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Cross-resistance to phage infection in *Listeria monocytogenes* serotype 1/2a mutants

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ABSTRACT:

Bacteriophage-based biocontrols are one of several tools available to control *Listeria monocytogenes* in food and food processing environments. The objective of this study was to determine if phage-resistance that has been characterized with a select few *Listeria* phages would also confer resistance to a diverse collection of over 100 other *Listeria* phages. We show that some mutations that are likely to emerge in bacteriophage-treated populations of serotype 1/2a *L. monocytogenes* can lead to cross-resistance against almost all types of characterized *Listeria* phages. Out of the 120 phages that showed activity against the parental strain, only one could form visible plaques on the mutant strain of *L. monocytogenes* lacking rhamnose in its wall teichoic acids. An additional two phages showed signs of lytic activity against this mutant strain; although no visible plaques were observed. The findings presented here are consistent with other studies showing mutations conferring phage resistance through loss of rhamnose likely pose the greatest challenge for phage-based biocontrol in serotype 1/2a strains.

1. Introduction

The Gram-positive foodborne pathogen *Listeria monocytogenes* is the causative agent of listeriosis. Listeriosis can cause serious health problems for susceptible populations, including septicemia, bacteremia, meningitis, and meningoencephalitis (Vázquez-Boland et al., 2001). Illness due to listeriosis leads to a high rate of hospitalizations (approx. 91%) and deaths (approx. 16%). Listeriosis in humans is contracted almost exclusively through consumption of contaminated foods (Scallan et al., 2011). The largest L. *monocytogenes* outbreak to-date occurred in South Africa and was caused by contamination of polony, a ready-to-eat meat, leading to over 1000 illnesses and 200 deaths from 2017 to 2018 (Salama et al., 2018; Allam et al., 2018). *L. monocytogenes* can be particularly problematic due to its abilities to replicate at refrigeration temperatures (Saldivar et al., 2018) and to persist for years in food processing facilities (Møretrø and Langsrud, 2004; Orsi et al., 2008). *L. monocytogenes* is categorized into at least thirteen different serotypes. Serotype 1/2a, 1/2b, and 4b are the most commonly associated with human illness (Orsi et al., 2011). From 1998 to 2008, serotype 1/2a caused 40% of the confirmed *L. monocytogenes* outbreaks in the U.S. (Cartwright et al., 2013). The largest outbreak of *L. monocytogenes* in the U.S. occurred in 2011 and was caused by cantaloupe contaminated with serotype 1/2a and 1/2b *L. monocytogenes*. Illnesses associated with the outbreak occurred in 28 states and were responsible for 143 hospitalizations and 33 deaths (McCollum et al., 2013).

Bacteriophages (or "phages"), viruses that specifically target bacteria, have been approved for use in the U.S. since 2006 for control of *L. monocytogenes* in foods and on food processing surfaces (Bren, 2007). There are currently two commercial *Listeria* phage products approved for food safety applications, and, more recently, products have been approved to target *Salmonella enterica*, *Escherichia coli, and Shigella* spp. (Moye et al., 2018). Previous work has shown that phage-resistance is consistently selected for in *L. monocytogenes* populations infected with *Listeria* phages (Denes et al., 2015). A screen of *L. monocytogenes* strains that were isolated from European processing facilities from 1987 to 2012 found that phage-resistant *L. monocytogenes* was only found after those processing facilities had used or experimentally tested a *Listeria* phage product; strains that were isolated prior to the intentional introduction of phage were all found to be sensitive (Fister et al., 2016). In L. *monocytogenes* serotype 1/2a strains 10403S and EGDe, mutations conferring phage-resistance were consistently found in genes that affect known *Listeria* phage binding receptors: terminal N-acetylglucosamine (GlcNAc) and rhamnose (Rha) decorations of the wall teichoic acids (WTA) (Denes et al., 2015; Eugster et al., 2015). Surprisingly, no other binding receptors have been conclusively shown to be used by *Listeria* phages that are able to infect *L. monocytogenes* serotype 1/2a strains.

Abbreviations: SM, Saline Magnesium; BHI, Brain Heart Infusion; LB, Lysogeny Broth; PBS, Phosphate Buffered Saline; WTA, Wall Teichoic Acids; GlcNAc, N-acetyl glucosamine; Rha, Rhamnose

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Here, we measure activity of a diverse collection of 120 *Listeria* phages, representing four of the five known orthoclusters, against three mutant strains of *L. monocytogenes* 10403S (serotype 1/2a). *Listeria* phages have been shown to form five distinct genomic clusters, referred to as orthoclusters. Each orthocluster has unique morphological characteristics; four contain siphoviruses and one contains myoviruses (Denes et al., 2014; Casey et al., 2015). None of the phages from orthocluster IV that were available to us showed activity against our serotype 1/2a strains, so they were not included in this study. The lysis profiles of a majority of the phages we tested were previously described (Vongkamjan et al., 2012). The three mutant strains represent three distinct phenotypes of phage resistance. Two of these mutants were previously sequenced and characterized (Denes et al., 2015) and the third is characterized here. We aimed to determine if these characterized phage-resistant mutants were broadly resistant to *Listeria* phages and if any phages could overcome these common resistance types.

2. Materials & methods

2.1. Growth of bacterial strains

Propagation host strains 10403S and 10403S-derived mutants (Table 1) were prepared from stocks stored at −80 °C in Brain Heart Infusion (BHI; Becton Dickinson, Sparks, MD) broth with 15% (wt/vol) glycerol and incubated at 30 °C on 1.5% (wt/vol) BHI agar plates. Overnight cultures were prepared by inoculating BHI broth with a single colony from a streak plate, and then incubated for 16 ± 2 h at 30 °C shaking at 160 RPM. Bacterial lawns were prepared using an agar overlay method as described previously (Tokman et al., 2016) with modifications. The agar underlay used here was prepared in square 6 × 6 grid plates (Simport Scientific, Beloeil, QC, Canada). Forty μL of overnight culture was aliquoted into 4–4.5 mL of 0.7% (wt/vol) modified lysogeny broth (LB) morpholino-propane sulfonic acid (MOPS) overlay agar supplemented with 0.1% (wt/vol) glucose and 1 mM each $MgCl_2$ and $CaCl_2$, after equilibrating to 56 °C. The mixture was vortexed briefly, poured onto the agar underlay and allowed to solidify for 20–30 min. UTK P1-0001 was isolated from a 10403S culture infected by LP-048 under previously described conditions (Denes et al., 2015).

2.2. Phage amplification

Phage stocks used for the experiment (Supplemental Table 1) were first amplified to a titer of at least 1×10^7 pfu/mL using the plate lysate method. All phages used in this study, except for A511, were previously isolated from NY dairy farms (Vongkamjan et al., 2012). Plates were prepared in duplicate using an agar overlay method as described previously (Vongkamjan et al., 2012) with modifications. Thirty μL of overnight culture and 100 μL of the phage dilution in saline magnesium (SM) buffer with gelatin (Fisher, Fair Lawn, NJ) was aliquoted into 3–3.5 mL of modified LB MOPS overlay agar after equilibrating to 56 °C. The mixture was vortexed briefly, poured onto the agar underlay and allowed to solidify for 20–30 min. Plates were then incubated for 18–24 h at 25 °C. Five mL of sterile SM buffer with gelatin was aliquoted onto each plate with confluent lysis and allowed to sit for 1–2 h. The overlay agar was then carefully broken into pieces and scraped to the side of the plate using a sterile cell scraper (VWR, Radnor, PA). The phage-containing buffer was then siphoned off the plate using a serological pipette, centrifuged at 3000 RPM for 15 min at 4 °C to remove debris, and then filter sterilized into an amber vial using a 0.20 μm-pore size surfactant-free cellulose acetate (SFCA) syringe filter (Corning, Incorporated, Corning, NY). Phage stocks were stored at 4 °C.

2.3. Screening for phage activity against Listeria monocytogenes 10403S

From the lab collection, phage stocks with a titer of at least 1×10^7 pfu/mL whose propagation host was not *L. monocytogenes* Mack (ST 1/2a) were tested for activity against *L. monocytogenes* 10403S (ST 1/2a) as described previously (Denes et al., 2015). Five μL of undiluted phage stocks were spotted onto bacterial lawns of 10403S in duplicate. All phages with a titer $\geq 1 \times 10^7$ pfu/mL, with a propagation host of Mack or with observed activity (visible inhibition of bacterial growth) against 10403S, were included in the study (120 total).

2.4. Preparation of concentration standardized working phage stocks

Stocks of each phage were diluted to a concentration of 1×10^7 pfu/mL based on recent (< 1 month old) titers.

2.5. Phage dilution spot assays

Phages were tested for activity and/or plaque formation against 10403S and the three mutant strains relative to their propagation host as described above. Ten μL of phage, either directly from a standardized stock or serially diluted in phosphate buffered saline (PBS) were spotted onto bacterial lawns. All phages were spotted within 4 h of bacterial lawns being poured. Plates were incubated at 30 °C for 18–24 h. Phage titers were calculated after observation of visible plaques. Phage activity against each strain was measured as the greatest phage dilution where either visible plaques were observed or visible inhibition of bacterial growth was observed (if no countable plaques were seen) as compared to the propagation host. Inhibition of bacterial growth was confirmed by visual comparison to controls of 10 μL spots of phage-free SM buffer with gelatin or PBS. Three replicates were conducted.

2.6. Adsorption assay

The adsorption of *L. monocytogenes* strains 10403S, FSL D4-0119 and UTK P1-0001 to bacteriophages LP-048 and LP-125 was determined as described previously (Denes et al., 2015). Working phage stocks of LP-125 and LP-048 at a titer of 1×10^9 pfu/mL were prepared using SM buffer with gelatin as the diluent. Sterile microcentrifuge tubes (2 mL) were prepared with 912 μL BHI, 20 μL of phage (at

Table 1
Listeria monocytogenes strains used in this study.

Strain	Features	Reference or Origin
Propagation Strains:		
Mack	Lineage II; 1/2a Serotype	Hodgson (2000)
F2365	Lineage I; 4b Serotype	Linnan et al. (1988); Wesley and Ashton (1991)
FSL J1-175	Lineage I; 1/2b Serotype	Bergholz et al. (2010); Stasiewicz et al. (2010)
FSL J1-208	Lineage IV; 4a Serotype	den Bakker et al. (2012)
Model Strains:		
10403S	Lineage II; 1/2a Serotype	Bishop and Hinrichs (1987)
FSL D4-0014	10403S mutant; nonsense mutation in *LMRG_00541*; deficiency of N-acetyl glucosamine in WTA	Denes et al. (2015)
FSL D4-0119	10403S mutant; nonsense mutation in *LMRG_00542*; deficiency of Rhamnose in WTA	Denes et al. (2015)
UTK P1-0001	10403S mutant; deletion in *LMRG_00544*; predicted disruption of L-Rhamnose synthesis pathway	This study

1×10^9 pfu/mL) and 9 μL each 1 M $MgCl_2$ and 1 M $CaCl_2$ before the addition of either 50 μL bacterial overnight culture (as described above) or 50 μL sterile BHI (negative control). After addition of the overnight culture or sterile BHI, tubes were incubated for 15 min at 30 °C with shaking at 160 RPM. Samples were then centrifuged for 1 min at 17,000 × *g* and 4 °C. The supernatant was filter sterilized (0.20 μm-pore size SFCA syringe filter; Corning, Incorporated, Corning, NY), serially diluted in PBS, and enumerated on duplicate plates using the agar overlay method (described above in section 2.2) to determine the number of unbound phage present. Three replicate experiments were conducted.

2.7. Cluster analysis (phage spot assays)

Heatmaps were generated using the heatmap hierarchical clustering tool from the HIV databases website, which can be accessed at https://www.hiv.lanl.gov/content/sequence/HEATMAP/heatmap.html. Clustering parameters included a complete method using Euclidean distances, which were calculated by taking the square root of the usual sum of squared differences distances between elements of vectors X [columns (*L. monocytogenes* strains)] and Y [rows (*Listeria* phages)]. Cluster stability was calculated using standard bootstraps with 100 iterations.

2.8. Statistical analysis (adsorption assays)

Log_{10} reduction was analyzed using JMP (version 13; SAS Institute Inc., Cary, NC). Linear models were constructed with the means of three replicates using strain as a factor. Log_{10} reduction was used as the model response. A Fit Y by X platform was used to compare means by one way ANOVA using Tukey's HSD test. Values in Fig. 3 represent the log_{10} reduction of phage in the supernatant. This was calculated by subtracting the log-transformed concentration of phage in the supernatant by the log-transformed concentration of phage in the negative control. An alpha of 0.05 was used to determine significant differences.

2.9. Sequencing and variant analysis of 10403S and UTK P1-0001

DNA was extracted using a QIAamp extraction kit (Qiagen, Hilden, Germany) as previously described (Denes et al., 2015). NexteraXT Library preparation (Illumina, San Diego, CA) and sequencing was conducted at the University of Tennessee Genomics Core. Sequencing was performed on an Illumina MiSeq instrument using 300 bp paired-end read chemistry. Raw reads were uploaded to the NCBI sequencing read archive (SRA IDs SRR9115406 and SRR9115405) and then trimmed with Trimmomatic version 0.35 (Bolger et al., 2014). Trimmed reads were then quality-checked with FastQC version 0.11.7 (Andrews, n.d.). McCortex (Turner et al., 2018) was used for variant calling in the *L. monocytogenes* mutant (UTK P1-0001) and control (10403S) isolates. The L. *monocytogenes* 10403S RefSeq assembly (RefSeq ID 376088) was downloaded and used as the reference. The McCortex pipeline was run with joint calling, the "vcfs" target, and a kmer size of 57 [optimum kmer size determined with KmerGenie version 1.7048 (Chikhi and Medvedev, 2014)]. SnpEff version 4.3t (Cingolani et al., 2012) was used to annotate the vcf output files.

3. Results & discussion

3.1. Listeria phages isolated from NY dairy farms almost all require rhamnose in their serotype 1/2a hosts' wall teichoic acids

Out of 120 phages tested, only LP-018 formed visible plaques on FSL D4-0119, the mutant strain of 10403S lacking rhamnose in its WTA (Fig. 1), and only two other phages from the collection showed any phage activity against the strain (Fig. 2). This supports previous observations that serotype 3 strains were largely resistant to a collection of 16 *Listeria* phages (Loessner et al., 1990). Although a mutant of a serotype 1/2a strain, FSL D4-0119 would be expected to be classified as a serotype 3 strain. Serotype 3 strains are known to possess only N-acetylglucosamine decorations in their WTA (Uchikawa et al., 1986; Fiedler, 1988). It has also been shown that loss of rhamnose due to phage selection in a serotype 1/2a strain converts that strain to serotype 3, and that serotype 3 strains typically resemble serotype 1/2 strains that have accumulated a mutation or mutations affecting rhamnosylation genes (Eugster et al., 2015). As spontaneous mutations affecting rhamnosylation of WTAs are consistently selected for in *L. monocytogenes* 1/2a populations infected with bacteriophages (Denes et al., 2015), it may be common for phage-resistance to emerge under the selective pressure of a single phage that confers cross-resistance to a majority of phages. This suggests that it will be a challenge for phage-based biocontrol to prevent the emergence of phage-resistant mutants in treated environments; however, the risk of *L. monocytogenes* contamination causing human illness may still be considerably reduced as rhamnose has been shown to promote virulence of serotype 1/2 strains by increasing resistance to host antimicrobial peptides (Carvalho et al., 2015) and by promoting association virulence factors to the cell wall (Carvalho et al., 2018). This suggests that rhamnosylation-affecting mutations may reduce the virulence of *L. monocytogenes,* which is further supported by the rarity of serotype 3 strains being associated with outbreaks or illnesses (Orsi et al., 2011; Todd and Notermans, 2011; Cartwright et al., 2013). If *Listeria* phage applications are expected to remain effective in the same environment for an extended period of time against all *L. monocytogenes* strains, it may be critical to include a phage like LP-018 that can infect mutants lacking rhamnose in their WTA. Future studies should be conducted to characterize LP-018 and to determine its binding receptors.

Mutations conferring phage resistance through loss of GlcNAc in WTA (Denes et al., 2015) are less problematic for *Listeria* phage applications than those that confer resistance through the loss of rhamnose, as there are many available phages that can infect the GlcNAc deficient 1/2a strain. Thirteen out of the 120 *Listeria* phages could form visible plaques on FSL D4-0014, the mutant strain of 10403S lacking GlcNAc in its WTA (Fig. 1), and one additional phage showed phage activity against the strain (Fig. 2). Phages that can infect FSL D4-0014 include the well-characterized LP-048, which is a myovirus from the *P100virus* genus (Krupovic et al., 2016) that may serve as an important model phage for studies on *Listeria* phage applications that aim to reduce the emergence of phage-resistance. Specifically, previous studies of LP-048 infection kinetics showed consistent bursts of approximately 13 phage particles under laboratory conditions (Denes et al., 2015), and plaquing efficiency was shown to increase by up to 50% at cooler temperatures that are more relevant to food and food processing conditions (Tokman et al., 2016).

L. monocytogenes strain UTK P1-0001 was included in this study as a phage-resistant mutant of 10403S because it showed a phenotype different from the mutants lacking rhamnose and GlcNAc in their WTA, i.e., UTK P1-0001 showed resistance to phage LP-048 and susceptibility to LP-125 (Figs. 1 and 2). Adsorption assays show UTK P1-0001 does not support binding of LP-048 (Fig. 3a), which likely uses rhamnose as its primary receptor. However, UTK P1-0001 does support a reduced level of binding of LP-125 (Fig. 3b), which is unable to bind to the rhamnose deficient mutant. Sequencing revealed that the only mutation present in UTK P1-0001 was a deletion in *LMRG_00544* (GAATA to G at nucleotide position 1,098,886; this variant was supported by 146/146 sequencing reads that covered the position) that would cause a frameshift and early termination, leading to a truncated protein product. *LMRG_00544* encodes RmlC, which is part of the dTDP-L-Rhamnose pathway (Giraud et al., 2000). It is possible that loss of function of RmlC would result in incorporation of dTDP-6-deoxy-D-xylo-4 hexulose (the substrate of RmlC) being incorporated into the WTA instead of L-rhamnose; this would be consistent with our observation that this mutant has a distinct phage susceptibility phenotype from FSL D4-

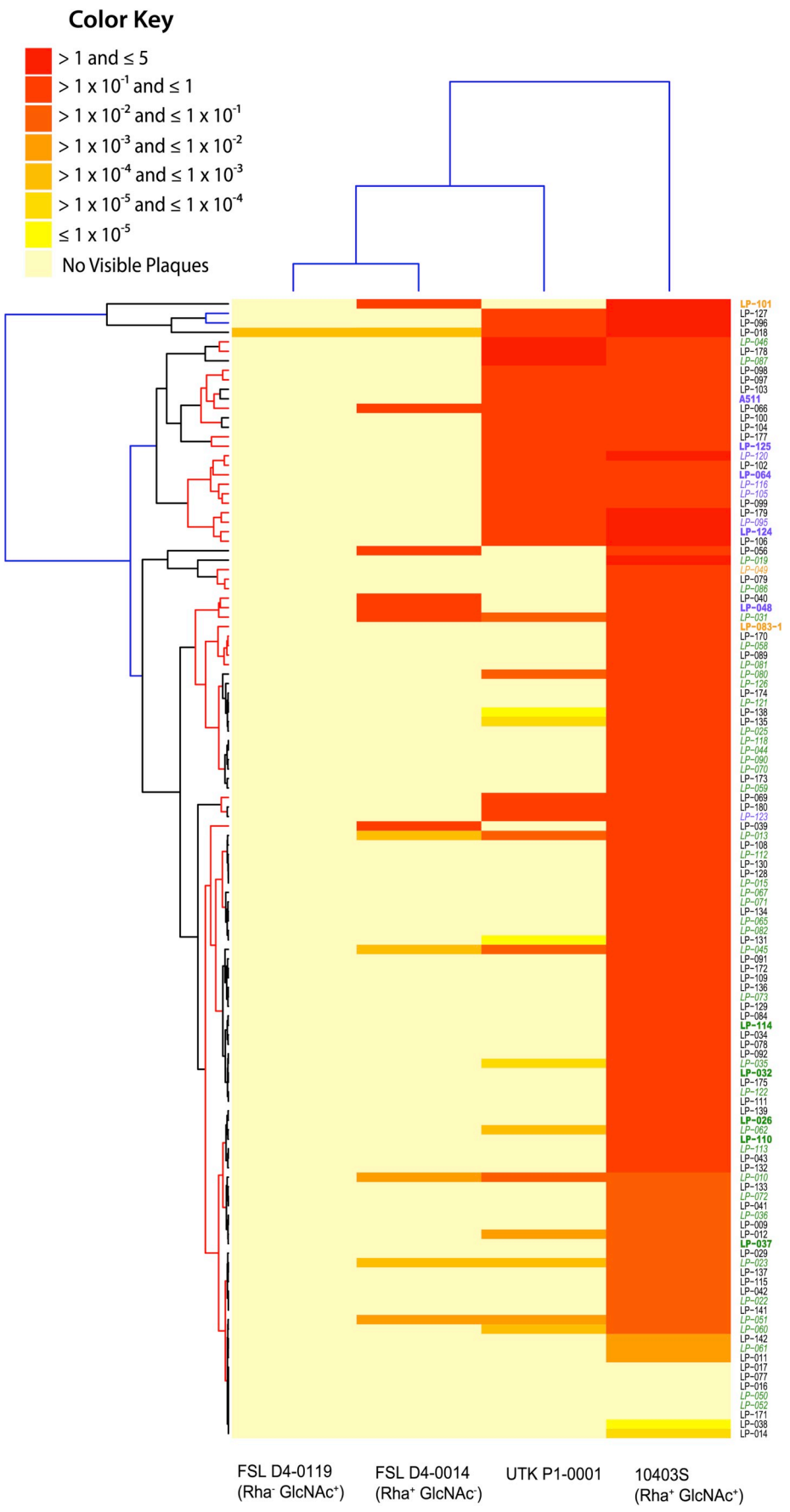

Fig. 1. Mean Efficiencies of Plaquing Heatmap. Values represent the titer of each *Listeria* phage on each bacterial strain compared to the titer on the phage's propagation host. "No visible plaques" indicates that no titer was obtained, but does not necessarily indicate no activity (Fig. 2). Colored dendrogram lines indicate stability of clustering. Bootstrap probabilities of ≥70% are highlighted in red; bootstrap probabilities of ≥90% are highlighted in blue. *Listeria* phage names are colored to indicate genome size categories. Purple indicates a large genome size (approx. 97–140 kb), predicted to be within Orthocluster I; green indicates a medium genome size (approx. 57–70 kb), predicted to be within Orthocluster V; orange indicates a small genome size (approx. 31–43 kb), predicted to be within Orthoclusters II-IV. **Bold** font indicates genome size obtained by sequencing; *italic* font indicates genome size obtained by PFGE. 10403S possesses WTA with terminal GlcNAc and Rha; FSL D4-0014 possesses WTA with terminal Rha; FSL D4-0119 possesses WTA with terminal GlcNAc. The means of three replicates are shown (See Supplemental Fig. 1 for values from each replicate). . (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

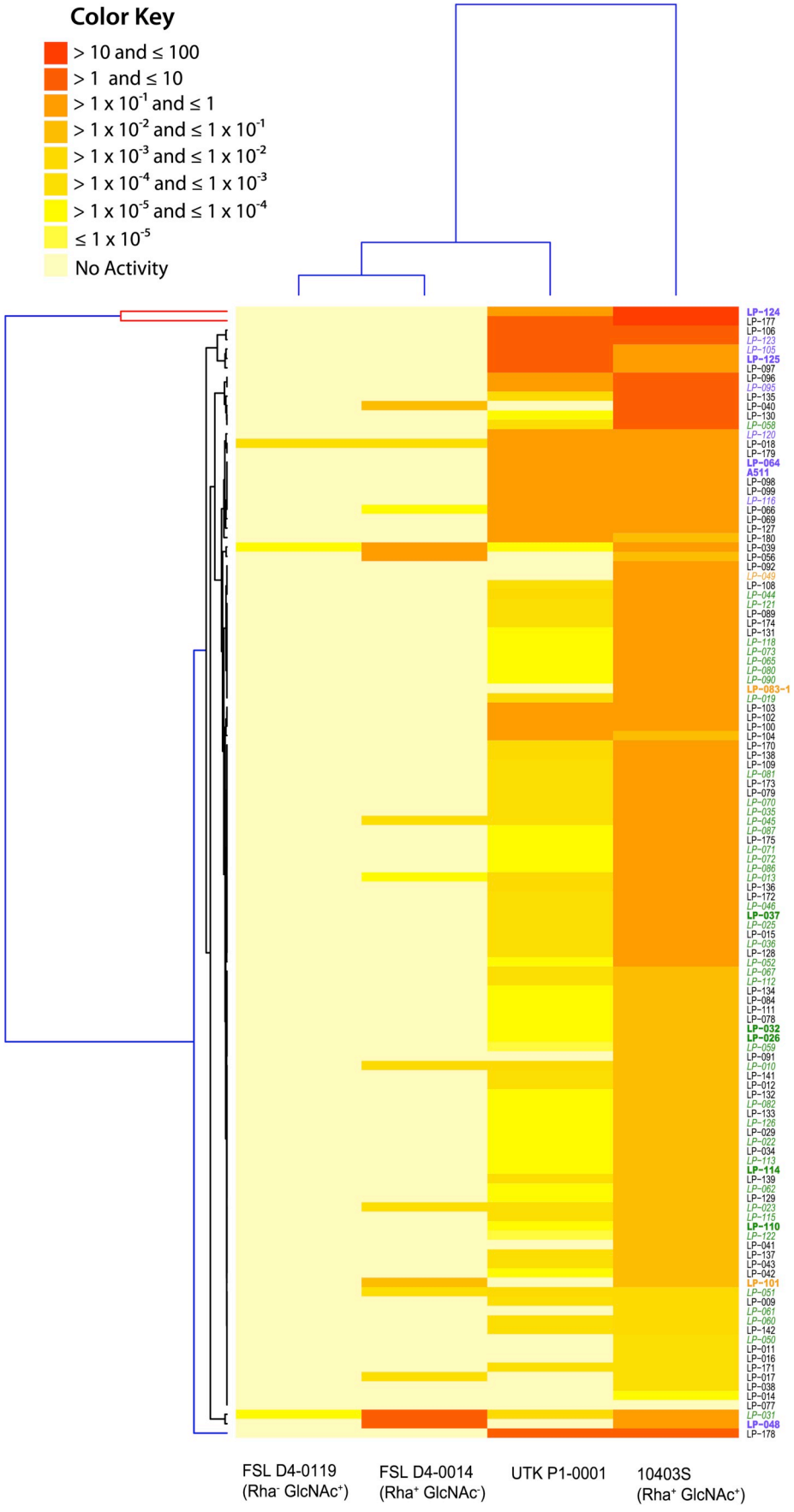

Fig. 2. Mean Relative Phage Activities Heatmap. Values represent the highest dilution of *Listeria* phage with visible activity against the specified bacterial strain compared to the highest dilution of that phage showing activity against its propagation strain. Colored dendrogram lines indicate stability of clustering. Bootstrap probabilities of ≥70% are highlighted in red; bootstrap probabilities of ≥90% are highlighted in blue. *Listeria* phage names are colored to indicate genome size categories. Purple indicates a large genome size (approx. 97–140 kb), predicted to be within Orthocluster I; green indicates a medium genome size (approx. 57–70 kb), predicted to be within Orthocluster V; orange indicates a small genome size (approx. 31–43 kb), predicted to be within Orthoclusters II-IV. **Bold** font indicates genome size obtained by sequencing; *italic* font indicates genome size obtained by PFGE. 10403S possesses WTA with terminal GlcNAc and Rha; FSL D4-0014 possesses WTA with terminal Rha; FSL D4-0119 possesses WTA with terminal GlcNAc. The means of three replicates are shown (see Supplemental Fig. 2. for values from each replicate). . (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

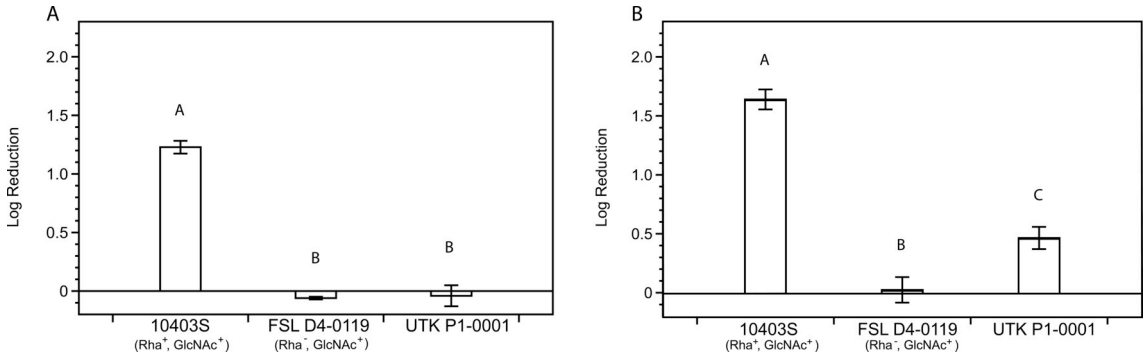

Fig. 3. Phage binding of LP-048 (a) and LP-125 (b) to 10403S and mutant strains. Panel A shows the binding of LP-048 (requiring rhamnose for binding serotype 1/2a strains) to parental 10403S, 10403S mutant strain FSL D4-0119 (deficient in WTA rhamnose), and 10403S mutant strain UTK P1-0001 (encodes a truncated RmlC; an enzyme in the dTDP-L-Rhamnose pathway). Panel B shows the binding of LP-125 (requiring rhamnose and GlcNAc for binding serotype 1/2a strains) to the same strains. Values represent the $\log_{10}$ reduction of phage in the supernatant, which indicates phage binding. In each panel, bars that do not share letters are significantly different from each other. Error bars represent the standard error of the mean (n = 3).

0119, which lacks L-rhamnose in the WTA. Forty-two of the *Listeria* phages showed visible plaques on UTK P1-0001, and an additional 61 phages showed activity against the mutant strain. This specific type of mutation is likely not a great challenge to *Listeria* phage applications as only a few *Listeria* phages are fully affected by it; however, the mutant strain may be useful for differentiating and better understanding receptor requirements of *Listeria* phages.

3.2. Phage activity was frequently observed without the formation of visible plaques

Efficiency of plaquing (or "plating") experiments are often conducted to evaluate the host range of bacteriophages (Kutter, 2009). Each phage-resistant mutant we used in this study showed several phages that failed to produce visible plaques, yet showed phage activity (Figs. 1 and 2). These phages were capable of lysing or significantly inhibiting the growth of the target strains; however, if we only performed efficiency of plaquing experiments, this activity would not be observed. The formation of a visible plaque is a complex biological process and absence of plaque formation does not necessarily indicate virion inviability (Abedon and Yin, 2009). If evaluating phages for potential in biocontrol applications, visible plaque formation on all target strains may not be necessary. For example, if constructing a cocktail that is designed to limit the emergence of phage-resistance, it may be effective to include a phage that only shows activity against a phage-resistance type (such as rhamnose deficient WTA); those specific mutations are likely to be rare in the target *L. monocytogenes* population, so as long as the cocktail exerts selective pressure on those mutants, they are unlikely to grow to problematic concentrations.

4. Conclusions

We have identified only one bacteriophage, LP-018, capable of infecting all three of the phage-resistant mutants of serotype 1/2a *L. monocytogenes* that were used in the study. We conclude that mutations conferring phage-resistance through loss of rhamnose likely pose the greatest challenge for phage-based biocontrol in serotype 1/2a strains, as we found that they confer resistance to almost all of the *Listeria* phages (119/120) in the diverse collection tested. These results have the potential to aid in the rational design of *Listeria* phage cocktails that aim to reduce the emergence of phage-resistance to ensure long-term efficacy.

Declarations of interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2019.06.003.

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