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Mutant and Recombinant Phages Selected from *In Vitro* Coevolution Conditions Overcome Phage-Resistant *Listeria monocytogenes*

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ABSTRACT Bacteriophages (phages) are currently available for use by the food industry to control the foodborne pathogen *Listeria monocytogenes*. Although phage biocontrols are effective under specific conditions, their use can select for phage-resistant bacteria that repopulate phage-treated environments. Here, we performed short-term coevolution experiments to investigate the impact of single phages and a two-phage cocktail on the regrowth of phage-resistant *L. monocytogenes* and the adaptation of the phages to overcome this resistance. We used whole-genome sequencing to identify mutations in the target host that confer phage resistance and in the phages that alter host range. We found that infections with *Listeria* phages LP-048, LP-125, or a combination of both select for different populations of phage-resistant *L. monocytogenes* bacteria with different regrowth times. Phages isolated from the end of the coevolution experiments were found to have gained the ability to infect phage-resistant mutants of *L. monocytogenes* and *L. monocytogenes* strains previously found to be broadly resistant to phage infection. Phages isolated from coinfected cultures were identified as recombinants of LP-048 and LP-125. Interestingly, recombination events occurred twice independently in a locus encoding two proteins putatively involved in DNA binding. We show that short-term coevolution of phages and their hosts can be utilized to obtain mutant and recombinant phages with adapted host ranges. These laboratory-evolved phages may be useful for limiting the emergence of phage resistance and for targeting strains that show general resistance to wild-type (WT) phages.

IMPORTANCE *Listeria monocytogenes* is a life-threatening bacterial foodborne pathogen that can persist in food processing facilities for years. Phages can be used to control *L. monocytogenes* in food production, but phage-resistant bacterial subpopulations can regrow in phage-treated environments. Coevolution experiments were conducted on a *Listeria* phage-host system to provide insight into the genetic variation that emerges in both the phage and bacterial host under reciprocal selective pressure. As expected, mutations were identified in both phage and host, but additionally, recombination events were shown to have repeatedly occurred between closely related phages that coinfected *L. monocytogenes*. This study demonstrates that *in vitro* evolution of phages can be utilized to expand the host range and improve the long-term efficacy of phage-based control of *L. monocytogenes*. This approach may also be applied to other phage-host systems for applications in biocontrol, detection, and phage therapy.

KEYWORDS *Listeria monocytogenes*, bacteriophage recombination, bacteriophage resistance, bacteriophages, biocontrol, coevolution, food safety, genomics

Listeria monocytogenes is an opportunistic bacterial foodborne pathogen (1) that is ubiquitous in many natural environments, such as plant-soil environments (2). *L. monocytogenes* has been isolated from diverse environmental samples, such as soil (3),

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water (4), silage (5), and animal feces (6–8). *L. monocytogenes* is primarily a saprophyte; however, it possesses a set of virulence genes that are transcriptionally regulated to be expressed upon ingestion by a susceptible host, which allows it to thrive as an intracellular pathogen (9, 10). *L. monocytogenes* is a particularly problematic foodborne pathogen due to its persistence in the environment, ability to grow at refrigeration temperatures, and ability to survive under stress (11, 12). Listeriosis, caused by *L. monocytogenes*, can result in high hospitalization and mortality rates. The annual global number of listeriosis cases is estimated at 23,150, with a 24% mortality rate (13). The United States has an estimated 1,591 to 2,518 listeriosis cases that occur each year, with a mortality rate of 16 to 20% (14, 15).

L. monocytogenes strains are often characterized by serotype, a subtyping scheme that groups strains by their bacterial surface antigens, which correlates with wall teichoic acid (WTA) composition (16, 17). Of the 12 known serotypes of *L. monocytogenes*, serotypes 1/2a, 1/2b, and 4b are the three most commonly associated with human listeriosis cases (18). Reported foodborne outbreaks of *L. monocytogenes* in the United States over a 10-year period (1998 to 2008) were primarily caused by serotype 4b (10 outbreaks) and serotype 1/2a (8 outbreaks) (19). Outbreaks caused by serotype 1/2a strains have continued to increase in frequency and prevalence since 2000 in North America and Europe (20). Serotype 1/2a is most often isolated from food samples (21) and was involved in the largest listeriosis outbreak in the United States, which originated from contaminated cantaloupes (22, 23). Phylogenetic analysis of outbreak strains shows that serotype 1/2a strains cluster with serotypes 1/2c and 3a and 3c (lineage II strains), whereas 1/2b serotypes cluster with serotypes 3b and 4b (lineage I) (24). It has been suggested that serotype 3 and 7 strains have descended from serotype 1/2 genetic backgrounds through a series of loss of function mutations (25). Differences in gene content between lineage I and II isolates include genes that are involved in rhamnose biosynthesis and glycosylation of teichoic acids (26, 27).

Lytic bacteriophages are viruses that infect a specific bacterial host. They exploit the host bacterium's cellular machinery to express their genomes and replicate, producing a new generation of progeny phages that lyse their host upon release into the environment (28). Phages that infect *L. monocytogenes* can be isolated from the environment and then prepared for use in the food industry as a biocontrol (29–31). For example, *Listeria* phages of the genus *Pecentumvirus*, such as P100 (32) and A511 (33), are effective at reducing the growth of *L. monocytogenes* on a variety of food matrices (34). However, one challenge facing the longevity of phage biocontrol products is that the application of phage treatments may select for phage-resistant bacterial populations in the food processing environment (35–37).

Previous studies on the selection of phage-resistant *L. monocytogenes* by *Pecentumvirus* phages show that mutations in bacterial genes involved in phage adsorption to the host, which result in deficiencies of the WTA sugars *N*-acetylglucosamine and rhamnose, are consistently selected for in phage-treated populations of *L. monocytogenes* by the process of natural selection (38, 39). Loss of rhamnose in WTAs cause 1/2a strains to convert to serotype 3 strains (39), which are known to be broadly phage resistant (40, 41). Genetic mutants of a 1/2a strain with rhamnose deficiencies in WTAs were also found to be resistant to all but one of a diverse collection of 120 *Listeria* phages isolated from the environment (42). The single phage that could infect the phage-resistant mutant, *Homburgvirus* LP-018, may not be broadly useful for biocontrol applications, as it demonstrated poor infection kinetics in liquid medium. Interestingly, phage-resistant mutants selected for by LP-018 were found to confer phage resistance through a mechanism independent of adsorption inhibition (43). Although isolation of novel phages from the environment with the ability to overcome phage resistance remains a viable option, the isolation of phages and subsequent characterization and preparation for application purposes can be a time- and resource-consuming process (44, 45). Other approaches that have been explored to improve phage host range include genetic engineering (46) and *in vitro* adaptation of phages (47).

Coevolution in phage-host systems is known to promote diversity (48) in the

TABLE 1 *Listeria monocytogenes* strains used in this study

L. monocytogenes strain	Lineage	Serotype	Description[b]	Reference or source
10403S	II	1/2a	Lab strain	102
MACK	II	1/2a	Lab strain	103
F2365	I	4b	Food	27
FSL J1-175	I	1/2b	Water	24
FSL J1-208	IV	4a	Animal	104
FSL C1-115	II	3a	Human	105
FSL J1-094	II	1/2c	Human	105
FSL F2-695	IIIA	4a	Human	104
FSL F2-501	IIIA	4b	Human	104
FSL J2-071	IIIA	4c	Animal	104
FSL W1-110	IIIC	4b	Unknown	106
FSL J1-158	IV	4b	Animal	106
FSL J1-169	I	3b	Human	105
FSL J1-049	I	3c	Human	105
FSL D4-0014(GlcNAc$^-$)	II	1/2a	Lab strain (GlcNAc$^-$)	38
FSL D4-0119(Rha$^-$)	II	3[a]	Lab strain (Rha$^-$)	38
FSL R9-0915	II	7		38
10403S mutants				
UTK P1-0003			NS mutation in LMRG_00542, MS mutation in LMRG_01466	This study
UTK P1-0004			MS mutation in LMRG_00545, MS mutation in LMRG_02979	This study
UTK P1-0006			MS mutation in LMRG_00545	This study
UTK P1-0007			FS deletion mutation in LMRG_00544, FS deletion mutation in LMRG_02528	This study
UTK P1-0013			NS mutation in LMRG_01697, FS deletion mutation in LMRG_01762	This study
UTK P1-0017			MS mutation in 02583, NS mutation in LMRG_01698	This study

[a]Strain has not been serotyped; serotype was inferred based on genetic and published information (39, 42).
[b]NS, nonsense; MS, missense; FS, frameshift.

bacterial host and in the host ranges of phages (49). The study of this process can contribute to a more comprehensive knowledge of phage-host interactions (50). Here, we explored the short-term coevolution of *L. monocytogenes* with *Listeria* phages. We aimed to isolate laboratory-evolved phages with improved host ranges adapted to infect generally phage-resistant strains of *L. monocytogenes*, as well as characterize the genetic variation that occurs in *Listeria* phages and the phage-resistant bacterial isolates they select for. The *Listeria* phages used, LP-048 and LP-125, are both members of the genus *Pecentumvirus*, which are tailed phages with double-stranded DNA (dsDNA) genomes of ~135 kb (51). *Pecentumvirus* phages are the only *Listeria*-infecting phages that are currently used in food safety applications (29, 32, 34, 52, 53). LP-048 and LP-125 were selected for use in this study because they have been well characterized (38, 40, 51, 54) and are known to differ in their binding receptor requirements. LP-048 binds rhamnose in WTAs, and LP-125 binds rhamnose and *N*-acetylglucosamine in WTAs (38).

RESULTS

Coevolution dynamics of *Listeria* phages and *L. monocytogenes* 10403S. To observe the lytic effects of phages on the growth of *L. monocytogenes*, phage-infected and uninfected 10403S cultures (Table 1) were monitored by spectrophotometry for up to 60 h (Fig. 1A); this was conducted for nine biological replicates. The goal was to obtain (i) lab-evolved phages with improved host ranges and (ii) phage-resistant bacterial survivors. All cultures infected with phages, either single infections or coinfections, showed nearly complete lysis and inhibition of growth around 6 h postinfection and for up to 24 h. Regrowth of phage-resistant *L. monocytogenes* was consistently observed between 24 and 26 h in LP-048-infected cultures, earlier than for either LP-125-infected cultures or coinfected cultures. LP-125-infected and coinfected cultures had similar regrowth patterns; however, the regrowth of phage-resistant subpopulations occurred anywhere between 26 and 36 h. Although regrowth of 10403S was observed in all phage-infected cultures, this was followed by a subsequent reduction in optical density at 600 nm (OD_{600}), indicating a possible secondary lysis event by

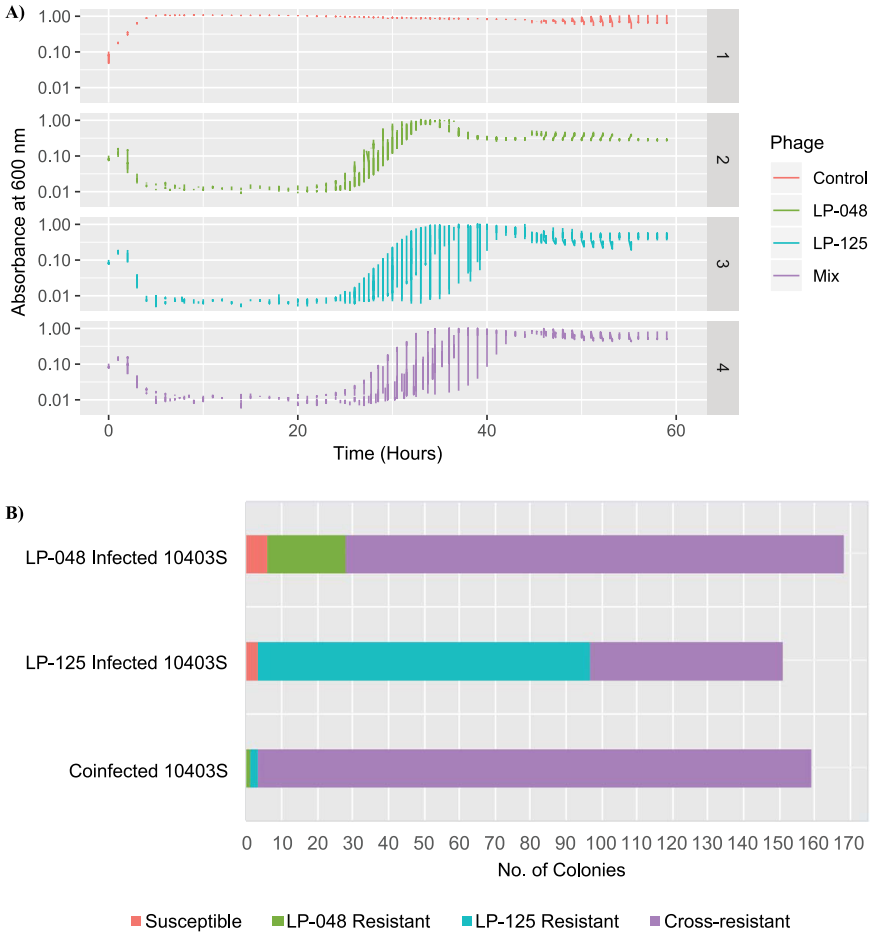

FIG 1 Coevolution dynamics and resulting phage-resistant phenotypes. (A) Growth curves representing optical density readings for *L. monocytogenes* 10403S cultures infected with LP-048, LP-125, LP-048 and LP-125 (Mix), or a phage-free control ($n = 9$) and grown at 30°C. Bacterial isolates were recovered at 6 to 8 h postinfection and before cultures regrew to an optical density (OD) of 0.1 (at 20 to 25 h) (B) Results from phenotypic testing of coevolved bacterial isolates for phage resistance against LP-048 and LP-125 using streak spot assays. A total of 478 colonies were evaluated; 10 colonies from each condition, time point, and replicate were selected, but 10 isolates were not always recovered, so a total of 478 colonies were evaluated.

adapted phages. *L. monocytogenes* survivors were recovered following the initial lysis period (6 to 8 h postinfection) and during the early stages of regrowth (samples were taken before readings reached an OD_{600} of 0.1). Sampling time points for the regrowth phase varied for each infected culture. Phage lysates from each phage-infected culture were recovered from the end of the experiment (55 to 60 h).

Phenotypic analysis of coevolved *Listeria* phages and *L. monocytogenes* 10403S. To assess whether coevolution resulted in adapted phages with an altered host range, we isolated phages from crude lysates obtained from the end of the coevolution experiment. Phage lysates that had a notable change in host range (e.g., that gained ability to form plaques against the rhamnose-deficient [Rha^-] strain) were triple plaque purified and further characterized with an efficiency of plaquing (EOP) assay. Phage lysate from one coinfected culture, LP-Mix_6, showed two plaque morphologies. These were further purified and designated LP-Mix_6.1 and LP-Mix_6.2. Phage-resistant mutants of 10403S were then selected for with LP-Mix_6.1 and LP-Mix_6.2 to identify mutations that confer resistance against these two mutant phages. Two of these isolates, one selected for by LP-Mix_6.1 (UTK P1-0013) and one selected for by LP-Mix_6.2 (UTK P1-0017) were used in the EOP assay described below. We also aimed to determine the phage susceptibility of bacterial survivors, and thus a streak

spot assay was performed to test for phage resistance against parental phages LP-048 and LP-125 (Fig. 1B). As expected, the majority of bacterial survivors from coinfected cultures were resistant to both parental phages. LP-048-infected cultures selected for a larger proportion of bacterial survivors resistant to both parental phages compared to that in LP-125-infected cultures.

Efficiency of plaquing assay for lab-evolved phages against diverse *L. monocytogenes* serotypes. In order to assess the altered host range of lab-evolved phages, an EOP assay was performed. Select coevolved phages were tested against wild-type (WT) 10403S (serotype 1/2a), the phage propagation host *L. monocytogenes* strain MACK (serotype 1/2a), phage-resistant 10403S mutants that lack either *N*-acetylglucosamine (GlcNAc$^-$) or rhamnose (Rha$^-$) in their wall teichoic acids, and a panel of representative *L. monocytogenes* serotype strains (Table 1). Parental phages LP-048 and LP-125 were included as controls, along with the models *Pecentumvirus* A511 (33) and *Homburgvirus* LP-018 (43). EOP assay results showed altered host ranges of coevolved phages compared to those of parental phages (Fig. 2). One consistent observation is that a phage's inability to form plaques against a strain is not necessarily an indication of that phage's ability to lyse a strain; as phages showed a broader capability of activity against strains (see Fig. S1 in the supplemental material) compared to plaque formation (Fig. 2). This is consistent with observations from Trudelle et al. (42). Two coevolved phages, LP-125_m8 and LP-Mix_6.1, were able to infect the 10403S (Rha$^-$) strain FSL D4-0119 and the serotype 3c strain FSL J1-049. Both of these strains have been found to generally resist phage infection (42, 55). One coevolved LP-048 phage, LP-048_m5, showed a decreased host range against the bacterial strains tested, in which clear plaques were only observed against the propagation host (MACK), and only minimal phage activity was observed against other strains (Fig. S1).

Identification of mutations in coevolved phages with altered host range. To identify genes with acquired mutations that may be involved in improved host range, whole-genome sequencing and variant analysis were performed on select lab-evolved phages (Table 2). In order to ensure accurate identification of mutations in lab-evolved phages, the parental phages LP-048 and LP-125 were resequenced as a control. We also sequenced and analyzed A511 to ensure that accurate comparison could be made. McCortex results for A511 showed three mutations compared to the NCBI submission (GenBank accession number NC_009811.2) (33). Two mutations were single-nucleotide polymorphisms (SNPs), one in *gp161* (nucleotide position 106/579), which would replace a serine with a glycine, and one in *gp165* (nucleotide position143/729), which would replace a serine with a cystine. The third mutation was a deletion of a 5-nucleotide repeat ("GGATA") in a tandem repeat region spanning nucleotide positions 126,380 to 126,454 in the A511 genome. This mutation is not expected to affect any coding regions. Since the two missense mutations identified were found in hypothetical proteins, we have no reason to suspect that they would alter the host range of A511; however, this is a possibility that must still be considered. LP-125_m8, one of the phages with an improved host range, had one mutation in *LP125_117* (Fig. 3A), a homolog to *gp108* in *Listeria* phage A511, which codes for a host receptor binding protein in the form of short tail fibers (56). The mutation was a missense variant and occurred at nucleotide position 1,036/1,293, resulting in a lysine rather than a glutamic acid. Another phage with an improved host range, LP-Mix_6.1, had two mutations (Fig. 3), one in *LP125_108* and one in *LP125_118*. Gene *LP125_108* is homologous to *gp99* in A511, which encodes a baseplate protein (57); it contained a missense mutation (T→C) at nucleotide position 1,023/1,533. This mutation would lead to a substitution of an isoleucine residue for a threonine residue in the peptide product. The second mutation observed in LP-Mix_6.1 was a missense mutation in *LP125_118* (C→A; nucleotide position 396/411), annotated as a putative tail fiber assembly chaperone protein. This mutation would lead to a substitution of a glutamic acid residue for an aspartic acid residue in the peptide product. The homolog gene in A511 is *gp109*, a putative assembly chaperone protein (56). Phenotypic analysis showed that LP-Mix_6.1

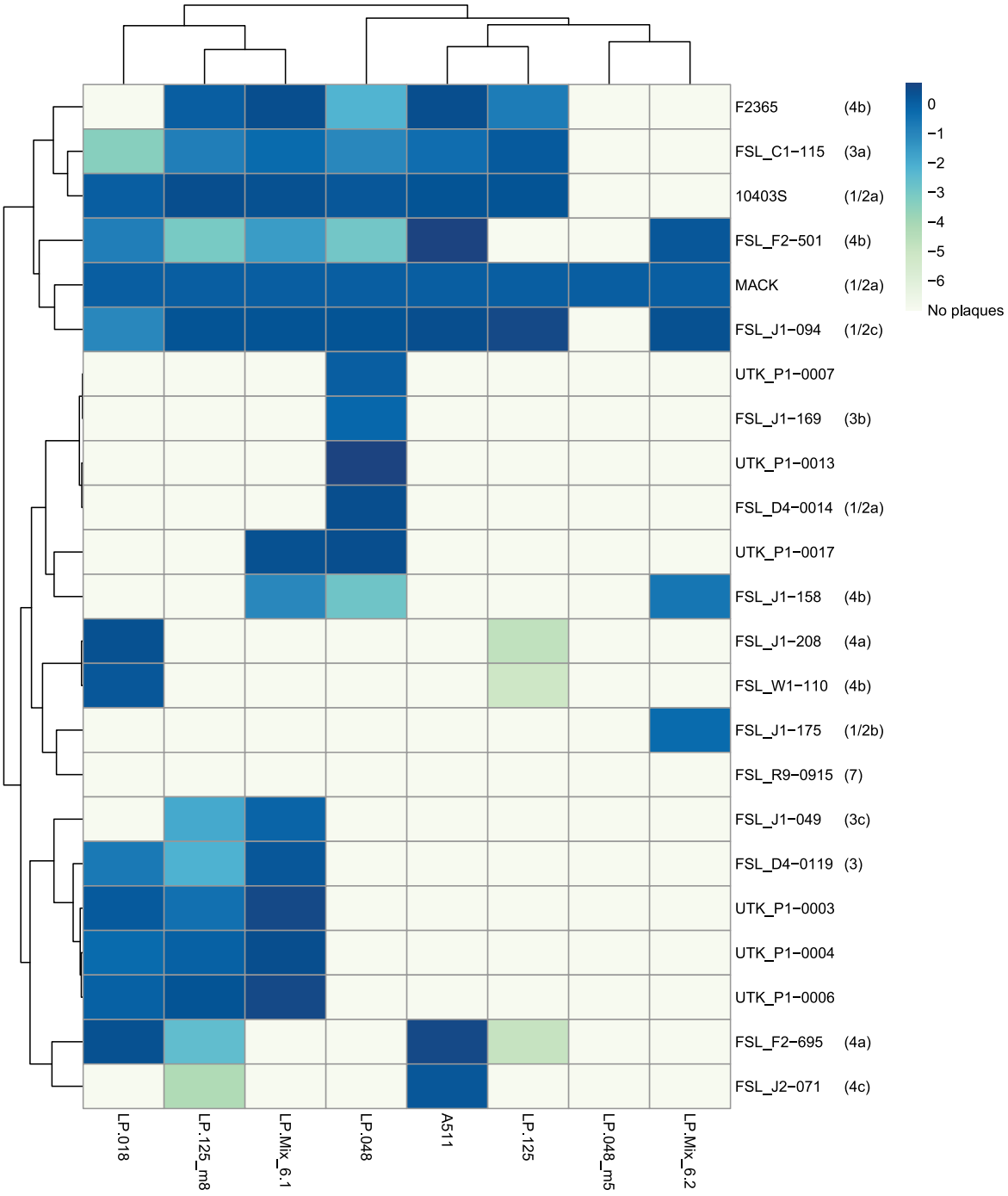

FIG 2 Host range analysis of lab-evolved phages. Heatmap shows the efficiency of plaquing of *Listeria* phages on a panel of different *L. monocytogenes* serotype strains and phage-resistant 10403S mutants ($n = 3$). Values are the log-transformed efficiencies of plaquing of phages against each strain relative to that against MACK. Parental phages LP-048 and LP-125 and wild-type 10403S were included as controls.

could infect the 10403S (Rha$^-$) mutant and serotype 3c strain J1-049 but not the 10403S (GlcNAc$^-$) mutant (Fig. 2). A loss of function mutation was identified in phage LP-048_m5, a phage that was observed to have a decreased host range; the sole mutation identified was located in a gene that codes for a tail fiber protein (Table 2).

Recombination occurred between LP-048 and LP-125. Two phages recovered from a coinfected culture of 10403S, LP-Mix_6.1, and LP-Mix_6.2 were found to be recombinants of LP-048 and LP-125 (Fig. 4). Nucleotide positions of possible regions of

TABLE 2 Mutations identified in lab-adapted phage isolates

Nucleotide position[a]	Gene homologs			Description[c]	Mutation type[d]	Nucleotide			Amino acid		Phage sample	Characteristic(s)[f]
	LP-125	LP-048	A511			LP-125	LP-048	Alt[e]	WT	Alt		
70807	LP125_108	LP048_098	gp99	Baseplate protein	MS	T	T	C	Ile	Thr	LP-Mix_6.1	Infects Rha$^-$, Infects ST 3c
83734 (76232)[b]	LP125_117	LP048_107	gp108	Short tail fiber (RBP)[c]	MS	C	C	A	Asn	Lys	LP-048_m5	Decreased host range
83744	LP125_117	LP048_107	gp108	Short tail fiber (RBP)	MS	G	G	A	Glu	Lys	LP-125_m8	Infects Rha$^-$, Infects ST 3c
83922	LP125_117	LP048_107	gp108	Short tail fiber (RBP)	MS	C	C	A	Thr	Lys	LP-Mix_6.2	Cannot infect Rha$^-$
84334	LP125_118	LP048_108	gp109	Putative assembly chaperone protein	MS	T	T	C	Val	Ala	LP-Mix_6.2	Cannot infect Rha$^-$
84428	LP125_118	LP048_108	gp109	Putative assembly chaperone protein	MS	C	T	A	Asp	Glu	LP-Mix_6.1	Infects Rha$^-$, Infects ST 3c

[a]Nucleotide position is relative to LP-125 (RefSeq accession number NC_021781).
[b]Nucleotide position in LP-048 (RefSeq accession number NC_024359) is indicated in parentheses.
[c]RBP, receptor binding protein.
[d]MS, missense.
[e]Alt, alternate allele.
[f]ST, serotype.

recombination were predicted by Recco and RDP4 (see Table S1 in the supplemental material). One region of recombination detected in LP-Mix_6.1 was located in gene *LP125_070*, which is annotated as a DUF2828 domain-containing protein. This gene is located downstream of the tRNA genes and upstream of *LP125_066*, a tRNA-splicing ligase. Phyre2 results for LP125_070 returned hits for an RNA binding protein (87% coverage and 100% confidence). Another region of recombination was predicted for both recombinant phages LP-Mix_6.1 and LP-Mix_6.2. This region (Fig. 5A) is located near genes that code for a synaptonemal complex 1 domain containing protein LP125_144 and a putative DNA binding protein, LP125_145. The junction between the two regions where recombination was detected in both phages occurs in *LP125_144*. There are two SNPs (nucleotide positions 582/789 and 585/789) that differ between the parental phages LP-048 and LP-125. Phage LP-Mix_6.1 has the LP-125 SNPs, with "G" and "T" at these positions (supported by sequencing read agreements of 1,032/1,032 and 1,001/1,003, respectively). Phage LP-Mix 6.2, however, has the LP-048 SNPs, with nucleotides of "A" and "C" at these positions (supported by sequencing read agreements of 876/882 and 846/849, respectively).

Since we observed strong evidence that there were two independent recombination events within the same four gene locus, we hypothesized that this region may be recombination hot spot in *Pecentumvirus* phages. To investigate the occurrence of recombination within this four-gene region for natural *Listeria* phages, recombination detection analysis using RDP4 was conducted on 14 *Pecentumvirus* phage genomes that are available in the NCBI database (see Table S2 in the supplemental material),

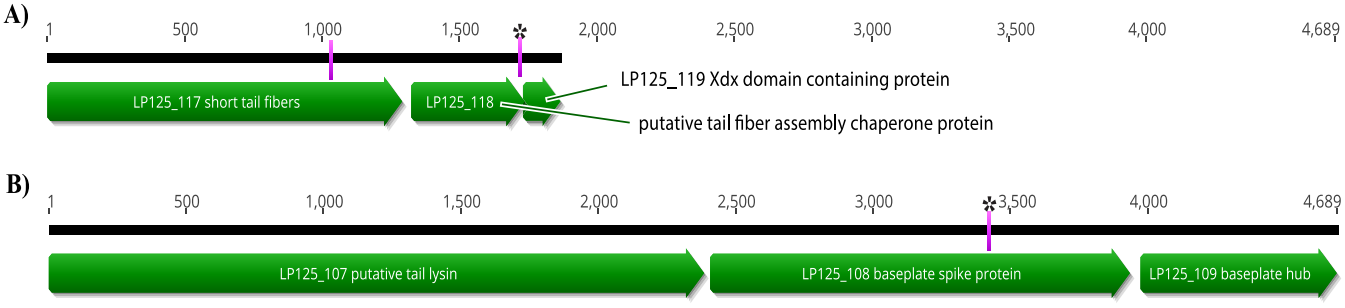

FIG 3 Mutations identified in coevolved phages able to infect broadly phage-resistant strains. Lab-evolved LP-125_m8 and LP-Mix_6.1 could infect the generally phage-resistant strains 10403S (Rha$^-$) and J1-049. Missense mutations were identified in (A) LP125_117 and LP125_118 and (B) LP125_108. Missense mutations are designated by pink marks. Mutations in recombinant phage LP-Mix 6.1 are designated by asterisks (*).

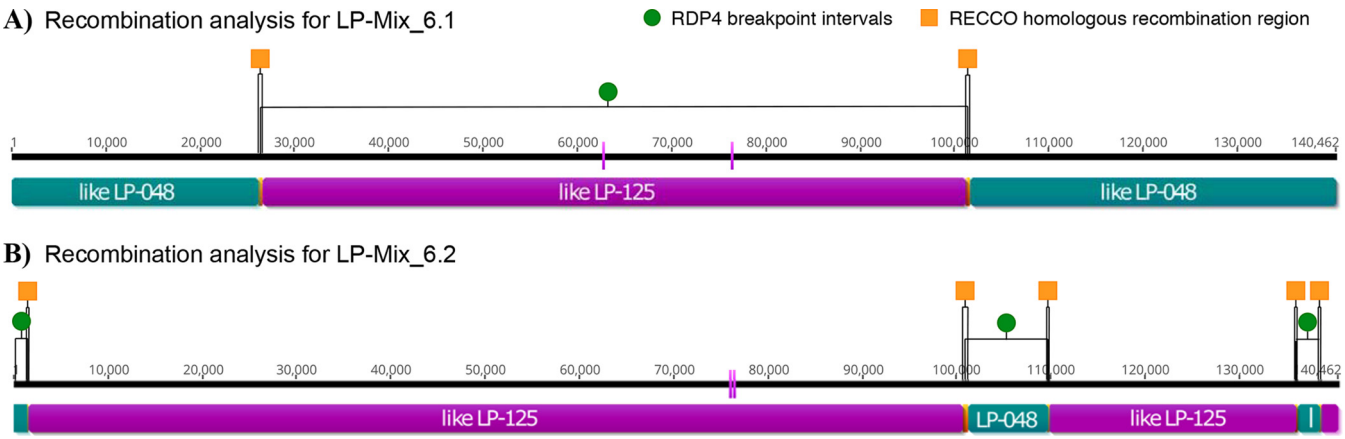

FIG 4 Recombination analysis of (A) LP-Mix_6.1 and (B) LP-Mix_6.2 compared to wild-type (WT) LP-048 and LP-125. Homologous regions of recombination were predicted by Recco (orange squares). Breakpoint intervals were predicted by RDP4 (green circles). Unique mutations different from either ancestral WT phage are designated by pink marks.

including parental phages LP-048 and LP-125. Analysis was performed on the four-gene region containing *LP125_143*, *LP125_144*, *LP125_145*, and *LP125_146*. Predicted recombination events were removed if not detected by at least five of the seven methods implemented in RDP4. Nine of the 14 wild-type *Pecentumvirus* phages were found to have a recombination event detected within or spanning the four-gene synaptonemal domain-containing region that we identified as a putative hot spot for recombination in our lab-adapted recombinant phages (Fig. 5B and Fig. S2 in the supplemental material). When the region in question was extended to analyze additional downstream genes, including *LP125_150*, which encodes a recombinase, recombination events were detected in all 14 *Pecentumvirus* phages (Table S2).

Identification of mutations in phage-resistant *L. monocytogenes* isolates selected by LP-048 and LP-125. We were also interested in identifying genes with acquired mutations that may be involved in phage resistance; therefore, whole-genome sequencing and variant analysis were performed on select coevolved bacterial mutants (Table 3). We sequenced representative isolates that showed different resistance phenotypes to LP-048 and LP-125. We were particularly interested in isolates resistant to LP-048 and susceptible to LP-125, a phenotype that was not previously well-characterized (38). Isolates resistant to both phages and those resistant to LP-125 and susceptible to LP-048 were included for comparison. Variant analysis revealed that the majority of identified mutations were located in genes related to WTA biosynthesis, consistent with previous research (38). Genes are described here using their *L. monocytogenes* EGD-e homologs (refer to Tables 3 and 4).

Four isolates showed resistance to LP-048 and susceptibility to LP-125. All four isolates each had one mutation in the same locus. One isolate (UTK P1-0001) had a four-nucleotide deletion frameshift mutation in *lmo1082* (*rmlC*; dTDP-sugar epimerase), two isolates (UTK P1-0005 and UTK P1-0006) had missense mutations in *lmo1083* (*rmlB;* dTDP-D-glucose-4,6-dehydratase), and one isolate (UTK P1-0002) had a nonsense mutation in *lmo1084* (*rmlD*; dTDP-L-Rha synthetase). These genes are part of the wall teichoic rhamnosylation locus *rmlACBD*, which is known to be associated with phage susceptibility (38, 39, 58, 59).

One isolate, UTK P1-0007, was susceptible to LP-048 and resistant to LP-125. This isolate was also resistant to both lab-evolved phages LP-125_m8 and LP-Mix_6.1. UTK P1-0007 shared the same four-nucleotide deletion frameshift mutation in *rmlC* as UTK P1-0001; however, this isolate also had two other mutations. One mutation was located upstream of *lmo1249*, annotated as a hypothetical protein. The other mutation identified was a single-nucleotide frameshift mutation (position 27/459) in *lmo1743*, a hypothetical protein, resulting in a truncated protein. Phyre2 results indicate a two-

FIG 5 Recombination analysis results from (A) a four-gene region in lab-adapted recombinant phage genomes that includes the genes *LP125_144*, encoding a synaptonemal complex 1 domain-containing protein, and *LP125_145*, encoding a putative DNA binding protein, and (B) aligned wild-type *Pecentumvirus* phage genomes with recombination detected in the same four-gene region for nine of 14 phages.

domain protein product with top results that include a secretion chaperone-like protein (56% coverage and 79.7% confidence), a transferase (51% coverage and 75.0% confidence), or a glutamine synthetase/guanido kinase (51% coverage and 69.9% confidence) (60).

TABLE 3 Mutations identified in phage-resistant 10403S mutants selected by LP-048, LP-125, or both

Nucleotide position	Gene homolog		Description	Mutation type[a]	WT	Alt[b]	Strain	No. of mutations	Phage selection	Susceptibility to:	
	10403S	EGD-e								LP-048	LP-125
1097413	LMRG_00542	lmo1080	Rhamnosyltransferase (RmlT)	NS	C	T	UTK P1-0003	3	LP-048	−	−
1098886	LMRG_00544	lmo1082	dTDP-4-dehydro-rhamnose 3,5-epimerase (RmlC)	FS	GAATA	G	UTK P1-0001	1	LP-048	−	+
1098886	LMRG_00544	lmo1082	dTDP-4-dehydro-rhamnose 3,5-epimerase (RmlC)	FS	GAATA	G	UTK P1-0007	3	LP-048	+	−
1099454	LMRG_00545	lmo1083	dTDP-glucose 4,6-dehydratase (RmlB)	MS	C	T	UTK P1-0006	1	LP-048	−	+
1099515	LMRG_00545	lmo1083	dTDP-glucose 4,6-dehydratase (RmlB)	MS	A	G	UTK P1-0004	2	LP-048	−	−
1099619	LMRG_00545	lmo1083	dTDP-glucose 4,6-dehydratase (RmlB)	MS	G	A	UTK P1-0005	1	LP-048	−	+
1099958	LMRG_00546	lmo1084	dTDP-4-dehydro-rhamnose reductase (RmlD)	NS	T	G	UTK P1-0002	1	LP-048	−	+
1235730	LMRG_00695	lmo1249	Hypothetical protein	Intergenic	T	TA	UTK P1-0007	3	LP-048	+	−
1493524	LMRG_01466	lmo1504	Alanyl tRNA synthetase (AlaS)	MS	C	T	UTK P1-0003	3	LP-048	−	−
1770042	LMRG_02528	lmo1743	Hypothetical protein	FS	CT	C	UTK P1-0007	3	LP-048	+	−
1890882	LMRG_02979	lmo1859	Peptide-methionine (R)-S-oxide reductase (MsrB)	MS	C	T	UTK P1-0004	2	LP-048	−	−
2573008	LMRG_01710	lmo2537	UDP-*N*-acetyl-glucosamine 2-epimerase	Intergenic	C	T	UTK P1-0003	3	LP-048	−	−

[a]NS, nonsense; MS, missense; FS, frameshift; the first nucleotide is maintained from WT to Alt in frameshift mutations.
[b]Alt, alternate allele.

TABLE 4 Mutations identified in phage-resistant 10403S mutants selected by LP-Mix_6.1 or LP-Mix_6.2

Nucleotide position	Gene homologs		Description	Mutation type[a]	WT	Alt[b]	Strain	No. of mutations	Phage selection	Susceptibility	
	10403S	EGD-e								LP-048	LP-125
309662	LMRG_02583	lmo0288	Cell wall metabolism sensor histidine kinase (YycG or WalK)	MS	A	G	UTK P1-0017	2	LP-Mix_6.2	+	−
396639	LMRG_00062	lmo0370	Alkylphosphonate utilization operon protein	FS	T	TA	UTK P1-0009	4	LP-Mix_6.1	+	−
975464	LMRG_02059	lmo0960	U32 family peptidase	SN	C	T	UTK P1-0009	4	LP-Mix_6.1	+	−
1021645	LMRG_02110	lmo1010	LysR family transcriptional regulator	SN	G	A	UTK P1-0012	3	LP-Mix_6.2	+	−
1093551	LMRG_00541	lmo1079	Glycosyltransferase (YfhO)	FS	G	GA	UTK P1-0009	4	LP-Mix_6.1	+	−
1093565	LMRG_00541	lmo1079	Glycosyltransferase (YfhO)	Conservative in-frame 48-nucleotide deletion		G	UTK P1-0011	1	LP-Mix_6.1	+	−
1093907	LMRG_00541	lmo1079	Glycosyltransferase (YfhO)	FS	TA	T	UTK P1-0018	2	LP-Mix_6.2	+	−
1094356	LMRG_00541	lmo1079	Glycosyltransferase (YfhO)	FS	TA	T	UTK P1-0012	3	LP-Mix_6.1	+	−
1094918	LMRG_00541	lmo1079	Glycosyltransferase (YfhO)	NS	C	T	UTK P1-0010	1	LP-Mix_6.1	+	−
1096556	LMRG_00542	lmo1080	Rhamnosyltransferase (RmlT)	FS	TA	T	UTK P1-0015	3	LP-Mix_6.2	−	−
1097397	LMRG_00542	lmo1080	Rhamnosyltransferase (RmlT)	FS	TA	T	UTK P1-0014	2	LP-Mix_6.2	−	−
1282575	LMRG_00745	lmo1295	RNA chaperone (Hfq)	FS	CT	C	UTK P1-0009	4	LP-Mix_6.1	+	−
1878265	LMRG_00992	lmo1845	Xanthine-uracil permease	MS	C	T	UTK P1-0016	2	LP-Mix_6.2	+	−
2322483	LMRG_01556	NA	*N*-acetyltransferase	FS	CT	C	UTK P1-0015	3	LP-Mix_6.2	−	−
2442088	LMRG_01832	lmo2416	Threonyl tRNA synthetase (ThrS)	MS	G	A	UTK P1-0014	2	LP-Mix_6.2	−	−
2515523	LMRG_01762	lmo2486	DUF4097 family beta strand repeat protein	FS	AT	A	UTK P1-0012	3	LP-Mix_6.1	+	−
2515523	LMRG_01762	lmo2486	DUF4097 family beta strand repeat protein	FS	AT	A	UTK P1-0013	2	LP-Mix_6.1	+	−
2515523	LMRG_01762	lmo2486	DUF4097 family beta strand repeat protein	FS	AT	A	UTK P1-0015	3	LP-Mix_6.2	−	−
2515523	LMRG_01762	lmo2486	DUF4097 family beta strand repeat protein	FS	AT	A	UTK P1-0018	2	LP-Mix_6.2	+	−
2579547	LMRG_01698	lmo2549	Putative flippase (GtcA)	NS	C	T	UTK P1-0017	2	LP-Mix_6.2	+	−
2580198	LMRG_01697	lmo2550	Glycosyltransferase (CsbB)	MS	C	T	UTK P1-0016	2	LP-Mix_6.2	+	−
2580621	LMRG_01697	lmo2550	Glycosyltransferase (CsbB)	NS	C	A	UTK P1-0013	2	LP-Mix_6.1	+	−

[a]NS, nonsense; MS, missense; FS, frameshift (the first nucleotide is maintained from WT to Alt in frameshift mutations); SN, synonymous.
[b]Alt, alternate allele.

Identification of mutations in phage-resistant *L. monocytogenes* isolates selected by LP-Mix_6.1 and LP-Mix_6.2. To further evaluate mutations involved in phage resistance, lab-evolved recombinant phages LP-Mix_6.1 and LP-Mix_6.2 were used to select for five phage-resistant 10403S isolates each. Whole-genome sequencing and variant analysis was then performed on these isolates (Table 4). Variant analysis revealed that all 10 isolates contained at least one of the following mutations in genes known to be involved in WTA biosynthesis (61) and phage resistance (38): *lmo1079* (*yfhO*; glycosyltransferase), *rmlT*, *lmo2550* (*csbB*; glycosyltransferase), and/or *lmo2549* (*gtcA*; putative flippase).

Four of the 10 individual isolates had the same frameshift mutation in *lmo2486*, a DUF4097 family beta strand repeat protein. This mutation was a single-nucleotide deletion of the last thymine in an 8-nucleotide homopolymeric thymine [poly(T)] tract at the 5′ end of a coding sequence. This would introduce an early stop (position 12/1,229), resulting in a truncated protein (see Fig. S3 in the supplemental material). BLAST results show that this gene is conserved across *L. monocytogenes* strains and is annotated as a phage shock protein C (PspC) domain-containing protein (62). Phyre2 prediction analysis returned results for cell adhesion proteins (44 to 67% coverage; 98.6 to 99.4% confidence) (60). One isolate that contained the frameshift mutation in *lmo2486*, UTK P1-0013, was resistant to lab-evolved phages LP-125_m8 and LP-Mix_6.1 (Table 4 and Fig. 2). This isolate also had a nonsense mutation in the glycosyltransferase *csbB* (nucleotide position 226/948).

Another isolate, UTK P1-0017, showed resistance to LP-Mix_6.1 but remained susceptible to LP-125_m8. This strain was found to have two mutations. One mutation was in *lmo0288* (*walK* or *yycG*; cell wall metabolism sense histidine kinase) (63). This was a missense mutation at position 1,277/1,833. WalK histidine kinases are also known to be involved in transcription regulation of autolysins and their inhibitors (64). The second mutation was a nonsense mutation in *gtcA* (nucleotide position 356/438).

DISCUSSION

In this study, we observe the *in vitro* coevolution between *Pecentumvirus* phages and *L. monocytogenes*. We show that (i) the process of coevolution can be utilized *in vitro* for the isolation of *Listeria* phages with improved host ranges, (ii) coevolution drives genetic diversity of both *Listeria* phages and their host, and (iii) recombination occurs *in vitro* during coinfection with similar phages and may be a common driver of genetic variation in *Pecentumvirus* phages.

Coevolution can be utilized for isolation of phages with improved host ranges. We were able to isolate lab-evolved phages that can infect strains that are broadly phage resistant. A typical strategy to overcome phage-resistant bacterial strains is to isolate novel phages from environmental sources; however, this can be a laborious process (65). Few *Listeria* phages isolated from the environment have previously been observed to infect *L. monocytogenes* strains deficient for rhamnose in their WTAs. Although a recent study found one *Homburgvirus*, LP-018, that could form plaques against the 10403S (Rha$^-$) strain (42), this phage did not have favorable binding efficiency, so may not be suitable for use as in phage biocontrol (43). The serotype 3c strain J1-049 was previously found to be insensitive to all known wild-type phages isolated from environmental sources, including one study that evaluated the host range of 114 phages (40, 66). In this study, we procured two lab-evolved phages that were able to infect both the rhamnose-deficient strain and the serotype 3c strain J1-049. Lytic bacteriophages are of increasing interest in controlling bacterial pathogens in food production, as well as for use in phage therapy to treat bacterial infections. Methods have been implemented to adapt therapeutic phages to overcome resistance in other foodborne pathogen phage-host systems, such as with antibacterial-resistant *Staphylococcus aureus* strains over six rounds of laboratory coevolution (67) and *Pseudomonas aeruginosa* strains over 30 rounds (47). Additionally, laboratory evolution was shown to improve the lytic ability of a phage infecting *Escherichia coli* O157:H7, which was ascribed to the acquisition of point mutations in tail fiber genes (68). One

study did not find coevolution to improve the host range of a phage that targets *Salmonella enterica* subsp. *enterica* serovar Enteritidis under the laboratory conditions tested (69). Taken together, these results show that phages isolated from natural environment can be lab evolved to overcome phage-resistant strains of target bacteria and can specifically be used to improve the host range of *Listeria* phages with the ability to infect a serotype 3c strain.

Coevolution drives genetic diversity in the *Listeria* phage-host system. Our results show that *in vitro* coevolution is a powerful approach to study genetic variation in the *Listeria* phage-host system. We saw bacterial regrowth of phage-resistant subpopulations after 24 h in both single phage-infected and coinfected 10403S cultures and found that different phage infection conditions select for different populations of phage-resistant *L. monocytogenes*, which also showed different regrowth times. The consistency observed in earlier regrowth times of LP-048-infected cultures may occur because of the sole binding requirement of LP-048, rhamnose in WTAs (38). Loss of this feature through point mutations in genes involved in WTA rhamnosylation pathways confer phage resistance (38, 39). Previous work showed that mutations in 10403S isolates that lack rhamnose in WTAs confer resistance to both LP-048 and LP-125 (38), which is consistent with our observation that LP-048 selects for a greater population of bacterial isolates resistant to both parental phages. In this study, we observed coevolution at 30°C; however, it has been previously shown that that *Listeria* phage binding and efficiency is influenced by temperature (54, 70). Therefore, it is possible that dynamics of coevolution may be affected by different growth temperatures.

As expected, many of the mutations that were identified in phage-resistant bacterial isolates were found in genes involved in glycosylation and rhamnosylation of WTAs. However, we also identified mutations in several genes that have not yet been linked to phage-host interactions and that generally have unknown functions in *L. monocytogenes*. Surprisingly, one deletion mutation was found to have been selected in four independent cocultures containing *L. monocytogenes* 10403S and either LP-Mix_6.1 or LP-Mix_6.2. As the bacterial inoculant for each coculture derived from a distinct individual colony, the mutations would have arisen independently. This deletion mutation was a one-nucleotide mutation in an eight-nucleotide-long 5′ poly(T) tract. This is consistent with the mechanism of phase variation known as slipped strand mispairing, which is characterized by the addition or deletion of nucleotides in simple repetitive sequences (71). Phase variation by slipped strand mispairing is a known mechanism of gene regulation in *L. monocytogenes*, such as in the *inlA* gene (72). Denes et al. previously identified evidence for phase variation by slipped strand mispairing in two loci involved in phage resistance in independent isolates that were sequenced (38). Although we did not observe a reversion to the wild-type state, our findings support the hypothesis that phase variation by slipped strand mispairing may play an important role in phage resistance in *L. monocytogenes* and is a direction for future study.

The genetic mutations identified in lab-evolved phages were located in genes that code for tail fiber proteins and are likely responsible for the improved host ranges observed. These findings are significant in regard to the lab-evolved strains adapting to infect the serotype 3c strain and the WTA rhamnose-deficient strain. The identification of mutations significant for improved host ranges through *in vitro* coevolution coupled with whole-genome sequencing may be useful to others in the field, particularly to inform genetic engineering strategies that target receptor binding proteins (73, 74). Although utilizing *in vitro* coevolution for modification of phages with desired characteristics is less precise than genetic engineering approaches, it may have advantages for certain applications, particularly in food production applications, where views of the safety and benefits of genetically modified organisms are still evolving (75). We also observed that mutations can occur in model phage strains that are frequently propagated for use in laboratory experiments. For example, we identified that there were three mutations in the A511 strain used in this study compared to the genome that was published in 2008. Accumulation of mutations over time is likely a common occurrence;

therefore, we recommend the routine resequencing of model phage strains to help compare current results with previously published work.

Recombination between *Listeria* phages occurs during coinfection. We showed that recombination can occur *in vitro* between similar phages during coinfection, and provide evidence that recombination occurred twice, independently, within the same four-gene region. These genes may serve functions similar to those of genes encoding other single-stranded DNA-annealing proteins and exonuclease (SSAPs/Exo) phage recombination systems (76–79). Generally, recombination can occur between homologous regions as short as 35 to 50 bp in length. These protein pairs usually consist of a 5′ to 3′ exonuclease, which binds to dsDNA ends and digests the 5′ strand to form a long 3′ overhang, and a protein that facilitates the annealing of single-stranded DNA to another region of homology (76). We also provide evidence that similar recombination events we observed under laboratory conditions have occurred in *Pecentumvirus* phages isolated from natural environments. Other *Listeria* phages, mainly temperate phages, have been described to have mosaic genomes shaped by recombination events with related phages (80). Although evidence of recombination in the *Pecentumvirus* genus has not been previously described, diversification of phage genomes due to recombination and substitutions has been confirmed to occur in a study of lytic *Lactococcal* phages (81). Recombination under laboratory conditions has been described as a significant contributor for the improvement of *Pseudomonas* phage host ranges (47). In our study, recombination did not seem to be the driving force for the improved host ranges observed, as similar improved phages were derived from single-infection cultures, nevertheless, recombination under short-term laboratory coevolution conditions has been demonstrated to be an additional tool to produce improved, lab-evolved phages with novel characteristics.

Conclusion. This study demonstrates how *in vitro* evolution of phages can be utilized to expand the host range of lytic phages and contribute to the improvement and long-term efficacy of phage-based control of *L. monocytogenes*. Genetic information on tail fiber genes of phages that can infect broadly phage-resistant *L. monocytogenes* strains can inform strategies for genetic engineering of phages. Identification of regions where recombination is likely to occur in *Pecentumvirus* phages, and confirmation that recombination between phages can be selected for *in vitro*, may provide a means to intentionally cross closely related phages to select for novel phenotypes with application potential. Additional knowledge of phage resistance gained here can be used to identify potential combinations of phages that may reduce the emergence of phage resistance. The findings of this study may also be applied to other phage-host systems to potentially improve applications in biocontrol and phage therapy to overcome and prevent the emergence of phage-resistant strains.

MATERIALS AND METHODS

Bacterial strains. All bacterial strains used in this study are listed in Table 1. The bacterial strain used for the coevolution experiment was *L. monocytogenes* 10403S. *L. monocytogenes* strains used in efficiency of plaquing assays include a panel representing various serotypes, mutant strains of 10403S that lack either rhamnose (Rha^-) or *N*-acetylglucosamine ($GlcNAc^-$) in WTAs, as well as strains sourced from this study. All strains were stored at −80°C in brain heart infusion broth (BHI; Becton, Dickinson, Sparks, MD) with 15% (wt/vol) glycerol, then prepared for use by incubation at 30°C overnight on 1.5% (wt/vol) BHI agar plates. Single colonies were used to prepare overnight cultures in 5 ml of BHI broth (incubated for 16 h ± 2 h at 30°C with shaking at 160 rpm).

Bacteriophage strains. All phage strains used in this study are listed in Table 5. The *L. monocytogenes* strain MACK was used as the propagation host strain. Titers of all phages were determined on LB-morpholinepropanesulfonic acid (MOPS) agar (1.5% wt/vol) plates supplemented with 0.1% (wt/vol) glucose and 1 mM $MgCl_2$ and $CaCl_2$ with a top agar overlay (0.7% wt/vol).

Coevolution of 10403S with LP-048 and LP-125. To investigate the coevolution dynamics of *L. monocytogenes* and *Listeria* phages, overnight cultures of 10403S were prepared in 5 ml LB-MOPS medium and incubated at 30°C overnight (16± 2 h) with shaking (160 rpm). Overnight cultures were used to inoculate 50 ml (1:50 dilution) of LB-MOPS medium supplemented with $MgCl_2$, $CaCl_2$, and glucose (final concentrations of 0.1 mM, 0.1 mM, and 6 mM, respectively). Cultures were grown to an OD_{600} of 0.05 and divided into four 5-ml aliquots. Each aliquot was infected with either LP-048 or LP-125 or was coinfected with both LP-048 and LP-125 at a multiplicity of infection (MOI) of 1; SM buffer was added to the 10403S control group. Nine biological replicates were conducted. Infected cultures and

TABLE 5 *Listeria* phages used in this study

Listeria phage	Taxon	Genome size (kbp)	Description	Reference(s) or source
LP-048	*Pecentumvirus*	135	Can infect GlcNAc$^-$, cannot infect Rha$^-$	40, 51
LP-125	*Pecentumvirus*	135	Cannot infect GlcNAc$^-$, cannot infect Rha$^-$	40, 51
LP-048_m5	LP-048 derived	135	Decreased host range	This study
LP-125_m8	LP-125 derived	135	Cannot infect GlcNAc$^-$, infects Rha$^-$	This study
LP-Mix_6.1	Recombinant	135	Cannot infect GlcNAc$^-$, infects Rha$^-$	This study
LP-Mix_6.2	Recombinant	135	Cannot infect GlcNAc$^-$, cannot infect Rha$^-$	This study
LP-018	*Homburgvirus*	65	Broad-host-range phage	40, 43
A511	*Pecentumvirus*	135	Model phage strain/commercially relevant	33

controls were monitored by spectrophotometry for 55 to 60 h at a wavelength of 600 nm (Fig. 1). Samples were streaked onto BHI agar to isolate bacterial survivors from phage-infected cultures at 6 to 8 h postinfection and at early stages of bacterial regrowth between OD_{600} readings of 0.05 to 0.1 (time differed between replicates and infection conditions) and incubated overnight at 30°C. Samples were taken at 55 to 60 h and filtered with 0.2-μm surfactant-free cellulose acetate membrane filters (VWR, Radnor, PA) to obtain a lysate of coevolved phages. Phages of interest were triple plaque purified as previously described (40) and amplified to high titers for further phenotypic characterization and DNA extraction.

Phenotypic characterization of coevolved 10403S. Coevolved bacterial and phage isolates were assessed for changes in phage resistance and improved host range, respectively. Bacterial survivors were tested for phage resistance against parental phages LP-048 and LP-125. For initial assessment of phage resistance, streak-spot assays were performed. Ten colonies from each sample taken from phage-infected cultures were streaked out on a BHI plate, then 5-μl spots of phages LP-048 and LP-125 were applied from working stocks at a concentration of 1×10^8 PFU/ml; plates were then incubated at 30°C overnight. *L. monocytogenes* 10403S wild-type (WT) and bacterial isolates from the control group (no phage added) were included as controls.

Phage selection of 10403S with lab-evolved LP-Mix_6.1 and LP-Mix_6.2 and phenotypic characterization. To obtain bacterial isolates resistant to LP_Mix_6.1 and LP-Mix_6.2, 10403S cultures were infected with either LP_Mix_6.1 or LP-Mix_6.2 as described above. At approximately 20 h postinfection or before regrowth reached an OD_{600} of 0.05 (whichever occurred first), samples were plated for bacterial survivors as described above. Isolated colonies were then phenotypically assessed for phage resistance against LP-048, LP-125, LP_Mix_6.1, and LP-Mix_6.2 using the streak-spot assay as described above, followed by initial EOP assays.

Efficiency of plaquing assays. Efficiency of plaquing (EOP) assays were used to assess lab-evolved phages for activity and plaquing against genetically confirmed phage-resistant mutants FSL D4-0014 (WTA *N*-acetylglucosamine deficient; GlcNAc$^-$) and FSL D4-0119 (WTA rhamnose deficient; Rha$^-$) (38), a panel of *L. monocytogenes* strains that represent different serotypes, and select coevolved phage-resistant bacterial isolates of interest from this study (Table 1). EOPs were performed as described by Trudelle et al. (42). The supplemented LB-MOPS underlay agar was prepared in 6×6 grid square plates (Simport Scientific, Beloeil, QC, Canada). Bacterial lawns were prepared by adding 40 μl of overnight culture to 4 ml of molten (56°C) 0.7% (wt/vol) supplemented LB-MOPS overlay agar, briefly vortexing, and pouring over the underlay; lawns were allowed to solidify for 20 to 30 min. Working stocks were prepared from purified phage samples at a concentration of 1×10^8 PFU/ml and then were serially diluted in 0.1 M phosphate buffer solution (PBS) to a 10^{-5} dilution. Aliquots (10 μl) of the working stock and dilutions were spotted onto the bacterial lawns. Spots were allowed to dry, and plates were incubated overnight at 25°C. Phage activity was recorded as the highest dilution at which activity was observed against a given strain relative to activity against the propagation host strain, MACK, compared to a PBS control. Phage titers were recorded as the highest dilution with countable plaques against a given strain relative to the number of plaques against MACK. Clustered heatmaps were generated with *pheatmap* using R (82).

DNA extraction and genomic analysis. Coevolved bacterial and phage isolates with noteworthy phenotypic differences were selected for genomic DNA extraction, sequencing, and analysis. DNA extractions for bacterial isolates were performed using Qiagen DNA minikits (Hilden, Germany) according to the manufacturer's instructions (38). Phage DNA was extracted from purified high-titer phage stocks using the phenol-chloroform method (83) with modifications. Phage samples were prepared with 2 mM $CaCl_2$, 5 μg/ml DNase I (Promega, Madison, WI), and 30 μg/ml RNase A (Sigma-Aldrich, Darmstadt, Germany), then incubated at room temperature for 30 min. Samples were then incubated for 10 min at 65°C, followed by the addition of proteinase K (2 mg/ml). Phase separation was completed using phase lock gel tubes (QuantaBio, Beverly, MA), and DNA was resuspended in 10 mM Tris buffer (pH 8), a modification from the original protocol. DNA concentration and quality were assessed using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Sequencing libraries were prepared using Nextera XT kits (Illumina, San Diego, CA) and/or native barcoding expansion and ligation kits (catalog no. NBD104 and SQK-LSK109; Oxford Nanopore, Oxford, UK) following the manufacturer's instructions. Long-read sequencing was performed in-house on an Oxford Nanopore Technology MinION platform. Illumina library preparation and sequencing were performed by the University of Tennessee Knoxville Genomics Core (MiSeq v3, 300- bp paired-end, 275 cycles) (Knoxville, TN) and by the Microbial Genome Sequencing Center (NextSeq 550, 150-bp paired-end) (MiGS; Pittsburgh, PA).

Raw Illumina reads were trimmed using Trimmomatic v0.35 (ILLUMINACLIP:NexteraPE-PE.fa:2:30:10 LEADING:3 TRAILING:3 SLIDINGWINDOW:4:15 MINLEN:36) (84) and checked for quality using FastQC v0.11.7 (85). Genome assembly was performed with either SPAdes v3.12.0 (default settings; "careful" option) using trimmed Illumina reads or through a hybrid assembly method with UniCycler v0.4.8-beta (86) using both Illumina and Nanopore reads. Assembly statistics were generated using BBMap v38.08 (87), SAMtools v0.1.8 (88), and QUAST v4.6.3 (89). Phage assemblies were reoriented such that direct terminal repeat regions were located at both ends of the genome (90) using Geneious Prime 2019.1.1 (91) as previously described (92).

For variant analysis of bacterial and phage mutant isolates, trimmed reads were analyzed in McCortex v.0.0.3 (93). Bacterial isolates were run using the 10403S RefSeq assembly (RefSeq identifier 376088; RefSeq assembly accession number GCF_999168695.2) as the reference with joint calling and links and a k-mer size of 57. Phage isolates from LP-048-infected cultures were run using the LP-048 RefSeq assembly (GenBank accession number NC_024359) as the reference with joint calling and links and a k-mer size of 61; isolates from LP-125-infected cultures were run with the LP-125 RefSeq assembly (accession number NC_021781) as the reference joint calling and links with a k-mer size of 91. Isolates from coinfected cultures (recombinant phages) were run using each of the parental phages independently as references, and outputs were compared to identify variants. Additionally, parental isolate reads for both bacterial and phage samples were included for comparison (10403S, LP-048, and LP-125). Variant call format (VCF) output files were annotated using SnpEff v4.3t (94). Pairwise alignments of adapted phages and parental phages were created using the MUSCLE v3.8.425 (95) algorithm in Geneious Prime v2019.1.1 (91) to verify identified genetic variants. Amino acid sequences of specific genes were submitted to Phyre2 with a modeling mode of "normal" (60). Sequences for genes of unknown function were run in NCBI-BLASTP with default settings using the nonredundant protein sequences database (62).

Recco v1.0 (96) and RDP4 v4.97-beta (97) were used to predict recombination sites. RDP4 was run with general options set to default with linear sequences and a *P* value cutoff of 0.05 using RDP, GENECONV, Chimaera, MaxChi, Bootscan, SiScan, and 3Seq. RDP4 was run as query versus reference sequence for analysis of lab-adapted recombinant with parental phages. RDP4 was run as full exploratory recombination scan for analysis of 14 *Pecentumvirus* phages (32, 33, 51, 92, 98–101) (see Table S2 in the supplemental material). RefSeq assembly genomes of *Pecentumvirus* phages were downloaded from NCBI. The four-gene recombination region was mapped to each *Pecentumvirus* phage genome, and this locus was aligned in Geneious Prime (4,570-bp aligned sequences) and used for recombination analysis in RDP4. Recombination events that were not detected by five of the seven methods implemented in RDP4 were eliminated.

Data availability. Raw read sequencing data for 10403S isolates and lab-evolved phages are available under NCBI BioProject accession numbers PRJNA544490 and PRJNA638358, respectively. Assemblies for recombinant phages LP-Mix_6.1 and LP-Mix_6.2 are available under GenBank accession numbers MT668623 and MT668624, respectively. Sequencing statistics for raw reads and assemblies are available in Tables S3 and S4 in the supplemental material.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 1.1 MB.

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