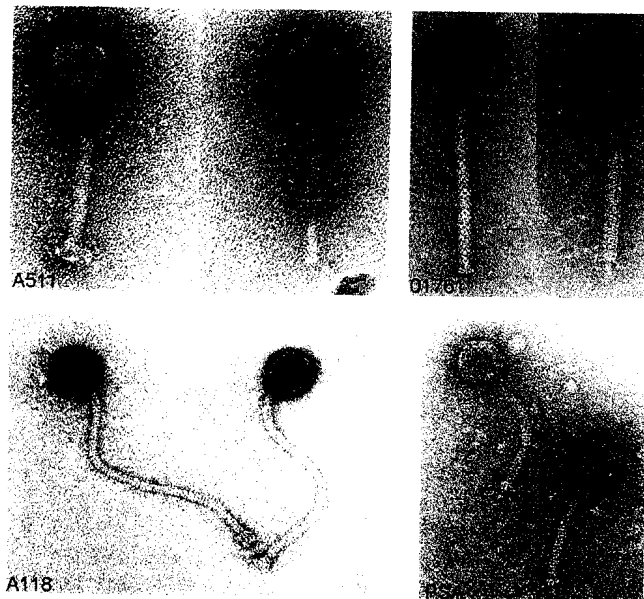
Figure 37-1 Electron micrographs of different *Listeria* bacteriophages. The phages shown belong to different *morphotypes* and species. A511 and 01761 are *Myoviridae* with contractile tails, while A118 and PSA are *Siphoviridae* with noncontractile, flexible tails (see table 37-1 and text).

galactose are important in serovar 4 strains (11, 62, 68). In contrast, teichoic acids are apparently not involved in binding of the polyvalent A511 phage. It is assumed that the peptidoglycan itself represents its receptor, possibly in conjunction with other, serovar-independent carbohydrates (68).

Virus Multiplication and Host Cell Lysis

Listeria phages seem to be well adapted to their host bacteria. Most phages can complete lytic cycles at 10–37 C, but some are more temperature-sensitive and only multiply at 25 C or below (25). Growth curves have been recorded for a few phages. At 30 C, the latent period of the lytic cycle of temperate phages infecting *L. monocytogenes* is between 60 and 70 minutes, followed by a rise phase of 40 to 65 minutes. An average of 25 progeny virions are released from lysed cells. In *L. ivanovii* as host, latent phases up to 115 minutes were observed, resulting in a burst size of up to 40 particles. The virulent nature of A511 agrees well with its shorter latent phase of 55 minutes and comparatively large burst size of 40 virions (unpublished data). (For an overview of phage infection-timing and burst-size characters, see chapter 5.)

Lysis of infected cells occurs through the combined action of a holin (Hol) and an endolysin (Ply), encoded by two immediately adjacent genes at the distal end of the late gene regions. In phage A511, the holin has not been identified. In phages A118, A500, and φ2438, Ply (the endolysin) was found to represent a new class of enzyme, an L-alanoyl-D-glutamate endopeptidase, whereas phages A511 and PSA feature (different) N-acetylmuramoyl-L-alanine amidases (44, 70, 73). An interesting aspect of these enzymes is their modular composition and their unusual substrate specificity, which is mediated by individual C-terminal domains recognizing unique cell wall carbohydrates (37). These cell wall binding domains (CBD) have distinct binding abilities: The CBD of phage Ply500 endolysin recognizes cell surfaces belonging to serovars 4, 5, and 6, and binds to a receptor evenly distributed in the wall. In contrast,

Table 37-1 Present Status of *Listeria* Phage Taxonomy, and Main Virion Characteristics

Family	Species	Other relevant members[a]	Approximate[b] virion size (in nm) (head diameter[c]/tail length[d])	Approximate genome sizes (in kb)	References
Myoviridae	A511		88/200	130–140	31, 38, 44, 71 (unpublished)
	4211	B054 01761	62–66/230–270	41–44	33, 51, 71
Siphoviridae	P35[e]		56–60/110	36	25, (unpublished)
	2389	PSA	58–62/170–180	38	2, 33, 70
	H387		58–62/190–200	36–40	49
	2685	B025	58–62/230–260	37–41	33, 54, 71
	2671	A118 A500 A006	58–62/270–310	38–42	25, 33, 36, 54, 71, (unpublished)

[a]Phages investigated and sequenced in our laboratories.
[b]Different phages within the species, different isolates, and different staining methods yield variable results.
[c]Measured from apex to apex.
[d]Measured including base plates.
[e]Proposed new species.

the CBD of phage Ply118 binds only to serovar 1/2 and 3 cells, with preference for septal regions and the poles. These distinctions indicate fundamental differences in carbohydrate composition among the cell walls of different strains of bacteria as well as cell wall differences along the contours of individual bacteria.

Immediately preceding cell wall hydrolysis, Hol is proposed to form lesions in the cytoplasmic membrane, enabling access of Ply to the murein (reviewed in chapter 10). Although the primary sequences of Hol118 and Hol500 (the Hol proteins from phages A118 and A500, respectively) are completely different from the prototype phage λ S protein, the overall structures of these class II holins are conserved (44). However, timing and regulation of Hol118 function is clearly different from the S paradigm (67). Although it features a dual-start motif, recent findings have demonstrated that Hol118-mediated regulation of lysis timing represents a novelty since it occurs via a cotranscribed intragenic inhibitor lacking the first transmembrane domain and thereby interfering with pore formation (66).

Temperate *Listeria* Phages

Genomics

The first *Listeria* phage completely sequenced and analyzed in detail was A118, a temperate bacteriophage specific for *L. monocytogenes* serovar 1/2 strains (36). Its genome is a circularly permuted collection of terminally redundant dsDNA molecules of an average length of 43.3 kb, which indicates 6% redundancy of the unit size of 40,834 bp. This has been confirmed by partial denaturation maps and electron microscopy, also showing that the right end of DNA is attached to the phage tail and that the roughly 10-mer concatemers are sequentially packaged left to right. The A118 genome contains 72 ORFs, organized in three major, life-cycle-specific gene clusters. The genes required for lytic development show an opposite orientation and arrangement compared with the lysogeny control region. A function could be assigned to 26 genes, while the remaining 46 have no known counterparts.

The sequence of the prophage of *L. monocytogenes* serovar 4b strain ScottA, PSA, was recently completed (70). In contrast to A118, PSA features 10-nucleotide 3'-overhanging cohesive ends and packages exactly one unit genome of 37,618 bp. Although its 55 open reading frames are mostly unrelated to phage A118's (figure 37-2), their overall life-style-specific gene organization is relatively similar, except for the presence of some unique genes such as a primase and a helicase in PSA, and a recombinase in A118, each of which are required for the different mechanisms of genome replication.

Proteome analysis of PSA revealed an unusual form of translational frameshifting which yields different-length

forms of the major structural proteins of the capsid and tail, respectively (70). The proteins feature identical N-termini but different C-termini as a result of programmed +1 translational frameshifting. Frameshifting appears to be initiated by a slippery nucleotide sequence with overlapping proline codons near the 3' ends of both genes. This apparently redirects the ribosomes into the +1 frames. Different *cis*-acting factors (a shifty stop and a pseudoknot) are also present. This phage PSA attribute is the first case of +1 frameshift among double-standed DNA phages, and also is the prototype of a virus featuring a 3' pseudoknot to stimulate ribosomal frameshifts.

Attachment Sites and Integration

So far, two different classes of temperate phages in *L. monocytogenes* have been shown to integrate their DNA into the host chromosome. Phage A118 is an example of the first class (36). Sequence comparisons indicate that the A118 integrase enzyme is a serine recombinase related to Tn10 resolvase and Hin invertase. The A118 bacterial attachment site, *attB*, lies within an open reading frame closely related to *comK* of *Bacillus subtilis*. This gene encodes a transcriptional activator for various factors involved in competence for DNA uptake. Since *L. monocytogenes* is not easily transformable, the role of its *comK* gene is not immediately obvious. Integration of the A118 genome into *comK* changes most of the coding sequence, and no phage sequence reconstitutes this reading frame. The A118 phage and bacterial attachment sites display only 3 base pairs of homology near the point of crossover, as is common for serine recombinases. In contrast, phage PSA integrates into the tRNA$_{Arg}$ gene (29). PSA's phage attachment site contains identity to the 15 nucleotides at the 3' end of tRNA$_{Arg}$ plus two downstream nucleotides. After integration by PSA, the sequence of tRNA$_{Arg}$ is regenerated by prophage nucleotides. PSA's integrase is different from A118 Int, and is homologous to *Escherichia coli* XerD, a tyrosine recombinase that resolves dimeric circles of *E. coli* DNA and plasmids of the ColE1 family (60).

Prophages and Lysogeny

Lysogeny is widespread among strains of the genus *Listeria*: the percentage of strains producing infective phage has been estimated to range from 6% to 37%, depending on the species (50). Prophages are readily inducible using UV light or mitomycin C (34). Lysogenization can easily be provoked by a high multiplicity of infection, and lysogens are generally resistant to superinfection by the same or related phages but not by phages of different immunity groups (35). Many of the commonly used laboratory strains (e.g., 10403S, EGDe, L028, and others) carry an integrated functional or cryptic prophage at the *attB* within *comK*. However, *comK* and tRNA$_{arg}$ are only two of several
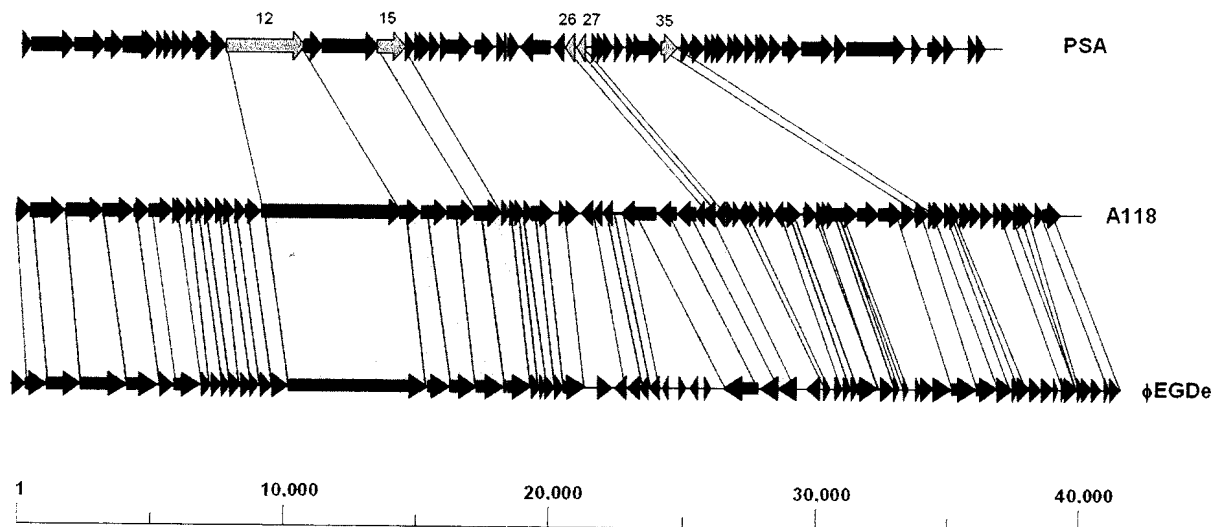
Figure 37-2 Alignment of the genetic maps from the temperate *Listeria monocytogenes* phages. Shown are phages PSA (70), A118 (36), and the cryptic prophage φEGDe (20). Open reading frames are shown as black arrows, pointing in the direction of transcription. The maps start with the terminase genes of the "late" region (left), and end with distal genes of the "early" gene region, involved in DNA replication and recombination. Lysogeny control regions (mostly leftward-pointing arrows) are located at approximate coordinates 20-30 kb on the ruler at the bottom of the figure. Genes encoding proteins of significant amino acid sequence similarity are linked by gray shading. For PSA, the few genes whose products feature significant similraity to A118 are indicated by numbers corresponding to the individual open reading frames (70).

existing *attB* sites in the *Listeria* genomes. since multiple lysogens can be created by subsequent challenge with different phages (35). and polylysogenic strains are frequently observed (see chapter 7 for a discussion of phage integration).

Despite the fact that most if not all strains carry functional or cryptic prophages. the potential influence of lysogeny on the host phenotype is unknown. No phenotype has been associated with *comK* inactivation by insertion of A118-like phage or by insertion of an integration vector (described below). No obvious association was yet observed between phage carrier state and *Listeria* phenotype. especially with regard to pathogenicity. Nevertheless, temperate phage may carry genes similar to host factors involved in bacteria–host interaction (36). Resistance to phage was sometimes found to be the result of changes in cell wall composition (62, 68), which could also be linked to decreased sensitivity to quaternary ammonium compounds (46). Moreover. the presence of phage-encoded methyltransferases such as M.*Lmo*A118I (8. 36) affects distribution of genetic material and may therefore influence the phenotype of lysogens.

Cryptic Prophages

Up to 71% (27) of all *Listeria* cultures produce substances inhibitory to other *Listeria* strains but not to other bacteria. These substances were termed "monocins" (7. 12. 22. 47. 61. 69). In monocin preparations. particles that

resemble phage tails or polyheads could be observed by electron microscopy (7. 73). It was later shown by genetic methods that these particles indeed result from incomplete. cryptic prophages and that their lethal effect is due to the presence of intact lysis genes (73). Similar to phage. monocins display a killing-from-without effect which to some degree is serovar correlated (72). A cryptic phage related to A118 was identified in the chromosome of *L. monocytogenes* EGDe (see figure 37-1). and five phage-like elements are present in the chromosome of *L. innocua* CLIP11262 (20).

Transducing Phages

Many of the temperate *Listeria* phages are capable of generalized transduction. that is they more or less randomly package host DNA and can therefore transduce functional genetic markers into other cells (25). Tranduction frequencies range from 10^{-4} up to 5×10^{-2}. depending on the phage and host used. The ability to package non-phage DNA appears to correlate with the genome structure of the viruses: the terminally redundant A118 does transduce. whereas the *cos*-site phage PSA does not (25). This correlation seems to be the case for other *Listeria* phages as well (unpublished data) and is likely dependent on the different DNA packaging mechanisms employed by these viruses. which were shown to have unrelated terminase enzymes.

Virulent *Listeria* Phages

As mentioned above, most *Listeria* phages are temperate and, with the exception of A511, nothing is known about the few virulent phages for the genus. Phage A511, however, is a particularly interesting virus. It has a very wide host range and can infect approximately 95% of the relevant *L. monocytogenes* strains found to be implicated in foodborne disease. Of its large genome, only a fraction has yet been analyzed in detail, namely the lysis gene region (44) and a 10 kb fragment containing most of the morphopoietic genes (40). A useful finding were the powerful promoter sequences controlling expression of the major late genes, *cps* and *tsh*, which enabled design of a reporter phage vehicle (see below).

Relationships and Phage Evolution

Comparative genomics demonstrates that phage A118 is only very distantly related to phage PSA (figure 37-2), but highly similar to the cryptic phage, φEGDe, found in the chromosome of *L. monocytogenes* EGDe (20). Most differences between A118 and φEGDe are found in the early gene region, whereas the late gene region is, with the exception of the major capsid genes, extremely conserved. Only a single gene, encoding a part of the virus tail structure, is conserved among these three *Listeria*-phage genomes. In addition, these three phages were shown to contain portions resembling functional regions of other phage genomes, in particular those infecting lactic acid bacteria and other members of the low G-C content subbranch of Gram-positive eubacteria (36, 70).

Of interest is the relatedness of the *Listeria* phage A511 to *Staphylococcus aureus* phage Twort. Both belong to a group of morphologically basically indistinguishable, obligately virulent myoviruses that infect various Gram-positive hosts (21). Phage A511 was reported to have significant homologies in its late gene region to an intron-containing sequence of Twort (28), raising the possibility of the presence of self-splicing introns in *Listeria* phages. Also, in contrast to the situation of the temperate phages, it seems more difficult to explain the presence of almost identical sequence elements in viruses that do not exist in a prophage state, and do not infect a common host. We have previously observed an unusually high rate of recombination in A511 (39), which suggests that these (and other) viruses may use some specially adapted mechanisms that augment their ability to participate in the genetic mix-and-match game.

Additional indications exist that point to a relatedness of *Listeria* phages to viruses of other closely related Gram-positive bacteria, such as *Brochothrix thermosphacta*. Several short regions of high DNA homology were identified in morphologically unrelated phages of the two genera

(unpublished data). One such region contains an *ssb* gene that is identical in the viruses of different origin but is flanked by unrelated portions of the genomes, which may reflect a good example of a limited, modular exchange.

Taken together, even the limited data on *Listeria* phages which are available to date clearly support the "mosaics" model of phage genome building, where phage genomes are built from genetic modules. Functional segments are accessible through different mechanisms from a large gene pool (23, 24). Horizontal exchange in phages is obviously dependent on the genetic material of their hosts, which may restrict promiscuous exchange. Nevertheless, *Listeria* phage genomic analyses add to the growing evidence that individual phages likely have evolved from a limited number of ancestral phages. This suggests a divergent evolution of bacterial viruses which is strongly influenced by continuous adaptation and genetic exchange of significant portions of their genomes (see chapters 4 and 27 for further discussion of these concepts).

Applications

Typing Phage

Various systems have been reported for phage typing of *Listeria* (4, 6, 15, 16, 19, 31, 32, 45, 52, 53, 64). Typability (sensitivity to at least one phage of a given set) is heavily dependent on the bacteria: serovar 3 strains are mostly resistant, in contrast to the high phage sensitivity of serovar 4 strains. Virulent phage with a broad lytic range such as A511 increased overall typability from around 70% to more than 90% (32, 65). Currently this technique provides the simplest and most widely used *Listeria* typing method (45), and it provides a sensitive means for tracing the origin and course of foodborne outbreaks of listeriosis.

Reporter Phage

The potential of genetically engineered phage is widely acknowledged in cloning procedures involving well-characterized bacteriophages such as phage λ. Because of its broad host range, phage A511 was selected as a candidate for construction of a reporter bacteriophage. A genetic fusion of *Vibrio harveyi* *luxA* and *luxB* genes was introduced into the A511 genome, under control of the powerful *cps* promoter (39). Following infection of *Listeria* cells by A511::*luxAB*, viral gene expression results in bioluminescent bacteria which can easily be detected and quantitatively monitored even in a mixed bacterial flora. A thorough evaluation of the system confirmed its usefulness as a quick and sensitive method for detection of viable *Listeria* in a variety of foods (40). See chapter 46 for further discussion of this reporter phage approach to bacteria identification and diagnosis.

Killer Phage

The potential usefulness of phage in bio-disinfection measures against *Listeria monocytogenes* on solid surfaces and production equipment was reported (56). However, this application faces many potential problems, and much more research is needed with respect to the potential of phage for eradicating *Listeria* in immensely complex environments such as food and feed. See chapter 48 of additional consideration of the use of intact phages as antibacterial agents.

Lytic Enzymes

Listeria phage endolysins can be produced in *E. coli*, and the recombinant enzymes retain high activity after affinity purification (42, 43). The enzymes evolved to exhibit stringent substrate specificity, that is they only lyse the *Listeria* cell peptidoglycan, with very few exceptions among closely related Gram-positive bacteria. Although the virus uses them from within the cell, Ply enzymes work equally well when added exogenously. A tiny amount of enzyme is sufficient to clear a dense suspension of *Listeria* cells within seconds. The enzymes are active in a pH range from 6 to 10, they are insensitive to common protease inhibitors and chelating agents, and moderate concentrations of detergents even increase their activity. Based on these properties, they have found a number of applications, such as rapid in vitro lysis (13, 41, 42, 44), removal of extracellular bacteria in eukaryotic cell invasion assays (58), selective release of intracellular metabolites such as ATP (59), and programmed self-destruction of intracellular *Listeria* cells with in the cytosol of macrophages (14). A novel approach for biological control of *Listeria monocytogenes* in fermented milk products is the production and secretion of N-terminally modified Ply511 by recombinant, lactose-utilizing *Lactococcus lactis* (18).

Another very interesting possibility is the use of the recombinantly produced cell wall binding domains of these enzymes (see above). The high-affinity, specific CBD polypeptides can be used for immobilization of host cells on solid surfaces such as coated microplates or magnetic beads. When fused to a fluorescent label, they have properties similar to a labeled antibody and allow specific decoration and efficient separation of *Listeria* cells from mixed bacterial populations and even within infected bacterial cells (30, 37).

Integration Vectors

On the basis of cloned integrase genes, two chromosomal integration vectors have been constructed. The first uses the integrase and phage attachment site of the A118-related phage, U153, and the second uses the analogous elements of phage PSA (29). These vectors are propagated in *E. coli*

and can be transferred to *L. monocytogenes* by conjugation or by electroporation. Since these plasmids cannot replicate in *L. monocytogenes*, retention of the drug-resistant phenotype requires chromosomal integration. Using the U153*int*-based vector to integrate the genes for Listeriolysin O (LLO) and ActA, Lauer and coworkers showed that these genes are expressed well from the *comK* attachment site (29). Using the PSA vector, it has been shown that the *secA2* gene can be expressed well from the tRNA^{Arg} attachment site (30).

Transducing Phage

A recent and important finding was the use of *Listeria* phage for generalized transduction of genetic material from one strain to others (25). Of particular use is the introduction of marker-tagged mutations and associated phenotypes into a clean genetic background, which enables detailed genetic mapping and characterization of the mutation. The most useful phages for this purpose are P35 and U153 (serovar 1/2 strains), and A500 (serovar 4 strains). Phages infecting *L. innocua* and *L. ivanovii* were not tested in this study, but it is conceivable that many of the temperate viruses infecting these species will also be generalized transducers.

Acknowledgments

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